Centro Internacional de Restauración Neurológica

Mecanismos fisiológicos en la modulación afectiva de procesos de plasticidad sináptica

Tesis presentada en opción al Grado Científico de Doctor en Ciencias

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SÍNTESIS

Los procesos neuroplásticos son la base funcional que sustenta las capacidades adaptativas del Sistema Nervioso, tanto para las actividades cotidianas como el aprendizaje en cualquiera de sus formas, como para enfrentar situaciones de agresión o daño al sistema, resistir y recuperar funciones. Una extensa base empírica sugiere que estos mecanismos pueden ser afectados por factores afectivos (emoción, motivación). Sin embargo, no se conocen los mecanismos por los cuales se lleva a cabo esa modulación. Utilizando el modelo de reforzamiento conductual de la potenciación sináptica duradera (LTP, del inglés:Long-Term Potentiation) hemos establecido que la modulación afectiva de procesos neuroplásticos se lleva a cabo mediante un sistema neural en el que intervienen estructuras límbicas como la amígdala y el septo medial, así como el locus coeruleus del tronco encefálico. Las proyecciones noradrenérgicas del locus y colinérgicas del septum parecen activar procesos celulares metabólicos (síntesis de proteínas) que conducen al reforzamiento en el tiempo de procesos de potenciación sináptica. Estos mecanismos se afectan por la edad asociada al deterioro cognitivo y parecen tener un carácter universal considerando los resultados obtenidos con el uso de otros modelos en otras regiones cerebrales. El conocimiento ganado sustenta, sobre firmes bases fisiológicas, la relación entre afectividad y cognición, así como entre estos y la restauración neurológica.

INTRODUCCIÓN

Esta tesis esta conformada por un conjunto de artículos encaminados a determinar los mecanismos fisiológicos implicados en la modulación de procesos neuroplásticos por factores afectivos, como el estado emocional y la motivación.

Desde hace mucho tiempo existen evidencias de que esta relación existe. De hecho, a partir de evidencias empíricas, todos los sistemas pedagógicos reconocen y enfatizan la importancia de una buena motivación para lograr un aprendizaje más efectivo. De la misma forma, todos los sistemas de condicionamiento animal con fines científicos, de servicio, detectivescos o de espectáculo, reconocen que todo intento de enseñar algo a un animal será infructuoso si no existe una motivación adecuada según la etología de la especie.

El surgimiento de la Neurología Restaurativa en años recientes, ha puesto en evidencia que también el éxito de los progresos en la recuperación funcional, espontáneos o inducidos mediante un programa de rehabilitación, son influenciados fuertemente por el estado afectivo de los pacientes.

A pesar de la importancia que tiene comprender cuales son las bases fisiológicas de estas interacciones entre afectividad y neuroplasticidad, poco se ha avanzado hasta hoy en el tema. Nosotros hemos empleado un modelo de neuroplasticidad: la potenciación sináptica duradera y su reforzamiento por factores motivacionales (reforzamiento conductual) para estudiar estos procesos y establecer sus bases funcionales, tanto al nivel sistémico con la identificación de qué estructuras están implicadas en esos mecanismos, como al nivel celular, identificando neurotransmisores, receptores y procesos metabólicos involucrados.

Los resultados obtenidos son de absoluta novedad científica y contribuyen a la comprensión de las bases fisiológicas de la relación entre los procesos neuroplásticos que sustentan funciones de memoria, adaptación y recuperación del Sistema Nervioso; y el estado afectivo, determinado por el Sistema Límbico.

Estos resultados ayudan a interpretar efectos conocidos, como la relación entre el aprendizaje y la motivación; o la relación entre el estado afectivo y el resultado de la rehabilitación. El conocimiento de las bases neuroquímicas puede también sustentar el desarrollo de una farmacoterapia de apoyo a estos procesos en el marco de tratamientos neurorrestaurativos.

Los resultados son la culminación de un esfuerzo conjunto entre el Departamento de Neurofisiología Experimental –que dirijo en el CIREN- y el Departamento de Neurofisiología del Instituto Leibniz de Neurobiología de Magdeburgo, Alemania – dirigido por la Prof. J.U. Frey. La mayor parte de los trabajos fueron realizados en Cuba. Los que se hicieron en Alemania fueron realizados personalmente por mi durante estancias de trabajo en ese país. Todos los trabajos fueron planificados, organizados y dirigidos por mi, con la colaboración de la Prof. Frey. De la misma forma, todos los artículos que conforman la tesis fueron escritos por mi.

MARCO TEORICO

Los antecedentes científicos necesarios para facilitar la comprensión de esta tesis y su ubicación dentro del contexto del problema que se aborda, aparecen recogidos en dos artículos de revisión publicados por el optante.

El primero de ellos presenta una revisión amplia del tema de la neuroplasticidad en todas sus dimensiones y facetas, con particular énfasis en la plasticidad sináptica, que es el modelo sobre el cual se trabaja en los artículos que conforman los resultados de la tesis. Aunque fue publicado en el año 2000 considero que conserva, en lo esencial, actualidad y vigencia.

El segundo aborda el tema de la plasticidad sináptica y su relación con el deterioro funcional que acompaña al envejecimiento cerebral y ofrece un marco referencial adecuado para los tres artículos donde se abordan problemas relacionados con el envejecimiento.

Mecanismos celulares de la neuroplasticidad

J.A. Bergado-Rosado, W. Almaguer-Melian

CELLULAR MECHANISMS OF NEUROPLASTICITY

Summary. Objective. *To present a unified vision of the principal known mechanisms of neuroplasticity, emphasizing their universality.* Development. *The concept of the central nervous system as an immutable entity has been considerably modified during the second half of the 20th century. Neuroplasticity, that is the ability of the brain regarding change and repair is expressed in different ways, from functional modifications of existing structures to the formation, by growth and proliferation, of new structures and neurons. This study considers the molecular and cellular mechanisms of neuroplastic phenomena and classifies them into two main groups: plasticity due to growth, including the mechanisms of axonal regeneration, collateralization and reactive synaptogenesis; and functional plasticity, which includes changes in the efficacy of synaptic transmission such as long-term potentiation and the activation of silent synapses. We also describe some of the relations of neuroplastic phenomena with disease of the central nervous system, together with examples of physiological, physical and pharmacological factors which may be used in future as therapeutic tools to stimulate and modulate neuroplasticity.* Conclusion. *Neuroplastic mechanisms show a high degree of phylogenetic and ontogenetic conservation. They are important both in the genesis of disorders and disease of the nervous system and for its repair after different types of damage and trauma. Modulation of neuroplastic mechanisms by physical and chemical agents would appear to be one of the most powerful therapeutic tools of restorative neurology. [REV NEUROL 2000; 31: 1074-95]* ***[http://www.revneurol.com/3111/j111074.pdf]***

Key words. *Collateralization. Complex environment. Cortical plasticity. Gangliosides. Long term potentiation. Neurogenesis. Neuroplasticity. Neurotrophic factors. Orotic acid. Pathology. Physical exercise. Regeneration. Steroids. Synaptogenesis.*

LA NEUROPLASTICIDAD. CONCEPTO

Durante muchos años se consideró al sistema nervioso central (SNC) como una estructura funcionalmente inmutable y anatómicamente estática. El dogma 'no nuevas neuronas', insidiosamente extendido, significó también en todo ese tiempo: no nuevas conexiones. El sistema, una vez concluido su desarrollo embrionario, era una entidad terminada y definitiva, mutable sólo por lesión o degeneración e irreparable por su propia naturaleza. Ramón y Cajal escribió en su obra *Degeneración y regeneración en el sistema nervioso: '...la especialización funcional del cerebro impone a las neuronas dos grandes lagunas: incapacidad de proliferación e irreversibilidad de la diferenciación intraprotoplasmática. Es por esta razón que, una vez terminado el desarrollo, las fuentes de crecimiento y regeneración de los axones y dendritas se secan irrevocablemente. En los cerebros adultos las vías nerviosas son algo fijo, terminado, inmutable. Todo puede morir, nada puede regenerarse'*. Pero Don Santiago no sería el Maestro si no hubiera escrito al final del párrafo: *'Corresponde a la ciencia del futuro cambiar, si es posible, este cruel decreto'* [1].

En los últimos 40 años, el dictamen ha cambiado radicalmente. El rígido esquema de circuitos invariables, tanto en el número de sus unidades como en las conexiones entre ellas, ha sido sustituido progresivamente por un sistema en que la modificación dinámica de sus propiedades, en respuesta a cambios en su ambiente y sus ingresos, constituyen la noción fundamental para comprender sus extraordinarias propiedades. Esta nueva visión se sustenta en el concepto de la neuroplasticidad y es hoy un elemento unificador esencial para comprender procesos tan aparentemente diferentes como el aprendizaje y la recuperación de funciones tras una lesión.

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De acuerdo con esta concepción, el SNC es un producto nunca terminado, es el resultado, siempre cambiante y cambiable, de la interacción de factores genéticos y epigenéticos.

Tal vez la importancia de la concepción neuroplástica del SNC radique en la nueva mentalidad que impregna actualmente el amplio espectro de las Neurociencias, tanto experimentales como aplicadas. Del fatalismo del 'nada puede hacerse' se transita hoy aceleradamente hacia la búsqueda y ensayo constante de nuevas formas de estimular los cambios plásticos que permitan la restauración de funciones alteradas por traumas, accidentes vasculares o enfermedades degenerativas (Tabla I), no sólo por la sustitución, sino buscando también la recuperación de las áreas dañadas [2]. Comienza a constituirse una Neurología Restaurativa que ha de ser, sin duda, la Neurología del nuevo siglo.

MECANISMOS DE LA NEUROPLASTICIDAD

Los mecanismos de la neuroplasticidad son muy diversos y pueden abarcar desde modificaciones morfológicas extensas, como las que se observan en la regeneración de axones y formación de nuevas sinapsis, hasta sutiles cambios moleculares que alteran la respuesta celular a los neurotransmisores [3]. En esta revisión enfatizaremos los mecanismos neuronales de la plasticidad, aunque no debe perderse la perspectiva de que cada neurona del SNC es sustentada por una unidad trófica formada por otras neuronas, células de la glía, vasos sanguíneos y moléculas de la matriz celular [4], y a ellas nos referiremos en aquellos casos en que su papel está mejor establecido. Hemos omitido algunos mecanismos considerados clásicamente como neuroplásticos, por ejemplo la diasquisis, porque no lo son. La diasquisis resulta del enmascaramiento de funciones por un desequilibrio transitorio entre excitación e inhibición [2] y nada tiene que ver con la neuroplasticidad.

La regeneración, formación de colaterales axónicas y de nuevas sinapsis, constituye la base de la reorganización y recuperación de funciones perdidas por daño a las neuronas. De sus características esenciales nos ocuparemos brevemente en la primera parte.

Tabla I. La neuroplasticidad está vinculada a las enfermedades más importantes que afectan al sistema nervioso. Los procesos neuroplásticos son responsables, en buena medida, de la recuperación de las funciones en los pacientes que sufren las consecuencias de trastornos cerebrovasculares. Procesos de neuroplasticidad aberrantes están implicados en la progresión y, tal vez, en la propia génesis de muchas formas de epilepsia. En las enfermedades neurodegenerativas, como la demencia tipo Alzheimer y el morbus Parkinson, el agotamiento de las capacidades neuroplásticas podría ser el responsable de algunas de las consecuencias más invalidantes de estos trastornos. (Datos tomados de Price DL. Nature 1999; 399 (Suppl)).

Enfermedad	N.º de casos en Estados Unidos
Enfermedad cerebrovascular	1,5 millones de casos nuevos por año
Epilepsia	2,5 millones de casos
Enfermedad de Alzheimer	5 millones de casos
Enfermedad de Parkinson	500.000 casos
Esclerosis múltiple	300.000 casos

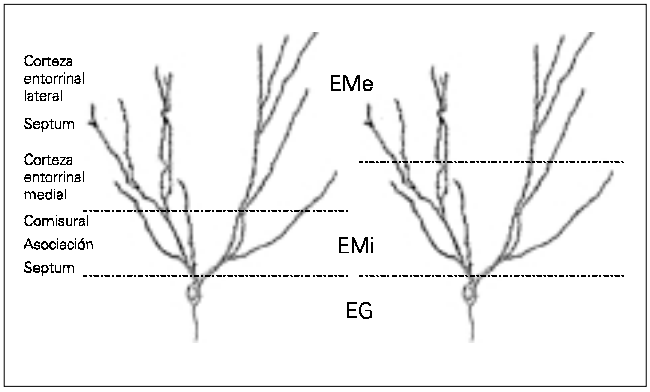

Figura 1. Modelo corteza entorrinal-giro dentado para el estudio de mecanismos neuroplásticos. A la izquierda se menciona el origen de las aferencias y se indica la zona en que se distribuyen en el estrato molecular del giro dentado sobre una neurona granular. a) Patrón normal. Los ingresos al giro dentado siguen un patrón de distribución ordenado. El estrato molecular externo recibe a las fibras de la vía perforante lateral en su región más distal y de la corteza entorrinal medial en su zona proximal. Al estrato molecular interno llegan fibras comisurales (del hipocampo contralateral) y asociativas (del mismo hipocampo). Las fibras de origen septal se distribuyen en ambos estratos. b) Después de lesionar la corteza entorrinal. La lesión de la corteza entorrinal destruye el ingreso al estrato molecular externo. La colateralización y sinaptogénesis reactiva conducen a la expansión del estrato molecular interno. EG: estrato granuloso; EMi: estrato molecular interno; EMe: estrato molecular externo.

La modificación de las capacidades funcionales de sinapsis existentes puede contribuir a la compensación funcional a expensas de sinapsis poco activas o silentes que están en la base de lo que se ha dado en llamar plasticidad conductual. Estos cambios de conectividad sináptica también se consideran hoy en día fundamentos fisiológicos de los procesos de aprendizaje y memoria, como fuera anticipado por Donald Hebb y Hansjürgen Matthies [5-7]. Revisaremos sus mecanismos fundamentales en el hipocampo y su expresión en otras áreas, en particular en los procesos de maduración funcional de la corteza cerebral.

Plasticidad por crecimiento

Regeneración axonal

Desde el siglo pasado se conoce que los axones del sistema nervioso periférico pueden regenerarse por crecimiento a partir del cabo proximal. Ello no ocurre en el SNC de los mamíferos, aunque sí en vertebrados más primitivos [8]. Al parecer, la ausencia de regeneración no se debe a una incapacidad esencial de las neuronas centrales, por cuanto cerca de las neuronas dañadas se encuentran signos de regeneración abortiva, llamada gemación *(sprouting)* regenerativa [9]. Existen evidencias de que la mielina central y los oligodendrocitos que la producen contienen sustancias que inhiben la regeneración axonal [10,11].

La regeneración axonal sería útil sobre todo para la reparación de tractos de fibras largas, como los del nervio óptico –que no es un nervio periférico– o los que actúan en la médula espinal. Actualmente se experimentan nuevas estrategias para promover su regeneración: puentes de nervio periférico, factores tróficos o anticuerpos monoclonales diseñados para bloquear los factores inhibidores gliales (Ver resumen en [8]).

Colateralización o gemación colateral

La ausencia de regeneración axonal no significa que no ocurran cambios regenerativos ante la pérdida de inervación. Estos cambios, además, pueden tener profundas influencias en la recuperación de funciones perdidas.

Una forma bien estudiada es la llamada colateralización o gemación *(sprouting)* colateral. La colateralización se diferencia de la regeneración en que el crecimiento ocurre a expensas de axones sanos, que pueden provenir de neuronas no afectadas por la lesión o de ramas colaterales de los mismos axones dañados que la lesión no llegó a afectar. Aunque suele distinguirse esta segunda variante con el nombre de efecto de poda *(pruning)*, los mecanismos de ambas formas de crecimiento axonal colateral parecen ser muy similares [9] a pesar de agentes diferentes los inician.

La colateralización puede ocurrir a partir de axones del mismo tipo de los dañados (colateralización homotípica) o de otro tipo (colateralización heterotípica). El proceso de colateralización normalmente concluye con la formación de nuevas sinapsis que reemplazan a las que se han perdido por la degeneración retrógrada de los axones destruidos. Este proceso se ha llamado sinaptogénesis reactiva, para distinguirlo de la sinaptogénesis que normalmente sucede en las etapas intermedias del desarrollo embrionario; no obstante, no parece existir diferencia alguna entre los mecanismos de una y otra.

La mayor parte de los estudios experimentales sobre los mecanismos de colateralización se ha realizado utilizando el modelo de lesión de la vía perforante que proyecta de la corteza entorrinal al giro dentado del hipocampo. Esta proyección es glutamatérgica y las fibras que la integran se distribuyen en el campo dendrítico de las neuronas granulares del giro dentado siguiendo un patrón regular y ordenado (Fig. 1). Las regiones más distales del campo dendrítico reciben las fibras que se originan en la parte lateral de la corteza entorrinal, mientras que aquellas que proceden de la porción medial de la corteza entorrinal terminan sobre el tercio medio del campo dendrítico. Estas dos subregiones se corresponden anatómicamente con el llamado estrato molecular externo. El tercio interno del campo dendrítico, correspondiente al estrato molecular interno, recibe fibras colinérgicas y gabérgicas del septum, noradrenérgicas y serotoninérgicas desde núcleos de la formación reticular, así como fibras asociativas y comisurales de otras regiones del hipocampo ipsi y contralateral, respectivamente. La lesión de la corteza entorrinal provoca una reducción significativa del estrato molecular externo que se acompaña de la expansión del estrato molecular interno.

En este modelo, los primeros signos de crecimiento de cola-

terales axónicas aparecen seis días después de la lesión y son muy intensos en la segunda y tercera semana [12]. Los agentes que inician este crecimiento no se conocen con precisión y se han formulado varias hipótesis, no alternativas, que podrían desencadenar procesos de colateralización:

- Especializaciones post-sinápticas vacantes. Los axones sobrevivientes tras la degeneración de los cabos distales de las fibras transectadas detectan la presencia de 'plazas vacantes' y ello estimula su crecimiento.
- Ausencia de inhibición competitiva. La densidad de inervación de una neurona podría estar controlada por señales inhibitorias que limitan el crecimiento axonal. La pérdida de una cantidad sustancial de terminales eliminaría este freno al crecimiento axonal.
- Cambios en la actividad sináptica. La pérdida de aferentes altera la actividad de las neuronas. Ello, a su vez, podría conducir a la liberación de factores tróficos del crecimiento axonal.
- Presencia de terminales en degeneración. Las terminales que degeneran liberan sustancias que estimulan la colateralización.
- Las células gliales que fagocitan los axones degenerados liberan factores tróficos que estimulan el crecimiento colateral [9].

La acción cooperativa de varios de los factores antes enunciados contribuye a crear lo que se ha dado en llamar un ambiente promotor de crecimiento que pone en marcha la gemación y extensión de los axones o ramas intactas.

La importancia de algunos factores para la colateralización se ha puesto de manifiesto en hallazgos recientes. Así, por ejemplo, la activación de receptores de tipo N-metil-D-aspartato (NMDA) en las neuronas post-sinápticas parece necesaria para promover el crecimiento axonal. El bloqueo de estos receptores impide la inducción de la proteína GAP-43 y el crecimiento colateral [13,14].

La fosfoproteína GAP-43 (*Growth Associated Protein*), también llamada F-1 o B-50, se relaciona con las terminales axónicas y podría tener alguna función en la transmisión sináptica normal, pero su expresión se incrementa dramáticamente en axones que se elongan [15,16]. Los niveles más altos de GAP-43 se encuentran siempre en neuronas que colateralizan [17] y se considera, por lo tanto, como un marcador específico de axones en crecimiento. Estudios con microscopía electrónica indican que GAP-43 se transporta predominantemente hacia los axones que sufren remodelación. Se desconoce la función específica de esta proteína en el proceso de crecimiento axonal y cómo se modifica su función al ser fosforilada por la proteinocinasa C. Por otra parte, GAP-43 no parece ser la única proteína sináptica vinculada al crecimiento axonal. Existen evidencias de que la proteína SNAP-25 también participa en el crecimiento axonal colateral [21] y otras como la sinapsina I, cuyo gen se activa sólo en el momento de establecer el contacto sináptico [22,23]. A pesar de esto, GAP-43 es un marcador esencial para el estudio experimental de la colateralización.

Otro aspecto que parece importante para la iniciación y desarrollo de la colateralización son las interacciones gliales. Como mencionamos anteriormente, las células gliales son necesarias para eliminar las terminales axónicas degeneradas. Existe una secuencia de activación glial que involucra primero a la microglía y luego incluye a los astrocitos. La respuesta microglial es evidente ya a las 24 horas de la lesión. La activación astrocítica únicamente es evidente dos días después, mientras que los primeros signos de colateralización se observan a los tres días. La activación microglial se mantiene durante cuatro semanas después de lesión y la astrocítica durante tres semanas [24]. Esta reacción no incluye, sin embargo, a los linfocitos T [25] u otros componentes de la cohorte inmune (Fig. 2).

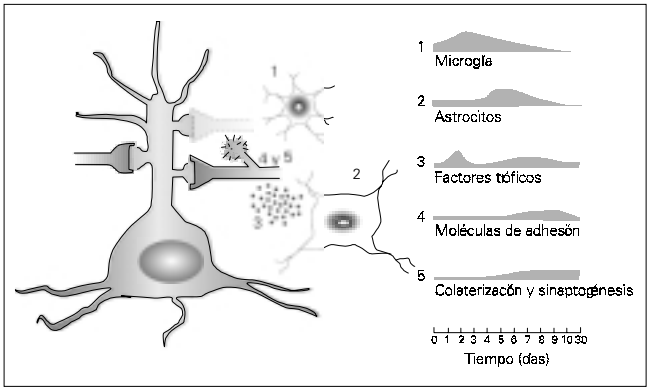

Figura 2. Mecanismos de colateralización. (1) La presencia de células dañadas atrae a la microglía, responsable principal de eliminar el detrito celular. Más tarde (2), se produce una reacción astrocítica que libera factores tróficos (3) estimuladores del crecimiento axonal colateral, el cual se acompaña de expresión de moléculas de adhesión (4) que guían y estabilizan las neuritas en crecimiento. El proceso culmina con la formación de nuevos contactos sinápticos (5).

La función fagocítica corresponde predominantemente a la microglía, mientras que los astrocitos parecen responsables de la producción de factores tróficos que estimulan el crecimiento axonal. La lesión induce en los astrocitos la expresión de formas truncadas del receptor tirosinocinasa B (TrkB) que, se piensa, actúan como 'presentadoras' de neurotrofinas a los axones en crecimiento [26], y de moléculas de adhesión celular, como la NCAM *(Neural Cell Adhesion Molecule)* [27-29] y la tenascina C [30], que participan en la guía de los axones en crecimiento hacia sus blancos y forman verdaderas 'pistas' de elongación [31-33].

El proceso de colateralización, seguido de la formación de nuevos contactos sinápticos, puede desempeñar un papel muy importante en la recuperación de funciones perdidas como consecuencia de lesión o en el retraso de la aparición de trastornos manifiestos en las enfermedades neurodegenerativas.

Si la colateralización es homotípica, su valor restaurativo resulta evidente, pero aun una colateralización heterotípica puede ser beneficiosa. Primero, porque la presencia de fibras aferentes es necesaria para el mantenimiento dendrítico; y, por otra parte, la colateralización heterotípica puede contribuir al equilibrio excitación-inhibición y con ello a una restauración parcial de la función neural.

En el modelo de lesión entorrinal antes citado, se ha descrito colateralización de fibras asociativas y comisurales (homotípica), así como de fibras colinérgicas septales o noradrenérgicas (heterotípica) [34,35]. Esto se expresa morfológicamente en la expansión del estrato molecular interno [36] y funcionalmente en la recuperación de funciones de memoria afectadas por la lesión [37].

También se han demostrado procesos de colateralización en otros modelos de desnervación hipocampal, como la lesión de fimbria-fórnix que interrumpe la aferencia colinérgica procedente del septum. En este modelo se produce un crecimiento heterotípico, principalmente noradrenérgico, y un crecimiento homotípico [38], reacción que, aunque está bien descrita en roedores, no parece tan importante en los primates [39].

Sinaptogénesis reactiva

El brote y extensión de nuevas ramas axónicas serían totalmente inútiles si no culminasen con la formación de nuevos contactos

sinápticos. La sinaptogénesis reactiva es parte indisoluble de un solo proceso que comienza con la colateralización y concluye con la formación de nuevos contactos funcionales.

En el modelo de lesión entorrinal, se ha demostrado que la desnervación inicial conduce a la pérdida de más del 85% de las sinapsis en el estrato molecular, que es seguido por un período de sinaptogénesis acelerada [40,41]. El elemento presináptico es aportado por las colaterales axónicas crecidas como consecuencia de la desnervación [42]. Las nuevas sinapsis muestran, en un principio, una talla reducida de los elementos que la integran, similar a las de sinapsis recién formadas durante el período embrionario de sinaptogénesis. Con el paso del tiempo, el tamaño de los elementos sinápticos aumenta y adquiere características 'adultas' [40].

En este proceso de sinaptogénesis no sólo es importante la colateralización de los axones, sino que también las dendritas, que aportan el elemento post-sináptico, sufren modificaciones como consecuencia de la desnervación y participan activamente en el proceso de reconstitución.

En zonas como la corteza cerebelosa o el núcleo geniculado, donde no ocurren procesos de colateralización, las dendritas muestran dos tipos de respuesta a la desaferentización: un proceso de axonización, en el cual aparecen en las dendritas especializaciones presinápticas y formación de sinapsis dendrodendríticas; o, en su defecto, las dendritas sufren un proceso de atrofia gradual que conduce al incremento relativo de la densidad de inervación a expensas de los axones sobrevivientes, no colateralizados. Ambos fenómenos contribuyen a cierta recuperación funcional, a pesar de la ausencia de colateralización.

Los mecanismos dendríticos implicados en la sinaptogénesis reactiva no se conocen con detalle, aunque desde hace tiempo se sabe que ésta requiere una síntesis de proteínas activa [43]. En correspondencia, se ha demostrado la existencia de transporte dendrítico de ARN mensajero (p. ej., de la subunidad R1 del receptor NMDA) y un incremento notable de síntesis dendrítica de proteínas que sólo se manifiesta en las regiones desnervadas [44]. El transporte dendrítico de ARNm involucra también modestos incrementos de proteínas dendríticas de importancia funcional, como el de la proteína relacionada con microtúbulos 2 (MAP-2, del inglés *Microtubule Associated Protein*) y la proteinocinasa II dependiente de calcio-calmodulina (PK-II) [45].

La MAP-2 es una proteína regulada por fosforilación que sólo se localiza en las dendritas (a diferencia de tau que es exclusivamente axónica), forma parte del citoesqueleto dendrítico y podría tener un papel en el proceso de remodelación dendrítica [46,47].

Neurogénesis

La producción de nuevas células nerviosas en el cerebro adulto se ha demostrado en todas las clases de vertebrados [1]. En las aves se piensa que está directamente implicado en los procesos de maduración posnatal y en funciones estacionales como el canto [48,49]. En roedores se conocen dos áreas donde la neurogénesis se mantiene activa hasta edades muy avanzadas de la vida: la zona subventricular (ZSV) de los ventrículos laterales y el giro dentado del hipocampo [50,51].

Las células progenitoras son capaces de generar neuronas, astrocitos y oligodendrocitos, y su diferenciación parece controlada por señales ambientales que incluyen al ácido retinoico, a la adenosina monofosfato cíclico (AMPc) y factores tróficos. La depleción de serotonina reduce la producción de células nerviosas en el giro dentado y la ZSV [52], y lo mismo ocurre por deficiencia en hormonas tiroideas [53]. Por otra parte, las crisis epilépticas aceleran la neurogénesis y la formación de circuitos aberrantes [54] que son importantes en la progresión del trastorno [55]. El establecimiento de circuitos aberrantes significa que las nuevas neuronas pueden formar sinapsis, a lo cual contribuye la expresión de moléculas de adhesión como la NCAM [56]. Un hallazgo interesante es el hecho de que ratas viejas que habitan en un ambiente complejo muestran un incremento de la neurogénesis [57]. Las células nerviosas recién formadas pueden migrar a regiones distantes [58], lo que añade un posible valor terapéutico a este interesante mecanismo.

Una comunicación reciente va más allá al proponer que neuronas ya diferenciadas pueden recuperar sus capacidades mitóticas si se colocan en un ambiente adecuado [59]. Se trata de un único estudio *in vitro* que debe ser confirmado, pero extraordinariamente provocativo.

Aunque no está resuelta la controversia sobre si existe neurogénesis en el cerebro adulto de los primates [60], es indudable que poder modular la formación de nuevas células nerviosas es una promesa de enormes potencialidades para la Neurología Restaurativa, tanto para la recuperación *in situ* de neuronas perdidas, como para el trasplante de células precursoras en zonas dañadas.

Plasticidad funcional

Plasticidad sináptica

Las sinapsis son especializaciones anatómicas y funcionales mediante las cuales la información, que circula en forma de pulsos eléctricos, es transferida de una neurona a otra. Las características funcionales de estas estructuras y los mecanismos de suma espacial y temporal que realizan las neuronas post-sinápticas son la base de las propiedades integradoras del sistema nervioso.

La importancia de las sinapsis en los procesos de almacenamiento de información se ha postulado desde la época de Ramón y Cajal y más recientemente en los trabajos de Hebb y Matthies [5,61-65]. Estos modelos 'conectivistas' de la memoria predicen cambios en la eficacia de la transmisión sináptica, en los circuitos neuronales implicados en la adquisición de nuevos contenidos de memoria. Atribuyen, por lo tanto, propiedades plásticas a las sinapsis y rompen con los conceptos primitivos que consideraban a las sinapsis inmutables en sus propiedades funcionales, como puntos de soldadura entre los componentes de un circuito eléctrico.

Formas de plasticidad

Las capacidades plásticas de las conexiones sinápticas pueden expresarse de formas diversas por su duración y por los mecanismos implicados [66].

Existen mecanismos que conducen a cambios transitorios, del orden de milisegundos a minutos, de la eficacia sináptica. La facilitación o inhibición por pulsos pareados y la llamada potenciación postetánica son ejemplos de estas formas efímeras de plasticidad [67], que parecen depender de la acumulación de Ca^{2+} residual en la terminal presináptica [68]; asimismo, su duración es limitada por los mecanismos de tampón que reducen la concentración de este ion [69].

Sin embargo, existen formas mucho más duraderas de plasticidad sináptica. En 1973, se publicaron dos artículos simultáneos en el *Journal of Physiology* (Londres), en los que se describía un fenómeno de modificación a largo plazo de la eficacia de la transmisión sináptica [70,71]. Este fenómeno se ha llamado potenciación a largo plazo (LTP, del inglés *Long-Term Potentiation*) y se considera, hasta hoy, como el mejor modelo de cambio funcional en la conectividad sináptica dependiente de la

actividad. Desde su descubrimiento se le vinculó a los procesos de memoria, aunque en la actualidad se propone también como un mecanismo importante en la maduración funcional de las sinapsis y en los procesos de remodelación que conducen a la recuperación de funciones perdidas como consecuencia de lesiones o trastornos degenerativos.

La plasticidad sináptica a largo plazo puede también expresarse en una disminución de la eficacia en la transmisión. Si el cambio se produce en una población previamente potenciada, suele llamársele despotenciación, en otro caso se denomina depresión a largo plazo (LTD, del inglés *Long-Term Depression*). La LTP y la LTD pueden ocurrir en las mismas sinapsis dependiendo de la frecuencia de estimulación utilizada [72]. Frecuencias bajas, entre 1 y 5 Hz, conducen a LTD, mientras que frecuencias mayores de 25 Hz producen LTP [73]. En ambos casos se ha probado la participación de receptores de tipo NMDA [74] y corrientes de Ca^{2+} a la terminal post-sináptica. En el caso de la LTP, ello conduce a la activación de proteinocinasas, mientras que en la LTD, donde el incremento de Ca^{2+} es menor, se activan fosfatasas que tienen una función antagónica [75-77]. Aunque ambos fenómenos, en interacción dinámica, parecen implicados en los procesos de memoria [78], nos ocuparemos en lo sucesivo de la LTP cuya importancia funcional y mecanismos están mejor estudiados.

Mecanismos de la LTP. La LTP fue descrita inicialmente en el hipocampo. Si bien hoy sabemos que no es un fenómeno exclusivo de las sinapsis de esta estructura cerebral, la mayoría de los estudios acerca de los mecanismos de la LTP se han realizado en las sinapsis del giro dentado y de la región 1 del cuerno de Ammon (CA1). Por esa razón, describiremos a continuación los mecanismos de la LTP en estas poblaciones y después comentaremos la LTP en otras regiones del SNC.

Receptores glutamatérgicos. Existen dos tipos fundamentales de receptores glutamatérgicos. Los receptores ionotrópicos forman canales iónicos y son responsables de la despolarización de la membrana post-sináptica. En ellos se distinguen los de tipo AMPA, kainato y NMDA, según el nombre del agonista más afín. El otro tipo son los llamados receptores metabotrópicos, una familia de ocho miembros conocidos que –como su nombre indica– se relacionan con cambios metabólicos más que con conductancias iónicas [79,80]. Los receptores AMPA/kainato son los responsables de la transmisión sináptica normal, pues median corrientes de sodio que despolarizan la membrana post-sináptica. Sin embargo, cuando el nivel de despolarización alcanza un valor umbral en presencia de glutamato, se produce la activación de los receptores de tipo NMDA. Este ionóforo abre canales de Ca^{2+} y su activación es imprescindible para la inducción de la LTP [81,82] (Fig. 3). Los receptores glutamatérgicos metabotrópicos también parecen implicados en la inducción de la LTP [83-85], aunque los resultados han sido contradictorios [86]. La entrada de Ca^{2+} parece ser el hecho decisivo. Los receptores AMPA son importantes porque posibilitan la activación NMDA, como indican estudios recientes en mutantes carentes de estos receptores [87], y también porque el resultado final de todos los procesos celulares implicados en la LTP podría ser el aumento en la densidad de estos receptores [88].

Papel del calcio. La entrada de calcio en la terminal post-sináptica es condición necesaria para iniciar los procesos de potenciación sináptica [89]. El ingreso de Ca^{2+} se realiza normalmente por los ionóforos NMDA, aunque también puede lograrse por la vía de canales de activación lenta dependientes de voltaje (VDCC) [90]. Este ion, como segundo mensajero, media todas las formas de plasticidad sináptica a largo plazo, LTP y LTD. La diferencia parece depender de la cantidad de calcio que penetra y, por consiguiente, del incremento en su concentración intracelular [91,92]. Si el incremento sobrepasa un nivel umbral, lo que normalmente ocurre con estimulación de alta frecuencia, el Ca^{2+} conduce a la activación de proteinocinasas que son responsables, por una parte, de mantener transitoriamente el estado de respuesta incrementada y, por otra, de activar procesos de transcripción/traducción que conducen a la estabilización del cambio sináptico. Algunas de estas proteinocinasas pueden actuar sobre los reservorios de Ca^{2+} mitocondriales y del retículo endoplasmático y liberar calcio al citoplasma, lo cual contribuye a la elevación de la concentración intracelular de este ion.

Proteinocinasas. La adición de grupos fosfato a las proteínas es un importante mecanismo de regulación de su función. Esta función está a cargo de enzimas especiales conocidas como proteinocinasas. Existen dos grandes grupos de proteinocinasas según el residuo aminoacídico blanco de la fosforilación: las tirosinocinasas (como es el caso de los receptores de neurotrofinas) y las serinaˆ-treonina proteinocinasas. Aunque existen evidencias recientes que involucran a algunas tirosinocinasas en la LTP, su papel no está bien establecido. Nos referiremos, por lo tanto, a las tres serina-treonina proteinocinasas con funciones comprobadas en los procesos de plasticidad sináptica.

La PK-II es activada por iones de Ca^{2+} y calmodulina por lo que también es llamada Ca-CamK-II. La activación se produce por fosforilación del residuo de treonina en posición 286 y ello conduce a un mecanismo multiplicador por autofosforilación [93-96].

Inhibidores de la PK-II, como el calmidazolio, bloquean la LTP desde etapas tempranas (<30 minutos) [97]. Se han encontrado resultados coincidentes en ratones mutantes carentes de la subunidad alfa de la PK-II [98,99], lo que demuestra la importancia de una activación temprana de la PK-II para el mantenimiento de la LTP.

La PK-II es muy abundante en las densidades post-sinápticas y se relaciona con las subunidades NR-1 y NR-2B del receptor NMDA, una posición estratégica para su activación inmediata por el calcio que penetra a través de este ionóforo [100]. Este hecho coloca a esta enzima en la vecindad de su principal sustrato: el receptor AMPA, responsable de la despolarización post-sináptica y, por lo tanto, de la eficacia de la transmisión. De este modo, la PK-II puede mediar la potenciación de sinapsis glutamatérgicas, incrementando la conductancia de canales AMPA existentes en la membrana post-sináptica [101].

Otra serina-treonina cinasa implicada en la LTP es la proteinocinasa C (PKC). Esta proteinocinasa se activa por Ca^{2+} y diacilglicerol (DAG). El calcio penetra por los canales NMDA. La fuente de DAG implica la activación previa de fosfolipasas, que actúan sobre los lípidos de membrana liberando DAG e inositol-3-P (IP3). El IP3 puede incrementar la concentración citoplásmica de Ca^{2+} y activar su liberación desde almacenes intracelulares.

La importancia de la PKC para la LTP está bien establecida. La administración de bloqueadores de la PKC no afecta la inducción de la LTP; sin embargo, la eficacia sináptica retorna a su valor basal en aproximadamente dos horas [102-104]. Por otra parte, activadores de la PKC como los ésteres de forbol prolongan una LTP débil [105,106]. La PKC es fosforilada y activada de forma duradera

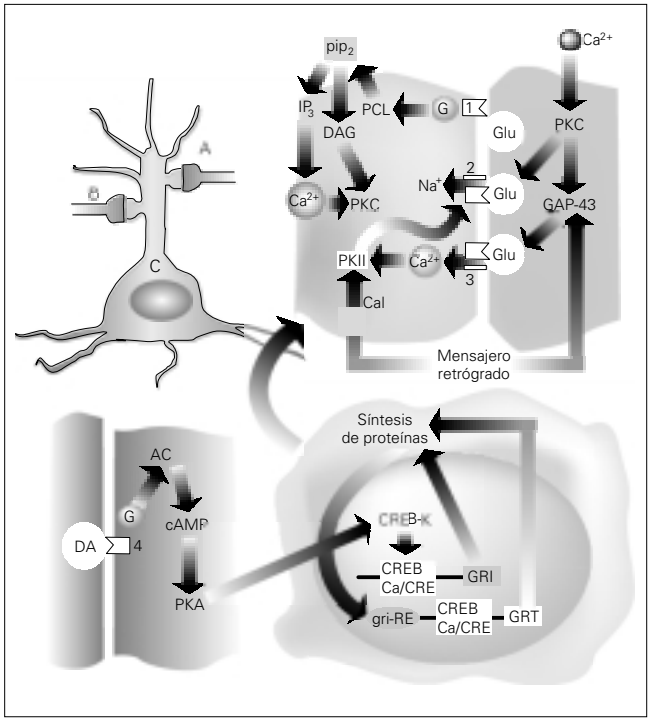

Figura 3. Mecanismos de potenciación sináptica. a) Sinapsis instructivas: la llegada de un tren de impulsos de alta frecuencia a las terminales axónicas glutamatérgicas provoca la liberación del neurotransmisor. Los receptores AMPA (2) activados permiten la entrada de sodio y la despolarización local que activa los receptores NMDA (3). Ello posibilita la entrada de calcio a la región post-sináptica. El calcio, junto con la calmodulina, activan la proteinocinasa II, responsable de mantener el estado de eficacia sináptica incrementada por fosforilación de proteínas existentes (potenciación a largo plazo temprana). Receptores metabotrópicos glutamatérgicos (1) en las propias terminales, junto con la proteína G, activan las fosfolipasas, las cuales degradan lípidos de membrana, producen diacilglicerol y liberan calcio de reservorios intracelulares que activan otras cinasas (proteinocinasa C), las cuales puede actuar localmente y a nivel nuclear. Presuntamente, la región post-sináptica libera un mensajero retrógrado que actúa a nivel presináptico aumentando la liberación de glutamato. b) Sinapsis moduladoras: la activación concurrente de sinapsis moduladoras metabotrópicas (p. ej., dopamina) activa la adenil ciclasa que cataliza la síntesis de adenosín monofosfato cíclico, un potente activador de la proteinocinasa A. c) Eventos nucleares: la proteinocinasa A se transloca al núcleo donde activa a la cinasa de CREB, un factor de transcripción para genes de respuesta inmediata; los productos de estos genes son, a su vez, factores de transcripción de genes estructurales de respuesta tardía. Como resultado, se produce una oleada de síntesis de proteínas que se distribuyen a las sinapsis instructivas marcadas, consolidando la potenciación (potenciación a largo plazo tardía). PKC: proteinocinasa C; GAP-43: Growth Associated Protein; Gl: glutamato; DA: dopamina. 1: receptor glutamatérgico metabotrópico; 2: receptor AMPA; 3: receptor NMDA; 4: receptor dopaminérgico; G: proteína G; PLC: fosfolipasa C; pip_2: fosfoinosítido de membrana; IP3: inositol 3 fosfato; Cal: calmodulina; AC: adenil ciclasa; DAG: diacilglicerol; AMPc: adenosín monofosfato cíclico; PK-II: proteinocinasa II; PKA: proteinocinasa A; CREB-K: cinasa de CREB; CREB: Cyclic AMP Responsive Element Binding protein; Ca/CRE: CAlcium and Cyclic AMP Responsive Element; GRI: genes de respuesta inmediata; gri-RE: elemento de respuesta a los genes de respuesta inmediata; GRT: genes de respuesta tardía.

durante la fase de mantenimiento de la LTP [107-109]. Todas estas evidencias indican que la activación de la PKC también es necesaria para el mantenimiento de la potenciación [110]. Sin embargo, la PKC interviene en una fase algo más tardía que la PK-II [97].

Dos problemas no tienen aún una respuesta satisfactoria en relación con el papel de la PKC en la LTP. La primera tiene que ver con su activación, en particular la activación de fosfolipasas para la producción de DAG. La secuencia lógica NMDA-calcio-PK-II, se ve interrumpida en este punto y hace necesaria la participación de otros factores. Los receptores metabotrópicos glutamatérgicos se relacionan con proteínas G que pueden activar fosfolipasas. Estudios farmacológicos demuestran la participación de receptores metabotrópicos, pero no NMDA, en la activación de la PKC [111]. Teniendo en cuenta la localización periférica de estos receptores en la hendidura sináptica, es cuestionable que niveles fisiológicos de activación produzcan un derrame suficiente de glutamato como para activarlos. Una alternativa –que progresivamente gana importancia– es la necesidad de refuerzo heterosináptico por terminales no glutamatérgicas con función metabotrópica, como la acetilcolina, dopamina, noradrenalina y otros. Más adelante volveremos sobre este punto.

El otro aspecto es la función particular de la PKC dentro del concierto de procesos implicados en la expresión de una transmisión sináptica incrementada. La proteína neurogranina es un sustrato conocido de la PKC, abundante en las dendritas y se sabe que su fosforilación se incrementa en las fases intermedias de la LTP [112,113], así como que la administración de anticuerpos contra ella bloquean la LTP [114]. Sin embargo, no está clara la forma en que éste u otros sustratos de la PKC, como la proteína GAP-43, intervienen en la LTP. La PKC puede ser translocada al núcleo y se piensa puede actuar allí como factor de transcripción [115], lo cual es una alternativa, si se tiene en cuenta la conocida relación de las fases tardías de la LTP con la síntesis de nuevas proteínas.

La proteinocinasa A (PKA), también llamada AMPc dependiente porque requiere de este nucleótido para ser activada, es el miembro más antiguo de la familia de las proteinocinasas.

Las evidencias que vinculan a la PKA con la LTP son más recientes y apuntan a su participación en fases aún más tardías de la cadena de eventos. El reclutamiento de la PKA en la LTP requiere patrones de activación fuertes, capaces de desencadenar una LTP duradera [116]. Ello parece indicar que, al igual que la activación de la PKC, la PKA necesita la participación moduladora de receptores metabotrópicos, glutamatérgicos o no, que activen la adenil ciclasa (AC), aunque ésta puede ser activada sinergísticamente por la PK-II [117,118].

La inhibición de la PKA produce la declinación de la LTP en aproximadamente cuatro horas [119]. Hallazgos similares se han comunicado en mutantes carentes de la subunidad catalítica de la PKA [120-122]. Por otra parte, activadores de la PKA pueden inducir cambios de eficacia sináptica similares a los de la LTP tardía [123].

Si bien la PKA puede influir sobre la eficacia sináptica modulando por fosforilación canales iónicos pre y post-sinápticos [124], su principal contribución a los procesos de plasticidad parece relacionado con su acción como activador de expresión génica y síntesis de proteínas. Esta se lleva a cabo por fosforilación de la proteína CREB (del inglés, *Cyclic AMP Responsive Element Binding protein*), que actúa como promotor de la expresión de proto-oncogenes. La expresión de estos genes se incrementa notablemente después de la inducción de una LTP duradera [125,126], pero no si el estímulo utilizado sólo induce una LTP transitoria. Además, inhibidores de la PKA bloquean tanto la LTP como el aumento en la expresión génica acompañante [127]. La proteína CREB es también esencial para la memoria a largo plazo [128].

Las proteinocinasas aparecen como mediadores importantes de diferentes fases de la LTP que actúan localmente en las sinapsis activadas [129], así como en mecanismos de regulación nuclear.

Síntesis de proteínas. Las primeras comunicaciones que vinculan la LTP con la síntesis de proteínas demostraron que antibióticos,

como la anisomicina, bloqueadores de la síntesis de estas macromoléculas, no afectan la inducción inicial del fenómeno plástico pero limitan su duración a unas 4-6 horas [130-132]. Dendritas separadas del soma desarrollan, pero no mantienen, la LTP [133]. Estos estudios dieron origen al concepto de que la LTP se desarrolla en fases dependientes de mecanismos moleculares diversos, como la memoria [134,135].

Ha resultado muy difícil identificar qué proteínas particulares son sintetizadas tras la inducción de la LTP [136,137], dada la multiplicidad de proteínas que son activadas como resultado de la activación nuclear. En una primera oleada, se activan proto-oncogenes (también conocidos como genes de respuesta inmediata) [138]; entre ellos se han identificado varios como el *zif-268*, la subunidad alfa de la PK-II, *c-fos, c-jun, jun B, jun D y krox-20* [139-142]. Los productos de muchos de estos proto-oncogenes, a su vez, constituyen factores de transcripción para otros genes estructurales. Tal es el caso de las proteínas Fos y Jun que se dimerizan para constituir el factor de transcripción AP-1. Ello conduce a una segunda oleada, más tardía, de síntesis de proteínas que también participan en los procesos de plasticidad. Se ha calculado que existen entre 500 y 1.000 genes relacionados con la plasticidad [143], lo que sin dudas dificulta la identificación de esos agentes. No obstante, trabajos recientes apuntan a un incremento en el número de receptores glutamatérgicos post-sinápticos [144], particularmente de tipo AMPA [145].

Un problema derivado de la relación LTP-síntesis de proteínas es su distribución. La LTP es específica y sólo las sinapsis activadas se potencian. ¿Cómo reconocen las proteínas responsables del cambio plástico aquellas sinapsis que fueron previamente activadas? Frey y Morris han demostrado recientemente que la inducción de la LTP en una sinapsis no sólo aumenta temporalmente su eficacia de transmisión por cambios locales (p. ej., fosforilación de receptores AMPA), sino que establece en ella una marca temporal que sirve de señal de identificación para las proteínas responsables del cambio duradero de conectividad (p. ej., inserción de nuevos receptores AMPA) [146,147]. La naturaleza molecular de la marca sináptica se investiga actualmente.

Cambios morfológicos. Los mecanismos antes citados explican la LTP en términos de modificaciones moleculares que conducen a cambios funcionales. Existen evidencias de que además, especialmente en fases más tardías (>8 horas), pueden aparecer cambios detectables en la morfología de las sinapsis que también podrían estar implicadas en la LTP.

Así, por ejemplo, se ha observado un aumento del número de sinapsis perforadas, con zonas de transmisión divididas que más tarde se convierten en espinas dendríticas dobles [148,149], las cuales, al parecer, representan un proceso de proliferación sináptica local. El incremento de espinas dendríticas cortas y gruesas después de la potenciación [150] podría ser expresión de este fenómeno.

La participación en la LTP de moléculas de adhesión, como la NCAM [27,151,152], que sirven de guía para el crecimiento axonal, o de proteínas presinápticas como la SNAP-25 [153,154] como parte de la intensa síntesis proteica relacionada con la LTP, son compatibles con la idea de que respuestas de diferenciación y proliferación puedan participar en la LTP [155].

Estos hallazgos permiten suponer que la sinaptogénesis podría ser la base de las fases más tardías de la LTP (días, semanas) [156]. La sucesión de mecanismos implicados en el sustento temporal de la LTP demuestra la estrecha imbricación de los mecanismos neuroplásticos. Comienza por cambios en el área funcional y culmina con procesos de crecimiento. En su aparente diversidad y complejidad, este universo fenomenológico es uno, simple y parsimonioso.

Cambios presinápticos. Puede lograrse mayor eficacia sináptica mediante:

1. El aumento de la cantidad de neurotransmisor liberado por la terminal presináptica.
2. El aumento de la afinidad de los receptores post-sinápticos por el neurotransmisor.
3. El aumento de la densidad de los receptores post-sinápticos.

Los mecanismos descritos hasta ahora tienen lugar, y afectan, principalmente los componentes post-sinápticos, lo que no excluye la participación de elementos presinápticos.

Aunque existen evidencias de incrementos en la liberación de glutamato en la LTP [157-159], ésta no ha podido demostrarse en etapas tardías de la LTP. Evidencias indirectas, como el aumento de proteínas relacionadas con la fusión vesicular y la liberación del neurotransmisor, 5 horas después de la inducción de la LTP hablan en favor de esta hipótesis [160]. Relacionado con ello se encuentra el hecho de que la LTP aumenta la confiabilidad de la transmisión sináptica en el hipocampo [161], que es normalmente poco fiable porque algunos potenciales de acción no provocan liberación de neurotransmisor.

El componente presináptico de la LTP requiere, sin embargo, la activación de las neuronas post-sinápticas para producirse. Se ha planteado que la neurona post-sináptica libera algún mensajero que difunde retrógradamente hasta la terminal presináptica y allí provoca los cambios mencionados. Este mensajero hipotético no ha sido identificado y se han propuesto diversos candidatos, entre ellos la adenosina [162], el factor neurotrófico derivado del cerebro (BDNF, del inglés, *Brain Derived Neurotrophic Factor*) [163] el óxido nítrico y el monóxido de carbono [164-166], el factor activador de plaquetas [167] y el ácido araquidónico [168].

Interacciones heterosinápticas. Para inducir una LTP duradera se requieren estímulos intensos y de alta frecuencia que provoquen la activación simultánea de un gran número de aferentes glutamatérgicas. Este ha sido uno de los argumentos más fuertes en contra de la LTP como mecanismo fisiológico de plasticidad sináptica. Ello, unido al hecho antes citado de que algunas cinasas implicadas en la LTP no parecen ser activadas eficazmente por la vía de los receptores NMDA, ha concedido relevancia al posible papel cooperativo de otras sinapsis en el desarrollo y mantenimiento de la LTP.

Existen evidencias de que, en efecto, la LTP puede ser modulada por sinapsis no glutamatérgicas con efectos metabotrópicos [169,170].

La deprivación del hipocampo de aferentes colinérgicas bloquea la LTP en el giro dentado [171,172] y el trasplante de tejido fetal colinérgico a esos animales restaura la LTP [173]. La inducción de la LTP es facilitada durante la actividad theta en el hipocampo por un ritmo colinérgico [174,175]. La administración de agonistas muscarínicos es capaz de inducir una potenciación que se desarrolla lentamente, y mimetiza las fases tardías de la LTP [176,177]. Los receptores muscarínicos, relacionados con la proteína G, promueven la activación de proteinocinasas que actúan sinérgicamente con los mecanismos dependientes de NMDA en los procesos de plasticidad sináptica [178].

La administración de antagonistas dopaminérgicos bloquea las fases tardías de la LTP [179,180]. La administración de agonistas, por el contrario, induce una potenciación retrasada que también

depende de la síntesis de proteínas [181]. Asimismo, en este caso, la dopamina parece actuar a través de cinasas como la PKA [119].

La depleción de sodio afecta la LTP en el giro dentado del hipocampo [182,183] y viceversa, la administración de sodio induce una potenciación lenta, similar a la descrita para la acetilcolina y dopamina, que es bloqueada por inhibidores de la síntesis de proteínas [184]. Estas evidencias sostienen la hipótesis de que la sodio ejerce una acción moduladora sobre los procesos de plasticidad sináptica [185].

Otras aminas biógenas como la serotonina [186,187] o la histamina [188] también muestran efectos moduladores sobre la LTP.

Todas estas aferencias, colinérgicas, dopaminérgicas, adrenérgicas, etc., ejercen sus efectos a través de la PKA [170]. De este modo, la activación de aferentes glutamatérgicas de origen cortical (instructivas) induce procesos de plasticidad en el hipocampo que son facilitados por aferentes de origen subcortical (moduladoras); un posible ejemplo de cómo interactúan, a nivel celular, el intelecto y las emociones.

Interacción amígdala-hipocampo. Una manifestación de lo anteriormente comentado es la interacción entre la amígdala y el hipocampo en la LTP.

La amígdala es una estructura del sistema límbico que constituye el sustrato neurológico de las emociones [189] y de la memoria emotiva [190,191]. También se ha demostrado que modula el almacenamiento de memoria en otras estructuras cerebrales como el hipocampo [192].

En el ámbito funcional, la estimulación de la amígdala favorece la inducción de la LTP en el hipocampo [193,194]. Recientemente, hemos demostrado que también la fase de mantenimiento tardía de la LTP se beneficia de la estimulación de la amígdala. Este efecto fue bloqueado por antagonistas muscarínicos y noradrenérgicos y por lesión de la fimbria-fórnix, lo que sugiere una mediación del septum en esta acción (Frey, Bergado et al [remitido]; Jas, Almaguer et al [en prensa]). De este modo, una LTP débil y de corta duración, inducida por la estimulación de las aferencias glutamatérgicas, puede ser convertida en una LTP fuerte y duradera por coactivación, dentro de una ventana temporal, de la amígdala. Este modelo funcional explica, en el ámbito celular, las relaciones amígdala-hipocampo en la memoria y la forma en que los factores emocionales-motivacionales influyen en ella.

Plasticidad sináptica y memoria. Se ha considerado a la LTP no sólo como una forma de plasticidad sináptica, sino también como un modelo celular de memoria [195]. Realmente, no existe una prueba definitiva y concluyente [196], pero muchas evidencias coinciden en indicar que dicha relación es verdadera.

Para demostrar que la LTP es un mecanismo celular de memoria se han intentado diversas estrategias. Una de ellas, la saturación, se basa en el siguiente razonamiento: si la LTP es la base funcional de la memoria, inducir una LTP saturada en el hipocampo debe impedir la adquisición de nuevos contenidos de memoria. Los resultados han sido contradictorios [197-199]. La debilidad fundamental de este enfoque es que nada asegura que las sinapsis en estudio estén necesariamente implicadas en el modelo de aprendizaje utilizado. Una aproximación correlacional ha sido, hasta ahora, más fructífera; utilizando la estimulación de la vía perforante como estímulo condicionado en una prueba de evitación activa, se encontraron cambios sinápticos similares a la LTP sólo en aquellos animales que aprenden bien la prueba [200].

Otras evidencias señalan que los receptores de tipo NMDA, cuya importancia para la inducción de la LTP ya hemos comentado, también participan en los procesos de adquisición de nuevos contenidos de memoria [201-203]. Asimismo, la PK-II también interviene en la formación de nuevos trazos de memoria, según demuestran los resultados de trabajos con mutantes deficientes de esta enzima [204]. Finalmente, se han encontrado cambios en la morfología de las sinapsis hipocampales, similares a los que ocurren en la LTP después del entrenamiento [205].

Estas coincidencias entre los mecanismos de la memoria y la plasticidad sináptica sustentan la convicción de que existe una relación funcional entre ambos procesos y validan los modelos conectivistas de aprendizaje.

Plasticidad funcional en otras regiones cerebrales. Los mecanismos de plasticidad sináptica funcional que acabamos de describir en el hipocampo no son exclusivos de esta estructura. Fenómenos de tipo LTP o LTD se han documentado experimentalmente en otras regiones del SNC.

Plasticidad cortical. El estudio de potenciales de campo inducidos por estimulación de la sustancia blanca subyacente ha demostrado LTP en diversas regiones corticales [206].

Las neuronas en la corteza muestran dos patrones de organización; un patrón laminar clásico que queda determinado prenatalmente y una organización funcional de tipo columnar que se desarrolla posnatalmente a partir de la estimulación que recibe [207] según la experiencia particular del individuo.

Los estudios en la corteza visual han documentado la importancia de procesos plásticos en el desarrollo de las capacidades funcionales de este sistema [208]. Mecanismos similares operan en otras áreas como la corteza somatosensorial y motora [209,210], auditiva [211,212] y áreas de asociación [213,214]. Las sinapsis talamocorticales e intracorticales que se establecen durante la embriogénesis son funcionalmente inmaduras, tal vez silentes, y su maduración dependiente de la estimulación parece implicar mecanismos similares, si no idénticos, a los descritos en la LTP hipocampal.

La activación de receptores NMDA y metabotrópicos [213,214], la entrada de Ca^{2+} [215], la activación de proteinocinasas [216] y de factores de transcripción de proto-oncogenes [217], seguida de la síntesis de proteínas funcionales [218] y, a más largo plazo, cambios morfológicos en las espinas dendríticas y formación de nuevas sinapsis [210], forman parte de la cadena funcional de la plasticidad cortical. A estas coincidencias se añade que los procesos de plasticidad cortical también son modulados heterosinápticamente por aferencias subcorticales [219].

El desarrollo de remodelaciones neuroplásticas puede modificar la representación cortical de funciones. Por ejemplo, la región ectosilviana de la corteza de asociación parietal es un área de relación polimodal con regiones visuales, auditivas y somatosensoriales. Tras la deprivación visual bilateral, la representación visual ectosilviana es 'tomada' por aferencias de las otras modalidades [220].

Pero también las sinapsis corticales muestran formas de plasticidad sináptica, incluyendo, además de LTP, la LTD e interacciones a corto plazo [209]. Estas pueden intervenir en la recuperación de funciones perdidas por daño o degeneración sin que, necesariamente, se produzcan modificaciones importantes en la cartografía cerebral de esas funciones [221].

En un interesante estudio se comprobó que la reorganización de la corteza auditiva por estímulos acústicos se potenciaba de forma significativa cuando se apareaba con estimulación eléctrica del núcleo basal magnocelular, proveedor de inervación colinérgica a la corteza [222]. Ello indica que los mecanismos de plasticidad cortical,

tal como describimos para la LTP hipocampal, son también modulables por señales metabotrópicas subcorticales; una analogía que confirma la universalidad de los mecanismos de neuroplasticidad.

Las capacidades plásticas corticales disminuyen con la edad, sobre todo en las áreas cerebrales primarias, pero siempre se conserva algún grado de plasticidad, especialmente en las áreas asociativas [223]. Al margen de las implicaciones terapéuticas de este hecho, esta diferencia podría contribuir a resolver la vieja disputa entre localizacionistas y antilocalizacionistas [224].

El cierre aparente de las capacidades neuroplásticas corticales ha dado origen al concepto de los períodos críticos del desarrollo después de los cuales se pierde la posibilidad de modificación funcional. Los períodos críticos no son absolutos y pueden reabrirse cuando se activan mecanismos neuroplásticos. Aunque no se conocen con exactitud los agentes moduladores, existen evidencias de que los receptores glutamatérgicos post-sinápticos también son importantes en esta función [225].

El estudio de los determinantes celulares y moleculares de los períodos críticos es un objetivo importante de la Neurología Restaurativa.

Plasticidad en otras estructuras. También se ha descrito LTP en la amígdala [226]. La estimulación eléctrica de núcleos talámicos produce potenciales evocados en la región lateral de la amígdala [227]; esta aferencia desarrolla una LTP [228] relacionada con el desarrollo de reacciones de miedo condicionado [229] y podría constituir la base de una memoria emocional. La proyección del hipocampo a la amígdala es potenciable por mecanismos dependientes de NMDA [230].

En el cerebelo se han descrito fenómenos plásticos de tipo LTD relacionados con el aprendizaje motor [231] dependientes de receptores metabotrópicos [232] y que pueden conducir a cambios morfológicos en las dendritas de las células estrelladas [233].

La LTP constituye la forma normal de plasticidad dependiente del uso en el estriado [234], en el accumbens (donde depende de receptores NMDA) [235,236] y en la médula espinal [237,238].

Sinapsis silentes

La existencia de sinapsis no funcionales, llamadas sinapsis silentes, se ha demostrado en especies tan lejanas como peces y mamíferos [239,240]. Las sinapsis silentes representan una reserva funcional que puede ser importante para la expresión de fenómenos neuroplásticos [239].

Estudios recientes han demostrado que, en el hipocampo, estas sinapsis sólo expresan receptores NMDA, circunstancia que las hace no funcionales a los valores normales de potencial de membrana en reposo [240]. Su conversión en sinapsis activas implica la aparición de receptores AMPA funcionales, bien por síntesis *de novo* o por alguna modificación molecular que los capacita [240,241]. La activación repetitiva, tal como ocurre en la inducción de la LTP, desencadena este proceso. De modo que la LTP puede ser considerada como un mecanismo de maduración sináptica [241,242], tanto en el adulto como durante la ontogénesis [243]. Takumi et al [244] han calculado que en la región CA1 del hipocampo adulto aproximadamente el 25% son sinapsis silentes y que la coexpresión de receptores NMDA y AMPA se relaciona con un aumento del tamaño de la densidad post-sináptica, correspondiendo a un cambio funcional.

La existencia de sinapsis silentes y su maduración por incorporación de receptores AMPA, mediada por activación repetitiva de NMDA, también ha sido demostrada en la corteza cerebral [245].

El mecanismo de activación de sinapsis silentes muestra similitudes llamativas con la LTP. Ambas comienzan con la activación de receptores NMDA y terminan (¿terminan?) con la incorporación de receptores AMPA a la membrana [156]. En qué medida la activación de sinapsis silentes podría ser parte del fenómeno de potenciación sináptica es una pregunta razonable que deberá resolverse en el futuro. Lo que queremos resaltar es el hecho de que parece existir un *continuum* de modificaciones, desde sutiles cambios de eficacia sináptica hasta la formación de nuevas sinapsis, pasando por la activación de contactos silentes, sustentados por mecanismos moleculares comunes. Esta simplicidad dentro de la aparente complejidad es uno de los fundamentos teóricos de la esperanza de lograr, en breve plazo, una comprensión de los fenómenos neuroplásticos que permita dirigirlos con éxito en el marco de nuevos esquemas terapéuticos y neurorrestaurativos.

Universalidad de los mecanismos de plasticidad

Los mecanismos de neuroplasticidad en adultos son, en esencia, idénticos a los que ocurren durante el desarrollo embrionario del SNC y operan en especies tan lejanas como moluscos y ratas. La conservación ontogenética y filogenética de los mecanismos de neuroplasticidad es una prueba de su valor adaptativo.

Estudios recientes sobre las moléculas de reconocimiento neural han revelado funciones similares para estas moléculas durante el desarrollo embrionario y la plasticidad en el adulto [246]. La diferencia fundamental estriba en que durante la primera se establece la circuitería nerviosa, vías y blancos, que es común a todos los miembros de una especie; mientras la segunda garantiza el desarrollo fino de patrones funcionales altamente precisos [247], individualizados según la experiencia particular de cada sujeto.

Desde el punto de vista filogenético se descubren cada día nuevas coincidencias.

En *Aplysia californica*, un molusco, existe LTP [248] que es dependiente de la síntesis de proteínas [249] y está relacionada con la adquisición de reacciones condicionadas simples [250], que son las formas de aprendizaje presentes en estos animales.

La mosca *Drosophila melanogaster* es capaz de aprender y desarrollar procesos neuroplásticos que son mediados por PK-II [251] y AMPc [252].

Finalmente, la universalidad de los mecanismos de plasticidad también se expresa en la identidad de los mismos en regiones diferentes del SNC, como el hipocampo y la corteza [253]. La naturaleza es parsimoniosa y no desdeña mecanismos eficaces por antiguos que sean.

Envejecimiento

Envejecer no es una enfermedad, sin embargo, en el proceso de envejecimiento normal se descubren cambios en las capacidades neuroplásticas que pueden estar relacionadas con las afectaciones de memoria que caracterizan al anciano.

Las ratas viejas aprenden lentamente y olvidan de manera rápida, posiblemente debido al deterioro de sus capacidades neuroplásticas [254]. De forma coincidente, en estos animales la LTP se desarrolla lentamente [255] y decae con rapidez [256,257]. También la LTD se encuentra afectada [258]. El deterioro de estas capacidades obedece, al menos en parte, a déficit en los mecanismos moleculares que los sustentan [259].

También la plasticidad por crecimiento se afecta por el envejecimiento. La colateralización de fibras comisurales y de asociación en el giro dentado, posterior a una lesión de corteza entorrinal está disminuida en ratas viejas [260], así como la colateralización

Tabla II. Factores neurotróficos. La muestra no pretende ser exhaustiva, pues tan sólo recoge una selección de algunos factores proteicos con acción neurotrófica probada. Los grupos o familias de factores tróficos se definen según el grado de homología estructural de sus miembros. Nótese que en todos los casos el mecanismo de acción implica receptores de membrana específicos para cada factor y fosforilación de tirosina (mediada por el propio receptor o por moléculas relacionadas) y la activación de cascadas celulares del tipo MAP-cinasa (una serina-treonina cinasa) y otras con actividad de regulación nuclear. (Se han mantenido las siglas en inglés, impuestas por el uso).

Grupo	Siglas	Nombre	Receptores y cascadas
Neurotrofinas	NGF	Factor de crecimiento nervioso	Tirosinocinasas unidas a membrana (Trk), MAP-cinasa y otros reguladores nucleares
	BDNF	Factor neurotrófico derivado del cerebro	
	NT-3	Neurotrofina 3	
	NT-4/5	Neurotrofina 4/5	
	NT-6	Neurotrofina 6	
Factores hematopoyéticos	CNTF	Factor neurotrófico ciliar	Receptores de membrana sin actividad enzimática intrínseca (CNTF-Rα) que atraen a tirosinocinasas solubles, MAP-cinasas y otros reguladores nucleares
	GDNF	Factor neurotrófico derivado de la glía	
Factores de crecimiento	EGF	Factor de crecimiento epidérmico	Tirosinocinasas unidas a membrana (EGFR), MAP-cinasa y otros reguladores nucleares
	FGF	Factor de crecimiento de fibroblastos	
	TGF	Factor de crecimiento transformante	
	TNF	Factor de necrosis tumoral	
	PDGF	Factor de crecimiento derivado de plaquetas	
	IGF	Factor de crecimiento similar a la insulina	

de fibras simpáticas en la misma región, consecutiva a lesión de la fimbria-fornix [261], aunque las fibras colinérgicas septales parecen conservar cierta capacidad de respuesta [38].

La reducción no es absoluta. En ratas viejas no lesionadas se observa una expansión discreta del tercio interno del estrato molecular por colateralización [262].

Enfermedades neurodegenerativas

Los déficit de memoria son más graves en desviaciones patológicas del proceso de envejecimiento, como la demencia de Alzheimer. Las causas de la enfermedad no son conocidas, como tampoco sus consecuencias funcionales, pero entre las hipótesis emitidas algunas atribuyen el deterioro cognitivo a una pérdida de plasticidad [263]. Ratones mutantes para la ApoE muestran una plasticidad sináptica alterada en el hipocampo [264] al igual que animales deficientes en proteína precursora del amiloide [265,266]. Se ha sugerido que la presencia de neuritas anormales en las placas seniles son el resultado de intentos regenerativos fallidos [267].

Otras entidades neurodegenerativas como la enfermedad de Parkinson y la corea de Huntington también se vinculan a formas alteradas de plasticidad [268,269]. Ratones portadores de la forma mutada del gen para la huntingtina muestran una reducción significativa de la LTP [270].

Epilepsia

La neuroplasticidad puede relacionarse con procesos patológicos no solo por defecto. La epilepsia es el ejemplo mejor caracterizado de cómo procesos de plasticidad excesivos y aberrantes pueden afectar la función normal del SNC.

Las crisis epilépticas provocan muerte neuronal por apoptosis y necrosis que es seguida, en las neuronas que sobreviven, por el desencadenamiento de fenómenos plásticos [271].

Tras crisis epileptógenas en el giro dentado, se produce una extensión y colateralización axonal de las neuronas principales [272] que se expresa por aumentos en la expresión de GAP-43 [18,273] y otros marcadores de crecimiento axonal [274] y dendrítico [275]. Los axones en crecimiento son guiados por moléculas de adhesión de tipo NCAM [276,277].

El crecimiento axonal se dirige fundamentalmente, en forma recurrente, al tercio interno del estrato molecular [278] donde se establecen sinapsis excitatorias, incluso autapsis, que son responsables del estado de hiperexcitabilidad [279-282]. Como ocurre en otros casos de sinaptogénesis reactiva, también se producen cambios en las dendritas y espinas dendríticas que reciben las colaterales recurrentes [283,284].

Esta misma secuencia de acontecimientos se manifiesta en la región CA1 con idénticos resultados [282]. Fenómenos de este tipo ocurren desde etapas tempranas del desarrollo posnatal [285] y existen buenas razones para creer que están realmente implicadas en la epileptogénesis en humanos [286-289].

FACTORES EPIGENÉTICOS MODULADORES DE LA PLASTICIDAD

Los mecanismos de neuroplasticidad pueden contribuir, de modo notable, a la recuperación de funciones nerviosas. La pregunta que se deriva es: ¿cómo podemos estimular, modular y controlar los procesos neuroplásticos para lograr una mejoría más completa? Existe una variada gama de agentes que pueden modificar, de alguna manera, los procesos de neuroplasticidad; aprender a uti-

lizarlos adecuadamente es una de las tareas más importantes de la Neurología Restaurativa.

Factores neurotróficos

Desde el descubrimiento del factor de crecimiento nervioso (NGF, del inglés *Nerve Growth Factor*) la relación de moléculas proteicas de origen natural, capaces de estimular y promover la supervivencia y desarrollo de las células nerviosas ha crecido continuamente. Hoy, además de las neurotrofinas propiamente dichas, se conocen una variada gama de moléculas con capacidades neurotróficas (Tabla II). Estos factores neurotróficos se agrupan en familias según el grado de homología molecular de sus miembros y el tipo de receptor que utilizan para lograr sus efectos tróficos, y muestran un alto grado de conservación filogenética [290], una evidencia evolutiva de su importancia.

La hipótesis neurotrófica atribuye a estos factores una acción principal en la supervivencia de las neuronas. De acuerdo con esta concepción, los factores neurotróficos producidos por el blanco son captados por las terminales presinápticas y transportados retrógradamente por las neuronas. El suministro continuo de un factor neurotrófico específico resulta imprescindible para mantener la vida y expresión fenotípica de las neuronas [291,292]. Esto parece particularmente importante durante el desarrollo, período en el cual un gran número de neuronas muere por apoptosis, aparentemente porque no pudieron proveerse de una cantidad suficiente de factor trófico [293].

Los factores tróficos ejercen sus efectos a través de receptores de membrana que conectan con diferentes cascadas moleculares intracelulares, como la MAP-cinasa, la PKC y la fosfatidil inositol 3-cinasa (PI3-K), capaces de modificar la expresión génica y la síntesis de proteínas (Fig. 4) [294-296]. Ello, a su vez, les capacita para inducir y modular los procesos de neuroplasticidad por crecimiento o funcional. Otros factores con acción neurotrófica, como los factores de crecimiento (EGF, FGF, PDGF e IGF), a través de receptores diferentes, activan las mismas cascadas y pueden ejercer, por lo tanto, acciones similares a las de las neurotrofinas [296].

La producción de factores tróficos en el hipocampo puede ser inducida por crisis epilépticas provocadas [297] o por estímulos inductores de LTP [298-300]. Por otra parte, la administración de algunos factores tróficos, en particular el BDNF, es capaz de inducir potenciación sináptica de tipo LTP [301], lo cual se ha visto reforzado por estudios posteriores con mutantes BDNF(-) [302-304] y otros paradigmas experimentales [305,306]. Los mecanismos de esta LTP inducida por neurotrofinas dependen de la fosforilación de receptores NMDA [307], la activación de proteinocinasas [308,309] y la síntesis de proteínas [156,310], sin olvidar el papel de hipotético mensajero retrógrado en la inducción de cambios presinápticos. En este sitio también el mecanismo involucra fosforilación mediada por MAP-cinasa [309].

Las neurotrofinas también pueden sustentar procesos de plasticidad sináptica indirectamente y reforzar la influencia de aferentes no glutamatérgicas moduladoras de la LTP [255,311].

Los factores tróficos tienen un papel importante en los procesos de plasticidad cortical que conducen a la maduración funcional –dependiente de la experiencia– de las conexiones talamocorticales; ello ha sido bien estudiado en el sistema visual [312-314] y en el somatosensorial [315]. Por esa razón, se les confiere importancia especial en la determinación de los períodos críticos del desarrollo [316].

El BDNF y el NGF potencian la transmisión excitadora en la corteza visual [317] y la LTP de estas conexiones sinápticas [318]. Se ha propuesto que la actividad visual incrementa la síntesis de BDNF por activación de promotores específicos y esto, a su vez, conduce a elevar la eficacia de la transmisión por mecanismos dependientes de NMDA [319]. Sin embargo, aunque los efectos de las neurotrofinas exógenas son dramáticos, su acción en condiciones fisiológicas es aún dudosa [320].

Evidencias experimentales sugieren una acción neuroprotectora de las neurotrofinas ante distintos insultos que comprometen la integridad y supervivencia de las neuronas, mediante la activación de sistemas enzimáticos implicados en la defensa celular [321-325].

Por todas estas razones, los factores neurotróficos han sido, durante más de una década, la gran esperanza para encontrar un tratamiento efectivo que combatiera las devastadoras consecuencias de las enfermedades neurodegenerativas. Su uso, avalado por experiencias en modelos animales, se ha propuesto para el tratamiento de la demencia de Alzheimer [255,300,311,326-328], la enfermedad de Parkinson [329-331], la esclerosis lateral amiotrófica [332,333] y la corea de Huntington [334], entre otras.

Pero dichas esperanzas no han sido satisfechas por muchas razones. La diversidad de factores con acción neurotrófica específica dificulta encontrar el más adecuado para una población neuronal dada. En la actualidad, se conocen más 20 factores tróficos capaces de sustentar a las neuronas dopaminérgicas de la sustancia *nigra* [329]. ¿Cuál de ellos, o combinación de ellos, es más eficaz? El panorama se complica aún más si consideramos que la sensibilidad a los factores tróficos varía en diferentes etapas del desarrollo, fenómeno conocido como conmutación neurotrófica *(neurotrophin switching)* [335]. En aquellos casos en que la sensibilidad a un factor trófico está demostrada, faltan estudios sistemáticos de dosis-respuesta, lo cual es importante para evitar o minimizar efectos secundarios y porque el propio efecto del factor trófico puede variar, incluso invertirse, dependiendo de la dosis. Por ejemplo, el exceso de BDNF tiene efectos proconvulsivos en regiones límbicas [336] y altas concentraciones de NGF detienen el crecimiento neurítico en neuronas periféricas [337]. Finalmente, comienza a comprenderse que el tipo de efecto trófico de un agente puede modificarse dependiendo de factores ambientales. La inhibición de la fosfolipasa-γ convierte al NGF, un antimitógeno clásico, en un factor mitogénico [338]; otro ejemplo, el BDNF y la NT-4/5 protegen a las células de Purkinje aisladas en cultivo, pero el efecto se invierte si se añaden células granulares [156].

Pero el pecado original de los factores tróficos sigue siendo su naturaleza proteica y, por lo tanto, su incapacidad para atravesar la barrera hematoencefálica. La implantación de células manipuladas genéticamente para producir una neurotrofina [332,334,339-343], o el uso de moléculas modificadas para aumentar su permeabilidad en la barrera hematoencefálica [344], han sido las alternativas más favorecidas.

En menor medida se ha intentado el uso de agentes periféricos que estimulen la producción intracerebral de factores tróficos, aunque algunas evidencias sugieren que agentes químicos [345-348] o fisiológicos (la actividad física) [349] y el estrés [350], pueden tener efectos moduladores de la producción endógena de factores tróficos.

En consecuencia, los ensayos clínicos han sido escasos y sus resultados poco prometedores [351]. Muchas preguntas y factores técnicos deben resolverse antes de que la promesa del uso farmacológico de los factores tróficos se convierta en realidad.

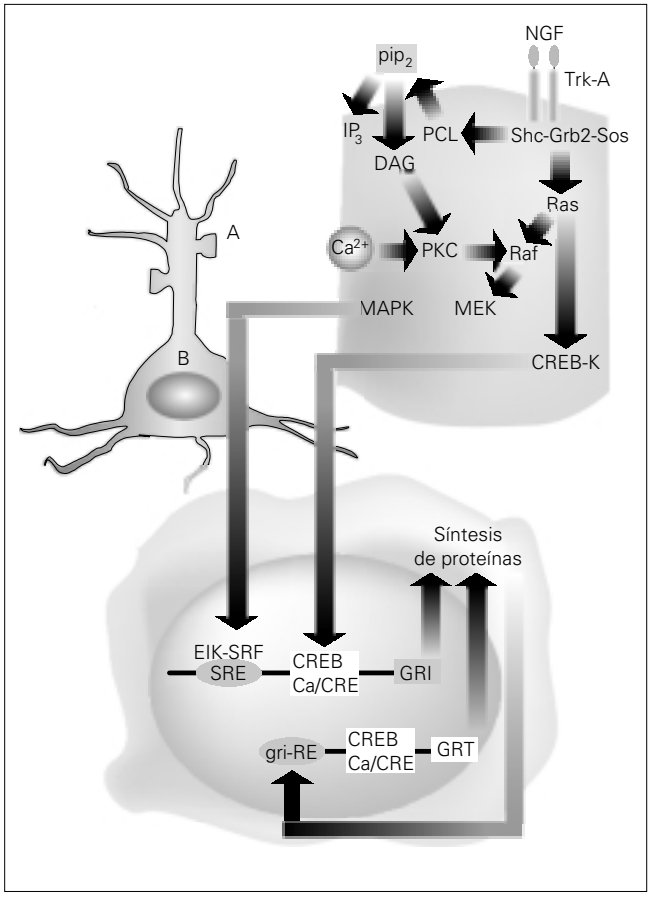

Figura 4. Mecanismo de acción de las neurotrofinas. a) La unión del factor de crecimiento nervioso al receptor tirosinocinasa A provoca su dimerización y autofosforilación en residuos de tirosina. Ello promueve la formación de un complejo tetramérico compuesto además por la proteína Shc fosforilada, el Grb2 y SoS que, de esta forma, son capaces de activar a Ras, una proteína G pequeña, por intercambio de GDP por GTP. En su forma activa, Ras atrae a Raf, una serina-treonina proteinocinasa, hacia la membrana, lo que provoca su activación. Otras serina-treonina cinasas son activadas secuencialmente por fosforilación (MEK, MAPK). Otras cascadas enzimáticas cooperan o complementan la anterior. La activación de la fosfolipasa C conduce a la de proteinocinasa C, que tiene a Raf entre sus sustratos. Por otra parte, Ras puede conducir, indirectamente, a la activación de CREB, un factor de transcripción cooperativo tanto para los genes de respuesta inmediata como para los genes de respuesta tardía. b) Eventos nucleares: la cinasa de la proteína relacionada con microtúbulos y la CREB-K se translocan al núcleo donde activan factores de transcripción de genes de activación inmediata. Los productos de estos genes (gri) son, a su vez, potentes factores de transcripción para genes estructurales de respuesta tardía. Las proteínas sintetizadas son responsables finales de los procesos de crecimiento y diferenciación inducidos por las neurotrofinas. NGF: factor de crecimiento nervioso; Trk-A: tirosinocinasa A, receptor de NGF; Shc: Src homology and collagen; Grb2: Growth factor receptor-bound protein 2; SoS: Son of Sevenless; Ras: proteína G pequeña relacionada con la membrana; Raf: serina-treonina proteinocinasa; MEK: cinasa de MAPK; MAPK: cinasa de proteína relacionada con microtúbulos (MAP, Microtubule Associated Protein); PLC: fosfolipasa C; pip_2: fosfolípidos de membrana; IP3: inositol-3-fosfato; DAG: diacilglicerol; SRF: Serum Response Factor; Elk: factor de transcripción; SRE: Serum Response Element; CREB: cAMP Regulatory Element-Binding protein; Ca/CRE: CAlcium and CREB Response Element; GRI: genes de respuesta inmediata; gri-RE: elemento de respuesta a los genes de respuesta inmediata; GRT: genes de respuesta tardía.

Soporte metabólico

Todos los procesos de neuroplasticidad descritos, tanto los que implican crecimiento como aquellos que tienen un carácter más funcional, implican procesos de remodelación que demandan síntesis de nuevas macromoléculas: proteínas, glicoproteínas y glicolípidos. Teóricamente, es posible que la disponibilidad limitada de algunos precursores biosintéticos reduzca las potencialidades neuroplásticas. Revisaremos algunos de estos precursores y las evidencias que les confieren atractivo como potenciadores de neuroplasticidad.

Ácido orótico

El ácido orótico es una sustancia natural, precursora de nucleótidos de pirimidina (UMP y CMP). Las concentraciones de pirimidinonucleótidos en el cerebro de los animales adultos son muy bajas [352], lo cual podría limitar la síntesis de macromoléculas (ARN, proteínas, glicoproteínas y glicolípidos) necesarias para la expresión de procesos neuroplásticos.

Estudios *in vitro* han demostrado que una mayor provisión de nucleótidos de pirimidina estimula el desarrollo de neuroblastos [353,354]. De la misma forma, el ácido orótico acelera el crecimiento neurítico [355,356], la migración y diferenciación neuronal [357] y el desarrollo mitocondrial de las neuronas en cultivo [358].

La administración de ácido orótico favorece los procesos neuroplásticos que subyacen en el aprendizaje [359], hecho que favorece particularmente a los animales viejos [360] o pobremente capacitados [361]. Estudios complementarios demostraron que el aprendizaje realmente acelera la síntesis macromolecular y que el ácido orótico favorece estos procesos [362].

Por otra parte, el ácido orótico también beneficia la expresión de procesos de plasticidad sináptica como la LTP [361,363-365].

Dos estudios recientes sugieren que el ácido orótico podría también tener un papel neuroprotector [366,367] en modelos de lesión isquémica del SNC, circunstancia que añade interés al estudio de la posible función de esta sustancia en los procesos de neuroplasticidad, sobre todo si consideramos que esta sustancia posee efectos cardiovasculares que mejoran la tolerancia al ejercicio [368,369] y reducen el daño vascular aterosclerótico [370].

Gangliósidos

Los gangliósidos son glucoesfingolípidos anfifílicos que contienen ácido siálico y se encuentran en grandes concentraciones en las membranas sinápticas [371].

In vitro los gangliósidos muestran efectos neuronotróficos, neuritogénicos [261] y neuroprotectores [372], por lo que se consideró que podrían ser beneficiosos en los procesos de reparación nerviosa central y periférica [373,374].

La administración de algunos gangliósidos mejora la memoria [375] y la LTP en animales de experimentación [376], aunque este hecho ha sido cuestionado por resultados posteriores [377]. Su administración en modelos animales mostró efectos neuroprotectores sobre neuronas dopaminérgicas nigrales, colinérgicas septales, así como serotoninérgicas y noradrenérgicas del tallo cerebral [378], tal vez las poblaciones moduladoras más importantes en la Neuropatología actual.

Los gangliósidos pueden administrarse por vía sistémica, pero no oral, pues son destruidos en el tracto digestivo. Se ha medido que sólo el 1-3% de la dosis administrada alcanza el cerebro, aunque en condiciones de trauma, la ruptura de barrera puede aumentar esta proporción [378].

Los estudios clínicos realizados no han confirmado estas expectativas [379]. A pesar de ello, los gangliósidos siguen siendo un grupo interesante por sus propiedades, aunque se requieren estudios moleculares y metabólicos [380] más profundos para una mejor evaluación de su uso como moduladores de neuroplasticidad.

Esteroides

Las hormonas esteroideas incluyen hormonas sexuales, glucocorticoides y mineralocorticoides. El mecanismo clásico de acción de estas hormonas se basa en su interacción con un receptor proteico intranuclear que desenmascara su región de unión al ADN y promueve la transcripción y síntesis de proteínas. Las hormonas esteroideas, por sus propiedades físico-químicas, atraviesan con facilidad la barrera hematoencefálica y la membrana plasmática. Sin embargo, en el cerebro, añaden a su mecanismo clásico de acción genómica efectos extragenómicos que parecen depender de receptores de membrana [381]. Existen receptores nucleares clásicos para los estrógenos en muchas zonas del sistema límbico como el hipocampo y el hipotálamo [382], aunque para su acción neuroplástica los estrógenos requieren la cooperación de receptores de membrana, entre los que se han invocado el receptor IGF-I [383] y los receptores NMDA [381,384].

Los estrógenos tienen efectos neuritogénicos que en algunas células se restringe a los axones [385]; no obstante, su acción más prominente en el hipocampo parece ser el aumento en la densidad de espinas dendríticas [386] por mecanismos dependientes de NMDA [384], que estimulan la formación de nuevas sinapsis y actúan sobre botones terminales preexistentes [387].

Los estrógenos favorecen o estimulan los mecanismos de neuroplasticidad sináptica [388] y por crecimiento [381], probablemente a través de la cascada AMPc-PKA-CREB [389].

La alta sensibilidad a los estrógenos de los mecanismos de neuroplasticidad se evidencia en la demostración de que las capacidades neuroplásticas de ratas hembras varía en diferentes etapas del ciclo estral; así, es alta cuando son elevados los niveles de estrógenos y decae rápidamente cuando éstos disminuyen [390,391].

Los glucocorticoides, las principales hormonas del estrés, han mostrado efectos contrarios y en parte antagónicos [392]. En el modelo de lesión de corteza entorrinal, la administración de corticosterona reduce la reinervación por colateralización y sinaptogénesis reactiva [393]. Ello sucede a pesar de que los receptores para glucocorticoides son regulados negativamente por la deaferentación [394].

Los efectos antineuroplásticos de los glucocorticoides parecen acentuarse con la edad [4,395].

Recientemente se ha demostrado que puede ocurrir síntesis de esteroides en el propio tejido cerebral. Sustancias como la pregnanolona y la alopregnanolona, denominadas genéricamente neuroesteroides, actúan principalmente a través de receptores de membrana. Actualmente se evalúan sus posibles acciones neuroplásticas [296].

Factores ambientales y estilo de vida

Desde hacía tiempo se sospechaba que la deprivación de estimulación y actividad retarda y compromete el desarrollo del sistema nervioso [396], pero sólo en las décadas recientes se han comenzado a entender los mecanismos y a conocer cómo estos mismos factores pueden operar en la recuperación de funciones del cerebro adulto dañado.

Las primeras evidencias experimentales de plasticidad dependiente del uso o la actividad provienen de los trabajos clásicos de Rosenzweig y Bennet en los años 60. Ratas criadas en ambientes enriquecidos (cajas grandes con abundantes laberintos, escaleras y objetos) mostraron tener una corteza cerebral más gruesa, más contactos sinápticos, mayor número de dendritas y espinas dendríticas [397]. Este experimento fue el inicial impulso trascendente de un gran número de trabajos experimentales para comprender qué, cuánto y cómo la actividad, la estimulación y la experiencia contribuyen a la formación funcional del sistema nervioso.

Estudios ulteriores demostraron que la vida en ambientes enriquecidos incrementa la talla de las neuronas, la ramificación de sus dendritas y la densidad de las espinas dendríticas, el número de sinapsis por neurona y el tamaño de los contactos sinápticos, así como la vascularización tisular y la talla de astrocitos, oligodendrocitos y las mitocondrias [398].

La permanencia por tiempos más o menos prolongados en ambientes enriquecidos mejora las capacidades de memoria y la actividad de la PKC [399], protege contra las consecuencias de lesiones vasculares provocadas y mejora la expresión de otras proteínas de plasticidad como el BDNF y la PK-II [400]. También se ha comprobado que este procedimiento incrementa el número de espinas dendríticas múltiples en el núcleo caudado [156] y reduce algunas consecuencias negativas del envejecimiento, como la reducción del número de sinapsis en diversas áreas cerebrales [401] y la neurotoxicidad por glucocorticoides [402]. La función glial, expresada en una mayor arborización astrocítica, también se beneficia [398]. La estancia en ambientes enriquecidos también induce cambios neuroplásticos en el cerebro de ratas viejas [398]. Recientemente se ha demostrado que vivir en ambientes enriquecidos estimula la neurogénesis en ratas viejas [57].

Los mecanismos de neuroplasticidad, que conducen a la recuperación por lesión del SNC, también se benefician de ambientes enriquecidos. Tanto en animales jóvenes como adultos, los mecanismos de soporte trófico de tipo glial o por medio de factores neurotróficos (FGF, NGF) muestran mejorías por exposición a ambientes enriquecidos, hecho que se acompaña de un incremento en los procesos de colateralización y sinaptogénesis reactiva en áreas vecinas a la lesión [398].

Es interesante destacar que cambios similares a los que provoca el ambiente enriquecido se han demostrado en animales sometidos a entrenamiento cognitivo o en la recuperación después de daño cerebral [156]; una evidencia de la universalidad de los mecanismos de neuroplasticidad.

Para una rata de laboratorio, vivir en un ambiente enriquecido significa, sobre todo, mayor estimulación sensorial, motora y cognitiva. Todos los experimentos que utilizan el paradigma de ambiente enriquecido vienen a significar que la estimulación neural de cualquier tipo, en cualquier etapa de la vida, estimula mecanismos de plasticidad importantes para la maduración morfofuncional del sistema y su reparación en caso de daño.

En efecto, los mecanismos de plasticidad son modulables por estimulación sensorial. La LTP en hipocampo y corteza piriforme es modulada por estímulos olfatorios [403]. Otras formas de plasticidad funcional pueden modificarse transitoriamente en la corteza somatosensorial por asociación de estímulos táctiles [404]. La LTP está exacerbada y la LTD disminuida en la corteza visual de ratas privadas de luz, situación que puede revertirse después de sólo dos días de exposición a la luz [405]; esta circunstancia también modifica la expresión de proteínas de plasticidad como AP-1 y zif-268 [406]. Cambios equivalentes se describen en áreas motoras [407] y ya algunos de estos conocimientos comienzan a comprobarse en la clínica [408]. El uso determina el número, morfología y tipo de sinapsis, aun con períodos relativamente cortos de activación [409].

La participación exacta de las neurotrofinas está por establecerse, pero existen evidencias de que su producción, en diversas

estructuras nerviosas centrales y periféricas, es modificada por estímulos fisiológicos [410].

Nuevamente se demuestra la participación de mecanismos similares a los anteriormente descritos en la plasticidad inducida por la experiencia, incluida la mediación de receptores NMDA [411], que confirman la universalidad de los mecanismos de neuroplasticidad.

La utilización en la práctica clínica de modelos de estimulación requiere, sin embargo, un mayor conocimiento de los mejores patrones de estimulación, ya que comienza a comprenderse que ligeros cambios en el modo de estimulación [412] y el modo de vida [413] pueden tener efectos muy diferentes en los procesos de plasticidad.

Actividad y ejercicio físico

El ejercicio físico es fuente de desarrollo no sólo del cuerpo, sino que también el cerebro y la mente pueden beneficiarse de la actividad física mediante la inducción de cambios plásticos. Después de lo comentado en el epígrafe anterior este acerto puede parecer obvio. Cierto es que la realización de cualquier tarea motora genera patrones de estimulación sensorial propioceptiva y puede ser fuente de modulación neuroplástica en áreas motoras y somatosensoriales. En este caso, nos referimos a efectos más generales e inespecíficos de la actividad física, provocados no sólo por patrones de estimulación sensorial, sino por la interacción de cambios físicos, hormonales y otros que mejoran o potencian los procesos involucrados en las remodelaciones neuroplásticas.

La actividad física, por ejemplo, mejoró la memoria y paralelamente la actividad de PKC en el hipocampo de ratones entrenados en una estera rodante durante ocho semanas [414]. También la expresión de neurotrofinas, en particular el BDNF y su receptor, aumentan en el hipocampo por el ejercicio [415,416]; la producción de nuevas células nerviosas y la LTP en el giro dentado se benefician por el ejercicio [417], y el entrenamiento repetido ayuda a reparar los daños por lesiones provocadas en ratas [3].

Es posible que la estimulación específica de áreas cerebrales y el ejercicio físico difieran en algunos aspectos con relación a los cambios neuroplásticos que uno y otro producen. Hasta hace sólo unos años, se consideraba que la actividad física modificaba sobre todo la vascularización cerebral y no la densidad sináptica [407,418,419]. Los experimentos citados en el párrafo anterior están ayudando a cambiar esa opinión, pero la combinación de actividad física y estimulación sensorial y motora específica sigue pareciendo la más eficaz para la inducción de procesos de remodelación neuroplástica del cerebro dañado.

CONCLUSIONES

Los mecanismos de la neuroplasticidad son universales. En la escala filogenética de las especies animales, mecanismos basados en patrones de activación y eventos moleculares similares o idénticos participan tanto en la construcción del sistema nervioso durante el desarrollo embrionario, como en su reconstrucción durante la vida posnatal.

Esta reconstrucción puede darse por medio de sutiles modificaciones funcionales, por ejemplo en el aprendizaje, o mediante procesos de crecimiento axonal, dendrítico y la formación de nuevas sinapsis en respuesta al daño.

En esencia, plasticidad por crecimiento y plasticidad funcional no son tampoco procesos separados. Como se ilustra en el caso de la LTP, estos procesos operan formando un *continuum* que va desde modificaciones moleculares en sinapsis existentes hasta la formación de nuevas sinapsis, según el tipo de estímulo aplicado.

La neuroplasticidad comienza a ganar un lugar en los esquemas terapéuticos más modernos de la Neurología Restaurativa [420]. Conocer mejor sus mecanismos y modulación por agentes físicos y químicos será una contribución de enorme utilidad para hacer de ellos un uso más eficiente y eficaz.

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MECANISMOS CELULARES DE LA NEUROPLASTICIDAD

Resumen. Objetivo. *Presentar, de manera unificada, una visión de los principales mecanismos de neuroplasticidad conocidos, destacando su universalidad.* Desarrollo. *La concepción del sistema nervioso como una entidad inmutable ha sufrido modificaciones sustanciales durante la segunda mitad del siglo XX. La neuroplasticidad, es decir, la capacidad de cambio y reparación del cerebro, se expresa de formas diversas, desde modificaciones funcionales de estructuras ya existentes, hasta la formación por crecimiento y proliferación de nuevas estructuras y neuronas. El presente trabajo aborda los mecanismos celulares y moleculares de los fenómenos neuroplásticos y los clasifica en dos grandes grupos: plasticidad por crecimiento, donde se incluyen los mecanismos de regeneración axonal, colateralización y sinaptogénesis reactiva; y plasticidad funcional, que abarca cambios en la eficacia de la transmisión sináptica como la potenciación a largo plazo y la activación de sinapsis silentes. Se presentan además algunas relaciones de fenómenos neuroplásticos con enfermedades del sistema nervioso, así como ejemplos de factores fisiológicos, físicos y farmacológicos que pueden, en el futuro, convertirse en herramientas terapéuticas para estimular y modular la neuroplasticidad.* Conclusiones. *Los mecanismos neuroplásticos muestran un alto grado de conservación filogenética y ontogenética, y son importantes tanto en la génesis de trastornos y enfermedades del sistema nervioso, como en su reparación tras sufrir traumatismos y daños muy diversos. La modulación de los mecanismos neuroplásticos por agentes físicos y químicos se vislumbra como una de las más potentes herramientas terapéuticas de la neurología restaurativa. [REV NEUROL 2000; 31: 1074-95]* ***[http://www.revneurol.com/3111/j111074.pdf]***

Palabras clave. *Ácido orótico. Ambiente complejo. Colateralización. Ejercicio físico. Esteroides. Factores neurotróficos. Gangliósidos. Neurogénesis. Neuroplasticidad. Patología. Plasticidad cortical. Potenciación a largo plazo. Regeneración. Sinaptogénesis.*

MECANISMOS DA NEUROPLASTICIDADE

Resumo. Objetivo. *Apresentar, de forma unificada, uma visão dos mecanismos principais de neuroplasticidade conhecidos, destacando sua universalidade.* Desenvolvimento. *A concepção do sistema nervoso, como uma entidade imutável, sofreu modificações significativas, durante a segunda metade do século XX. A neuroplasticidade, ou seja, a capacidade de mudança e reparação do cérebro, é expressada de modos diversos, desde modificações funcionais de estruturas já existentes, até a formação por crescimento e proliferação de novas estruturas e neurônios. O presente trabalho aborda os mecanismos celulares e moleculares dos fenômenos neuroplásticos e os classifica em dois grandes grupos: plasticidade po crescimento, onde são incluídos os mecanismos de regeneração axonal, colateralização e sinaptogênesis reativa. E plasticidade funcional que abarca mudanças na efetividade da transmissão sináptica como a potenciação a longo prazo e a ativação de sinapsis silentes. Apresentam-se, também, algumas relações de fenômenos neuroplásticos com enfermidades do sistema nervoso, como também exemplos de fatores fisiológicos, físicos e farmacológicos que podem, no futuro, tornar-se ferramentas terapêuticas para estimular e modular a neuroplasticidade.* Conclusões. *Os mecanismos neuroplásticos mostram u alto grau de conservação filogenética e ontogenética e são tão importantes na gênese de transtornos e enfermidades do sistema nervoso como em sua reparação, depois de sofrer traumatismos e danos muito diversos. A modulação dos mecanismos neuroplásticos por agentes físicos e químicos desponta como uma das mais potentes ferramentas terapêutica na neurologia restaurativa. [REV NEUROL 2000; 31: 1074-95]* ***[http://www.revneurol.com/3111/j111074.pdf]***

Palavras chave. *Acido orótico. Ambiente complexo. Colateralização. Esteróides. Exercício físico. Fatores neurotróficos. Gangliósidos. Neurogênesis. Neuroplasticidade. Patologia. Plasticidade cortical. Potenciação a long prazo. Regeneração. Sinaptogénesis.*

Aging and Synaptic Plasticity: A Review

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SUMMARY

Aging affects all systems, but the brain seems to be particularly vulnerable to the action of negative, age-dependent factors. A gradual loss of memory functions is one of the earliest and most widespread consequences of brain aging. The causes for such impairment are still unclear. Long-term potentiation (LTP) is one form of neural plasticity, which has been proposed as the cellular correlate for memory. LTP is affected by aging, and such alteration might be causally related to memory dysfunction. In the present paper, we review the evidence sustaining the existence of a causal link between cognitive and LTP impairments, as well as the possible mechanisms involved. New results indicate a possible involvement of a deficient reinforcement of LTP by affective influences.

INTRODUCTION

Aging, in biological or cosmological dimension, is the most evident consequence of the passing of time. Time is one of the coordinates (along with space) that form the referential frame in which we exist. It is difficult to assess whether time itself has any influence on living organisms. More probable are thermodynamically irreversible processes that occur in a timely organized sequence, those responsible for aging and for giving us the sense of the passing of time. In biological terms, the aging process is accompanied by a progressive multi-systemic deterioration which, in the absence of other factors (like accidents or disease), inevitably leads to death.

Although there appears to be little hope at present of reaching immortality, major improvements in living conditions and medical care during the last century have brought a steady and significant lengthening of human life (at least for a part of mankind). Consequently, one of the main goals of modern science is to provide a better quality to those years added to life by retarding, reducing, or eliminating (when possible) the negative consequences of aging. Any progress in this direction will be based on a better understanding of the mechanisms involved in the progressive impairments accompanying aging.

The preservation of memory abilities, in both senses, namely, the ability to learn new information and to retrieve old contents, is among the most desired because it is precisely one of the more severely affected by aging. A widely accepted view at present attributes memory formation and consolidation to functional modifications at synapses in different regions of the brain. Whereas the brain system serving particular forms of memory (declarative, motor skills, emotional, classical conditioning, and others) might be different, the basic mechanism modifying synaptic efficacy seems to be common (Matthies, 1998; Medina et al., 2002; Milner et al., 1998).

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Long-term potentiation (LTP) of synaptic efficacy (Bliss & Gardner-Medwin, 1973; Bliss & Lomo, 1973) is a long-lasting increase in synaptic efficacy after high frequency (tetanic) stimulus, and appears as the best candidate mechanism for explaining the learning-induced changes in synaptic connectivity. A growing body of evidence links LTP and memory. This evidence is based, not only on the suggestive and numerous analogies between both phenomena but also on direct experiments demonstrating a relation between changes in synaptic efficacy and memory in different brain structures as well (Bergado et al., 1988; Matthies et al., 1986; Rioult-Pedotti et al., 1998; Rogan et al., 1997) (for a recent review on the subject see Martin & Morris, 2002). It is plausible, therefore, that age-related disorders in synaptic plasticity might be functionally linked to memory impairment (Shapiro, 2001). These concepts are beginning to gain consideration for the effective prevention or treatment of such impairments (Rosenzweig, 1996).

In the present review we will focus our attention on describing how LTP is affected by aging, stressing new results linking memory and emotional disorders with the modulation of LTP in aged animals.

THE GENERAL PROBLEM OF AGING

Several hypotheses have been forwarded to explain the causes of aging. General theories emphasize the role of alterations in biological membranes, the attack of free radicals, calcium dysregulation, and (particularly in mammals) the negative effect of glucocorticoids and stress (Lynch, 1998b) Obviously, all the mentioned factors strongly interact with each other and might potentiate reciprocally, obscuring the possibility to develop a testable hypothesis regarding which might be the primary events in aging.

What appears clear is that aging is a process, and that aging is caused by the action of multiple factors. Aging affects all systems: Muscles weaken, bones become fragile, skin shrinks, arteries harden, hormones (particularly sex hormones) decay, immunity fades and memory fails.

Aging and the nervous system

The alterations in nervous system function as a consequence of aging are diverse. Aged persons (and animals) move different, sleep different, and show alterations in mood and memory. Neuron death and loss of afferents have been proposed as primary events leading to brain dysfunction (Morrison & Hof, 1997; Ward et al., 1999). Although neuron death in specific brain regions is common in neurodegenerative diseases (Barili et al., 1998; Gerlach & Riederer, 1996; Graeber et al., 1998; Landfield et al., 1992; Morrison & Hof, 1997; West, 1993), the relative contribution of this factor to impairment in normal aging is a matter of debate (Rapp & Gallagher, 1996; West, 1993). Non-neuronal alterations like an impaired blood flow (Ajmani et al., 2000) or glial dysfunction (Sykova, 2001) should also contribute to a generalized brain malfunction.

Aging and memory impairment

The decline in cognitive capacities in humans is one of the earliest, most dramatic, and generalized consequences of aging. Even in normal aged persons, memory dysfunctions limit intellectual abilities. In several pathological, age-related conditions, whether vascular or neuro-degenerative like Alzheimer's disease and other dementias, the impact of age on cognition is devastating. The literature showing memory alterations as a consequence of aging in rodents (Ando & Ohashi, 1991; Kadar et al., 1990; Luparini et al., 2000; Ward et al., 1999; Ward et al., 1999), as well as in humans (Albert, 1997; Langley & Madden, 2000; Soininen

et al., 1994) and non-human primates (Bachevalier, Landis et al., 1991) is extensive. The cholinergic hypothesis of geriatric memory dysfunction (Bartus, 2000; Bartus et al., 1982) stresses the importance of the cholinergic afferents arising from the basal forebrain in memory functions (Baxter et al., 1999; Ikegami, 1994; Ikegami et al., 1992; Pedigo Jr, 1994; Russell, 1996; Shen et al., 1996; Smith et al., 1995) and has inspired some promising efforts in the search for effective treatments to overcome the age-related cognitive impairments (Fernández et al., 1994; Fischer, 1994; Fischer et al., 1994; Flood et al., 1996; Garrone et al., 1998; Levin & Torry, 1996; Scali et al., 1994; Sirvio, 1999; Smith et al., 1999; Vannucchi et al., 1997). Despite the intensive effort and literature supporting the cholinergic hypothesis, no clear picture has emerged on how acetylcholine might act to support memory processing.

When the methodological basis of animal learning models was established in the early years of the 20th Century, it was immediately recognized that for an animal to learn something a strong motivation must be present. Much less attention has been paid, however, both clinically and experimentally, to the possible impact of dysfunctional emotional-motivational reactions on cognitive performance. Such neglect might appear surprising considering that aged related alterations in mood are widespread (Blazer, 1987) and often associated with neurodegenerative diseases (Harwood et al., 2000).

AGING AND SYNAPTIC PLASTICITY

Mechanisms of plasticity

Decades of intensive research have contributed to clarify the mechanisms involved in the induction, development, and maintenance of LTP. Although different forms of LTP seem to exist—according to the induction mechanisms involved—the most common form requires the activation of NMDA-type glutamate receptors (Bashir et al., 1991; Bashir et al., 1990; Collingridge, 1987; 1992). The NMDA ionophore allows the entrance of calcium to the postsynaptic region, an event that seems decisive to the strength and direction of the plastic modification. When the increase in Ca^{++} is above certain values, an increase in synaptic efficacy (i.e., LTP) develops, but lower values could lead to the opposite, a reduction in synaptic efficacy (the so called Long-Term Depression, LTD) (Cormier et al., 2001; Foster & Norris, 1997; Teyler et al., 1994). In LTP, Ca^{++} activates protein kinases; whereas phosphatases are activated in LTD.

Several kinases have been proved to participate in the event cascade involved in LTP; among them, the Ca^{++}-calmodulin dependent protein kinase II (PKII), the Ca^{++}-phospholipid dependent protein kinase C (PKC), and the cyclic AMP dependent protein kinase A (PKA) (Abel et al., 1997; Colley et al., 1990; Fukunaga et al., 1996; Huang & Kandel, 1994; Matthies & Reymann, 1993; Reymann et al., 1988a; 1988b; Silva et al., 1992; Stevens et al., 1994). These enzymes can modify synaptic efficacy by acting pre- and post-synaptically. For example, PKII can increase the conductance of the AMPA-glutamate receptors (Derkach et al., 1999), and consequently, increase the level of post-synaptic depolarization. But they can also act at distant regions, like the nucleus, regulating gene expression and protein synthesis (Nguyen & Kandel, 1996; 1997; Pontzer et al., 1990).

The dependency of late phases of LTP (L-LTP, >4 h) on protein synthesis is well established (Frey et al., 1988; Krug et al., 1984; Otani & Abraham, 1989). Such dependency has led to a multi-phase model of LTP, similar to that proposed for memory (Matthies et al., 1990). Yet, the identification of specific proteins required for LTP late maintenance has proven difficult. It seems that in a first step, proto-oncogenes are activated (Abraham et al., 1991; Bishop et al., 1994; Roberts et al., 1996), which can, in turn, activate structural genes; like

those coding subunits of AMPA receptors (Desmond & Weinberg, 1998).

The mechanism to guarantee LTP specificity after massive, non-specific protein synthesis requires the setting of a tag at the activated synapses, so that arriving proteins can be 'captured' only by those synapses that have been previously activated above a certain limit (Frey & Morris, 1997; 1998).

Although the incorporation of new AMPA receptors might well subserve an increased synaptic efficacy in potentiated synapses, one can't help but wonder why so many other proteins are needed. One likely explanation is that really long, long-lasting changes in synaptic efficacy are accompanied by morphological changes at the existing synapses—a possibility raised in early studies (Desmond & Levy, 1986a; 1986b; 1988; Fifkova, 1985)—or the growth and establishment of new functional synaptic contacts. Electron microscope studies reveal signs of dendritic spine division and axonal sprouting days after induction of LTP by strong stimuli (Geinisman, 1993; Geinisman et al., 1989; Yuste & Bonhoeffer, 2001).

Recent developments have shown that the plastic processes initiated by the activation of specific glutamatergic synapses can be modulated by synapses different from those previously involved in LTP induction (i.e., hetero-synaptically). This point is conceptually important because LTP has been considered a homosynaptic phenomenon, despite evidence indicating the need for hetero-synaptic cooperation. An impaired LTP has been reported in animals bearing lesions of the fimbria-fornix, the fiber system providing the hippocampus with cholinergic and aminergic fibers of sub-cortical origin (Bergado et al., 1996; Buzsáki & Gage, 1989). Cooperation between the perforant pathway (mainly glutamatergic) and the septum (mainly non-glutamatergic) and the locus coeruleus (mainly noradrenergic) has also been documented (Harley & Sara, 1992; Kitchigina et al., 1997; Robinson & Racine, 1982; 1986).

More recently, a group of studies has shown that the activation of the amygdala—a limbic structure related to emotion (Quirk et al., 1996)—can influence the induction of LTP at the dentate gyrus (Akirav & Richter-Levin, 1999; Ikegaya et al., 1995; Ikegaya et al., 1996). We have recently shown that the electrical stimulation of the amygdala also contributes to the maintenance of LTP, converting a short lasting early-LTP (E-LTP <4h) into a late-LTP (L-LTP >4h) (Frey et al., 2001). Interestingly, the effect is protein-synthesis dependent and requires the activity of noradrenergic and cholinergic inputs. Lesioning of the fimbria-fornix, the main source of subcortical aminergic and cholinergic input to the hippocampal formation, impairs the reinforcing effect of the amygdala stimulation on L-LTP (Abe et al., 1998; Jas et al., 2000). The activity of the amygdala also seems crucial for the so-called behavioral reinforcement of LTP. According to this paradigm, E-LTP can also be converted into an L-LTP by giving the animals a behavioral stimulus with a strong motivational content (i.e. drinking after 24-h water deprivation) shortly before, or after, inducing LTP using a weak tetanus (3x15 impulses at 200 Hz) (Seidenbecheret al., 1995; 1997). We have recently obtained evidence that lesioning, or blocking the amygdala with lidocaine, abolishes the behavioral reinforcement of LTP (Almaguer et al., in press).

AGE-RELATED CHANGES OF SYNAPTIC PLASTICITY

Developmental changes

Like many other functions, synaptic plasticity presumably shows developmental changes during the early stages of postnatal life, followed by a period of relative stability in adulthood and then deteriorating slowly at older ages.

LTP is difficult to induce before postnatal day 5. At P15 a level similar to adults can be reached (Teyler et al., 1989), but outlasting in time the duration of LTP in adults under similar induction paradigms (Bronzinoet al., 1994; 1995). Younger animals, on the other hand, seemed to be more prone to develop LTD (Dudek & Bear, 1993; Wasling et al., 2002).

Older animals show impaired synaptic plasticity

Although earlier reports pointed to a reduced synaptic plasticity accompanying age-dependent memory impairment (see for example (Landfield et al., 1972), a demonstration of LTP deterioration with aging came from a series of elegant studies from Barnes and McNaughton (1985) (according to the references available to us). These authors demonstrated an impressive parallelism between the slower rate of development and the faster decay of memory and LTP among aged rats, compared with young controls (Barnes, 1979; Barnes & McNaughton, 1985). This result was later confirmed by others (Bergado et al., 1997; de Toledo-Morrell & Morrell, 1991; deToledo-Morrell et al., 1988; Diana, De Carolis et al., 1994; Geinisman, deToledo-Morrell et al., 1995) showing that behavioral, electrophysiological, and histological alterations were more profound in old rats with memory impairments than in similarly old animals without cognitive impairments. Later studies showed that aged rats were also more prone to develop LTD or to reverse LTP (depotentiation) by low frequency stimulation (Norris et al., 1996).

The former doesn't mean that aged rats are unable to develop LTP. When adequately stimulated, aged rats can reach an increase in synaptic efficacy, similar to that of young animals (Diana et al., 1994; Moore et al., 1993)—even those showing memory impairment—but the former seem to require stronger stimulation, and the decay rate is faster. This phenomenon has been attributed to an impaired "ability of aged rats' synapses to provide the sustained depolarization necessary to activate the LTP-induction cascade" (Rosenzweig et al., 1997).

POSSIBLE MECHANISMS OF IMPAIRMENT

Impaired induction?

Considering the importance of NMDA receptor activation for LTP induction, it seems plausible that any impairment in glutamatergic systems would have an impact on synaptic plasticity. In patients with Alzheimer's disease, neuropathology studies have shown that even in the early stages, the number of neurons in the entorhinal cortex is reduced (Braak & Braak, 1992; Palmer, 2002). The evidence from aged animals is not so clear, at least regarding the total number of cells originating the perforant pathway (Gazzaley et al., 1997; Merrill et al., 2000; 2001). In the rat, however, there are indications of a reduced glutamatergic influence on the EPSP (Barnes et al., 1997; Billard et al., 1997). This decline might be related to a reduction in glutamate receptors in the cortex and in the hippocampus—particularly of the NMDA type- that have been repeatedly reported in rats (Kito et al., 1990; Liang & Lu, 1992; Magnusson, 1998; Magnusson & Cotman, 1993; Tamaru et al., 1991; Wenk et al., 1991) and probably associated with a change in the subunit composition (Clayton & Browning, 2001; Clayton et al., 2002; Magnusson et al., 2002) and subtle subregional variations within hippocampal subfields (Wenk & Barnes, 2000). The activity-dependent redistribution of NMDA receptors is also affected among aged rats (Clayton et al., 2002). Nevertheless, the basic mechanisms of NMDA-receptor induction mechanism for LTP seemed to be preserved (Barnes et al., 1996).

Aging seems to provoke a shift from NMDA-

receptor calcium influx to Voltage Dependent Calcium Channels (VDCC) (Shankar et al., 1998), with the probable consequence that the increase in intracellular calcium will not reach the level required to activate Ca^{++}-dependent kinases, but sufficient to activate phosphatases (Foster, 1999; Foster & Norris, 1997). This situation would lead to the prediction that LTD and depotentiation are facilitated in aged rats, for which there is some experimental support (Norris et al., 1996). That blockade of VDCC attenuates this altered plasticity is in line with this hypothesis (Norris et al., 1998).

Other indications point to impaired regulation of protein kinases in aged animals. For PKII, a dysfunctional regulation of the alpha subunit has been reported in aged animals showing a decremental LTP (Davis et al., 2000), in contrast to those maintaining LTP beyond 3 h. An age-related decrease of cortical plasticity was found in mutant mice lacking the αCaMKII subunit (Kirkwood et al., 1997). Similarly, a reduced PKC activity and its substrates has been experimentally documented in aged animals (Casoli et al., 1996; Chang et al., 1997; Mons et al., 2001; Okuma et al., 2000).

Impaired maintenance?

The temporal course of LTP in aged animals, particularly its faster decay, suggests an absent protein-synthesis dependent L-LTP. Several reports show alteration in protein synthesis and expression pattern or early genes among aged rats after LTP induction (Lanahan et al., 1997; Mullany & Lynch, 1997). An impaired gene activation and protein synthesis might, in turn, affect the ability of axons and dendritic spines to divide, grow, and form new contacts that apparently are required for very long-lasting LTP. In aged rats, electron micro-scopic studies have evidenced that this capacity might be quantitatively altered (Chang et al., 1991; Geinisman et al., 1992). In line with this evidence are findings showing that the expression of cell adhesion molecules required for axonal growth is impaired in aged rats (Ronn et al., 2000), indicating a deficient maintenance of LTP (Lynch & Voss, 1994).

Impaired heterosynaptic reinforcement?

As mentioned previously, the activation of heterosynaptic afferents (i.e. behavioral or amygdala-induced reinforcement) to a neuronal population, in which an E-LTP has been induced through a mild tetanus, can prolong the duration of the plastic process, converting it into an L-LTP that is protein-synthesis dependent. This effect suggests that the activation of such afferents is able to induce the activity of molecular cascades, which complement the insufficiently activated cascade by the previous glutamatergic tetanus. Norepinephrine and acetylcholine have been implicated in those effects (Frey et al., 2001). Both transmitters have been shown to induce a slowly developing potentiation in *in vitro* studies, which might correlate with their effects on L-LTP. Norepinephrine and acetylcholine, when applied to slices obtained from young animals, induce a slowly developing potentiation (Segal & Auerbach, 1997; Stanton & Sarvey, 1987), which might represent an L-LTP. The effect of NE is protein synthesis dependent (Stanton & Sarvey, 1985). Acetylcholine is responsible for theta rhythm in the hippocampus. The magnitude of LTP induced by tetani delivered at the positive theta peak was reduced in aged animals rats (Orr et al., 2001).

Recent results from our group provided the first evidence that such heterosynaptic reinforcing mechanisms might be deficient in aged rats, particularly among those animals showing memory deficits in a spatial task. Both behavioral- and amygdala-induced reinforcement are impaired, not only in cognitively deficient aged rats but also among aged rats showing no memory impairment in the Morris water maze (Almaguer et al., 2002;

Bergado et al., 2001). Both noradrenergic and cholinergic systems in the brain are affected by aging (Baxter et al., 1999; Chouinard et al., 1995; Friedman & Duckles, 1994; Isacson et al., 2002; Luine et al., 1990; Powers et al., 1988; Sherman & Friedman, 1990; Stemmelin et al., 2000) and both can activate kinase systems involved in regulating protein synthesis (Bevilaqua et al., 1997; Stratton et al., 1988).

We assume that the deficient reinforcement of LTP by amygdala or by behavioral stimulation is caused by a reduction in noradrenergic and/or cholinergic afferents to the dentate gyrus, the brain area in which both studies were carried out.

The results of these studies provide a rationale, at the cellular level, for a possible functional link between two of the major consequences of aging: memory impairment and affective dystonia.

Additional factors might increase the negative impact of aging on synaptic plasticity. Chronic stress has been shown to lead to a disruption in LTP (Sapolsky, 1999) by the excessive production of glucocorticoids (Bodnoff et al., 1995; McEwen, 1994), to which aged rats seemed particularly sensitive. Also, inflammatory cytokines (Lynch, 1998a) have been shown to affect LTP in an age-dependent manner.

Whereas a general agreement seems to have been reached on the relation between age-related impairments in plasticity and memory, some debate remains about which of the factors mentioned plays the major role in the degenerative process. We sustain the view that, most likely, all factors can be involved and interact cooperatively, leading to a continuously deteriorating plastic ability that causes, in turn, the decay in mnesic capacities.

LINKS TO PATHOLOGY?

Another important aspect to consider is whether alterations in synaptic plasticity might be also involved in memory losses in pathological forms of aging like Alzheimer's disease. New developments in animal models have provided indications that such a relation might exist.

The RNA for the amyloid precursor protein (APP), one of the key molecules in Alzheimer's pathogenesis, is upregulated after LTP induction in young rats, but not among aged rats regardless of whether they were cognitively impaired or not (Stephan et al., 2002). On the other hand, mutant mice lacking the APP gene, and in mice expressing the carboxy terminus of APP, released after proteolytic cleavage of the protein, showed an altered LTP maintenance (Nalbantoglu et al., 1997; Seabrook, Smith et al., 1999). Moreover, mice overexpressing mutant forms of human APP developed not only the typical histopathology of Alzheimer's but also an altered plasticity (Chapman et al., 1999; Larson et al., 1999) (see however (Fitzjohn et al., 2001).

AN IMPACT ON THERAPEUTICS?

The studies on the alterations of synaptic plasticity in aging have also provided some results of potential therapeutic implications.

General factors like caloric restriction (Eckles-Smith et al., 2000) or dietary supplement with antioxidants (vitamin E or lipoic acid) (Murray & Lynch, 1998; McGahon et al., 1999) reverse aged-dependent alterations in LTP, and improve glutamatergic transmission in the dentate gyrus. The use of calcium regulators like nimodipine or nifedipine has shown beneficial effects on LTP among aged rats (De Jong et al., 1992; Norris et al., 1998), and cholinergic agonists have improved declining LTP in aged rats (Fujii & Sumikawa, 2001).

Trophic factors are among the greatest hopes of restorative neurology in the last decades. Some have shown a direct effect on synaptic plasticity. Early reports suggested an action for epidermal

growth factor (EGF) and fibroblast growth factor (FGF), but not nerve growth factors (NGF), promoting LTP in normal and lesioned rats (Abe et al., 1992; Ishiyama et al., 1991; Terlau & Seifert, 1990). More recently, the results of in vitro and in vivo studies have shown that brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3), but again not NGF, are able to induce an LTP-like plastic development in the hippocampus (Chen et al., 1999; Kang & Schuman, 1995a; 1995b; 1996; Kovalchuk et al., 2002; Lu & Chow, 1999), thus re-opening the issue for a potential use of BDNF for the treatment of age-related memory impairments. Although NGF cannot by itself induce LTP, chronic treatment with NGF restores the ability of aged, memory-deficient rats to develop LTP (Bergado et al., 1997; Bergado et al., 1998), a result that correlates with behavioral studies showing an improvement in memory function after similar treatment (Fischer et al., 1994). We interpret this effect as mediated by the protective and restorative function of NGF on the basal forebrain cholinergic projection to the hippocampus and cortex. Similar results have been reported after the transplantation of septal fetal neurons to aged rats, or in rats with lesion of the septo-hippocampal projection (Bergado et al., 1997; Björklund & Stenevi, 1977; Fernández et al., 1994; Leanza et al., 1998) Although neurotrophic administration to the brain is technically difficult, an intensive search for new strategies could bring some promising results to this important field.

CONCLUSIONS

The accumulated evidence seems reasonably sufficient to postulate the existence of a functional link between the age-dependent impairment in synaptic plasticity and the deterioration of memory function. The knowledge about the mechanisms of LTP can lead not only to a better understanding of the causality of memory decline with age but also to the development of new therapeutic strategies for treating it. Several lines of evidence also point to an impaired relation, at the cellular level, between affective and cognitive processes. Any progress in these important topics can represent a substantial contribution to reach the goal of successful aging, so that the years gained for life can also be an enjoyable period of existence.

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RESULTADOS

Los resultados que conforman esta tesis están recogidos en una serie de artículos científicos que aparecen a continuación.

A la copia de cada artículo antecede una breve descripción del contenido fundamental del mismo que facilitará al lector seguir el orden de pensamiento que guió este trabajo. Con ese mismo propósito los artículos aparecen en orden lógico y no cronológico ya que algunos, por imperativos materiales y de oportunidad, tuvieron que ser realizados antes y otros, por razones de igual índole, tuvieron que ser diferidos.

Capítulo 1: El reforzamiento conductual de la potenciación sináptica duradera es dependiente de la síntesis de proteínas

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Los procesos de plasticidad sináptica, incluida la LTP, dependen del metabolismo macromolecular. La activación de los procesos de síntesis de proteínas que se requieren para la consolidación del fenómeno neuroplástico puede ser provocada por el mismo agente inductor, o por algún evento modulador adicional.

En el modelo de reforzamiento conductual que empleamos, la tetanización de la vía perforante (inductor) no parece tener intensidad suficiente como para activar los mecanismos de síntesis de proteínas. De acuerdo con esa concepción formulamos la hipótesis que dio origen a este trabajo:

El reforzamiento conductual actúa como evento modulador que, por vías posiblemente heterosinápticas, activa la maquinaria biosintética celular y proporciona a las sinapsis potenciadas las proteínas necesarias para consolidar (prolongar) el estado de potenciación.

De aquí puede derivarse fácilmente la hipótesis que se explora directamente en este trabajo: Si el reforzamiento conductual actúa como evento modulador activador de la síntesis de proteínas, el bloqueo de la síntesis de proteínas debe abolir el efecto reforzador del evento conductual.

Para comprobar esta hipótesis aplicamos el modelo de reforzamiento conductual a un grupo de animales a los que se inyectó intraventricularmente un bloqueador de la síntesis de proteínas (anisomicina) una hora antes de inducir la LTP y permitir el acceso al agua

(reforzamiento conductual). Un grupo similar de animales, también privado de agua durante 24 horas, al que se inyectó solución salina sirvió como control.

El resultado fundamental fue que el bloqueo de la síntesis de proteínas abolió totalmente el efecto de reforzamiento conductual, como muestra la figura 1b, donde se comparan ambos grupos.

Este resultado confirma la hipótesis planteada y permite asumir que el reforzamiento conductual actúa como evento modulador de la plasticidad sináptica mediante la activación de los mecanismos celulares de biosíntesis que aportan las proteínas requeridas por el proceso neuroplástico para su consolidación.

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Behavioral reinforcement of long-term potentiation in rat dentate gyrus in vivo is protein synthesis-dependent

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Abstract

A transient, protein synthesis-independent long-term potentiation (early-LTP, <4 h) can be reinforced into a maintained protein synthesis-dependent late-LTP (>4 h) by specific electrical stimulation of limbic structures (J. Neurosci. 21 (2001) 3697). Similarly, LTP-modulation can be obtained by behavioral stimuli with strong motivational content. However, the requirement of protein synthesis during behavioral reinforcement has not been shown so far. Thus, we have studied here this specific question using a behavioral reinforcement protocol, i.e. allowing water-deprived animals to drink 15 min after induction of early-LTP. This procedure transformed early-LTP into late-LTP. Anisomycin, a reversible protein synthesis inhibitor, abolished behavioral LTP-reinforcement. These results demonstrate that behavioral reinforcement depends on protein synthesis.

Keywords: Behavioral reinforcement; Long-term potentiation; Late-long-term potentiation; Protein synthesis; Anisomycin; Dentate gyrus; Hippocampus

Long-term potentiation (LTP) is a long-lasting increase of synaptic efficacy which is considered to be a cellular correlate of memory formation [15]. LTP can be separated in at least two temporally distinct phases by the use of protein synthesis inhibitors during its induction: an early phase (early-LTP) lasting less than 4 h and a protein synthesis-dependent late-LTP [8,13]. Early-LTP in the dentate gyrus (DG), induced by a weak tetanus to the perforant pathway (PP), can be converted into late-LTP by heterosynaptic, modulating signals arising from other limbic regions like the basolateral amygdala (BLA) [6] or the septum [5]. Both effects are protein synthesis-dependent as they are abolished by the intraventricular administration of anisomycin. A reinforcement of LTP can also be observed when animals receive a behavioral stimulus with a strong motivational content, an effect we called behavioral reinforcement (BR) [19]. It remains unclear whether BR depends on protein synthesis as well. BR and amygdala-induced reinforcement have similar effective time windows and we have recently suggested that the BLA is an important structure of the neural system involved in BR [2]. Our findings led to the hypothesis that processes of BR on plastic events, such as prolonging LTP, require the synthesis of proteins in general. We have now tested this hypothesis. Male adult WISTAR rats (obtained from CENPALAB and weighing 250 g during electrode implantation) were housed individually after surgery in plastic, translucent cages. Water and food (Rat Chow, Cenpalab, Cuba) remained ad libitum throughout the experiment except for the series investigating the effect of BR of LTP in rats which were water-deprived for 24 h. Animals were handled according to the German Regulations for Using Laboratory Animals.

Surgery was performed under chloral hydrate narcosis (420 mg/kg, intraperitoneally) according to the procedure described elsewhere [1]. Electrodes for recording were implanted in the hilus of DG and for stimulation in the angular bundle of the PP. A guide cannulae was implanted to reach the right lateral ventricle [1]. One week after surgery the animals were habituated to the test chamber for at least 4 h before obtaining an input–output curve on which the stimulus intensity was determined for each animal (40% maximal population spike). Animals were then randomly

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assigned to one of the experimental groups. In the control-LTP group a weak tetanus (3 × 15 impulses at 200 Hz) was used to induce early-LTP (Fig. 1a, open circles). The BR group was water-deprived for 24 h before inducing early-LTP by the above protocol; 15 min later water was made available until the end of the recording session (Fig. 1a, filled circles). The same protocol was followed using a NaCl-treated BR group (NaCl-BR; NaCl used as the vehicle; Fig. 1b, open circles) and an anisomycin (ANI)-treated BR group (ANI-BR; Fig. 1b, filled circles). In the latter series 5 μl of the saline or anisomycin solution (0.905 mol, Sigma, St. Louis, MO) was injected, respectively, into the lateral ventricle (i.c.v.) 1 h before induction of the early-LTP and subsequent BR. Control experiments performed earlier in our laboratory revealed no effects of water deprivation on test potentials recorded in a control group

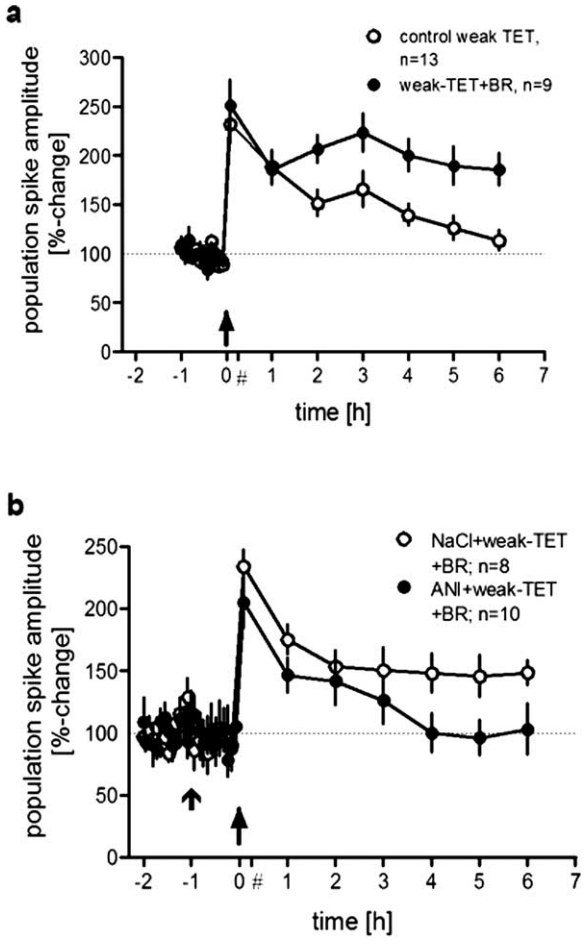

Fig. 1. The role of protein synthesis during behaviorally reinforced LTP. (a) Weak tetanization (strong arrow) resulted in a transient protein synthesis-independent early-LTP (open circles). Early-LTP was transferred into late-LTP if water-deprived animals were allowed to drink water 15 min (double cross) after tetanization (filled circles). (b) Early-LTP can also be reinforced into late-LTP by water access in water-deprived animals which received NaCl i.c.v. 1 h before tetanization (small arrow) and when drinking was allowed 15 min after tetanization (open circles). This reinforcement of early-LTP beyond 4 h was blocked when the reversible protein synthesis inhibitor anisomycin was applied i.c.v. instead of NaCl before tetanization (filled circles). Statistics: see text.

without tetanization [19]. Test recordings were made every 15 min after early-LTP induction. Each data-point presented in Fig. 1 represents an averaged value of five consecutive potentials recorded at a frequency of 0.1 Hz. All animals involved in BR protocols, even those receiving anisomycin, drank abundantly within 1 min after water was made available. No group differences were found in the total volume of water consumed at the end of the recording period (BR-control group: 15.8 ± 3.81 ml, NaCl-weak-TET-BR control group: 16.5 ± 3.53 ml, ANI-weak-TET-BR group: 17.0 ± 4.84 ml; the values represent the mean volume of water consumption ± standard deviation). After finishing the experiments the animals were perfused under narcosis and the correct location of the cannulae as well as electrodes was proven histologically.

Administration of anisomycin showed no effect on the population spike amplitude (PSA, Fig. 1b: baseline recordings, filled circles) during control baseline recordings taken before the induction of LTP. The two-way ANOVA showed no treatment ($F(1,21) = 0.125418$, $P > 0.05$) or time ($F(23,483) = 0.736305$, $P > 0.05$; data not shown) effect.

Tetanic stimulation resulted in a significant increase of the PSA for a duration of 4 h, when the data of the control LTP group were compared with its own pre-tetanus baseline values (Fig. 1a, open circles, control early-LTP; Student's *t*-test for paired samples). Other groups (BR, NaCl-BR and ANI-BR) showed a similar initial level of potentiation measured 5 min after tetanization (no differences detected by using the one-way ANOVA: $F(3,39) = 0.776967$, $P > 0.05$). However, the time course of LTP differed significantly among the latter two groups. The two-way ANOVA with treatment and time (repeated measures) as factors showed significant differences for both factors and their interaction (treatment, $F(3,39) = 3.15204$; time, $F(5,195) = 15.83220$; interaction, $F(15,195) = 3.14201$; $P < 0.05$ in every case). A post-hoc analysis (Duncan's test) proved statistically significant differences between the control BR-LTP group and the ANI-BR group (Fig. 1b, filled circles), respectively, but not the NaCl-BR group (Fig. 1, open circles). Further analysis of the NaCl-BR and ANI-BR groups (Duncan's test) revealed significant differences between groups only at later phases, i.e. after 4 h, corresponding to late-LTP (two-way ANOVA, $F(1,17) = 6.028451$, $P < 0.05$).

Our results demonstrate that also BR of LTP depends on protein synthesis. The requirement of protein synthesis has been consistently reported for long-term memory formation in several species and brain structures [3,11,18]. Also processes of functional plasticity like LTP [8,13,16], LTD [14,17] and other forms [4,10,12] are characterized by a similar requirement: a preserved induction but an impaired maintenance if protein synthesis is blocked during initial phases. Although one can easily conceive a general role for proteins in long-term plasticity mechanisms the identification of specific proteins involved has been shown to be difficult, perhaps because of the big number of proteins

synthesized in apparent correlation with processes of functional plasticity. Two related problems refer to the mechanisms of induction and synapse-specific distribution of newly synthesized plasticity-related proteins. Data from our group and others suggest that modulatory, non-glutamatergic transmitters like norepinephrine, dopamine and acetylcholine mediate the reinforcing effect of heterosynaptic afferents during LTP [5–7] by activating intracellular cascades resulting in the regulation of the synthesis of plasticity-related proteins within the neuron [7]. Regarding their distribution, the synaptic tagging hypothesis [9] offers a plausible explanation to maintain synapse-specificity during protein synthesis-dependent events. According to the hypothesis of synaptic tagging our results of protein synthesis-dependent BR of early- into late-LTP can be explained as follows: a weak tetanus used to induce early-LTP may tag specifically the activated synapse population. Modulatory, behaviorally reinforcing inputs which were activated by drinking 15 min after tetanization led to synapse non-specific synthesis of plasticity-related proteins. These proteins were captured by synapses with a tag set, thus transforming early- into late-LTP by activation of the rewarding system, probably involving the amygdala. When protein synthesis was blocked at the time of water presentation, the reinforcing effect was abolished. Our data strongly suggest the physiological significance of heterosynaptic reinforcement processes of plastic events in general.

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Capítulo 2: La amígdala es parte del sistema de reforzamiento conductual que modula la plasticidad sináptica duradera

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Además del problema del mecanismo celular implicado en el efecto de reforzamiento del proceso neuroplástico por factores afectivos, es necesario conocer cuáles son las estructuras nerviosas que median dicho efecto.

Conocido el papel de la amígdala como elemento central del Sistema Límbico en la determinación del estado afectivo, así como su acción reforzadora de procesos de memoria, resulta obvio suponer que:

La amígdala es parte del sistema neural implicado en el reforzamiento de procesos neuroplásticos por factores afectivos.

Para evaluar esta hipótesis empleamos nuevamente el modelo de reforzamiento conductual de la LTP en ratas. La participación de la amígdala en el fenómeno de reforzamiento conductual fue evaluado comparando los resultados de grupos de animales en los que se evaluó el reforzamiento conductual en condiciones de bloqueo temporal de (por inyección tópica de un anestésico local) o permanente de la amígdala (por lesión electrolítica). En ambos casos, el resultado fue similar, la inactivación temporal (fig. 1b) o permanente (fig. 2a) de la amígdala bloquea completamente el efecto reforzador del estímulo conductual aplicado después de la inducción de la LTP.

Este resultado confirma la hipótesis planteada y demuestra que la amígdala es parte del sistema neural implicado en el reforzamiento de procesos neuroplásticos por factores afectivos.

Lo anterior no significa, por supuesto, que otras estructuras cerebrales no tengan un papel en estos procesos. Más que un enunciado de cautela, tal reconocimiento es

particularmente importante en este caso, considerando que no existen proyecciones anatómicas directas desde la región bloqueada de la amígdala (amígdala basolateral), hasta la región donde se indujo y estudió el fenómeno de LTP (giro dentado) lo que necesariamente implica la participación de otros mediadores neurales.

Neuroscience 119 (2003) 319–322

LETTER TO NEUROSCIENCE

THE AMYGDALA IS PART OF THE BEHAVIOURAL REINFORCEMENT SYSTEM MODULATING LONG-TERM POTENTIATION IN RAT HIPPOCAMPUS

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Abstract—Long-term potentiation (LTP) in the dentate gyrus can be modulated and prolonged by emotional/motivational influences when concurrently activated. A similar effect on LTP can be obtained by stimulating the amygdala, suggesting that this limbic structure might be part of the neural system involved in behavioural reinforcement. To confirm this we have performed a series of experiments in which the basolateral amygdala was either temporary inactivated by injection of lidocaine or permanently lesioned electrolytically. Both manipulations completely blocked the reinforcing effect of a motivational stimulus (drinking after 24-h deprivation) on LTP at the perforant pathway-dentate gyrus synapses, whilst leaving intact the non-reinforced potentiation. These results demonstrate that the basolateral amygdala is a key structure within the system involved in the modulatory interaction between the affective status of the animal and the mechanisms of functional plasticity.

Key words: lidocaine, electrolytic lesions, synaptic plasticity, emotion, motivation, memory.

Long-term potentiation (LTP) has been considered as a cellular mechanism of learning and memory (Matthies, 1989, 1998). It is well known that learning and memory are influenced by the emotional/motivational status of the animal. A similar dependence has been recently demonstrated on LTP (Seidenbecher et al., 1997). Behavioural stimuli with a strong motivational content (behavioural reinforcement), are able to prolong a short-lasting LTP (2–4 h) into a long-lasting LTP (with a duration beyond 4 h). We have obtained coincident results by stimulating the basolateral amygdala (BLA) shortly before or after the induction of protein synthesis-independent early-LTP (E-LTP) (Frey et al., 2001). We were able to show that this E-LTP can be transformed into a protein synthesis-dependent late-LTP (L-LTP). This suggests that the BLA might be part of the neural system involved in behavioural reinforcement of LTP.

To prove this hypothesis we have performed a series of experiments based on the behavioural reinforcement protocol described by Seidenbecher and co-workers (Seidenbecher et al., 1997) combined with the temporal or permanent inactivation of the BLA by lidocaine injection or electrolytic lesion, respectively.

EXPERIMENTAL PROCEDURES

Male Sprague–Dawley rats, weighing 250–300 g at the time of surgery, were prepared stereotactically and implanted with a recording electrode at the dentate gyrus and a stimulating electrode at the perforant pathway (PP) to obtain monosynaptically evoked potentials. A guide cannula was also implanted in the BLA. Some animals received no cannulae; instead a monopolar electrode was lowered to the BLA in order to lesion this structure by passing 2.5-mA cathodal current during 10 s. All implant or lesion procedures were carried out during the same surgical session. Two weeks after surgery the animals were habituated to the recording chamber for at least 4 h and assigned randomly to the different experimental protocols. The electrophysiological studies included the induction of E-LTP using a weak tetanus (3×15 impulses at 200 Hz). Each impulse was a 0.1-ms square pulse adjusted in intensity to produce a population spike about 40% of its maximal value. In one series 1 μl of a lidocaine solution (2%) was injected into the BLA within 1 min to temporarily inactivate this nucleus. Lidocaine, or saline, was applied to the BLA 5 min after the tetanus. Behavioural stimulation, which lead to behavioural reinforcement of E-LTP under control conditions (Fig. 1a, closed circles), was applied 15 min after E-LTP induction and consisted of access to water after a 24-h deprivation period. All deprived animals drank abundantly, starting 1–2 min after water was made available. There were no noticeable differences in the volume consumed or the number of drinking bouts during the first 10 min. Water remained *ad libitum* during the rest of the experiment.

Efforts were made to minimise pain or discomfort of the animals according to the Cuban Regulations for the Use of Laboratory Animals. At the end of the experiments the animals were perfused with formalin under chloral hydrate narcose and the brains processed histologically to confirm the location of the cannulae or the success of electrolytic lesion, respectively. Only data from animals with a correct cannulae location or lesion were processed. The lesion of the BLA often compromised also the lateral or the central region of the amygdala.

Changes in the population spike amplitude (PSA) were measured and statistically evaluated. The relative value to baseline (% PSA) was calculated to each averaged recording of

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Abbreviations: ANCOVA, analysis of covariance; BLA, basolateral amygdala; E-LTP, early-long-term potentiation; EPSPs, slope of the excitatory postsynaptic potential; LTP, long-term potentiation; L-LTP, late-long-term potentiation; PP, perforant pathway; PSA, population spike amplitude.

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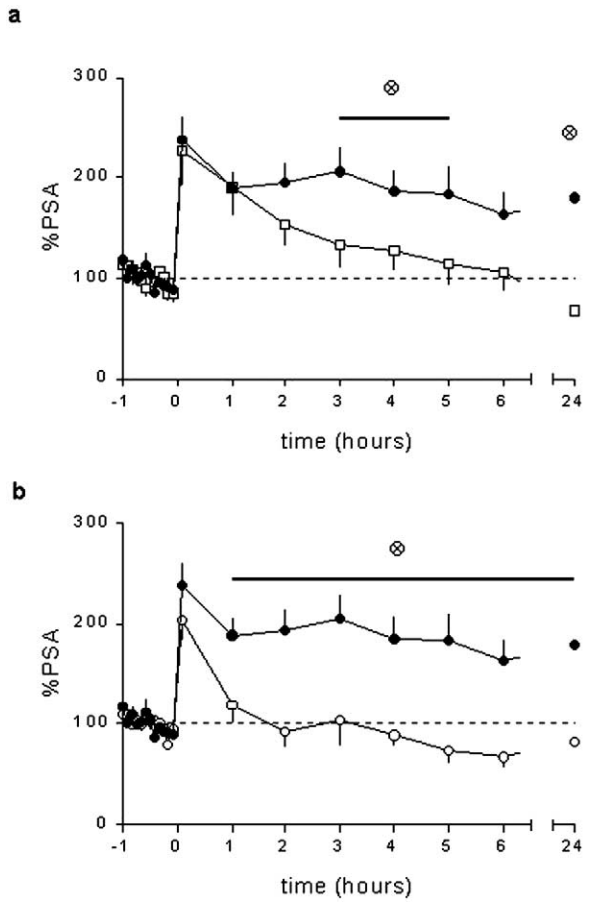

Fig. 1. (a) The weak tetanus used (3×15 impulses at 200 Hz) induced early-long-term potentiation (E-LTP) (control LTP, open squares, $n=8$) lasting about 2 h. When this stimulus is combined with access to water in deprived animals (LTP and behavioural stimulation, filled circles in a and b, $n=14$) LTP is prolonged up to the end of the follow-up period (Wilcoxon test). Statistically significant differences between curves were found at the time points under the symbol (⊗), *U* test, $P<0.05$). (b) Injection of lidocaine to the basolateral amygdala (LTP induction, lidocaine and behavioural stimulation, open circles, $n=8$) reduces the time of the E-LTP to less than 1 h (Wilcoxon test) and abolishes the behavioural reinforcement effect. Symbol (⊗) as in (a).

five consecutive potentials (f=0.1 Hz). The slope of the field excitatory postsynaptic potential (EPSP) showed a similar trend though the changes are smaller in value. The recording electrodes were positioned in the hilus, i.e. a location far away from EPSP generation; thus the PSA was normally used for analysis. Non-parametric tests were used because of the non-normal distribution of the values. The Wilcoxon paired measures test was used for within-group comparison of data obtained after tetanization with their own baseline values. The Kruskal-Wallis test was used for comparing whole curves obtained for two groups, whilst the Mann-Whitney's *U* test was used to compare isolated points between two curves. To compare the input-output curves between lesioned and non-lesioned animals linear regression curves were adjusted to the data in each group. The adjustment to linearity of each regression line was assessed with the runs test. To compare the slopes of both regression lines a lineal model of PSA versus EPSP was adjusted, introducing an interaction term (ANCOVA). In every case, $P<0.05$ was considered as statistically significant.

RESULTS

The weak tetanus to the PP induced an E-LTP which remained significantly different from baseline values for the first 2 h ($n=8$, Wilcoxon test; Fig. 1a, open squares). Behavioural stimulation reinforced, i.e. prolonged this potentiation to an L-LTP. Control experiments revealed that the injection of 1 μl saline into the BLA did not modify this effect (Kruskal-Wallis test; $n=6$, data not shown). Therefore, both groups were pooled together to form one behavioural reinforcement group ($n=14$, Fig. 1a, closed circles).

The injection of lidocaine into the BLA did not modify baseline potentials in the dentate gyrus evoked by stimulation of the PP ($n=5$, data not shown). Injection of lidocaine to the BLA 5 min after tetanization, did not change the time course of E-LTP ($n=8$, data not shown). However, when this protocol was combined with behavioural stimulation, i.e. drinking 15 min after induction of LTP, the inactivation of the BLA completely blocked the normally reinforcing effect of the appetitive stimulus ($n=8$, Fig. 1b, open circles). In fact, E-LTP even seemed to decay faster by inactivation of the BLA and behavioural reinforcement, and a small, but statistically significant depression was observed 5–6 h after tetanization (Wilcoxon test).

A second series of experiments investigated whether a complete electrical lesion of the BLA influenced the behavioural reinforcement of LTP in the dentate gyrus similarly to the temporal inactivation of the BLA by lidocaine. E-LTP in the dentate gyrus induced by the weak tetanus did not differ from that of non-lesioned animals ($n=9$, data not shown). However, when LTP induction was combined with behavioural stimulation ($n=6$) no reinforcement of E-LTP was detected (Fig. 2 filled squares), an effect identical to the one observed in the lidocaine treated animals (Fig. 2a, open circles). Both curves differed significantly from the one obtained in control animals after behavioural reinforcement (Fig. 1b, filled circles). Stimulation to obtain input-output curves before LTP-induction revealed that the PSA and the slope of the EPSPs were increased amongst lesioned animals. These changes were statistically significant when linear regression curves (EPSP versus PSA) were calculated using the EPSPs as independent and the PSA as dependent variables, respectively. Both curves adjust to linearity ($P>0.5$, runs test), but slopes differed significantly between lesioned and non-lesioned animals analysis of covariance (ANCOVA), $P<0.05$ (Fig. 2b). This could reflect an increased excitability within this group, though it does not seem to be an immediate consequence of the lesion because no such differences were observed in the control potentials recorded at the end of surgery.

DISCUSSION

The results obtained in intact, non-treated animals, confirm previous reports showing that behavioural stimuli with a strong motivational content were able to prolong LTP (Seidenbecher et al., 1995, 1997). The complete abolition of this effect after temporal or permanent inactivation of the BLA supports the hypothesis that this limbic structure is part of the neural system involved in the reinforcement of

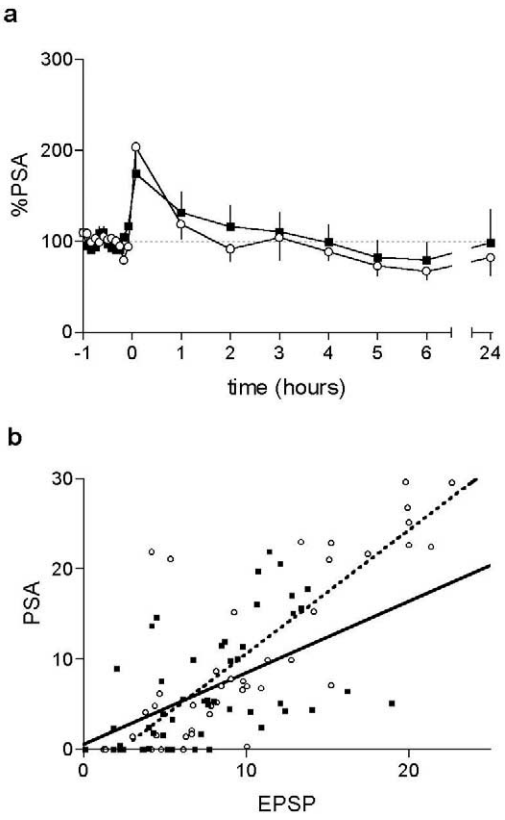

Fig. 2. (a) Lesioning the amygdala (basolateral amygdala lesioning, long-term potentiation [LTP] induction and behavioural stimulation, filled squares, $n=6$) or its temporary inhibition by lidocaine (LTP induction, lidocaine application and behavioural stimulation, open circles, $n=8$) have similar preventing effects on behavioural reinforcing of early-LTP. No differences between curves were found (Kruskal-Wallis test). (b) Lesioning the amygdala increases the slope of the input-output curve, when the slope of the field excitatory postsynaptic potential (EPSP) was plotted against the amplitude of the population spike (PSA). The adjusted regression lines (non-lesioned animals: continuous line; lesioned animals: interrupted line), and scatter plots of the data points (non-lesioned animals: filled squares; lesioned animals: open circles) are shown. The slopes of the respective lines (non-lesioned $=0.7901\pm0.1869$ ms; lesioned $=1.369\pm0.1252$ ms) differed significantly (ANCOVA).

functional plasticity in the hippocampus by motivational stimuli. However, it should be considered that both inactivation methods affect also the fibers crossing through the amygdala and therefore, a possible contribution of these fibers cannot be ruled out. On the other hand, as electrolytic lesions extended beyond the BLA to other nuclei of the amygdala, it could also be the case that the observed effects are not exclusively contributed to the BLA. As no differences were observed in the drinking or motor behaviour of the lesioned or lidocaine-injected animals with regard to controls, it is unlikely that the differences observed in the time course of LTP are caused by such unspecific factors.

The amygdala is part of a system related to emotion and motivation (Quirk et al., 1996) which modulates memory consolidation in other brain structures (Packard et al., 1994; McGaugh and Cahill, 1997). In earlier reports, we have demonstrated that comparable effects on processes of functional plasticity in the hippocampus can be obtained by direct, electrical stimulation of the amygdala (Frey et al., 2001). We hypothesise that the transition from E-LTP into L-LTP may represent a cellular correlate of memory consolidation. If so, behavioural reinforcement of LTP can represent a cellular mechanism and can be used as a model to study the effect of emotional/motivational influences on memory formation. Interestingly, both processes are dependent on protein synthesis and the induction of L-LTP as well as the transformation from E- into L-LTP requires heterosynaptic mechanisms (Frey and Morris, 1997a,b). Thus, synaptic inputs activated to induce E-LTP are different from those responsible for the reinforcement. In addition, the synapses involved seem to carry different neurotransmitters. In earlier reports, we have shown that noradrenergic and muscarinergic, but not glutamatergic receptor antagonists, prevented the reinforcing effect of direct electrical stimulation of the BLA on the granular cells of the dentate gyrus (Frey et al., 2001). In addition, we had shown that the pathway involved in the reinforcing BLA effects on dentate gyrus LTP requires the fimbria-fornix (Jas et al., 2000), a system which provides cholinergic and aminergic innervation to the hippocampus.

Furthermore, we have shown recently that aging affects both amygdala and behavioural reinforcement of LTP (Almaguer et al., 2002; Bergado et al., 2001). Interestingly the combination of E-LTP induction and behavioural reinforcement not only fails to prolong LTP amongst aged rats, but produced a depression of the PP-evoked potentials which was statistically significant beyond 3 h after tetanization. This result is similar in magnitude and the time course to the data obtained here after blocking the BLA with lidocaine. Furthermore, a depression also develops 5–6 h after BLA stimulation when the amygdala is stimulated in young animals without pairing with a PP-tetanization protocol. The mechanisms and significance of such effects remain to be investigated, but the long latency suggests the mediation of plastic mechanisms rather than a direct process of inhibition.

Finally, we found that the lesion of the amygdala increases excitability of the granule cells stimulated via the PP as shown by the changed input-output characteristics. Despite this, lesioned animals displayed normal E-LTP, which could not be reinforced by motivational stimulation. Such behaviour suggests that behavioural reinforcement and probably also that induced by the stimulation of the amygdala, is not dependent on changes in the general excitability of the target cells, but on adaptive modifications, likely at the synaptic level, mediated by intracellular signal cascades leading to the synthesis, distribution and processing of plasticity-related molecules (Frey and Morris, 1997a,b).

In summary, our findings demonstrate that the amygdala is part of a behavioural reinforcement system (Frey et al., 2001; Seidenbecher et al., 1997). This assumption is in line with the general concept of the function of this particular limbic structure as a neural substrate of emotions and motivation and their influence on learning and memory.

Our data may add further insights into the complex relationships between affective and cognitive processes, two of the most relevant abilities guiding behaviour.

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Capítulo 3: La estimulación de la amígdala refuerza los procesos de plasticidad sináptica duradera

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Este trabajo fue básicamente exploratorio. Su objetivo principal fue determinar si la estimulación eléctrica de la amígdala podía influir sobre el desarrollo de fenómenos neuroplásticos. La hipótesis que lo inspiró podría referirse como:

La amígdala es una estructura esencial en el procesamiento y establecimiento del estado afectivo(emoción) y el valor de las experiencias vividas (motivación). Sabiendo que factores afectivos pueden modular el curso temporal de la LTP y que la amígdala es parte esencial de este sistema de reforzamiento, debe ser posible lograr el mismo efecto estimulando adecuadamente la amígdala.

Los resultados mostraron que la estimulación de la amígdala en concurrencia temporal con la inducción de la LTP es capaz de prolongar el fenómeno neuroplástico, convirtiendo de hecho, una LTP temprana, con una duración inferior a 4 horas, en una LTP tardía. Esto fue cierto tanto para estímulos de alta frecuencia (200 Hz) como de baja frecuencia (1Hz) como muestran las figuras 2a, b y c, 3a y b. El efecto solo es posible dentro de una ventana temporal de ±30 min. También el reforzamiento conductual se comporta de manera similar aunque la ventana se extiende hasta una hora.

Otra similitud de la estimulación de la amígdala y el reforzamiento conductual es su dependencia de la síntesis de proteínas, como se demostró al observar el bloqueo del reforzamiento inducido por la amígdala en animales tratados con anisomicina intraventricular (fig. 6).

Se exploró además cuales neurotransmisores podrían estar involucrados en este efecto, mediante el empleo de bloqueadores específicos. El bloqueo de receptores de tipo

NMDA (usando AP-5, ver figura 4) o dopaminérgicos tipo D1 (SCH23390) no impidió el reforzamiento de la LTP por estimulación de la amígdala (fig. 5b). Esto sugiere que los sistemas glutamatérgicos y dopaminérgicos no intervienen en el mecanismo de ese reforzamiento en el giro dentado. Sin embargo el bloqueo de receptores noradrenérgicos (propranolol, fig. 5a) o colinérgicos de tipo muscarínico (atropina, fig. 5c) abolió completamente el efecto de reforzamiento lo cual sugiere que ambos neurotransmisores forman parte del mecanismo neural implicado en el efecto de reforzamiento descrito en este artículo.

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Reinforcement of Early Long-Term Potentiation (Early-LTP) in Dentate Gyrus by Stimulation of the Basolateral Amygdala: Heterosynaptic Induction Mechanisms of Late-LTP

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The basolateral amygdala (BLA) can influence distinct learning and memory formation. Hippocampal long-term potentiation (LTP), the most prominent cellular model of memory formation, can be modulated by stimulation of the BLA in its induction and early maintenance. However, it is not known how the late maintenance of LTP beyond its initial phases might be affected. Behavioral stimuli have been shown to result in a reinforcement of a transient early-LTP into a lasting potentiation. Here we show that BLA stimulation mimics the behavioral effects on early-LTP in freely moving rats when the BLA is activated within a time window of 30 min before or after tetanization of the perforant path. The reinforcement of LTP was blocked by inhibitors of muscarinergic and β-adrenergic but not dopaminergic receptors and was dependent on translation. Through these heterosynaptic associative interactions, hippocampal sensory information can be stabilized by amygdaloidal influences.

Key words: long-term potentiation; reinforcement; heterosynaptic LTP; associative LTP; late-LTP; basolateral amygdala; hippocampus; dentate gyrus

The hippocampus is important for the formation of certain kinds of memory. Although the information presented to and sent from the hippocampal network remains uncertain, hippocampal neurons exhibit a number of intriguing biophysical properties that enable them to participate in aspects of memory formation. These include mechanisms of synaptic plasticity that can respond to incoming information by detecting associative interactions between presynaptic, postsynaptic, and heterosynaptic activity and register these conjunctions as an increase in synaptic weights (Frey and Morris, 1998a). The latter process was named long-term potentiation (LTP) (Bliss and Lomo, 1973), which has become the best-studied cellular model of memory formation. Interestingly, hippocampal LTP exhibits similar temporal stages as described for certain types of hippocampus-dependent memory. An early-LTP (with a duration of ~4–6 hr) can be dissociated from late-LTP (beyond 6 hr) by inhibition of specific protein kinases, by protein synthesis, and partially by mRNA synthesis (Krug et al., 1984; Frey et al., 1988, 1996; Frey, 1997; Frey and Morris, 1997, 1998a).

In recent studies, Seidenbecher et al. (1997) reported that early-LTP in the dentate gyrus (DG) was reinforced if an appetitive or aversive stimulation was presented within 30 min after LTP induction. This reinforcement was dependent on the activation of β-adrenergic receptors. It was speculated that the consolidation of a memory trace in the hippocampal formation, as part of a more complex memory processing system, is reinforced if a modifying, most likely extra-glutamatergic input is active within a distinct time interval. Because late-LTP in the hippocampus requires similar heterosynaptic processes (Frey, 1997; Frey and Morris, 1998a), we hypothesize that the synergistic action of different transmitter systems and therefore different brain structures are needed for the induction of cellular processes leading to the long-lasting consolidation of a memory trace. However, the brain structures involved in reinforcing LTP in the DG remain unclear.

Interestingly, stimulation of the BLA, a structure thought to be part of an emotional memory system, can influence the induction and early maintenance of DG LTP (Ikegaya et al., 1995a; Akirav and Richter-Levin, 1999) by aminergic mechanisms (Ikegaya et al., 1997), whereas lesion of the amygdala attenuates DG LTP (Ikegaya et al., 1994). These results led us to investigate whether the maintenance of DG LTP can also be modulated by BLA stimulation. Here, we have studied the effect of BLA stimulation on the early-LTP in DG. If LTP subserves cellular mechanisms during declarative learning, then a hypothetical heterosynaptic associative LTP reinforcement by BLA stimulation could be of special importance. Behavioral experiments by others (for review, see Cahill and McGaugh, 1998) have shown similar processes with respect to emotional arousal and the maintenance of declarative memory. Electrophysiological studies on the induction and early transient stages of LTP in DG (Ikegaya et al., 1995a,b, 1997; Akirav and Richter-Levin, 1999) revealed modulatory effects of the BLA on hippocampal function, but it is not known whether protein synthesis-dependent late LTP is also affected, a prerequisite for long-lasting memory traces to be formed. Interestingly, it has been shown previously that hippocampus-dependent long-term memory processes are influenced by the amygdala (Bevilaqua et al., 1997; Bianchin et al., 1999; Izquierdo et al., 1999). Here

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we report that early-LTP in the DG *in vivo* can be influenced in its maintenance by stimulation of the BLA.

MATERIALS AND METHODS

Subjects and surgery. Experiments were performed on 8-week-old Wistar rats (200–250 gm). All experiments were performed in compliance with the relevant laws and institutional guidelines and have been approved by the Land Sachsen-Anhalt.

For chronical implantation of electrodes, the animals were anesthetized with pentobarbital [40 mg/kg, i.e., 40 mg was dissolved in 10 ml saline and 1 ml/100 gm was injected intraperitoneally as an initial dose, supplemented by 0.3 (1.2 mg)–0.5 ml (2 mg) intraperitoneal injections as necessary] and placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA). The scalp was incised and retracted, and head position was adjusted to place bregma 1 mm higher than lambda. Small holes were drilled in the skull for the placement of stimulating and recording electrodes. The electrodes consisted of insulated stainless steel wires 125 μm in diameter. A monopolar recording electrode was placed in the DG–granular cell layer [coordinates −2.8 mm posterior to bregma (AP), 1.8 mm lateral to midline (L), 3.2–3.5 mm ventral from dura (V); coordinates from the atlas of Paxinos and Watson (1998); a bipolar stimulating electrode was implanted in the medial perforant pathway (AP −6.9 mm, L 4.1 mm, V 2.4–2.7 mm)]. For stimulating the amygdala, a monopolar electrode was placed into the basolateral amygdala (AP −3.5 mm, L 5.0 mm, V 7.6 mm) with an indifferent electrode consisting of silver bar wire lowered on dura anterior to the stimulating electrode. The electrodes were adjusted to optimize the population spike (PS) amplitude in the perforant path–DG system. All rats were allowed 8–10 d recovery after surgery before the electrophysiological experiments in freely moving animals. The positioning of electrodes was checked in each animal histologically after the end of the experiment, and only those animals with a correct positioning of the electrodes were included in further analyses [interestingly, no effects of DG LTP were detected in experiments in which the histological confirmation of electrode positioning afterward revealed a placement of the amygdala-stimulating electrode in the central instead of the basolateral nucleus (data not shown)].

Recording. All electrophysiological recordings were performed in special experimental boxes, where animals were connected by a flexible cable to a 10-channel swivel that allowed them to move freely with *ad libitum* access to water and food (Frey et al., 1996; Seidenbecher et al., 1997). Biphasic current pulses (0.1 msec per cycle, 150–250 μA) were applied to the perforant path to evoke extracellular field potentials in the DG of ~40% of the maximal PS. PS recording and analysis were favored against the slope of field EPSPs because the latter is relatively unstable in the hilar region of the dentate gyrus in freely moving animals, especially if taken into consideration that the stimulation intensity was adjusted to obtain a population spike that influenced strongly the dipole of the field EPSP in the hilus. The spike, however, is required to induce LTP. A few experiments showed a reasonable, larger field EPSP that could be used for calculations, and we provide representative examples of the time course of the field EPSP for crucial experiments below. Analyses of these experiments revealed similar results as PS measurements, suggesting that the recorded and analyzed PS is not just a measure of changes in excitability but also represents adequate synaptic function. This is supported by the fact that, to our knowledge, LTP has never been reported to be associated with changes of excitability. The basolateral amygdala was stimulated by impulses with an intensity of standardized 300 μA independent of the stimulation protocol (biphasic constant current pulses, 0.2 msec duration per polarity). This stimulation intensity evoked an average BLA DG potential as shown in Figure 1*a*.

After a stable baseline was recorded for at least 30 min (recordings every 5 min), an "unsaturated" LTP was induced by three bursts of 15 impulses, 200 Hz, 0.2 msec pulse width each stimulus, interburst interval 10 sec ("weak tetanus"), resulting in a potentiation that decayed within 4–7 hr to pretetanus value. In the series with late-LTP, tetanization consisted of 20 bursts of 15 impulses, 200 Hz, 0.2 msec pulse width each stimulus, interburst interval 10 sec ("strong tetanus"). This stimulation paradigm resulted in late-LTP with a duration of 8 hr, the longest time point we have investigated. Averaged responses were recorded every 15 min for up to 8 hr after tetanization.

For estimation of the time window for "reinforcement" of the unsaturated DG LTP by stimulation of the basolateral amygdala, the following stimulating protocols were used. At various time points (5, 15, and 30 min) before or after tetanization of the perforant path, the basolateral amygdala was stimulated by high frequency [three bursts of 15 impulses, 200 Hz, 0.2 msec pulse width each stimulus, interburst interval 10 sec (weak tetanus) at 300 μA] or low frequency (45 impulses at 0.1 Hz, 0.2 msec pulse width, each stimulus at 300 μA).

Pharmacology. Substances were applied intraventricularly through chronically implanted cannulas [anterior horn of the right lateral ventricle, for detail, see Seidenbecher et al. (1997)]. Propranolol (6.76 nmol), SCH 23390 (3.08 nmol), and atropine (1 nmol) (Sigma, St. Louis, MO) were injected 5 min after tetanization of the PP, that is, 10 min before BLA tetanization. The selected doses of propranolol and SCH 23390 have been shown previously to be effective (Balschun et al., 1997; Seidenbecher et al., 1997). The used concentration of atropine was able to inhibit oxotremorine-induced hippocampal theta EEG activity (Malisch and Ott, 1982) (intraperitoneal application of 0.2 mg/kg oxotremorine sesquifumarate; data not shown). Application of the drugs 5 min (propranolol, SCH 23390, atropine) or 10 min (AP-5, 100 nmol, from RBI) after perforant path (PP) tetanization (control experiments without stimulation of the BLA) did not influence the time course of early LTP [for propranolol, see Seidenbecher et al. (1997); other data not shown]. All of the above substances had no effect on baseline evoked potentials nor on early-LTP, with the exception of AP-5, which blocked early LTP when applied before PP tetanization (Fig. 4*a*). The latter result confirms earlier results that compounds with similar biophysical properties can diffuse within 5 min to their place of action, i.e., from the ventricle to the dentate gyrus. Anisomycin (0.905 mol; ICN Biochemicals, Costa Mesa, CA) was injected 2 hr before the PP was tetanized. To avoid possible nonspecific side effects of the presence of the reversible protein synthesis inhibitor anisomycin after intracerebroventricular injection on recorded control field potentials in the DG, the time point was first determined at which anisomycin was still effective in inhibiting protein synthesis when applied sufficiently before induction of LTP. This was achieved by measuring the incorporation of radioactive-labeled amino acids into hippocampal proteins. It was found that anisomycin inhibited the incorporation of amino acids into hippocampal proteins by >90% for at least 2 hr after its application (data not shown), the time at which LTP was induced. In the series with anisomycin and LTP induction, only those experiments with normal post-tetanic potentiation were used for statistical evaluation. In all cases the injection was performed at 1 μl/min to a total volume of 5 μl.

Statistics. Data analyzed here are from non-Gaussian populations but show near identical shapes of distributions. Therefore, nonparametric tests were performed. Within-group comparisons were made using the Wilcoxon test for paired samples. For comparisons between groups the Mann–Whitney *U* test was used after performing the Kruskal–Wallis test for the different groups. Differences were considered statistically significant only when $p < 0.05$ in Kruskal–Wallis and the post test. For clarity when comparing data, the mean of percentage change of the PS amplitude measured in millivolts ± SEM is shown.

RESULTS

Our studies revealed that low-frequency control stimulation of the perforant path or the BLA did not dramatically influence DG potentials (Fig. 1). Weak tetanization of the perforant path resulted only in early-LTP decaying to baseline values within 8 hr (Fig. 1*b*, ●). Strong tetanization, in contrast, revealed late-LTP with a duration of at least 8 hr, the latest time point we have investigated (Fig. 1*b*, ○).

BLA stimulation before and after tetanization of DG

We then investigated whether stimulation of the BLA before, simultaneously with, or after tetanization of the perforant pathway influences early-LTP in the DG. As shown in Figure 2*a* (●), the duration of LTP in the DG, induced by a tetanization protocol that normally would lead only to early-LTP, can be influenced by a subsequent high-frequency stimulation of the BLA applied 15 min after the tetanization of the perforant path. Although short-term potentiation (STP) (<1 hr) was not influenced, the maintenance of LTP was changed significantly. The transient time course of LTP induced by weak tetanization of the perforant path was transformed (or reinforced) to a long-lasting potentiation with a duration of at least 8 hr. As mentioned earlier, for technical reasons the DG LTP reinforcement by BLA stimulation

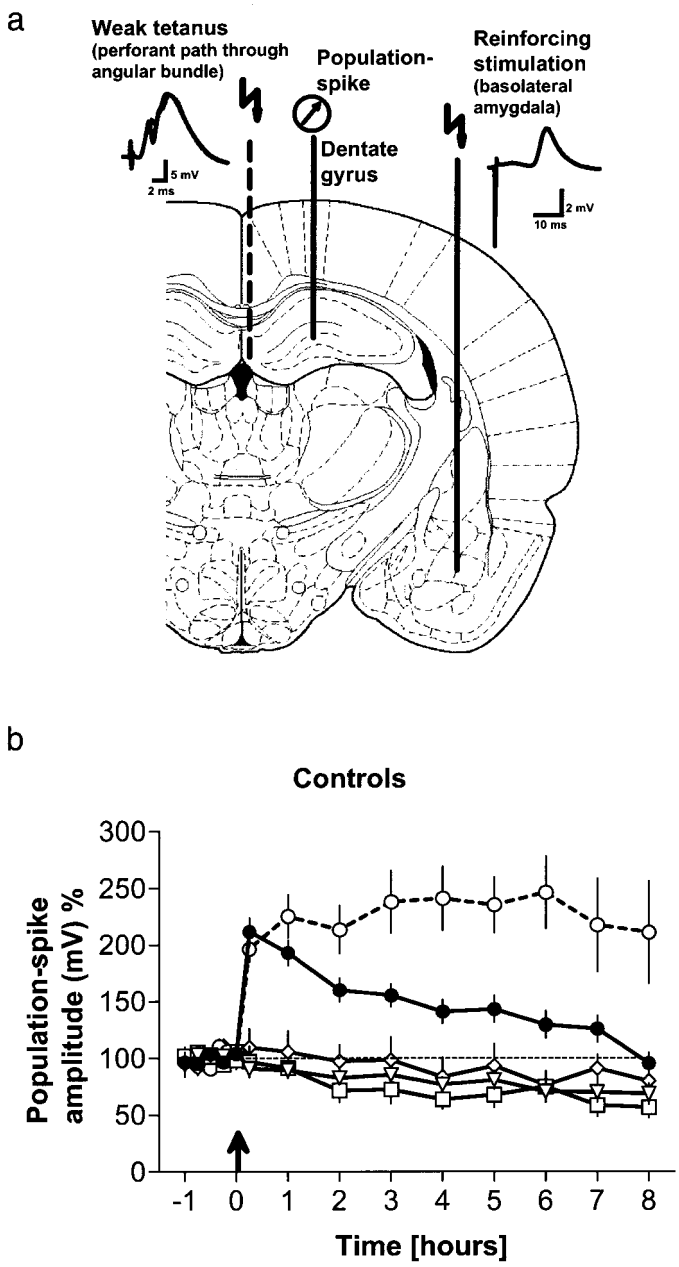

Figure 1. Reinforcement of hippocampal early-LTP by stimulation of the basolateral nucleus of the amygdala in freely moving rats. *a*, Schematic illustration of electrode localization. For clarity, in this section the DG stimulation electrode is shown activating the perforant pathway (*broken line*). Originally, this electrode was positioned in the angular bundle (see Materials and Methods). *Insets* show analog examples of recordings obtained before (*dotted line*) and after (*filled line*) LTP induction of the perforant path (*top left*) and after stimulation of the BLA (*top right*). *b*, Control recordings: induction of early-LTP (●, $n = 18$) or late-LTP (○, $n = 5$) by a weak or strong tetanus of the perforant path, respectively, or tetanization of the BLA (▽, $n = 11$), or after application of 45 impulses at an LFS in the BLA (□, $n = 11$), and finally after LFS of the perforant path alone (◇, $n = 6$). Low-frequency stimulation of the perforant path did not severely influence baseline potentials for the investigated 8 hr (Fig. 1*b*, ◇). A statistically significant difference was detected only at 6 hr after low-frequency stimulation when compared with prestimulation values (Wilcoxon test, $p < 0.05$). High-frequency (Fig. 1*b*, ▽) or low-frequency stimulation (Fig. 1*b*, □) of the BLA resulted in a slowly developing long-term depression at the DG synapses. At 2 hr a statistically significant depression of the initial baseline potentials was observed [82.9 ± 4.18% (millivolts; percentage change ± SEM) in the group with a high-frequency train to the amygdala and 71.6 ± 10.24% in the group with low-frequency stimulation, respectively] that remained at this level until the end of the experiment (Wilcoxon test, $p < 0.05$). However, statistical comparison of the BLA-stimulated control series revealed no significant difference (Mann–Whitney *U* test) with the exception at 7 hr (low-frequency perforant path control vs low-frequency BLA stimulation). The *arrow* indicates the time point of weak or strong tetanization or LFS, respectively.

was measured as percentage changes of the PS amplitude in general. However, the EPSP was similarly affected, as confirmed by the following example: the level of EPSP potentiation was 121.1% at 1 hr after tetanization and 84.7% at 8 hr after tetanization of the DG alone versus 119.2 and 124.6% after DG tetanization followed by BLA stimulation 15 min later.

Similar results were obtained when a low-frequency stimulation (LFS) pattern instead of a weak high-frequency train was applied to the BLA (Fig. 2*b*). Late-LTP induced by a strong tetanization protocol was not influenced by BLA stimulation 15 min after LTP induction (Fig. 2*c*, ■).

A series of experiments was then conducted exploring a possible time window in which the stimulation of the BLA and perforant pathway results in long-lasting associative plastic changes. Figure 3 summarizes the amount of potentiation obtained in the DG 15 min (Fig. 3*a*) and 8 hr (Fig. 3*b*) after tetanization of the perforant pathway when the BLA was stimulated either (1) before, (2) at the same time as, or (3) after tetanization of the perforant path. The time interval between stimulation of the two structures varied from 5 to 15 to 30 min. As shown in Figure 3*a*, the potentiation was transiently enhanced 15 min after LTP induction when either the BLA and perforant path stimulation were applied simultaneously or the BLA stimulation preceded perforant path tetanization.

Figure 3*b* shows the effect of BLA stimulation on the maintenance of LTP induced by a weak tetanus (after 8 hr). Simultaneous stimulation of the BLA and perforant path did not influence the time course of a weak potentiation in the DG. BLA tetanization 5 or 15 min after a weak tetanization of the perforant path resulted in a reinforced potentiation that was now long lasting (8 hr). Similar results were obtained when the BLA was tetanized at 5 and 15 but also at 30 min before perforant path tetanization, although with a decaying degree of reinforcement. The time course of the field EPSP paralleled the PS as shown by the following examples: the level of EPSP potentiation at the 15 min interval between BLA and DG tetanization ($n = 3$) was 116.7 ± 1.33% at 1 hr and 113.5 ± 3.47% at 8 hr after LTP induction; the 30 min interval ($n = 3$) results were 125.9 ± 4.09% at 1 hr versus 94.5 ± 9.47% at 8 hr after LTP induction. We do not know whether an increase in the time interval between BLA and perforant path tetanization beyond 30 min results in a reinforced long-lasting potentiation. Future experiments should be conducted to investigate this time window more thoroughly.

Involved transmitter systems

The next series of experiments was conducted to elaborate which transmitter systems are involved in the reinforcing effect of amygdala stimulation on early LTP in the DG. A number of transmitters, including dopamine, norepinephrine, acetylcholine, and opioids, are known to modulate LTP (Dunwiddie et al., 1982; Bliss et al., 1983; Krug et al., 1983; Stanton and Sarvey, 1985). We showed earlier (for review, see Frey, 1997; Frey and Morris, 1998a) that late-LTP requires the heterosynaptic activation of nonglutamatergic receptors during tetanization. In a control ex-

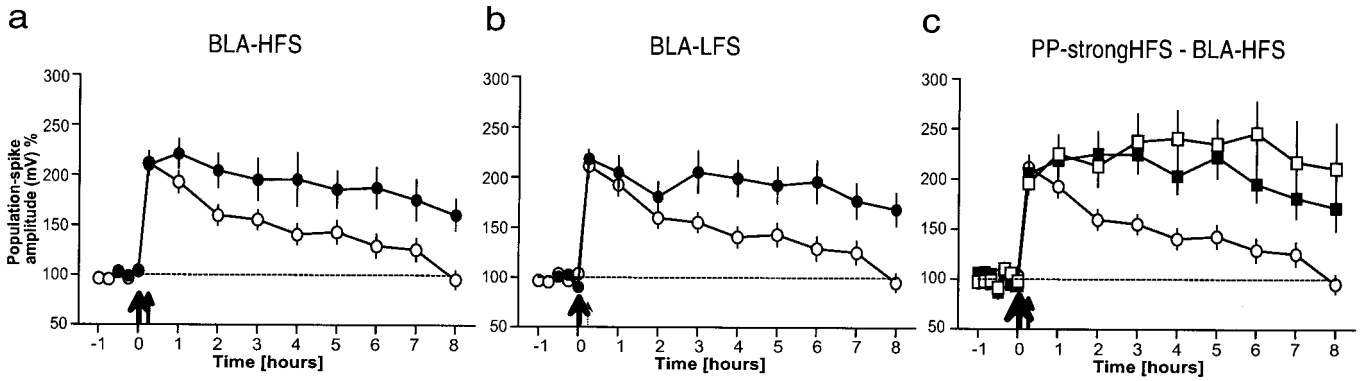

Figure 2. Reinforcement of early- but not late-LTP by high- and low-frequency BLA stimulation 15 min after tetanization of the DG. Reinforcement of early-LTP when the BLA was stimulated with either a high-frequency train *(a*, *thin arrow)* or a low-frequency stimulation *(b*, *dotted arrow)* 15 min after weak tetanization (*thick arrow*) of the DG (●, $n = 11$ for each series) is shown. *Open circles* indicate the time course of early-LTP when induced in the DG without BLA pairing ($n = 18$). LTP in the reinforced group was statistically significantly different from the nonpaired group from the second hour onward after tetanization of the DG (Mann–Whitney *U* test). *c* represents no influence on late-LTP when the BLA was tetanized 15 min after LTP induction in the perforant path DG by a strong tetanization protocol [□ represents a control late-LTP series of the DG alone; $n = 5$; ■ shows strong tetanization of DG (*larger arrow*) followed by BLA tetanization (*thin arrow*) 15 min after its induction; $n = 5$]. Strong DG LTP (□) and strong DG BLA LTP (■) were statistically significantly different from weak LTP (○) from the second hour onward (Mann–Whitney *U* test).

periment it was now investigated whether a direct, additional glutamatergic activation initiated by BLA stimulation was sufficient and responsible for the reinforcement of early LTP in the DG or whether additional transmitter systems are required. The possibility that subsequent tetanization of glutamatergic pathways can result in a greater level of potentiation attributable to a stronger depolarization and subsequent activation of NMDA receptors leads one to assume that the described reinforcement was triggered by such a mechanism (i.e., saturation of LTP by repeated tetanization of glutamatergic afferents). The NMDA receptor antagonist AP-5 was therefore applied intracerebroventricularly after tetanization of the perforant path. If AP-5 was injected 5 min after PP tetanus, the initial level of potentiation (late time points of post-tetanic potentiation) was influenced (data not shown). Therefore, AP-5 was injected 10 min after PP tetanus, i.e., 5 min before BLA stimulation, which was sufficient for the drug to perfuse to the DG. Control experiments revealed that AP-5 blocked LTP induction in the DG when applied intracerebroventricularly 5 min before tetanization (Fig. 4*a*). As shown in Figure 4*b*, the NMDA receptor antagonist did not influence the time course of reinforced LTP.

Figure 5 illustrates the action of the other tested receptor blockers on the reinforced potentiation. As shown in Figure 5, *a* and *c*, only the β-adrenergic receptor antagonist propranolol and the muscarinergic antagonist atropine, but not the dopaminergic D1 receptor antagonist SCH 23390 (Fig. 5*b*), were effective in blocking the reinforced potentiation. Similar time courses were obtained in experiments with measurable field EPSPs [e.g., propranolol (single experiment): 117.1% at 1 hr vs 90.7% at 8 hr after LTP induction; and atropine ($n = 2$): 116.5 ± 4.40% vs 91.1 ± 5.55%]. When one of the two blockers was delivered into the ventricle after LTP induction in the DG, but 10 min before tetanization of the BLA, only early-LTP was seen, as was the case in the control experiments with weak tetanization of the perforant path alone.

Protein synthesis dependence of reinforcement

A last series of experiments investigated whether the reinforcement of early-LTP was accompanied by protein synthesis (Fig. 6). The protein synthesis inhibitor anisomycin was applied 2 hr before tetanization to avoid possible effects on LTP induction (Fig. 6*a*). Under these conditions, early-LTP by weak tetanization of the perforant path could be induced, although the duration was shorter when compared with control early-LTP in nontreated groups (indicating a distinct requirement of protein synthesis during early-LTP). The reinforcing effect on early-LTP by subsequent BLA stimulation was prevented by anisomycin, suggesting a requirement of protein synthesis for the transformation from early- to late-LTP.

DISCUSSION

In summary, it was shown that only a weak transient form of LTP is affected by BLA stimulation in the intact animal. Induction of late-LTP by a strong tetanus in the DG is not influenced in either its induction or its maintenance, at least during the 8 hr after tetanization that we have investigated (Fig. 2*c*). Heterosynaptic, late associative effects on early-LTP occur when the amygdala is stimulated within a distinct time interval, before or after induction of LTP in the DG. Our pharmacological experiments using the NMDA receptor inhibitor AP-5 revealed that BLA stimulation does not interfere with the reinforcing effects in DG via a direct glutamatergic innervation, as would be expected during saturation experiments in which the same glutamatergic inputs are tetanized subsequently until an asymptotic level of potentiation is achieved (Barnes et al., 1994; Moser et al., 1998). However, direct activation of AMPA receptors in the DG by BLA stimulation cannot be excluded. The subsequent depolarization of granular cells and activation of voltage-dependent calcium channels therefore could be involved in the processes, resulting in a reinforced potentiation. However, the absence of morphological data describing a direct innervation of the DG by BLA makes this assumption unlikely (Pikkarainen et al., 1999).

We have proposed recently that consolidation of hippocampal LTP requires the synergistic activation of both glutamatergic inputs and an additional modulating transmitter system during the induction of LTP, during which coactivation of the latter is necessary to trigger the synthesis of plasticity-related proteins (for review, see Frey and Morris, 1998a). This is a prerequisite for the formation of late-LTP, i.e., its consolidation. Artificial

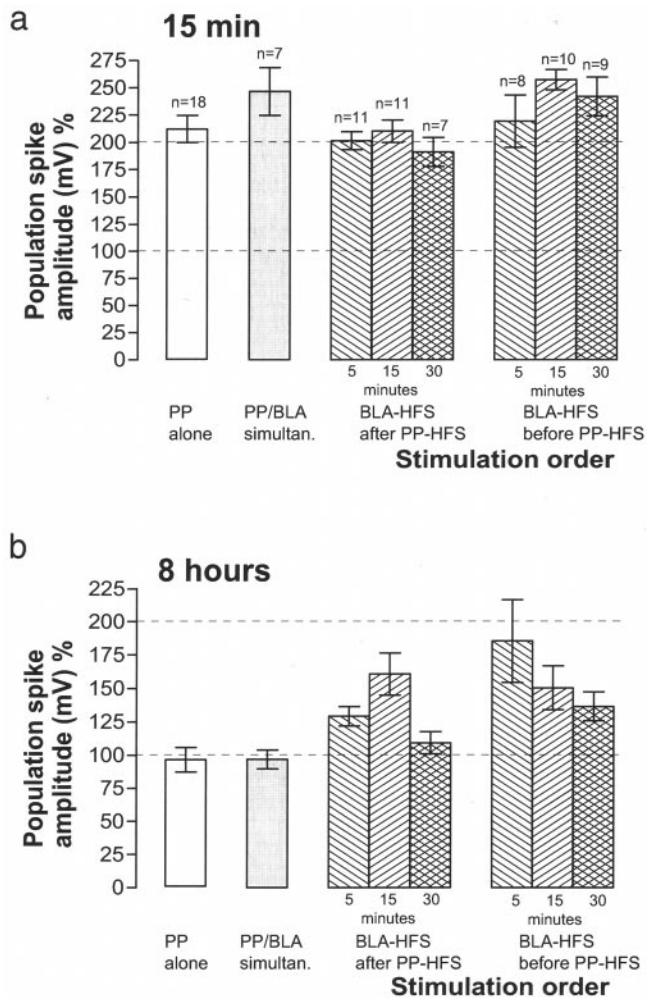

Figure 3. Time window of LTP reinforcement of DG LTP by BLA stimulation. Level of potentiation 15 min (*a*) and 8 hr (*b*) after induction of early-LTP in DG to illustrate the effect of BLA stimulation on the induction of DG LTP (*a*) and maintenance (*b*). Only the series of simultaneous stimulation of the BLA and DG and the series during which the BLA was stimulated 15 min before perforant path tetanization showed a statistically significant enhanced STP measured 15 min after LTP induction (*a*). The potentiation at 8 hr (*b*) returned to pretetanization levels when the perforant pathway was tetanized alone or simultaneously with BLA, but not when the BLA was stimulated within 15 min before or after LTP induction in DG. In the series during which LTP was induced in the DG after BLA stimulation with a 30 min interval, a remaining statistically significant potentiation was still observed, which is probably attributable to the initial effect on STP by BLA stimulation.

electrical field stimulation in the brain may lead to late LTP if the stimulus is strong enough to reach sufficient heterosynaptic inputs, as could be the case for the series with strong tetanization that is shown here. More subtle stimulation may involve homosynaptic inputs that initiate only early-LTP if not preceded or followed by activation of an additional input of another transmitter system. However, the latter protocol seems to be the more physiological way of neuronal functioning.

Early-LTP in the DG in the intact animal can be transformed into protein synthesis-dependent late-LTP by heterosynaptic and associative mechanisms when both the perforant pathway and the basolateral nucleus of the amygdala are stimulated within a time window of ~30 min. The order of "paired" stimulation is not important, i.e., whether tetanization of the DG or electrical stimulation of the BLA occurred first, and it is not important whether a high-frequency train or a low-frequency stimulation was delivered to the BLA. With respect to the order of stimulation, our data seem to be in contrast to earlier findings in which DG LTP was reinforced by behavioral stimuli only if the latter was presented after tetanization (Seidenbecher et al., 1997). A possible explanation could be that the behavioral and aversive stimuli used in these experiments involve the serial and parallel interaction of more complex structures at different times than the artificial direct stimulation of the BLA at a given time. However, regarding the order of stimulation, our results are in accordance with the properties of "synaptic tagging" (Frey and Morris, 1998b) (see below).

BLA action on DG LTP is neither direct (Pikkarainen et al., 1999) nor mediated by NMDA receptor stimulation but triggered through brain structures carrying norepinephrine or acetylcholine, or both, but not dopamine. However, because the receptor blockers were applied intraventricularly, a direct action of noradrenergic or muscarinergic processes, or both, in the BLA cannot be excluded. Therefore, the reinforcement of DG LTP by BLA stimulation can also involve additional mechanisms such as the action of "modulated" BLA stimulation on different receptor systems and the BLA-dependent regulation of stress hormones required for normal hippocampal function (Brinton and McEwen, 1989; Cahill and McGaugh, 1998; Ferry et al., 1999). This would resemble findings in which hippocampus-associated learning is strongly influenced by BLA modulation (Packard et al., 1994; Cahill et al., 1995; Cahill and McGaugh, 1998).

Questions remain such as why simultaneous tetanization of the DG and BLA does not influence the maintenance of early-LTP (data not shown). It can be speculated that the effect of BLA stimulation on DG LTP is triggered by synergistic actions of glutamatergic and nonglutamatergic mechanisms that require a sequence of processes to be activated. It is not important which of the systems was activated first, but a simultaneous activation prevents the induction of events leading to the reinforcement of LTP. Heterosynaptic, nonglutamatergic receptor activation during tetanization may negatively influence the required level of depolarization for late-LTP to occur [e.g., BLA-stimulated norepinephrine release activates hippocampal interneurons (Bergles et al., 1996)]. Another possibility might be that simultaneous heterosynaptic, i.e., glutamatergic and β-adrenergic, receptor activation in the dentate gyrus cannot sufficiently shift processes such as intracellular calcium transients (Stanton and Heinemann, 1986; Gray and Johnston, 1987) required for late-LTP. The different regulation of calcium may then influence the balance between activated kinases and phosphatases in favor of short-lasting plastic events (Coussens and Teyler, 1996).

A 5 min delay of subsequent stimulation of the two brain structures, however, revealed reinforced LTP. This could have been achieved by heterosynaptic stimulation of the cAMP/PKA cascade, a prerequisite for late-LTP to occur (Frey et al., 1993), or by hormone-dependent regulation of plasticity-relevant proteins in the hippocampus through cAMP/PKA-dependent processes initiated either in the hippocampus (Bevilaqua et al., 1997) or indirectly in the BLA (Frey and Morris, 1998a). In addition, it cannot be ruled out that other brain structures directly interact with the granular cells in the DG because the BLA does not directly innervate the DG granular cells (Pikkarainen et al., 1999). BLA activation requires the additional stimulation of as yet unidentified structures directly connected with the DG by adrenergic or muscarinergic fiber systems or influences the level

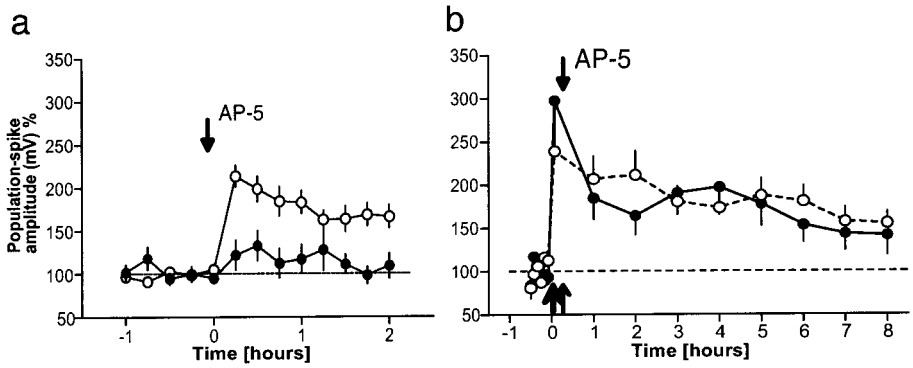

Figure 4. NMDA receptor activation for BLA-reinforced DG LTP is not required. The protocol with a 15 min time interval between perforant path and BLA tetanization resulted in a stable reinforced potentiation of DG LTP and therefore was chosen for the pharmacological studies (Figs. 4*b*, 5, ○). This figure illustrates that the NMDA receptor antagonist AP-5 is effective in blocking early-LTP if applied intracerebroventricularly 5 min (*thin arrow above*) before tetanization of the DG (●, drug-treated group, $n = 6$; ○, induction of control early-LTP, $n = 15$) (*a*) and ineffective in influencing the BLA-reinforced DG LTP [●, $n = 7$; the drug (*thin arrow above*) was applied intracerebroventricularly 5 min before BLA stimulation (*thin arrow below*)] (*b*). As a control, BLA-reinforced DG LTP, a series was performed in which physiological NaCl was applied instead of AP-5 (○, $n = 9$). Other symbols are denoted as in Figure 2.

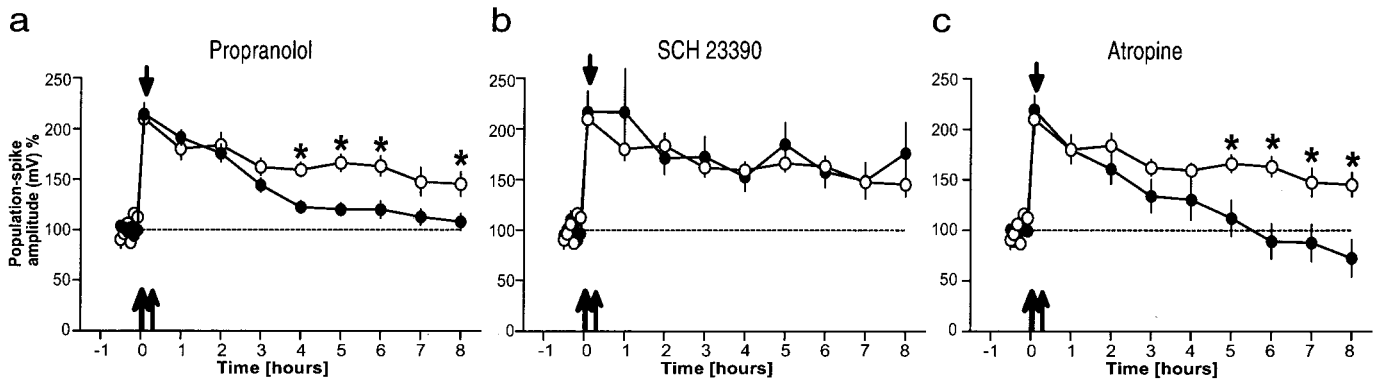

Figure 5. Pharmacology of BLA-reinforced DG LTP. Shown is the effect of the β-adrenergic-receptor antagonist propranolol (●, $n = 13$) (*a*), the dopaminergic D1 receptor antagonist SCH23390 ($n = 6$) (*b*), and the muscarinergic receptor antagonist atropine ($n = 8$) (*c*) on BLA-reinforced DG LTP. *Asterisks* illustrate a statistically significant difference of the drug-treated group when compared with NaCl controls (○, $n = 9$; Mann–Whitney *U* test). Other symbols are denoted as in Figure 4.

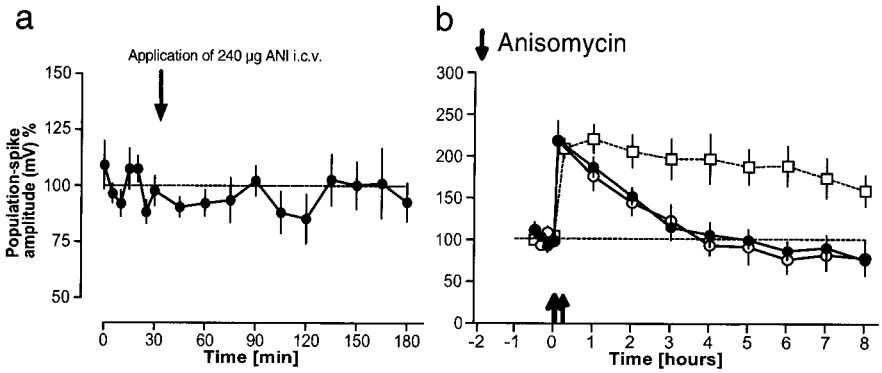

Figure 6. Protein synthesis and BLA-reinforced DG LTP. *a*, The effects of intracerebroventricular application of 240 μg of anisomycin on DG potentials are shown ($n = 7$). *b*, The reversible protein synthesis inhibitor anisomycin prevents the BLA reinforcement of DG LTP when applied intracerebroventricularly 2 hr before LTP induction (●, $n = 7$). The drug did not affect a control early-LTP that was not paired with BLA stimulation (○, $n = 6$) illustrating a specific effect of anisomycin on the reinforcing effect. *Squares* indicate a control series with reinforced LTP ($n = 10$). Other symbols are denoted as in Figure 4.

of stress hormones, which may then finally modulate neuronal plasticity in the DG.

Interestingly, our studies revealed a model to study early and late associative components of long-lasting plastic changes with an interaction of heterosynaptic components within the minute or even hour range, respectively. Further studies will determine the key players and the locus of action of BLA-dependent reinforcement of DG LTP.

The described time window, the protein synthesis dependence of the reinforcement, and the heterosynaptic associative components of these processes lead us to speculate that the described effects might be related to a phenomenon that we have recently described as synaptic tagging (Frey and Morris, 1997, 1998a). Synaptic tagging characterizes a late associative property of LTP, which requires the transient setting of a synaptic tag with the function of capturing and processing plasticity-related proteins, thus facilitating consolidation, from a short-term into a long-lasting synaptic plastic change. Interestingly, it has been shown that under distinct circumstances the setting of the tag is sufficient to result in late-LTP at that input, if a heterosynaptic input was stimulated within a specific time window.

Considering the proposed role of LTP in information processing and the described interaction of the hippocampus and amygdala during distinct learning tasks (LeDoux, 1993; Packard et al., 1994; Cahill et al., 1995; Izquierdo and Medina, 1997; Cahill and McGaugh, 1998; Roozendaal et al., 1999), the data presented

here may provide a hint for more detailed investigation of interstructural, associative interactions at the cellular level. We have shown that BLA stimulation can modulate hippocampus-specific long-lasting plasticity beyond its induction and early maintenance. However, future studies will show whether cellular consolidation under these conditions exceeds the investigated 8 hr. Our data support the hypothesis that describes the amygdala as a structure involved in the formation/modulation of declarative memory in other brain structures that might be related to emotionally arousing events (for review, see Cahill and McGaugh, 1998). The investigation and description of structures and processes that are functionally correlated may illuminate interneuronal mechanisms required for long-lasting plastic changes and the formation of declarative memory.

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Capítulo 4: La lesion de la fimbria-fornix afecta el reforzamiento de la plasticidad sináptica duradera inducido por estimulación de la amígdala

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La demostración del efecto reforzador de la estimulación de la amígdala basolateral sobre la LTP en el giro dentado plantea una interrogante acerca de cuáles estructuras pueden mediar dicho efecto, teniendo en cuenta que no se conocen proyecciones anatómicas directas entre ambas estructuras.

Existen dos posibles rutas principales: una a través de la proyección colinérgica del septo medial hacia el cuál la amígdala envía fibras excitadoras. Otra, por la vía de la corteza entorrinal y su proyección excitadora glutamatérgica a la formación del hipocampo.

El efecto farmacológico de bloqueo de la acción reforzadora de la amígdala por antagonistas de receptores muscarínicos, es un argumento a favor de una mediación por la vía septal. Nuestra hipótesis en este trabajo era la siguiente:

Si la proyección amígdala- septum-giro dentado es parte esencial del sistema neural implicado en el reforzamiento por estimulación, entonces la interrupción de esta vía por lesión de la fimbria-fornix debe bloquear dicho efecto.

Este trabajo a diferencia del resto, se realizó en animales anestesiados. La estimulación eléctrica de la amígdala basolateral se aplicó 15 minutos antes de inducir la LTP en la sinapsis vía perforante-giro dentado. En los animales con lesión de fimbria-fornix la inducción de la LTP no se vio alterada, pero si su mantenimiento, lo cuál apoya la hipótesis planteada.

Este resultado demuestra la importancia de la proyección fimbria-fornix como mediadora en el reforzamiento de procesos neuroplásticos por estimulación de la amígdala. Aunque

la proyección colinérgica septo-hipocampal alcanza el giro dentado por la fimbria-fornix, esto no significa que sea esa, y mucho menos solo esa, la proyección implicada en el efecto estudiado. A través de la fimbria-fornix llegan al giro dentado muchas otras fibras de origen subcortical, como la noradrenérgicas del locus coeruleus o serotonérgicas del núcleo del rafe.

El resultado tampoco excluye que la vía alternativa amígdala-corteza entorrinal-hipocampo tenga alguna participación en los procesos estudiados.

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Short communication

Lesioning the fimbria–fornix impairs basolateral amygdala induced reinforcement of LTP in the dentate gyrus

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Abstract

We have recently shown that early-long-term potentiation (LTP) in the dentate gyrus can be reinforced into late LTP by stimulation of the basolateral nucleus of the amygdala [Frey et al., submitted for publication]. The pathways and mechanisms for such interactions are unclear, considering that no direct projection from the amygdala to the dentate gyrus is known. To ascertain the possible mediation of the septo-hippocampal projection we have transected the fimbria–fornix (FF) fiber system in young adult (2 months) male rats. The electrophysiological evaluation a week later showed that the lesion does not modify the effects of pre-stimulation of the basolateral amygdala (BLA) on the induction of LTP at the perforant pathway (PP)-granule cells synapses, but impairs its maintenance 1 h later. This suggests that two different pathways might mediate different aspects of the amygdala–hippocampal interactions. One seemed to be anatomically independent from the FF and might influence LTP induction; while the second, probably through the septo-hippocampal fornical projection appeared important for LTP maintenance.

Keywords: Synaptic plasticity; Memory; Hippocampus; Limbic system; Rat

The amygdala and the hippocampus are limbic structures, both involved in memory functions [15]. While the hippocampus is considered a key structure for cognitive processes [24], the amygdala appears primarily related to emotions [18]. A plausible hypothesis is that amygdala acts on memory mediating the reinforcing effects of motivational/emotional stimuli temporarily associated with learning [23]. However, the cellular mechanisms, which may sustain such interactions, are poorly understood.

Hippocampal long-term potentiation (LTP) has been considered a cellular correlate of memory at synaptic level [4,16,22], and there are some evidence that amygdala might contribute to the induction of LTP in the hippocampal formation [11,12,14,27]. Behavioral stimuli with a strong motivational/emotional component have shown able to improve the maintenance of LTP [25], suggesting that the amygdala influence might also extend to later LTP phases.

Recently, we have shown that induction of an early-LTP, with a duration of about 4 h, can be converted into a protein-synthesis-dependent late-LTP if the basolateral amygdala (BLA) was stimulated within a distinct time window during LTP-induction (Frey et al., submitted for publication).

However, the anatomical pathways through which such functional interaction might occur, are unclear considering that no direct communication between the amygdala and the hippocampus [13,19] has yet been described. Two alternative pathways appear as candidates to mediate the proposed interaction: the entorhinal cortex–perforant pathway and the septum–fimbria–fornix system. There are both anatomical and electrophysiological evidence of a monosynaptic projection from the amygdala to each of these relay structures [6,7,19], but which of them could be responsible for mediating the BLA-induced reinforcement of LTP remains to be established.

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We have tried to approach this question using the fimbria–fornix (FF) lesion, a paradigm widely used as model for disconnecting the septal projections to the hippocampus [5], to determine whether the interruption of this pathway modifies the effects of BLA on hippocampal LTP.

Lesion of the FF was carried out on 2-month-old male Sprague–Dawley rats at the beginning of the experiments. Under chloral hydrate narcose (420 mg/kg, i.p.) a stereotactically guided bilateral knife transection at the coordinates AP: −1.4 mm, ML: ±0.8 to ±3.1 mm and DV: 5.0 mm was carried out. Control animals received the same treatment but no knife was inserted and no transection was performed. This procedure significantly reduce spatial learning in the Morris water maze and eliminates completely the septal cholinergic innervation to the hippocampus, as shown by immunochemistry for choline esterase [2]. Though all the electrophysiological studies were done in the right hemisphere, lesions were made bilaterally to prevent recovery by sprouting of the contralateral fibers.

One week after surgery, the animals were anaesthetized again and fixed at the stereotactic frame (David Kopf, Tujunga). One monopolar recording electrode was placed in the hilar region of the dentate gyrus, while stimulating electrodes were placed ipsilaterally on the perforant pathway (PP) and the BLA. Signals were filtered between 1.5 and 3000 Hz using a VC-11 memory oscilloscope and averaged using a QC-111J Averager. Stimuli were produced by a SEN-3301 via a SS-104J isolation unit (all from Nihon-Kohden, Japan). Once a convenient location of the electrodes was achieved, input/output curves were determined for both inputs. On the PP curve, a stimulus intensity producing 40% of the maximal population spike was selected for test recording and tetanization. On the BLA curve, an intensity able to evoke a potential of about 1 mV was determined for tetanizing this input. Three control recordings were made before the first tetanization (baseline values); and test recordings were made at different time points after tetanization. Each record consisted of four consecutive responses to square pulses (0.1 ms) averaged at 0.1 Hz. On the test recordings, the amplitude of the population spike (P) was measured (Fig. 1A) and expressed as percent of the baseline values.

A tetanus consisted of three bursts of 15 impulses at 200 Hz with 10-s interburst interval. In the main experiment, a group of lesioned animals (LBLA, $n = 7$) and a group of control rats (CBLA, $n = 9$) were first tetanized in the BLA ($t = -15$) followed by tetanization of the PP ($t = 0$). Test recordings were made in the interval between the two tetanizations: 10 min and immediately before the second tetanization, as well as 2, 5, 15, 30 and 60 min after PP tetanization. In control experiments, performed in independent groups of rats, single or double PP tetanization were applied to control and lesioned animals. In the case of double PP tetanization, the time schedule for tetanization and recordings was the same as described for the main experiment. When single tetanization of the PP was used, test recordings were made 2, 5, 15, 30 and 60 min after tetanization.

Transection of the FF does not alter the main features of the BLA evoked potentials in the dentate gyrus. A low amplitude (3 mV maximum) monophasic negative wave of about 20 ms latency was the response of the dentate granule cell population to single stimuli applied to the BLA, as it has been previously described [13,26]. Control experiments using single or double tetanization of the PP showed no difference in the amplitude or duration of LTP between lesioned and control animals (data not shown). This is consistent with previous findings showing that lesioning the FF does not impair the potentiation of the population spike [3].

In the groups in which, the BLA was tetanized before the PP, a small but significant (Wilcoxon T-test, $p < 0.05$) enhancement of the population spike develops after stimulating the amygdaloid nucleus (see Fig. 1B). The level of this increase in the population spike amplitude was similar in control and lesioned rats. The ability of the amygdala to induce LTP-like enhancements in the PP-granule cells synapses have been recently reported after stimulating the medial nuclear complex [1,11] though others failed to obtain such effect [13].

Tetanizing the PP 15 min later induces an initially similar LTP in both groups (Fig. 1C). However, a significant difference between lesioned and control groups appeared after 60 min. The level of potentiation at that time point was significantly reduced in lesioned rats when compared with control rats (Fig. 1D). Values in the lesioned rats were not significantly different from baseline values after 1 h. These data support recent findings [1] describing a similar involvement of the central amygdala on LTP induced in the PP-granule cells synapses and the role of FF mediating later stages.

Summarizing our results, FF transection does not affect normal synaptic transmission nor early synaptic plasticity (such as short-term potentiation) at the PP synapses in the dentate gyrus in vivo. However, the maintenance of LTP at PP synapses is significantly reduced and even blocked 1 h after LTP induction when the FF afferents have been previously interrupted.

LTP has been classically considered a homosynaptic phenomenon. Recent results [8,9,20], however, indicate that the maintenance of LTP requires the synergistic activation of non-glutamatergic pathways. The influence of heterosynaptic modulating inputs on later stages of hippocampal LTP, such as shown by the lesion of the septo-hippocampal pathway, may support this hypothesis. We do not yet know which transmitter system is involved in the effect reported here. The FF is the major input of subcortical afferents to the hippocampus. Noradrenergic, serotonergic and dopaminergic fibers arising from brain stem nuclei and acetylcholine from the septal nuclei are within the major components [5]. We have recently shown that

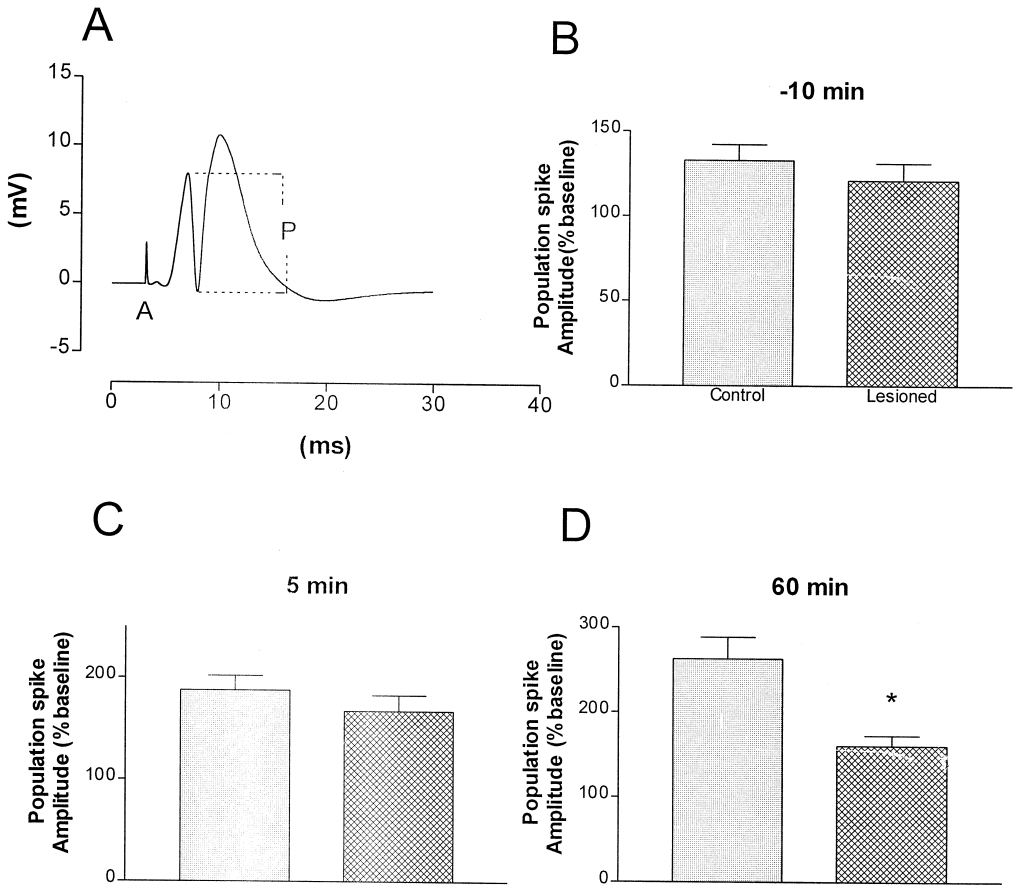

Fig. 1. (A) A test potential recorded in the dentate gyrus after stimulation of the PP. A: stimulus artefact; *P*: amplitude of the population spike. (B) Relative amplitude of the population spike after tetanizing the BLA to the baseline (%). The record was made 5 min after tetanizing the BLA, that is 10 min before tetanizing the PP. Control: non-lesioned animals. Lesioned: animals with bilateral transection of the FF. (C) Relative amplitude of the population spike 5 min after tetanizing the PP. (D) Relative amplitude of the population spike 60 min after tetanizing the PP, $^{*}p < 0.05$ Mann–Whitney *U*-test.

the conversion of an early-LTP into a late-LTP by stimulation of the BLA is greatly influenced by noradrenergic and cholinergic, but not dopaminergic inputs (Frey et al., submitted for publication). This modulating inputs, we hypothesize, are required to regulate protein synthesis necessary for late-LTP to occur [17,21]. Glutamatergic inputs set in motion a series of 'fast-acting plasticity processors' whose function is to induce and maintain LTP during its early, protein-synthesis independent stages. The synergistic action of glutamatergic and modulatory inputs may activate the long-lasting reinforcement of the tagged synapses [10] only when such associative event takes place within a distinct time window.

The preservation of early effects of BLA stimulation in FF lesioned rats suggests that an alternative pathway may mediate this interaction. A likely candidate is the entorhinal cortex, considering the physiological [26] and pharmacological evidence [1]. The existence of two independent pathways linking the amygdala and the hippocampus may allow the former to influence effectively the different steps involved in hippocampal LTP. Future studies should ascertain the specific function of the septo-hippocampal and entorhinal inputs on long-lasting neuronal plasticity.

The present results and the proposed interpretation stress the functional relevance of the amygdala influencing plastic mechanisms in the hippocampus and contribute to a better understanding of its role mediating emotional/motivational modulation of memory storage.

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Capítulo 5: La desaferentación subcortical afecta el reforzamiento conductual de la potenciación sináptica duradera

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La hipótesis que da origen a este trabajo se deriva directamente de la anterior.

Si el sistema de la fimbria-fornix es un mediador importante del efecto reforzador de la amígdala sobre la LTP en el giro dentado, y la amígdala es un componente esencial del mecanismo de reforzamiento conductual, entonces el sistema fimbria-fornix debe ser también mediador del reforzamiento conductual.

Para comprobarla se empleó el modelo de reforzamiento conductual en animales privados de agua a los cuales se les había lesionado la fimbria-fornix una semana antes.

Los grupos de control mostraron que esta lesión no afectó los valores basales del componente evaluado en los potenciales de campo y tampoco afectó el curso temporal de la LTP sin reforzamiento (fig. 3B). Sin embargo el efecto de reforzamiento conductual si fue abolido completamente por la lesión de la fimbria-fornix.

Si bien este resultado demuestra que esta proyección es importante, y confirman la hipótesis, deben tenerse en cuenta las mismas limitaciones y la misma cautela que en el análisis del resultado precedente, para no extrapolar su validez injustificadamente.

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SUBCORTICAL DEAFFERENTATION IMPAIRS BEHAVIORAL REINFORCEMENT OF LONG-TERM POTENTIATION IN THE DENTATE GYRUS OF FREELY MOVING RATS

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Abstract—Long-term potentiation is a form of neural functional plasticity which has been related with memory formation and recovery of function after brain injury. Previous studies have shown that a transient early-long-term potentiation can be prolonged by direct stimulation of distinct brain areas, or behavioral stimuli with a high motivational content. The basolateral amygdala and other subcortical structures, like the medial septum and the locus coeruleus, are involved in mediating the reinforcing effect. We have previously shown that the lesion of the fimbria-fornix—the main entrance of subcortical afferents to the hippocampus—abolishes the reinforcing basolateral amygdala-effects on long-term potentiation in the dentate gyrus *in vivo*. It remains to be investigated, however, if such subcortical afferents may also be important for behavioral reinforcement of long-term potentiation. Young-adult (8 weeks) Sprague–Dawley male rats were fimbria-fornix-transected under anesthesia, and electrodes were implanted at the dentate gyrus and the perforant path. One week after surgery the freely moving animals were studied. Fimbria-fornix-lesion reduced the ability of the animals to develop long-term potentiation when a short pulse duration was used for tetanization (0.1 ms per half-wave of a biphasic stimulus), whereas increasing the pulse duration to 0.2 ms per half-wave during tetanization resulted in a transient early-long-term potentiation lasting about 4 h in the lesioned animals, comparable to that obtained in non-lesioned or sham-operated control rats. In water-deprived (24 h) control animals, i.e. in non-lesioned and sham-operated rats, early-long-term potentiation could be behaviorally reinforced by drinking 15 min after tetanization. However, in fimbria-fornix-lesioned animals long-term potentiation-reinforcement by drinking was not detected. This result indicates that the effect of behavioral–motivational stimuli to reinforce long-term potentiation is mediated by subcortical, heterosynaptic afferents. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: synaptic plasticity, behavioral reinforcement, fimbria-fornix, dentate gyrus, basolateral amygdala, rat.

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Abbreviations: ACh, acetylcholine; AChE, acetyl cholinesterase; BLA, basolateral portion of the amygdala; BR, behavioral reinforcement; DG, dentate gyrus; EC, entorhinal cortex; E-LTP, early long-term potentiation; FF, fimbria-fornix; L-LTP, late long-term potentiation; LTP, long-term potentiation; NE, norepinephrine; NMDA, *N*-methyl-D-aspartate; PSA, population spike amplitude.

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Long-term potentiation (LTP) is a form of functional plasticity expressed as an increase of synaptic efficacy following brief, high frequency stimulation (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). The phenomenon is not unitary, but consists of temporarily sequenced, partially overlapping phases. An initial, early-long-term potentiation phase (E-LTP) lasting less than 4 h is followed by a late-long-term potentiation (L-LTP) which depends on protein synthesis (Krug et al., 1984; Frey et al., 1988; Reymann et al., 1988).

It has been shown that an E-LTP induced by mild stimulation patterns in the dentate gyrus (DG) of freely moving rats, can be converted into an L-LTP by the application of behavioral stimuli with a high motivational value within a limited time window before or after LTP-induction (Seidenbecher et al., 1997; Straube et al., 2003b). This effect seems to depend on the ability of such stimuli to activate modulatory brain regions projecting to the DG, resulting in the synthesis of plasticity-related proteins required for L-LTP to occur. This is documented by the fact that the application of anisomycin, a protein synthesis inhibitor, prevented behaviorally-induced LTP-reinforcement (Bergado et al., 2003; Straube et al., 2003a).

Similarly, direct electrical stimulation of the basolateral portion of the amygdala (BLA) is also able to reinforce E-LTP into L-LTP (Frey et al., 2001) suggesting a role for this limbic structure during behavioral reinforcement (BR). This effect was also dependent on protein synthesis (Frey et al., 2001) and the temporal or permanent inactivation of the amygdala impaired behavioral LTP-reinforcement (Almaguer-Melian et al., 2003), strengthening the hypothesis that the BLA might be part of the BR system.

The BLA might project to the DG via the entorhinal cortex (EC) or the fimbria-fornix (FF) (Canning and Leung, 1997; Thomas et al., 1984; Maren, 1996; Maren and Quirk, 2004). The EC sends an excitatory, glutamatergic projection to the DG (Deller and Frotscher, 1997; Vizi and Kiss, 1998). The FF afferents to the DG are of subcortical origin, and include a GABAergic and a cholinergic component from septal origin, as well as noradrenergic fibers arising from the locus coeruleus (Adelmann et al., 1996; Cassel et al., 1997; Vizi and Kiss, 1998). We have previously shown that interrupting the FF abolishes the reinforcing effects of stimulating the BLA on LTP (Jas et al., 2000), but it is not known whether that lesion will have similar effects on BR.

Here, we present evidence that lesioning the FF, i.e. depriving the DG from subcortical innervation, abolishes BR of LTP.

EXPERIMENTAL PROCEDURES

Animals

Male adult Sprague–Dawley rats (obtained from CENPALAB, Havana, Cuba) weighing 250–270 g during electrode implantation were housed individually after surgery in plastic, translucent cages. Water and food (Rat Chow, CENPALAB) remained *ad libitum* through the experiment, except for the series investigating the effect of BR of LTP, in which rats were water-deprived for 24 h. Animals were handled according to the Cuban Regulations for Using Laboratory Animals and the U.S. National Institutes of Health guidelines for the use of laboratory animals. All efforts were made to reduce the number of animals and their suffering.

Surgery

Surgery was performed under chloral hydrate narcosis (420 mg/kg, intraperitoneally) according to the procedure described elsewhere (Almaguer-Melian et al., 2005b). The animals were mounted on a stereotactic frame (David Kopf Inst., Saint Louis, MO, USA) and the skull was exposed after cutting the skin.

Lesioning of the FF

A bilateral window was opened at the skull at coordinates (AP: −1.4 mm, ML: ±0.5 to ±5.2 mm). A reduced #11 surgical blade was lowered at 15° (with respect to the vertical direction) and a bilateral knife transection was carried out at the coordinates AP: −1.4 mm, ML: ±0.8 to ±3.1 mm, and DV: 5.0 mm. Though all the electrophysiological studies were done in the right hemisphere, lesions were made bilaterally to prevent recovery by sprouting of the contralateral fibers. Sham-operated animals received the same treatment, but no knife was inserted and no transection was performed. Control animals were not operated.

Electrode implantation

Stainless steel, Teflon-isolated monopolar electrodes for recording were implanted in the hilus of the DG (coordinates AP: −3.8 mm, ML: 2.0 mm, DV: −3.5 mm from bregma) and bipolar electrodes of the same wire for stimulation at the angular bundle of the PP (coordinates AP: −7.5 mm, ML: 4.0 mm, DV: −3.7 mm). Miniscrews were attached in the right frontal and left parietal bones to serve as indifferent and ground electrode respectively.

Electrophysiology

One week after surgery the animals were habituated to the test chamber for at least 4 h before obtaining an input–output curve on which the stimulus intensity was determined for each animal at 40% of the maximal population spike amplitude (PSA). The test chamber consisted of individual plastic concave boxes, in which the animals could move freely while remain connected to the amplifier (AB 621G, Nihon Kohden, Japan) and stimulator (AM Systems 2100, AM Systems, Sequim, WA, USA). The filtered signals (1–5000 Hz) were digitized (AD converter 1401+, CED, Cambridge, Great Britain) fed into a PC and averaged and measured by a special computer program (Intracell, Magdeburg, Germany). Lesioned (lesion) sham-operated (sham) and control animals were then randomly assigned to one of the experimental protocols. In the LTP protocol a weak tetanus (3×15 impulses at 200 Hz) was used to induce an E-LTP. In the initial design we have planned to use 0.1 ms pulse duration per half-wave for LTP induction, but we noticed that the level of LTP induced was to low

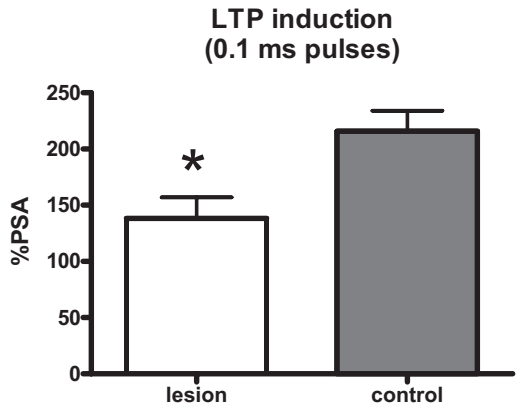

Fig. 1. Level of potentiation reached by FF-lesioned animals (lesion, $n=9$) using 0.1 ms pulse duration for the high frequency stimulation compared with a "historical" sample of non-lesioned animals (control, $n=24$). The percentual potentiation of the PSA (%PSA) is shown 5 min after LTP induction.

in comparison to the historical average in our laboratory (see Fig. 1). Therefore, we have changed to 0.2 ms pulse duration, restarted the group, and used it throughout the experiment. For the BR protocol animals were water deprived for 24 h before inducing E-LTP using the same stimulus pattern (0.2 ms). Fifteen minutes after LTP induction, water was made available until the end of the recording session. Control experiments, performed earlier in our laboratory, revealed no effects of water deprivation on test potentials recorded in a control group without tetanization [19].

Test recordings

Test recordings were made before and after induction of LTP. Twelve recordings were made during one hour before LTP. After LTP induction, test recordings were made 5 min after induction, and from 30 min on, up to 6 h, new test recordings were made every 15 min, as well as 24 h later. Each test recording represents the averaged value of five consecutive evoked potentials recorded at a frequency of 0.1 Hz.

Histology

After finishing the experiments the animals were perfused under narcosis, and the brains sectioned, and stained for the enzyme acetyl cholinesterase (AChE), to confirm the efficacy of the lesioning procedure.

Statistical analysis

A three-way ANOVA with one repeated measures factor (LESION, STIMULATION and TIME) was used. When significant differences were found a post hoc test using the Tukey honest significant differences was performed. Differences were considered significant only when $P<0.05$.

RESULTS

The histological examination confirmed a severe reduction of AChE in the hippocampus and the DG of lesioned animals, indicating that the lesion procedure was effective in provoking an intense subcortical denervation of the hippocampal formation (Fig. 2).

An initial ANOVA, considering the factors type of LESION (i.e. lesion, sham or controls), and the STIMULATION protocol (whether it includes BR or not) as independent

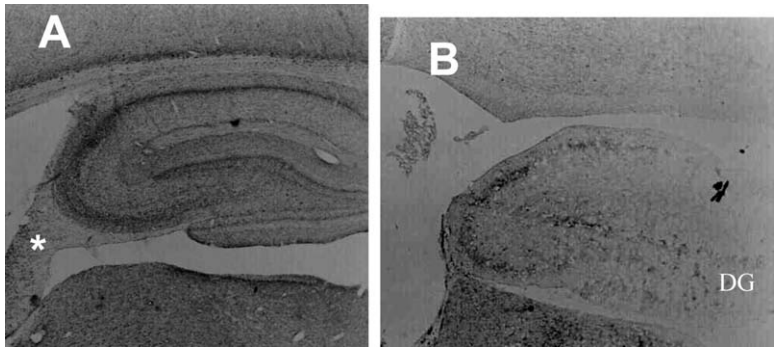

Fig. 2. Representative microphotographs of a control animal (A) and a FF-lesioned animal (B) stained for ACh esterase. Notice the intense reduction of staining in all subfields of the hippocampal formation including the DG in the lesioned animal. Notice also the absence of the fornix in B (marked with an asterisk in the control animal).

variables, with TIME as repeated measures factor dependent variable, confirmed significant differences due to LESION ($F_{2,\ 42}=3.91428$); the STIMULATION protocol ($F_{1,\ 42}=21.66280$); and TIME ($F_{7,\ 294}=33.19984$). A significant interaction between LESION and STIMULATION was also confirmed ($F_{2,\ 42}=6.98083$) as well as between LESION and TIME ($F_{14,\ 294}=2.50766$).

For the sake of simplicity in the presentation of results, we have separated both stimulation protocols in two graphs which are shown in the following paragraphs, with their corresponding ANOVA results.

Fig. 3 shows the results of the three lesion groups in the LTP stimulation protocol. A two-way ANOVA (LESION and TIME, repeated measures) showed no effect for LESION ($F_{2,\ 21}=0.46395$) a significant effect for TIME ($F_{7,\ 147}=32.11707$) and no interaction between factors ($F_{14,\ 147}=1.27327$) was also seen. The post hoc analysis for factor TIME showed that the records corresponding to 5 min and 1 h after LTP induction significantly differ from the rest. However, the value corresponding to 2 h do not differ from those of 3 and 4 h, but do differ from those taken from 5 h on.

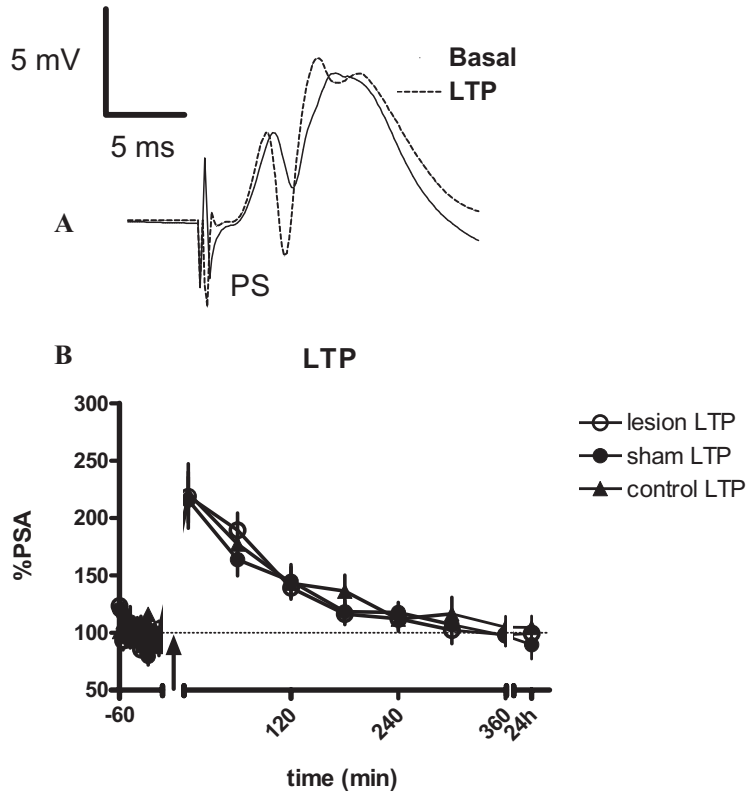

Fig. 3. (A) Representative recordings taken from one FF-lesioned animal 5 min before (basal: continuous line) and 5 min after (LTP: interrupted line) induction of LTP (PS: population spike). (B) Time course of LTP in FF-lesioned animals (lesion LTP, $n=9$), sham-operated (sham LTP, $n=10$) and control animals (control LTP, $n=10$). The ANOVA two way with repeated measures showed no differences due to the lesioning protocol. (The vertical arrow indicates the time of LTP induction.)

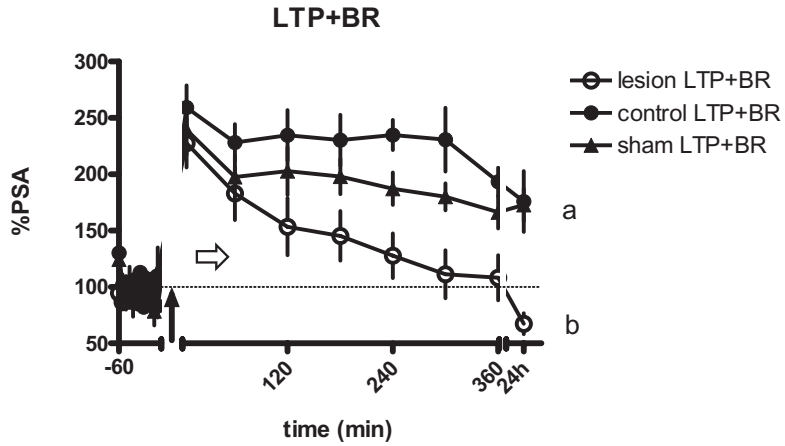

Fig. 4. Time course of LTP followed by BR (water access) 15 min later. The two way ANOVA with repeated measures confirmed differences among groups. FF-lesioned animals (lesion LTP+BR, $n=10$), sham-operated (sham LTP+BR, $n=10$) and control animals (control LTP+BR, $n=9$) are shown. The post hoc analysis showed no differences in the initial level of potentiation, but the reinforcing effect (a prolonged potentiation due to BR) was absent in lesioned animals. (The vertical arrow indicates the time of LTP induction; the open horizontal arrow indicates the time in which water was made available.)

These results confirm that the stimulation protocol induced a decaying E-LTP in all three groups of animals, and that the lesion of the FF induced no difference in the level and evolution of LTP.

Fig. 4 shows the results when behavioral motivational stimulus (drinking) was applied 15 min after inducing LTP with an identical train. A two-way ANOVA (LESION and TIME, repeated measures) showed significant effects for LESION ($F_{2,\ 21}=7.06803$) TIME ($F_{7,\ 147}=10.58999$) and the interaction between both ($F_{14,\ 147}=2.04525$). The post hoc analysis for the LESION×TIME interaction revealed no differences among groups during the first 2 h after LTP induction, confirming that a similar E-LTP was induced in the three groups irrespective of the fact that they were lesioned or not. However, after 3 h the lesioned animals differed significantly from the control ones, while from 4 h to 24 h they differed from both lesioned and sham-operated animals. These two groups (control and sham) do not show differences at any time point. This indicates that while the BR was able to transform the initial E-LTP into an L-LTP in animals bearing no or sham lesion, in those with a bilateral lesion of the FF such effect was abolished.

DISCUSSION

Our results confirm our hypothesis that the lesion of the FF should also abolish the reinforcement of LTP by motivational behavioral stimuli.

Application of the 0.2 ms pulse duration tetanization protocol resulted in the induction of a similar E-LTP in all groups. However, application of the weaker, 0.1 ms pulse duration tetanization protocol caused different plastic events in the different groups of animals. An early report about the effects of FF lesion on LTP showed an absence of potentiation in lesioned animals (Buzsáki and Gage, 1989). These authors employed 0.1 ms pulses at 200 Hz to induce LTP, but the number of impulses delivered was higher (eight trains of 20 pulses) than ours (three trains of 15 pulses). Similar results were reported later by a different group using the same frequency (Valjakka et al., 1991) although they do not mention the pulse duration employed. However, this impaired induction seems to be dependent on the stimulation protocol. We have studied LTP in FF-lesioned rats employing stronger stimuli (10 trains of 10 pulses at 400 Hz) and showed a normal induction of LTP of the population spike (but reduced in the slope of the EPSP) (Bergado et al., 1996). There is no clear interpretation for these results. It has been argued that a lower level of catecholamines might activate Ca^{2+}-dependent K channels, impairing thus the activation of *N*-methyl-D-aspartate (NMDA) receptors through high frequency stimulation. According to this, it is conceivable that a stronger stimulus (like a longer pulse) might overcome this deficit to reach a normal level of LTP.

Our LTP groups confirm this assumption. All three groups (lesion, sham and control) showed an identical level and time course of LTP when tetanized with the 0.2 ms protocol. This is consistent with the assumption that the stimulation pattern employed induced only an E-LTP (Seidenbecher et al., 1997; Frey et al., 2001).

The lesion of the FF had consequences on the mechanisms of induction of later phases of LTP (L-LTP) as demonstrated by our results combining high frequency stimulation and BR. The FF-lesioned animals develop an E-LTP which does not differ from the sham or control animals. However, while in two control groups the access to water prolonged LTP; in the FF-lesioned group LTP-reinforcement was completely absent. In fact the decay of LTP in these animals is identical to that of the groups in which no BR was applied. These data support the important role and the relevance of subcortical modulatory afferents for BR of the NMDA-dependent LTP induced by stimulation of the medial perforant pathway in the DG.

As L-LTP (Krug et al., 1984; Frey et al., 1988) and BR (Bergado et al., 2003) depend on protein synthesis, we

suggest to interpret our result in terms of the synaptic tagging hypothesis (Frey and Morris, 1997a,b). According to this, the induction of LTP sets a tag at the activated synapses which allows the recognition and insertion of the proteins required for a longer lasting enhancement of synaptic efficacy. BR might activate heterosynaptic afferents, releasing transmitter substances able to activate metabolic cascades leading to regulation of transcription, and therefore, the synthesis of plasticity proteins. The lack of reinforcement in the FF lesioned group, strongly suggests that these modulatory afferents reach the DG via the FF, and suggest further a subcortical origin for them.

Pharmacological studies have shown that propranolol, a β-adrenergic receptor antagonist, can effectively block BR (Seidenbecher et al., 1997; Straube et al., 2003a) and BLA-induced reinforcement (Frey et al., 2001), while atropine, a muscarinic receptor antagonist, only blocks the latter. Intraventricular administration of norepinephrine (NE) but not oxotremorine (a muscarinic agonist) reinforces LTP in a similar manner as after BR or BLA-stimulation (Almaguer-Melian et al., 2005b). The mentioned pharmacological studies lead to the hypothesis of an action of NE on the DG, converting an E-LTP into an L-LTP, while the possible role of acetylcholine (ACh) seemed not so clear with respect to the above mentioned forms of reinforcement. However, one should keep in mind that the i.c.v. administration opens the possibility that the effects of these substances may not necessarily take place within the DG. In a microdialysis study we have tried to solve this question studying changes in neurotransmitter release at the DG under BLA stimulation or BR. The results show an increase in the release of ACh and a reduction in NE under BLA-stimulation, while under BR, ACh and NE remained unchanged and a reduction in glutamate and GABA release was found (Almaguer-Melian et al., 2005a). While the large sampling time, and the low frequency of stimulation used, might have masked some short-term changes, this result suggests that effects of NE are indirect, acting perhaps on the amygdala to modulate its activity.

The amygdala appears as a key structure to mediate motivational effects on learning (Abe et al., 1998; Antoniadis and McDonald, 2000; Cahill, 1999; Hamann et al., 1999; Huff and Rudy, 2004; Izquierdo et al., 1997; McGaugh et al., 2002; McIntyre et al., 2003; Pare, 2003; Richter-Levin, 2004) and synaptic plasticity (Abe, 2001; Akirav and Richter-Levin, 1999, 2002; Almaguer-Melian and Bergado Rosado, 2002; Ikegaya et al., 1994, 1995), two processes that appear to be functionally linked. The lack of BR in FF-lesioned animals could be attributed to the interruption of one of the pathways through which, the BLA projects into the DG. However, other brain structures like the locus coeruleus (Harley et al., 1989; Berridge and Waterhouse, 2003; Kitchigina et al., 1997) sending a noradrenergic projection to the hippocampal formation via the FF should not be discarded. Experiments in progress, using topical application of substances within relevant limbic and forebrain structures, should contribute to a better understanding of the system and transmitters involved in the motivational modulation of LTP.

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Capítulo 6: La estimulación del septo medial modula la potenciación sináptica duradera en el giro dentado

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Varios de los resultados antes descritos sugieren que la región del septo medial puede ser parte de los mecanismos de modulación de procesos neuroplásticos en el giro dentado:

1. La mediación demostrada del sistema de fimbria fornix tanto en el reforzamiento conductual como en el iniciado por estimulación de la amígdala.
2. El efecto de bloqueo por atropina del reforzamiento por estimulación de la amígdala ya que la proyección colinérgica en toda la formación del hipocampo es de origen septal.

Basados en esta evidencias se puede proponer la hipótesis de que *la proyección septo-hipocampal es parte del sistema neural de reforzamiento de procesos neuroplásticos.*

Si esto es cierto, debe ser posible provocar un efecto de reforzamiento similar al obtenido con estimulación de la amígdala, mediante la estimulación de la región medial del septum. El objetivo de este trabajo fue comprobar esa hipótesis.

Los resultados muestran que es posible reforzar la LTP en el giro dentado mediante la estimulación de la región medial del septum con la aplicación de estímulos de alta frecuencia al septum (fig. 2A) pero no con estímulos de baja frecuencia (2B). Lo primero mimetiza el efecto de la estimulación de la amígdala pero no lo segundo. El estudio de los mecanismos celulares mostró la participación de los mecanismos de síntesis de proteínas (ver figura 4) mientras que el estudio farmacológico demostró la mediación de aferentes noradrenérgicas (fig. 3B). Inesperadamente, el bloqueo de la transmisión colinérgica no tuvo efectos (fig. 3C). Este último resultado parece contradecir el efecto antes encontrado para la atropina con estimulación de la amígdala y choca con el hecho

de que la principal proyección del septum al hipocampo es colinérgica, aunque la estimulación eléctrica puede activar también fibras que pasan por el septum, como las noradrenérgicas procedentes del locus coruleus.

Debe tenerse en cuenta que la aplicación de las sustancias bloqueadoras se realizó en el ventrículo cerebral derecho y, por esa razón sus efectos pueden ocurrir no solo en el giro dentado, que es la región de interés, sino en cualquier otra zona del encéfalo. Eso reduce las posibilidades de interpretación inequívoca de los resultados. Para lograr una caracterización más precisa de la contribución de esos neurotransmisores sería necesario su aplicación tópica en las regiones de interés como se mostrará más adelante.

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MODULATION OF LATE PHASES OF LONG-TERM POTENTIATION IN RAT DENTATE GYRUS BY STIMULATION OF THE MEDIAL SEPTUM

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Abstract—The prolonged maintenance of hippocampal long-term potentiation (LTP) seems to require heterosynaptic events during its induction. We have previously shown that stimulation of the basolateral nucleus of the amygdala (BLA) within a distinct time window can reinforce a transient early-LTP into a long-lasting late-LTP in the dentate gyrus (DG) in freely moving rats. We have shown that this reinforcement was dependent on β-adrenergic and/or muscarinergic receptor activation and protein synthesis. However, since the BLA does not directly stimulate the DG the question remained by which inputs such heterosynaptic processes are triggered. We have now directly stimulated the medial septal pathway 15 min after induction of early-LTP in the DG and show that this input is capable of reinforcing early into late-LTP in a frequency-dependent manner. This septal reinforcement of DG LTP was dependent on β-adrenergic receptor activation and protein synthesis. We suggest that the reinforcing effect of the BLA stimulation can, potentially, be mediated via the septal input to the DG, though it differs in its ability to induce or modulate functional plasticity.

Key words: late-LTP, hippocampus, reinforcement, memory formation, learning, septum.

Learning is a complex form of experience-dependent plasticity which allows animals, and humans, to modify and adapt their behaviour in response to changes in environmental conditions. Long-term potentiation (LTP) (Bliss and Lomo, 1970; Bliss and Gardner-Medwin, 1971) is an endurable change in synaptic efficacy produced by brief repetitive stimulation of specific afferents and has been considered as a cellular model of long-term memory. Best studied in the dentate gyrus (DG) and the CA1 region of the hippocampus, LTP has long been thought to be the result of the monosynaptic activation of glutamatergic receptors, mainly of the *N*-methyl-D-aspartate (NMDA)-receptor type (Bashir, et al., 1991). LTP, however, is not a unitary phenomenon. Like memory, LTP has phases that depend on different intracellular molecular mechanisms. Blocking protein synthesis with specific inhibitors allows the dissociation of a protein synthesis-independent early-LTP lasting less than 4 h, from a protein synthesis-dependent late-LTP (Krug et al., 1984; Frey et al., 1988). Whilst the role of NMDA-receptor activation for the induction of LTP seems to be well established, accumulating evidence suggests that other transmitter systems might exert important influences on the maintenance of LTP. Amongst others, acetylcholine (Segal and Auerbach, 1997) as well as catecholamines, like dopamine (Frey et al., 1990) and norepinephrine (Stanton and Sarvey, 1987) are able to influence LTP, mainly in its late components (Frey et al., 1990). Interestingly, these transmitters are thought to be part of the neural systems involved in evaluating and determining the emotional and motivational status of the individual (McGaugh and Introini-Collison, 1987; Hasselmo, 1995; McGaugh et al., 1996; Nader and LeDoux, 1999). Recently it was demonstrated that emotional–motivational processes can influence the duration of hippocampal LTP (Seidenbecher et al., 1995, 1997). The main findings of those studies showed that behavioural stimuli with a strong emotional–motivational content, like drinking after 24 h of water deprivation, were able to transform an early-LTP induced by a weak tetanus into a long-lasting LTP beyond 8 h. This effect was abolished by propranolol an antagonist of norepinephrine β-receptors.

One candidate brain structure to subserve such 'behavioural reinforcement' effect is the amygdala. This limbic structure is considered to be part of an emotional memory system (Quirk et al., 1996) and to mediate emotional reinforcement of memory in other brain structures like the hippocampus (Wang and Arvanov, 1998). A functional link between the amygdala and hippocampal LTP has been demonstrated as well (Ikegaya et al., 1994, 1995a,b; Akirav and Richter-Levin, 1999a,b; Kamiya and Ozawa, 1998). These studies, however, concentrated on the induction or very early stages of early-LTP. In a recent report, we have assessed whether amygdala stimulation can also influence the maintenance of LTP. The main findings showed that amygdala stimulation is able to transform a transient early-LTP at the perforant path (PP)–DG synapses into a late-LTP when applied within a 30-min time window, before or after the induction of LTP (with one exception: simultaneous tetanization did not lead to a reinforcement). The effect was protein synthesis-dependent and could be abolished by noradrenergic or muscarinergic

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Abbreviations: BDNF, brain-derived neurotrophic factor; BLA, basolateral nucleus of the amygdala; DG, dentate gyrus; EPSP, excitatory postsynaptic potential; hfs, high-frequency stimulation; lfs, low-frequency stimulation; LTP, long-term potentiation; MS, medial septum; MShfs, high-frequency stimulation of the medial septum; MSlfs, low-frequency stimulation of the medial septum; NMDA, *N*-methyl-D-aspartate; PP, perforant path; PPhfs, high-frequency stimulation of the perforant path; PSA, population spike amplitude.

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antagonists but not by dopaminergic or NMDA-receptor antagonists (Frey et al., 2001).

There are no known direct efferents linking the amygdala with the DG (Chen et al., 1999). Two alternative pathways, via the entorhinal cortex or the medial septum (MS), might sustain such interactions. We have shown that the interruption of the septo-hippocampal afferents by the lesion of the fimbria-fornix affects the late effects of stimulating the amygdala (Jas et al., 2000). However, this does not prove that stimulating the septum might reproduce the effects of stimulating the amygdala.

In the present paper we show results demonstrating the efficacy of the septal stimulation to prolong LTP at the DG.

EXPERIMENTAL PROCEDURES

Male Wistar rats (250–300 g at the time of preparation) were used. All experiments were performed in compliance with the relevant laws and institutional guidelines and have been approved by the Land Sachsen-Anhalt. After surgery for electrode implantation animals were housed individually in plastic translucent cages with free access to water and food.

Surgery was performed under pentobarbital narcosis (40 mg/kg, i.p.) supplemented with additional doses if required. The animals were mounted in a stereotactic frame (bregma 1 mm above lambda) and electrodes were lowered to their final positions under electrophysiological control of the evoked potentials to achieve the best sensitivity. A monopolar recording electrode was placed at the hilar region of the DG (coordinates, AP: −2.8 mm, ML: 1.8 mm, from bregma, DV: −3.5 mm from the dural surface) whilst bipolar stimulating electrodes were set at the PP (AP: −6.9 mm, ML: 4.1 mm from bregma, DV: −2.5 mm from the dura) the basolateral nucleus of the amygdala (BLA) (AP: −2.4 mm, ML: 5.0 mm from bregma, DV: −7.6 mm from the dura) and the MS (AP: +1.7 mm, ML: 1.8 mm, DV: −7.0 from the dura; tilted 15° toward the midline in the coronal plane). Miniscrews attached to the skull served as indifferent electrode and earth respectively.

After 2 weeks of recovery animals were habituated to the recording set for at least 4 h before starting the experiment.

LTP was studied by the evaluation of potentials evoked at the DG by stimulation of the PP. An input/output curve was constructed for each animal and the stimulation intensity required to induce a 40% of the maximal population spike amplitude (PSA) determined for each animal. This intensity was used for test recordings as well as for inducing LTP stimulating the PP. For stimulating the BLA or the MS 0.1-ms square pulses of constant intensity were used. The stimulation of BLA but not of the MS produced evoked potentials at the DG (Frey et al., 2001), but, as the recording electrode position was optimised for the PP input, they were small and showed flattened I/O relationships. Therefore a constant 400 μA current was used for both inputs. PSA-recording and analysis were favoured against the slope of field excitatory postsynaptic potentials (field-EPSPs) since the latter is relatively unstable in freely moving animals, especially considering that the PP stimulation intensity was adjusted to obtain an optimal population spike, which negatively influences the field-EPSP. During high-frequency stimulation (hfs), i.e. during LTP induction the spike is required to induce late-LTP. We have previously studied the relationships between PSA potentiation and EPSP potentiation and found a positive correlation between both variables over a wide range of tetanization frequencies (Frey et al., 2001; López Planes et al., 1999). In addition, in a few experiments with potentials containing a relatively large proportion of an "EPSP," we have analysed the time course of that response's initial positive slope after stimulus artefact. The slope of this potential followed the time course of the corresponding PSA, respectively (data not shown).

Test recordings of the PP–DG-evoked potentials were recorded during 1 h (baseline) before applying any of the stimulation protocols. Each recorded potential was the average of five consecutive single pulses (0.05 Hz). Hfs or low-frequency stimulation (lfs) protocols were applied to the PP, the BLA or MS after baseline recording. The averaged value of the PSA in baseline records was used to calculate the percentile change in this variable in test recordings taken during the following 8 h, and 24 h after stimulation.

Hfs consisted of three trains of 15 impulses at 200 Hz (10-s intertrain interval) whilst lfs consisted of 45 impulses at 0.1 Hz

The experimental protocol included the following stimulation groups

Single stimulation
- hfs of the PP (PPhfs), $n=8$
- hfs of the MS (MShfs), $n=6$
- lfs of the MS (MSlfs), $n=6$

Double stimulation (15-min interval)
- PPhfs followed by MShfs (PPhfs+MShfs), $n=5$
- PPhfs followed by MSlfs (PPhfs+MSlfs), $n=8$

Pharmacology

The application procedure for drugs were the same as described earlier (Frey et al., 2001) when DG–LTP reinforcement was investigated at an interval between LTP induction in the DG and reinforcing stimulation of 15 min. There were no non-specific behavioural effects detected during or after drug application. The following pharmacological experiments were carried out:
- PPhfs+MShfs and the application of NaCl, $n=6$
- PPhfs+MShfs and the application of the β-adrenergic receptor blocker propranolol (6.76 nmol), $n=4$
- PPhfs+MShfs and the application of the muscarinergic receptor blocker atropine (1 nmol), $n=4$
- PPhfs+MShfs and the application of the protein synthesis inhibitor anisomycin (0.905 mol), $n=5$

The animals were randomly assigned to the experimental groups. Some animals were used in more than one protocol. In those cases the second protocol was at least 1 week after the first, and after baseline values completely recovered their original level.

The statistical analysis was performed using the Wilcoxon *t*-test for paired samples for within group comparisons (test recording versus own baseline), whilst the Kruskal-Wallis non-parametric ANOVA was used for between-groups comparisons. In each case a two-tailed $P<0.05$ was established as statistically significant.

At the end of the experiments the brains of all animals were fixed by intracardiac perfusion under narcosis and the location of the electrodes was histologically confirmed. Only animals with a correct electrode location (i.e. within the structures of interest) were included in the final results.

All efforts were made to minimise the number of rats used and their suffering. All experiments have been performed with permission of the local legislatives authorities of the Land Sachsen-Anhalt.

RESULTS

The group with single PPhfs ($n=8$) was introduced as control of the classical LTP that can be induced in the DG after stimulating the glutamatergic fibres arising from the entorhinal cortex. As shown in Fig. 1B, this stimulus paradigm induces an increase of the PSA relative to baseline recordings. This LTP, however, is decremental as shown in Fig. 1B. The level of potentiation slowly declines to baseline values within

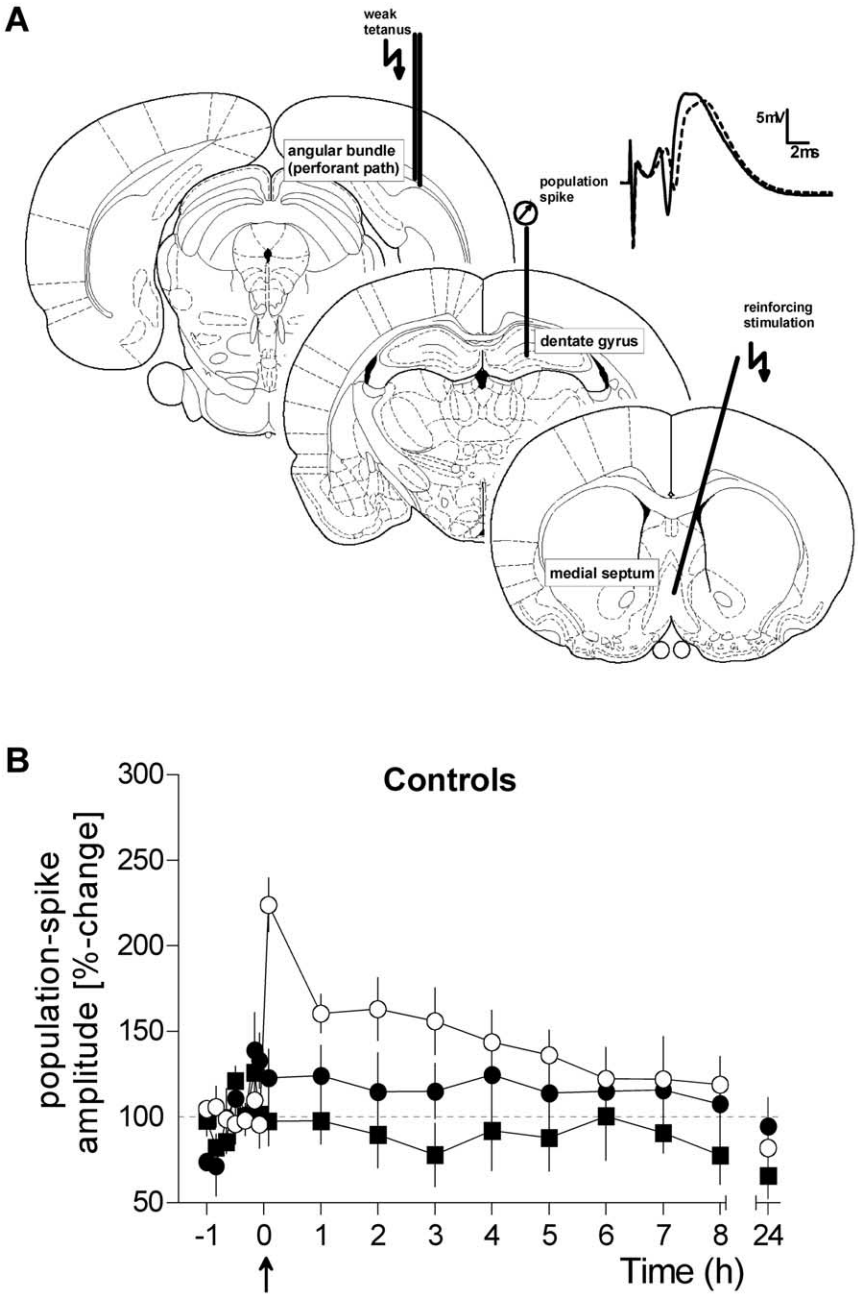

Fig. 1. Control experiments. (A) Schematic illustration of electrode localisation. Insets show analog examples of recordings obtained before (dotted line) and after LTP induction (filled line) of the perforant path (upper right). Notice the increase in the PSA (downwards spike-like deflection) and the small increase in the slope of the population EPSP (first upward deflection). The PSA was used as an indicator of potentiation. (B) Time course of early-LTP at the PP–DG synapses (control-LTP, open circles) and the influence of hfs (closed circles) or lfs (closed squares) of the MS on test potentials in the DG. The arrow indicates the time of tetanic stimulation or lfs of the PP or the MS, respectively.

the first 3–6 h. The Wilcoxon test confirmed a significant increase of the PSA only during the first 2 h after induction.

Hfs (n=6) or lfs (n=6) of the MS alone (Fig. 1B) produced no significant change on the PSA in test recordings during the same 24-h period. However, when the MS was stimulated with high frequency after the induction of LTP in DG (Fig. 2A, PPhfs+MShfs, n=5) a significant modification in LTP time course appeared (Kruskal-Wallis test). Instead of declining to non-significant levels after 2 h, the population spike remained statistically significantly potentiated even after 24 h. Such an effect of MS stimulation seemed to be frequency-dependent whilst lfs of this region was ineffective in influencing the maintenance of early DG–LTP (Fig. 2B, n=8). No significant differences between curves were found by the Kruskal-Wallis test. It is important to emphasise that no change was detected with respect to the level of initial potentiation when hfs or lfs was applied after induction of DG–early-LTP.

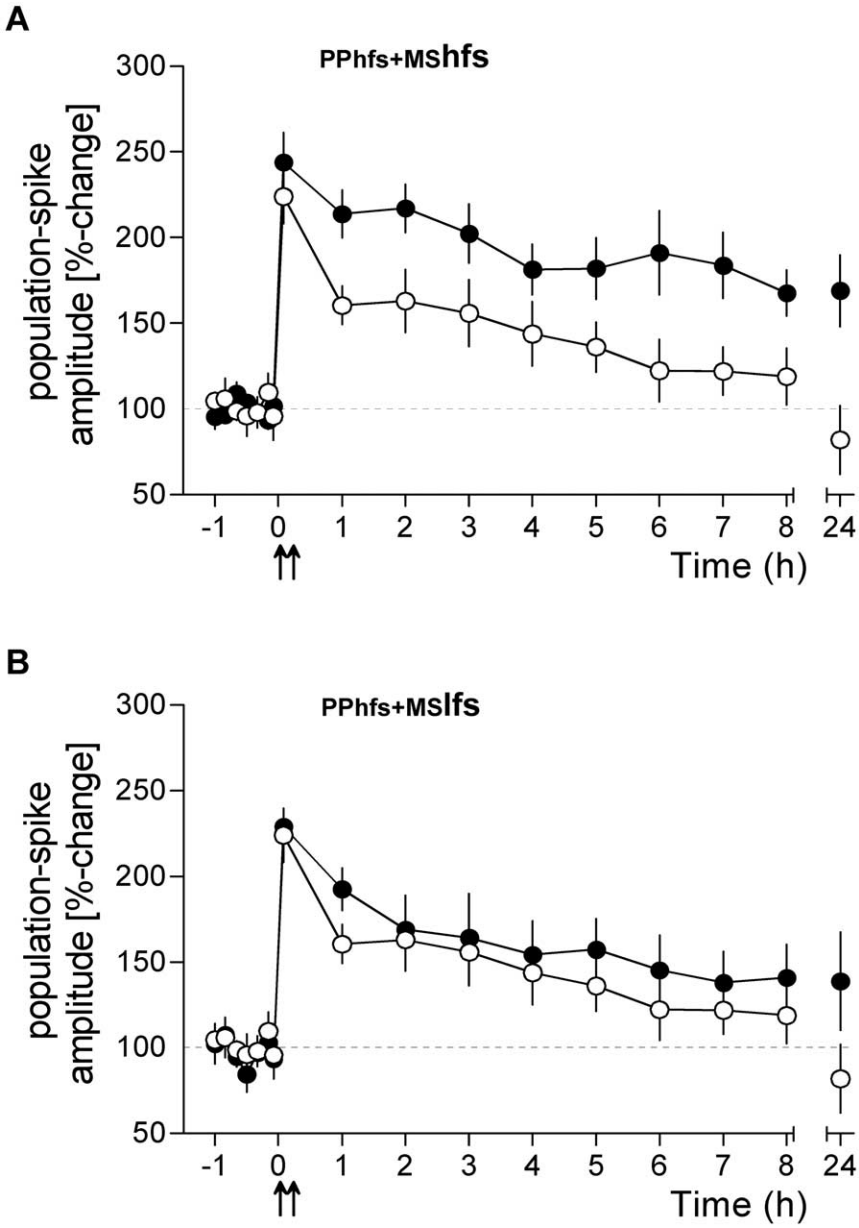

Fig. 2. MS stimulation can prolong LTP at the PP–DG synapses. (A) Hfs of the MS 15 min after induction of LTP at the PP–DG synapses (PPhfs+MShfs, filled circles) significantly prolonged the time course of LTP for at least 24 h. This curve was also significantly different from control early-LTP ($P<0.05$, Kruskal-Wallis test). For comparison, the time course of DG early-LTP is presented (open circles). (B) lfs of the MS (PPhfs+MSlfs, filled circles) has no reinforcing effect on LTP at the PP–DG synapses. No significant differences between this and the control LTP curve were observed (Kruskal-Wallis test). For comparison, the time course of DG early-LTP is presented in both graphs (open circles). The arrows indicate the time of stimulation of the PP or the MS.

Pharmacological interventions resulted in the following effects on a control reinforcement of DG–early-LTP by subsequent tetanization of the MS 15 after the DG tetanization ($n=5$): Control experiments with the application of saline instead of drug treatment (Fig. 3A, NaCl controls, $n=6$) revealed no difference when compared with untreated control reinforced animals. However, the application of the β-adrenergic receptor antagonist propranolol (Fig. 3B, $n=4$) but not of the muscarinergic receptor antagonist atropine (Fig. 3C, $n=4$), prevented the reinforcing effect of MShfs on DG–early-LTP. After the application of propranolol a statistically significant potentiation was only observed for the first 2 h after the tetanization (Wilcoxon test). Similar results as with propranolol where obtained with the reversible protein synthesis inhibitor anisomycin (Fig. 4, $n=5$). Application of anisomycin prevented LTP after 1 h onward (Wilcoxon test).

DISCUSSION

The tetanization paradigm we have used in the present experiments effectively induced LTP at the PP–DG syn-

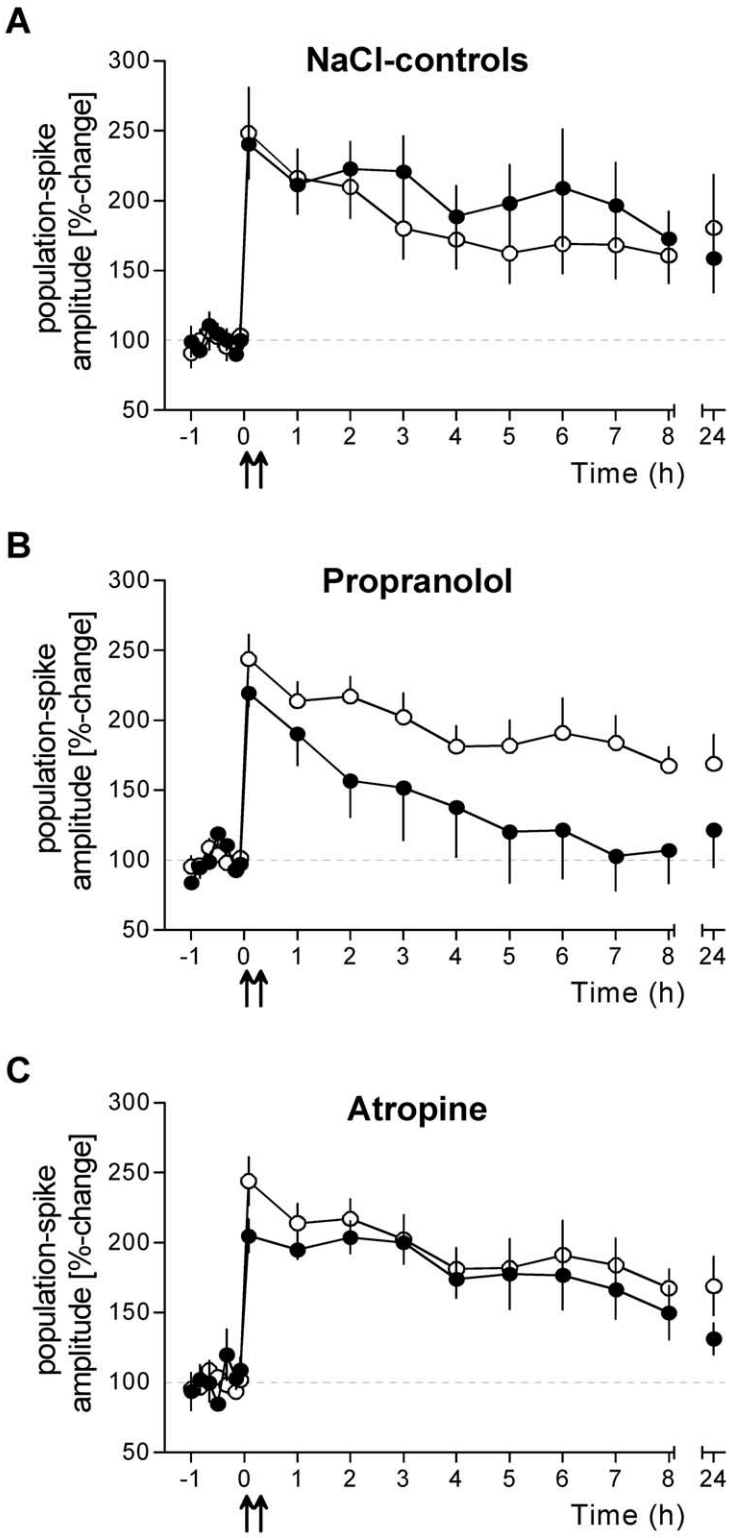

Fig. 3. The influence of pharmacological substances on the MS–hfs reinforcement of DG–early-LTP. (A) A repeated pattern of hfs of the MS 15 min after induction of LTP at the PP–DG synapses significantly prolongs the time course of early-LTP in untreated (open circles; see also Fig. 2A) as well as in animals in which saline (NaCl) was injected into the ventricle (filled circles). The curves were statistically significant different from control early-LTP ($P<0.05$, Kruskal-Wallis test) for at least 24 h. (B) The application of the β-adrenergic receptor antagonist propranolol i.c.v. prevented (closed circles) the PPhfs–MShfs reinforcement from 3 h onward. (C) The application of the muscarinergic receptor antagonist atropine (closed circles) was ineffective in influencing PPhfs–MShfs reinforcement. For comparison the time course of the PPhfs–MShfs-reinforced late-LTP (untreated and NaCl controls) is presented in all three graphs (open circles). The arrows indicate the time of tetanic stimulation of the PP or the MS.

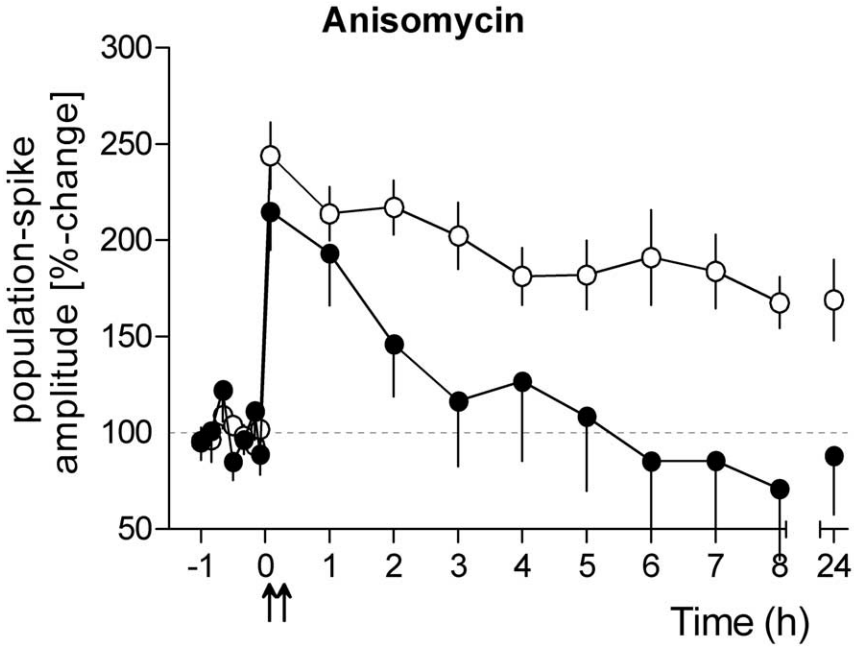

Fig. 4. The influence of anisomycin on PPhfs–MShfs-reinforced LTP. The application of the reversible protein synthesis inhibitor anisomycin i.c.v. prevented (closed circles) the PPhfs–MShfs reinforcement from 2 h onward. For comparison the time course of the PPhfs–MShfs-reinforced late-LTP (untreated and NaCl controls) is presented (open circles). The arrows indicate the time of tetanic stimulation of the PP or the MS.

apses. This potentiation was short lasting (<4 h) corresponding to early-LTP, similar to the one obtained in previous reports using an identical stimulation pattern (Seidenbecher et al., 1997; Frey et al., 2001). In contrast, an lfs pattern, as the one used in the present study, induced no increase of the PSA; instead a slight but significant slowly developing decrease (6 h) appeared after lfs of the PP alone (Frey et al., 2001). Interestingly, hfs or lfs of the BLA alone induced a slowly developing, slight depression of the PSA (Frey et al., 2001). Whether such effects might be causally related to the depression observed after lfs of the PP, and whether they could be considered a form of long-term depression deserves future examination.

Our results after hfs of the MS in the paired PPhfs–MShfs protocol (Fig. 2A) support our previous findings where we have shown that heterosynaptic, non-glutamatergic afferents can modulate the maintenance of LTP induced at a glutamatergic synaptic population. Septo-hippocampal interactions are well known to be involved in the production of θ rhythm in the hippocampal EEG (Bland et al., 1999), a cholinergic rhythm which can be induced by activation of the amygdala (Dringenberg and Vanderwolf, 1996). Septal stimulation can affect PP–DG-evoked potentials (Robinson and Racine, 1982; Carre and Harley, 2000) and hippocampal LTP (Robinson and Racine, 1982; Robinson, 1986) using a shorter interval between stimuli and restricted to the initial induction of LTP.

Electrical stimulation of the MS can activate local cholinergic fibres projecting to the hippocampus, but it can also activate noradrenergic, dopaminergic, serotoninergic, GABA-ergic and histaminergic fibres passing through the septum and reaching the hippocampus via the fimbria-fornix. In our previous work we have shown that the blockade of cholinergic (muscarinergic) and β-adrenergic, but not dopaminergic D1-receptors, abolishes the reinforcing effect of stimulating the BLA (Frey et al., 2001). Surprisingly, we found in this study only an effect of the β-adrenergic but not muscarinergic receptor blocker on the reinforcement of DG LTP by stimulating the MS. An explanation for that could be that the location of the stimulation electrode in the MS was optimal to modulate noradrenergic but suboptimal to stimulate cholinergic inputs to the hippocampus. Future experiments will further investigate this hypothesis. However, we could also show that the reinforcement of DG LTP by MS stimulation required protein synthesis thusly demonstrating a transformation of early into late-LTP by β-adrenergic receptor stimulation.

Acetylcholine and norepinephrine are both able to produce an LTP-like, slowly developing potentiation if applied to the DG (Stanton and Sarvey, 1985b; Auerbach and Segal, 1994) very similar to the potentiation produced by the neurotrophin brain-derived neurotrophic factor (BDNF) (Kang and Schuman, 1995). All these forms of potentiation are protein synthesis-dependent (Stanton and Sarvey, 1985a; Kang and Schuman, 1996). Our working hypothesis is that the prolonged maintenance of LTP in general depends on heterosynaptic activity: glutamatergic events are mainly required to store afferent information transiently (Morris and Frey, 1997; Frey and Morris, 1998), i.e. generating early-LTP and to mark the activated synapses in a specific way, which makes them able to capture plasticity-related proteins necessary to transform early into late-LTP. Heterosynaptic events are involved in regulating the availability of plasticity-related proteins thusly guaranteeing the potential transformation of a short- into a long-term memory trace. In distinct cases heterosynaptic activity such as

local increases in cyclic AMP may cause the non-specific activation ("tagging") of synapses which would explain the non-specific drug-induced potentiation by different modulatory substances such as norepinephrine, dopamine, BDNF, protein kinase A activators, etc. (Stanton and Sarvey, 1985a; Frey et al., 1993; Auerbach and Segal, 1994; Kang and Schuman, 1995; Frey, 2001). All of these processes are included in the synaptic-tagging hypothesis (Frey and Morris, 1997) which provides a rationale to understand the heterosynaptic requirement for long-lasting plastic changes to occur.

The same events might explain the reinforcing effects of our double-stimulation paradigm. The first tetanus to the PP, along with inducing LTP, tags the activated synapses. The effect of the second tetanus is not to increase the level of potentiation but to activate molecular cascades leading to macromolecular synthesis (Frey et al., 1993, 1995) for instance by the further accumulation of intracellular Ca^{2+} by glutamatergic or other transmitter systems which may activate processes inducing protein synthesis, and the distribution and insertion of the newly synthesised proteins at the tagged synaptic contacts (Frey and Morris, 1997, 1998). Previous and the current results support our hypothesis that field stimulation and induction of LTP in the PP–DG synapses can be modified by heterosynaptic events (Dunwiddie et al., 1982; Stanton and Sarvey, 1987; Bramham et al., 1997).

Therefore, the stimulation of the septum reproduced some aspects of the reinforcing effects of the BLA. There are, however some important differences which must be considered in future experiments. Only hfs of the MS is effective to prolong DG LTP, whilst lfs of the BLA also resulted in an effective reinforcement (Frey et al., 2001). On the other hand, only hfs of the PP is able to induce a potentiation by itself, hfs of the amygdala or the septum can act only on and modulate an existing potentiation elsewhere, such as at the PP–DG synapses. The concept of instructive (glutamatergic PP) and modulatory synapses (septal and entorhinal inputs) is well illustrated in this example (Matthies, 1989). The knowledge obtained in this study is not only of theoretical value. We have recently shown that amygdala–hippocampus interactions (Almaguer et al., 2002), as well as behavioural reinforcement of LTP (Bergado et al., 2001) are impaired in aged rats with cognitive deficits. Further studies on these mechanisms should contribute to the development of new strategies to treat memory disorders and thusly, could have a direct practical value.

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Capìtulo 7: La administración de norepinefrina, pero no de oxotremorina refuerza procesos de plasticidad sináptica

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En este trabajo intentamos una aproximación farmacológica diferente al tema que hemos venido desarrollando. En lugar de intentar bloquear determinados sistemas neurotransmisores con antagonistas, nos propusimos suplantar la estimulación directa de determinados núcleos por la administración de agonistas conocidos de los sistemas neurotransmisores seleccionados.

En este caso se trató de la transmisión noradrenérgica y colinérgica que fueron las que mostraron resultados de bloqueo del reforzamiento cuando se emplearon antagosnistas específicos.

La hipótesis de trabajo fue la siguiente: *Si el bloqueo de la transmisión noradrenérgica y colinérgica impide el reforzamiento conductual, entonces la administración de agonistas directos de estos sistemas puede mimetizar el efecto del reforzamiento conductual.*

Como agonista noradrenérgico empleamos norepinefrina, mientras que para la transmisión colinérgica empleamos oxotremorina, un agonista de los receptores muscarínicos.

La administración intraventricular de norepinefrina 15 minutos después de la inducción de la LTP en el giro dentado mostró efectos similares a los del reforzamiento. Este efecto fue dosis dependiente y al parecer muestra el clásico patrón de U invertida. La dosis más baja empleada fue poco efectiva y la dosis más alta fue inefectiva. La oxotremorina no mostró el mismo efecto de reforzamiento para ninguna de las tres dosis empleadas.

Es posible mimetizar el efecto de reforzamiento afectivo mediante la administración de agonistas de la neurotransmisión noradrenérgica en dosis adecuadas.

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Long-term potentiation in the dentate gyrus in freely moving rats is reinforced by intraventricular application of norepinephrine, but not oxotremorine

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Abstract

Growing evidence suggests that processes of synaptic plasticity, such as long-term potentiation (LTP) occurring in one synaptic population, can be modulated by consolidating afferents from other brain structures. We have previously shown that an early-LTP lasting less than 4 h (E-LTP) in the dentate gyrus can be prolonged by stimulating the basolateral amygdala, the septum or the locus coeruleus within a specific time window. Pharmacological experiments have suggested that noradregenergic (NE) and/or cholinergic systems might be involved in these effects. We have therefore investigated whether the direct intraventricular application of agonists for NE- or muscarinic receptors is able to modulate synaptic plasticity. E-LTP was induced at the dentate gyrus of freely moving rats using a mild tetanization protocol that induces only an E-LTP. NE or oxotremorine (OXO) were applied icv 10 min after the tetanus. Results show that low doses of NE (1.5 and 5 nM) effectively prolong LTP. A higher dose (50 nM) was not effective. None of the OXO doses employed (5, 25, and 50 nM) showed similar effects. These results stress the importance of transmitter-specific modulatory influences on the time course of synaptic plasticity, in particular NE whose application mimics the reinforcing effect of directly stimulating limbic structures on LTP.

Keywords: Long-term potentiation; Reinforcement; Norepinephrine; Oxotremorine; Early-LTP; Late-LTP; Functional plasticity

1. Introduction

Long-term potentiation (LTP) is a form of functional neural plasticity which has been considered a cellular correlate of memory formation (Bliss & Collingridge, 1993; Matthies, 1982). LTP has phases, as does memory and the molecular mechanisms implicated in LTP phases are also implicated in memory. An initial early-LTP (E-LTP) lasting less than 4 h and not depending on protein synthesis might eventually be converted into a late-LTP (L-LTP) extending beyond 4 h and requiring the synthesis of new proteins (Krug, Lössner, & Ott, 1984; Frey, Krug, Reymann, & Matthies, 1988; Matthies et al., 1989; Matthies, 1998).

Studies by Seidenbecher et al. for the first time showed that an E-LTP, elicited at the dentate gyrus of freely moving rats by a mild tetanus, is transformed into a L-LTP if, within a restricted time window, it is associated with a behavioral stimulus of strong motivational significance (Seidenbecher, Balschun, & Reymann, 1995; Seidenbecher, Reymann, & Balschun, 1997). This behavioral, or motivational reinforcement of LTP

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depends on the function of the amygdala (Almaguer-Melian, Martínez-Martí, Frey, & Bergado, 2003), is protein synthesis dependent (Bergado, Almaguer-Melian, Kostenko, Frey, & Frey, 2003) and is blocked by propranolol, a β-receptor blocker (Seidenbecher et al., 1997). These data supported our hypothesis that late-LTP requires heterosynaptic modulation—here by norepinephrine (NE)—in addition to previously activated glutamatergic synapses—for review see Matthies (1998) and Frey and Morris (1998).

A similar conversion of E-LTP into L-LTP as that seen with behavioral reinforcement can be obtained by a direct electrical stimulation of the amygdala shortly before or after the induction of an E-LTP by a mild tetanus. The effect is abolished by propranolol and by the muscarinic antagonist atropine (Frey, Bergado, Seidenbecher, Pape, & Frey, 2001). A further evidence for a role for cholinergic systems in the modulatory effect leading to reinforcement is the observation that lesioning the main cholinergic projection to the hippocampus abolishes the L-LTP promoting effects of the amygdala stimulation (Jas, Almaguer, Frey, & Bergado, 2000). However, propranolol, but not atropine, blocked the reinforcing effects on LTP of stimulating the septum (Frey, Bergado, & Frey, 2003).

All of the LTP-reinforcing actions—i.e., motivational, amygdalar or septal—seem to activate protein synthesis, a prerequisite for late-LTP to occur (Bergado et al., 2003; Frey et al., 2001, 2003). Both NE and acetylcholine (ACh) are able to activate protein synthesis via molecular cascades involving kinases regulating transcription, like MAPK (Greenwood & Dragunow, 2002; Hamilton & Nathanson, 2001; Massey, Bhabra, Cho, Brown, & Bashir, 2001; Rosenblum, Futter, Jones, Hulme, & Bliss, 2000; Stratton, Baraban, & Worley, 1988) or protein kinase A (Frey & Morris, 1998). It appears thus pertinent to test whether the direct administration of NE or a cholinergic agonist can mimic the reinforcing effects on LTP shown in the above-mentioned paradigms.

The results obtained showed that NE, but not oxotremorine (a muscarinic agonist), dose-dependently reinforce LTP in the dentate gyrus.

2. Materials and methods

2.1. Experimental animals

Male Sprague–Dawley rats, weighing 250 g by the time of surgery, were obtained from the Cuban national breeder (CENPALAB, La Habana) and kept in translucent Macrolon cages (five animals per cage before surgery) under controlled temperature (22–25 °C), humidity (lower than 60%), and light exposure conditions (12:12 h light:dark). All experiments were carried out during the light phase of the cycle (8:00 am to 6:00 pm). Access to food and water were ad libitum during the experiment. Animal handling and experimental procedures were performed under observance of the Cuban Regulations for the Use of Laboratory Animals.

2.2. Surgery

Under narcosis (chloral hydrate 420 mg/kg, ip) the animals were implanted with a monopolar recording electrode at the dentate gyrus (AP: −3.8 mm, ML: 2.0 mm, DV: −3–7) a bipolar stimulating electrode at the medial perforant pathway (AP: −7.5 mm, ML: 4.0 mm, DV: −3.9 mm) and a guide cannulae directed at the lateral ventricle (AP: −0.3 mm, ML: 1.3 mm, DV: −2.4 mm). All implants were placed in the right hemisphere using bregma as reference (bregma and lambda were at the same horizontal plane). Additionally, two miniscrews were fixed over the parietal and frontal bones to serve as reference electrode and connection to earth. Electrodes were then connected to minisockets and all implanted material was secured with dental cement. After surgery the animals were individually housed and treated with antibiotics during 7 days to prevent infection (solution of 250 mg tetracycline dissolved in 500 ml water).

2.3. Electrophysiology

A week after surgery the animals were carried to the recording chamber (between 8:00 and 8:30 am) and connected to the stimulation and recording set through a swivel, which allowed the free movement of the animals. Stimulation and recording of the evoked potentials was started no less than after 4 h of habituation.

Each recording consisted of five averaged consecutive responses of 0.1 Hz. Responses were bandpass filtered (1–5000 Hz) and amplified using an AB-621G (Nihon-Kohden, Japan) bioelectric amplifier and passed through a CED 1401+ A/D converter to an IBM-compatible computer which allow visualization of the evoked potentials and the automatic measurement of its components with the aid of a self-made program (IntraCell for Windows, IfN Magdeburg). The same program was used to control the delivery of electrical stimulation to the perforant path by an isolated pulse stimulator (A-M Systems Mod. 2001, USA).

The amplitude of the population spike (PSA), the component reflecting the synchronous discharge of action potentials by the granule cell population, was measured and the changes in its relative amplitude (percent from baseline) were used to evaluate changes in synaptic efficacy. We have preferred to base on the PSA rather than the population excitatory post-synaptic potential (pEPSP) because of technical reasons while working in vivo and freely moving animals. On the other hand,

changes in PSA reflect the modifications in the output from the neuronal population that we have investigated, which is of a great significance in functional terms.

An Input/Output curve was first obtained from each animal, by recording the evoked responses in the dentate gyrus to stimulation (100, 200, 400, 600, and 800 μA) of the perforant path. The intensity required to produce a 40% maximum PSA was determined and used for the rest of the experiments. Twelve recordings were taken at 5 min interval. The averaged PSA was used as baseline.

Long-term potentiation was induced by a mild tetanus consisting of three trains of 15 impulses at 200 Hz (10 s intertrain interval). Five minutes after induction of LTP a test recording was taken to evaluate the initial level of potentiation. Starting 30 min after tetanization test recordings were taken every 15 min for up to 6 h to follow the time course of LTP. A final series of four recordings separated by 15 min intervals was taken 24 h after LTP induction.

2.4. Substances and injection

NE, a natural ligand of α- and β-receptors, and oxotremorine, an agonist of muscarinic cholinergic receptors, were employed (both from Sigma, Saint Louis, USA). Both substances were tested for their efficacy before the beginning of the experiments using a different group of animals. NE was able to induce a significant increase in heart rate (ip 1 mg/kg) and oxotremorine induced sustained theta activity in the hippocampal EEG (ip 0.1 mg/kg). For the experiments the substances were applied in the right lateral ventricle in different doses. For NE, doses of 1.5, 5, and 50 nM were employed. NE was dissolved in saline solution containing ascorbic acid (2 mg/ml) to prevent oxidation. Oxotremorine, dissolved in saline, was prepared in concentrations of 5, 25, and 50 nM. The solutions were prepared fresh every day in a way that a final volume of 1 μl was injected in every case. The injection was performed over 1 min using an injection cannula (1 mm longer than the guide cannula) connected to a micro-syringe (Hamilton, USA). The cannula was left in place for 5 min to reduce retrograde flux of the injected substance.

2.5. Experimental groups

Experimental groups were constituted according to the drug applied, the dose, and the stimulation pattern applied.

To test the stability of the evoked potentials over the time period studied and the effect of the applied substances and the vehicles, a series of control groups were studied. In these groups no LTP was induced. Substances were applied 5 min after 1-h baseline recordings. One group (basal, $n = 10$) received no treatment. Two groups were injected with 1 μl saline solution (basal + NaCl, $n = 10$) or the vehicle containing ascorbate (basal + Veh, $n = 8$). Additionally, two groups received 1 μl of a solution containing 5 nM NE (basal + NE, $n = 9$) or the same volume of 25 nM oxotremorine (basal + OXO, $n = 8$).

To establish the time course of the LTP induced by the mild tetanus and the effects of the vehicles the following groups were created: a control LTP group ($n = 11$) in which LTP was induced and followed without injection, and two groups in which LTP was induced and 1 μl of saline (LTP + NaCl, $n = 8$) or vehicle with ascorbic acid (LTP + Veh, $n = 8$) was injected icv 10 min later.

To study the effects of NE on LTP we used three groups, corresponding to the different doses (1.5 nM, $n = 6$; 5 nM, $n = 7$; and 50 nM, $n = 7$) of NE-injected icv 10 min after the induction of LTP with a mild tetanus. In the same way, three groups injected with oxotremorine were studied corresponding to each of the doses employed (5 nM, $n = 8$; 25 nM, $n = 8$; and 50 nM, $n = 9$).

2.6. Histology

After finishing the electrophysiological studies all animals were intracardially perfused with formalin under narcosis. The brains were extracted and processed for histology with cresyl violet to assess the correct location of the cannulae. The microscopic observation of the slices confirmed the correct location—in the direction of the lateral ventricle—in all cases.

2.7. Statistics

Data were analyzed using a two-way ANOVA with Group (treatment) and Time (repeated measures) as factors. Post hoc studies with the Duncan's test were performed when significant differences between groups were confirmed. When the factor Time was significant, paired t tests were carried out to confirm within group differences to baseline. In every case differences were considered significant only if $p < .05$.

3. Results

The comparison among control groups, without LTP induction (Fig. 1) showed no differences between groups ($F_{4,36} = 0.775722$), suggesting that none of the control treatments with vehicle or substance induced significant deviations from baseline. However and unexpectedly, the factor Time showed significant differences ($F_{7,252} = 2.083680$). The within groups analysis (paired t test) showed that this was caused only by the group treated with 5 nM NE. The animals that received NE

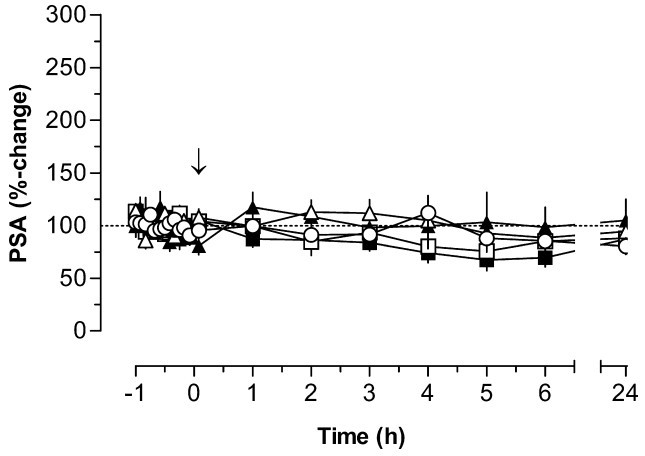

Fig. 1. Control recordings of the PSA and the influence of drugs investigated on baseline potentials measured as the population spike amplitude (PSA) during a period of 24 h. The mean PSA (±SEM) relative to baseline and expressed in percentile values (PSA % change) for each time point and for each group are shown. No differences between groups were found (ANOVA, two-way). Factor Time showed significant differences only for the group treated with norepinephrine (basal + NE, filled squares) during 4–6 h. Other symbols: open circles: basal group; open triangles: basal + NaCl group; filled triangles: basal + OXO group; and open squares: basal + Veh group. The arrow (↓) shows the time at which substances were applied, except for the group basal which received no treatment or handling at that time.

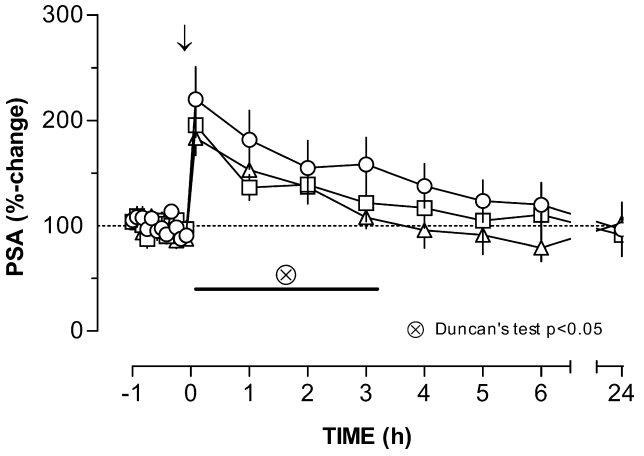

Fig. 2. Induction and maintenance of E-LTP and the influence of the vehicles employed. The mean PSA % change (±SEM) for each time point before (time −1 to 0) and after tetanization (times 0–24) and for each group are shown. In all the groups the pattern of tetanization applied at time 0 induced an increase in the PSA which remained significant only up to the third hour after induction (Duncan's test). No differences between groups were found (ANOVA, two-way). Symbols: open circles: Control LTP group; open squares: LTP + Veh group; and open triangles: LTP + NaCl. The arrow (↓) shows the time at which substances were applied, except for the group Control LTP which received no treatment or handling at that time.

developed a slight depression of the PSA that was significant between 4 and 6 h after injection. Other groups (basal, basal + NaCl, basal + OXO, and basal + Veh) showed no significant deviation from baseline over time.

The control groups in which LTP was induced (control LTP, LTP + Veh, and LTP + NaCl) showed no significant differences to each other (Fig. 2). The two-way ANOVA revealed no differences for the factor Group ($F_{2,21} = 0.52733$). The factor Time showed a significant effect ($F_{8,168} = 19.32421$) confirming that a relevant change in the PSA occurred in all groups after tetanization. This potentiation remained significant for 3 h (Duncan's test).

The administration of NE in the lower doses (1.5 and 5 nM) significantly prolongs LTP, but not the higher dose of 50 nM, as shown in Fig. 3. The two-way ANOVA revealed significant effects for both factors, Group ($F_{2,17} = 6.29625$) and Time ($F_{8,136} = 16.33383$). The tetanization paradigm employed induced an increase in the PSA in all groups, but the duration of the potentiation was different according to the dose of NE employed. The post hoc Duncan's test showed that the groups receiving 1.5 and 5 nM NE were significantly different from the vehicle treated group while the animals injected with 50 nM NE did not differ from vehicle. The *t* test for paired samples proved that LTP was prolonged in the 1.5 and 5 nM groups to 5 and 6 h, respectively.

Oxotremorine produced no effects on the magnitude or the duration of LTP (Fig. 4). Treatments using different doses of oxotremorine did not reveal pronounced

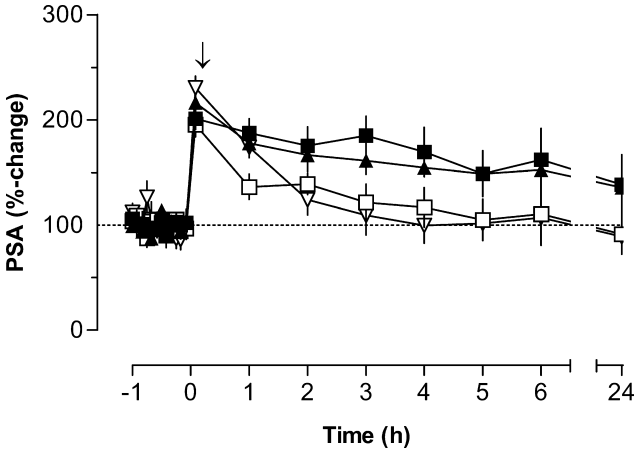

Fig. 3. Effect of norepinephrine on E-LTP. The mean PSA % change (±SEM) for each time point before (time −1 to 0) and after tetanization (times 0–24) and for each group are shown. Significant differences between groups were found (ANOVA, two-way). All groups reached the same level of PSA potentiation, but they differ in how long the potentiated state holds. Groups treated with 1.5 or 5 nM norepinephrine (NE 1.5 filled squares; and NE 5 filled triangles) differed significantly from vehicle treated animals (Veh open squares) or those receiving a higher dose of NE (NE 50 open inverted triangles) as confirmed by the post hoc Duncan's test. Significant differences from baseline values were found up to 5 h after LTP-induction and up to 6 h for the NE 1.5 and NE 5 groups, respectively. The arrow (↓) shows the time at which substances were applied.

changes on early-LTP when compared with the NaCl-injected control group as indicated by the two-way ANOVA ($F_{3,29} = 1.99304$). The factor Time, as expected

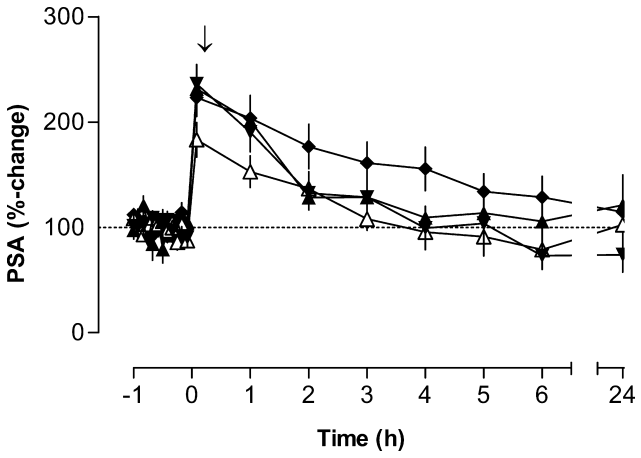

Fig. 4. Effect of oxotremorine on E-LTP. The mean PSA % change (±SEM) for each time point before (time −1 to 0) and after tetanization (times 0–24) and for each group are shown. No major differences among groups were found (ANOVA two ways). All groups reached the same initial level of potentiation after LTP induction which decline to baseline within 3 h. Only the group receiving the higher dose of oxotremorine (LTP + OXO 50 filled diamonds) showed a slight, but significant Group/Time interaction. Other symbols: open triangles: LTP + NaCl group; filled inverted triangles: LTP + OXO 5; and filled triangles LTP + OXO 25. The arrow (↓) shows the time at which substances were applied.

after tetanizing, resulted in significant changes in PSA ($F_{8,232} = 58.43984$). The interaction Group × Time ($F_{24,232} = 1.89460$) was also significant. The post hoc Duncan's test determined that this slight, time-dependent effect of oxotremorine was caused by the highest dose (50 nM) used.

4. Discussion

The results in the basal group of animals, without tetanization, vehicle or drug treatment showed that the evoked potentials in the dentate gyrus induced by stimulation of the perforant path were stable over time. This is important to rule out a major effect of circadian factors inducing transient modifications in synaptic transmission. Furthermore, neither the injection procedure nor the volume injected affected the evoked potentials. None of the injected vehicles or oxotremorine produced changes in the PSA. However, the control injection of NE induced a late and slight, but significant decrease of the PSA. Earlier reports have shown that the administration of NE to hippocampal slices was able to increase the amplitude of the PSA (Lacaille & Harley, 1985) developing a delayed onset potentiation of the PSA (maximum was reached after about 1 h). Additionally, this NE-induced potentiation in vitro was dependent on protein synthesis (Stanton & Sarvey, 1985b). Therefore, the authors concluded that this form of potentiation resembled a LTP-like phenomenon. What are the reasons for the obvious contradiction between results obtained in vitro or—as in our case—in vivo? There are a couple of possibilities such as the difference between the in vitro and in vivo conditions. Furthermore, the NE concentration employed was much higher (10 μM) than the ones employed in our study in vivo. Additionally, extrahippocampal activity may interact in vivo more dramatically on evoked potentials than in vitro. In any case the depression reported here seemed to be the consequence of an interaction of NE-receptor activation on long-lasting plastic events rather than a direct effect of NE on membrane potentials. Interestingly, in previous studies we have shown a similar depression of the PSA recorded in the dentate gyrus either after low- or high-frequency stimulation of the amygdala (Frey et al., 2001) or in aged animals submitted to a behavioral reinforcement paradigm. Whether there is a functional or mechanistic link between them remains to be determined.

The control LTP group confirmed that the pattern of mild tetanization employed was only able to induce E-LTP, decaying within 4 h. The saline and vehicle groups failed to reveal any non-specific effect of manipulation, injection or volume on the time course of E-LTP. Any modification in LTP after substance application should, therefore, be attributed to a specific effect of the applied agonist.

Interestingly, NE dose-dependently influenced the time course of induced E-LTP if the mild tetanus preceded NE-application. The lower doses showed a reinforcing effect, consisting in a prolongation of the potentiated state and not in an increase of the level of PSA potentiation. This effect was similar to the one observed after behavioral reinforcement by motivational stimuli (Seidenbecher et al., 1997), and after direct, electrical stimulation of the basolateral amygdala (Frey et al., 2001). Both, motivational and amygdala stimulation induced a reinforcement of LTP which was blocked by the β-1-receptor antagonist propranolol. The effect reported here strengthens the hypothesis that NE might specifically be a part of the mechanisms mediating such reinforcement effects finally expressed in the dentate gyrus.

The mechanism for this effect of NE on LTP might be explained in terms of a modulatory-metabolic action of NE. NE is a strong activator of the cAMP-PKA cascade (Gereau & Conn, 1994; Stanton & Sarvey, 1985a) which may activate the protein synthesis required for the expression of L-LTP. This β-adrenergic reinforcement seemed particularly important to reinforce LTP induced by relatively weak tetanic stimuli (Straube & Frey, 2003). A functional relationship like the one suggested here may support and explain the multiple experiments showing a modulating-reinforcing action of NE on memory (McGaugh & Cahill, 1997). The action of NE might occur directly on the dentate gyrus, but it might involve other brain structures, like the amygdala (McGaugh, 2002). This limbic structure plays a critical role in emotional reactions and the motivational modu-

lation of memory and LTP (Richter-Levin, 2004; Richter-Levin & Akirav, 2003).

Regarding the action of the muscarinic agonist oxotremorine, our findings surprisingly do not confirm the expectation of a reinforcing effect of cholinergic-receptor activation on E-LTP in the dentate gyrus. None of the employed doses of this substance was able to significantly modify LTP, only a mild Group × Time interaction was found with the a relatively high dose. Cholinergic mechanisms have been proposed to sustain memory functions (Baratti, Opezzo, & Kopf, 1993; Blokland, 1995; Conner, Culberson, Packowski, Chiba, & Tuszynsky, 2003; Ohno, Yamamoto, & Watanabe, 1994; Ramirez Lugo, Miranda, Escobar, & Bermudez-Rattoni, 2003; Warburton et al., 2003) and deterioration of cholinergic systems is considered as a major cause for age-related memory impairments (Bartus, 2000; Bartus, Dean III, Beer, & Lippa, 1982). Both lines of evidence have guided experimental and clinical trails to restore memory functions based on pharmaceuticals (Lyketsos, Corazzini, Steele, & Kraus, 1996; Sands, Katz, & Schneider, 1999) transplants (Fernández et al., 1994; Leanza, Martinez-Serrano, & Bjorklund, 1998) or trophic factors (Fischer, Sirevaag, Wiegand, Lindsay, & Bjorklund, 1994). The role of cholinergic mechanisms in LTP has been, however, controversial. While some reports describe a facilitatory, and even an inducing role (Auerbach & Segal, 1994; Boyd, Trepel, & Racine, 2000; Hess & Donoghue, 1999; Leung, Shen, Rajakumar, & Ma, 2003; Motooka et al., 2001; Segal & Auerbach, 1997; Ye, Qi, & Qiao, 2001) others found mild or no effects (Abe, Nakata, Mizutani, & Saito, 1994; Feasey-Truger, Li, & ten Bruggencate, 1992; Jouvenceau, Billard, Lamour, & Dutar, 1996; Kleschevnikov, Sinden, & Marchbanks, 1994). Previous results from our group have also provided contradictory results with respect to cholinergic influences via muscarinic receptors in LTP-reinforcement mechanisms. While atropine blocked reinforcement induced by stimulation of the basolateral amygdala (Frey et al., 2001), it did not block reinforcement induced by stimulation of the septal area (Frey et al., 2003) suggesting that some actions might involve other extrahippocampal structures. A targetted application instead of an icv injection might contribute to clarify this issue.

In summary, direct activation of β-adrenergic, but not muscarinic receptors, can convert early- into late-LTP in the dentate gyrus in vivo in freely moving animals. The possibility of modulating L-LTP by the direct application of agonistic drugs may lead to the development of drugs to treat memory disorders in the future.

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Capítulo 8: Efecto de paradigmas de reforzamiento de la plasticidad sináptica sobre la liberación de neurotransmisores en el giro dentado

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Los estudios farmacológicos previos habían mostrado la necesidad de participación de sistemas de neurotransmisión colinérgico y noradrenérgico. Sin embargo, en algunos trabajos aparecieron resultados contradictorios, sobre todo con relación al papel de la acetil colina. El estudio de estimulación de la amígdala muestra que la atropina bloquea el efecto. Sin embargo, no fue así en el caso de la estimulación del septum y tampoco la oxotremorina mimetizó los efectos del reforzamiento.

Con todas esas interrogantes en consideración, intentamos resolver las contradicciones midiendo directamente la liberación de estos y otros neurotransmisores en el giro dentado cuando se estimula la amígdala basolateral o cuando se someten los animales a reforzamiento conductual.

Este fue un estudio exploratorio. Más que testar una hipótesis intentaba encontrar pistas para interpretar las contradicciones antes mencionadas. Para ello empleamos la técnica microdialisis cerebral que permite la recogida de muestras de fluido del cerebro *in situ* e *in vivo*. Se aprovechó además la disponibilidad de ratas viejas con deficiencias cognitivas comprobadas en estudios conductuales para explorar los efectos de la edad sobre estos procesos.

Es justo reconocer que, en el interés de abarcar la mayor cantidad de sistemas neurotrasmisores posibles (aminoácidos, aminas biógenas y acetil colina) se hizo un diseño experimental que requería la recogida de muestras por períodos de tiempo relativamente largos. Eso, por una parte, puede "diluir" cualquier variación aguda provocada por el reforzamiento conductual o la estimulación, y por otra, obligó a emplear

frecuencias bajas de estimulación de la amígdala, muy diferentes a las utilizadas en los estudios anteriores. Tal vez por esas razones los resultados no arrojaron las respuestas esperadas y, en algunos casos hicieron aún más compleja la interpretación.

Las variaciones detectadas durante la estimulación de la amígdala mostraron un aumento en los niveles acetil colina y una disminución de noradrenalina y serotonina. Más sorprendente aún, el reforzamiento conductual disminuye los niveles de glutamato y GABA.

No obstante estos problemas, el trabajo tiene valor por cuanto muestra una tabla comparativa de los niveles basales de neurotransmisores y metabolitos asociados entre ratas jóvenes y ratas viejas con deficiencias cognitivas a partir de muestras directas de fluido cerebral y cuantificadas con las técnicas más modernas disponibles.

Otro resultado interesante fue que las ratas viejas no mostraron alteración alguna de los niveles de ninguna de las sustancias estudiadas, ni ante la estimulación de la amígdala, ni por efecto del reforzamiento conductual.

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Effect of LTP-reinforcing paradigms on neurotransmitter release in the dentate gyrus of young and aged rats

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Abstract

Long-term potentiation (LTP) is considered a cellular correlate of memory processing. A short-lasting early-LTP can be prolonged into a late-L TP (>4 h) by stimulation of the basolateral amygdala (BLA) or motivational behavioral stimuli in young, but not in aged, cognitively impaired rats. We measured the changes in transmitter release-induced by BLA or behavioral reinforcement—in young and aged cognitively impaired rats, after implanting a microdialysis cannula at the dentate gyrus. Samples were taken under baseline conditions and during stimulation of BLA. Rats were water deprived and tested again next day, taking samples after allowing access to water. Higher concentrations of choline, HIAA, aspartate, glutamate, and glycine were found in baseline samples from young animals compared to aged. In young animals, BLA stimulation increased the levels of ACh and reduced norepinephrine and serotonine, while behavioral reinforcement reduced the levels of glutamate and glycine. These effects were absent among aged rats, suggesting that this reduced neurochemical response might be linked to the impaired LTP-reinforcement reported previously.

Keywords: LTP; Amygdala; Behavioral reinforcement; Neurotransmitters; Microdialysis

The hippocampus is a cortical structure involved in explicit and spatial memory in rodents and humans [19,39,48]. The inputs to this limbic structure are highly organized according to origin and neurotransmitter system. An excitatory-glutamatergic input reaches the dentate gyrus, and other hippocampal subfields, via the perforant pathway (PP), while other, mainly modulatory, transmitters arising in the medial septum and brain stem nuclei converge in the fimbria–fornix system (FF) to enter the hippocampal formation [37,58]. The latter include acetylcholine (ACh), γ-aminobutyric acid (GABA), norepinephrine (NA), dopamine (DA), and serotonin (5-HT), among others.

Long-term potentiation (LTP)—first described in the early 1970s [13,14]—represents a long-lasting increase in synaptic efficacy and is considered a cellular correlate of memory [15,31,33,34]. In the dentate gyrus, the CA1 subfield, and other brain regions, LTP is initiated by the activation of NMDA–glutamatergic receptors which allows Ca^{2+} to enter the postsynaptic terminal [7]. The Ca^{2+}-mediated activation of protein kinases, like the calcium–calmodulin-dependent kinase (CaM-kinase), provokes local changes in receptor proteins, like the AMPA–glutamatergic receptor, which are considered to be responsible for the initial increase in synaptic efficacy [27,36,54]. At later stages, LTP requires the synthesis of new proteins for its maintenance [25,30,38].

Based on this background, a two phase model of LTP has been proposed. An early, protein synthesis

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independent phase, lasting for about 4 h (E-LTP), is followed by a longer-lasting, protein synthesis-dependent late-LTP (L-LTP) [30,32].

While glutamatergic synapses appear able to induce LTP (instructive synapses), this does not preclude a cooperative action of non-glutamatergic afferents to the same population (modulatory synapses) [31]. Such modulation appears to be of particular importance for L-LTP. In the CA1, a role for DA has been well documented [23,24,26,43]. In the dentate gyrus, behavioral manipulations known to be associated with the activation of modulatory inputs are able to prolong an E-LTP induced by a mild tetanus to the PP, into a L-LTP [47,53].

Our group has studied the neural and neurochemical mechanisms involved in LTP reinforcement. The basolateral amygdala (BLA) seems to play a key role because its temporal or permanent inactivation abolishes reinforcement [3]. Moreover, stimulating the BLA can produce a similar reinforcing effect to that seen with behavioral stimuli [22]. This effect is mediated by the FF system [28]. Pharmacological studies have consistently reported a role for NA [22,52], however, the results for ACh have been contradictory. While atropine blocks the reinforcing effects of BLA-stimulation [22], it does not influence the reinforcing effects of stimulating the medial septum [21].

All these studies have been carried out using pharmacological approaches, i.e., injecting antagonists in the cerebral ventricles, thus raising the possibility that the applied substances might be exerting their effects on the dentate gyrus indirectly through other brain structures.

We carried out the present study in an attempt to obtain direct evidence concerning which transmitter systems might be involved in both behavioral and BLA-stimulation reinforcement of E-LTP. We used in vivo microdialysis sampling to measure neurochemical release in the dentate gyrus under both reinforcing paradigms.

A main goal of our study was to compare the results obtained in young animals with those of aged, cognitively impaired rats. It is known that the main difference in LTP between young and aged rats is the faster decay, and consequent shorter duration, of LTP [4,5]. In line with this, our group has already shown that reinforcing paradigms are impaired in aged rats which have demonstrable memory deficits [2,9]. Changes in the pattern of activation of modulating inputs under both reinforcing paradigms could be responsible for their reduced efficacy.

Materials and methods

Animal housing and handling. Male Sprague–Dawley rats obtained from a local professional breeder (CENPALAB, Havana) were used. Young (2–3 months) and aged animals (24–27 months) obtained as retired breeders were kept in plastic translucent cages (five animals per cage) under controlled environmental conditions (temperature, humidity, and light cycle) with free access to water and food throughout the experiment, except for 1 day of water deprivation during the behavioral reinforcement paradigm. All efforts were made to reduce pain or discomfort of the animals under strict observance of the Cuban Regulations for the Use of Laboratory Animals.

Memory test. Young ($n = 6$) and aged animals were first tested for their spatial memory abilities using the Morris Water Maze (MWM) as described elsewhere [1]. The average escape latency of aged rats over the five days of training (8 trials daily) was compared with that of young animals. Aged rats showing values above the mean escape latency plus two standard deviations of the young controls ($n = 6$) were considered as cognitively impaired.

Surgery. Under ketamine narcosis (33.3 mg/kg, ip.) rats were implanted with a microdialysis cannula (CMA/12, Sweden) in the hilus of the dentate gyrus (AP: −3.8 mm, ML: 2.0 mm, DV: −3.5 mm). A stainless steel stimulating electrode was positioned at the BLA (AP: −2.8, ML: 5.0, DV: 8.5 mm). All co-ordinates were calculated from bregma, with bregma and lambda at the same height.

Sample collection. All samples were collected from freely moving animals. The day after surgery rats were placed in a plastic bowl and connected through a swivel to the microinfusion pump (CMA/100, CMA Microdialysis, Sweden). Artificial cerebrospinal fluid (125 mM NaCl, 2.5 mM KCl, 0.5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1 mM $MgCl_2 \cdot 6H_2O$, 0.2 mM ascorbic acid, and 1.2 mM $CaCl_2 \cdot 2H_2O$) was infused at 2 μL/min using a syringe pump (CMA Microdialysis, Sweden). Sample volumes of 90 μL fluid were collected over 45 min periods and stored at −80 °C until used for analysis.

Stimulation and reinforcing paradigms. After a 1 h habituation to the experimental set-up, three consecutive samples were collected to determine the baseline extracellular concentrations for each transmitter. Low frequency stimulation to the the BLA was then delivered at 0.1 Hz (0.1 ms square pulses, 400 μA) by a SS104-J Isolation Unit coupled to a SEN 3301 Electronic Stimulator (both Nihon Kohden, Japan) for 45 min.

The behavioral reinforcement paradigm was carried out the following day. A 1 h habituation was permitted and two new baseline samples were collected for that day. After 24 h of water deprivation, access to water was allowed and a sample was collected during the following 45 min.

Biochemical analysis. Amino acid levels in dialysates were determined by high performance liquid chromatography (HPLC) with fluorescence detection following automated derivatization (autosampler Gilson 231, Villiers-le-Bel, France) with *o*-phthaldialdehyde (OPA). The derivatization reaction was started by mixing 10 μL of the sample and 3 μL of the OPA solution (35 mM OPA containing 114 mM of 3-mercaptoethanol and 10% methanol in 0.4 M sodium tetraborate buffer, weekly prepared) for 2 min. Ten microliters of the derivatized sample was injected into a 15.0 cm × 3.2 mm Sphere-Clone ODS(2) reversed phase column with 3 μm particle size (Phenomenex, Macclesfield, UK) kept at 38 °C. A gradient of eluents at a 0.525 ml/min flow rate was accomplished by means of a binary pump (Beckman 126, Fullerton, CA, USA). Eluent A was 39.8 mM sodium acetate with 10% methanol at pH 5.7 and eluent B was methanol with 20% of eluent A at pH 6.7. The gradient started at 23% B, rose to 45% B in 0.5 min, to 70% B at 6.5 min, to 97% B at 10 min, and returned to 23% B at 16 min. The fluorescence detector (CMA/280, CMA Microdialysis, Sweden) was set at an excitation wavelength of 340 nm and an emission wavelength of 495 nm. Chromatograms were recorded and the amino acid content in the samples was quantified by means of the external standard procedure with the 32 Karatsoftware (Beckman, Fullerton, CA, USA).

Monoamines and metabolites were measured by HPLC with electrochemical detection. The detector (Waters, Milford, USA), with an adapted 6-mm glassy carbon cell (Bioanalytical Systems, West

Lafayette, USA), was set at +0.65 V for the oxidation of monoamines and metabolites. The mobile phase contained 10 mM NaCl, 80 mM sodium acetate, 0.8 mM octanesulfonic acid, 0.3 mM EDTA, and 5% methanol (pH 4.5). A flow rate of 0.210 mL/min was set by an isocratic pump (Gynkotek M480, Macclesfield, UK). Compounds were separated in a 15.0 cm × 2.0 mm SphereClone ODS(2) reversed phase column with 3 μm particle size kept at 43 °C. A refrigerated autosampler (CMA/200, CMA Microdialysis, Sweden) injected 9.6 μL of the samples. Monoamine concentrations were calculated with reference to external standards using GynkoSoft software (Gynkotek, Macclesfield, UK).

Acetylcholine concentrations were determined by HPLC with electrochemical detection using a wired electrode system and a microbore column and enzyme reactor (BAS, West Lafayette, USA). The mobile phase consisted of 85 mM NaH_2PO_4, 6.6 mM NaCl, and 0.5% Kathon (antibacterial agent) at pH 8.5. The fluid was pumped at 0.120 mL/min. by an isocratic liquid chromatograph (CMA/250, Stockholm, Sweden) through a UniJet microbore column sized 530 × 1 mm. At the end of the column, an enzyme electrode kit was attached. This device consisted of an immobilized-enzyme reactor where acetylcholinesterase and choline oxidase catalyzed-reactions produced hydrogen peroxide and a 6-mm glassy carbon electrode with coated horseradish peroxidase, which was replaced weekly. The signal derived from the reduction of the hydrogen peroxide by the peroxidase was detected by an electrochemical detector (LC-4C, BAS, West Lafayette, USA) set at 0 V reducing potential. A refrigerated autosampler (CMA/200) injected 7 μL of the samples. Chromatograms were recorded and the ACh concentrations in the samples were quantified by means of the external standard procedure with the GynkoSoft software (Gynkotek, Macclesfield, UK).

Statistical analysis. The values measured for each substance under the different stimulation or reinforcing protocols for young and aged rats were compared using Student's t test for independent samples.

Within group comparisons between the different stimulation protocols were referred to the baseline of the corresponding day and compared by Student's t test for paired samples. Differences were considered significant only if $p < 0.05$.

Results

Table 1 contains the mean baseline concentration for all of the substances measured. Statistical analysis confirmed significant differences between age groups for some of the measured substances. Basal concentrations of choline, 5-hydroxyindolacetic acid (5-HIAA), aspartate, glutamate, glycine, and GABA were significantly reduced in aged animals. There were similar trends towards age-associated reductions in DA, homovanillic acid (HVA), NA, 5-HT, citrulline, and taurine but these just failed to achieve significance. In the case of ACh a slight increase was apparent among aged rats, but this was associated with a greater variability as indicated by the standard error and was not significant. The reinforcing paradigms induced modifications in the release of several transmitters.

Fig. 1 shows the changes induced by the stimulation of the BLA. ACh release was significantly increased during the electrical stimulation of this structure. On the contrary, the release of NA and 5-HT was significantly reduced. Other transmitters and metabolites showed no significant alterations.

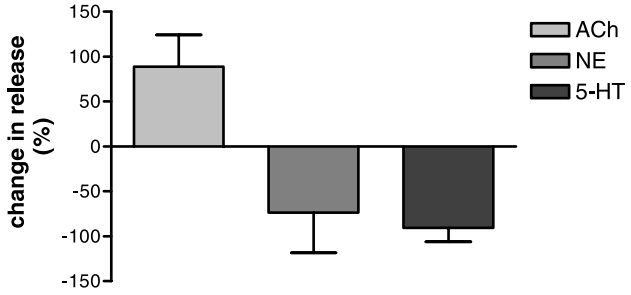

Fig. 1. Changes in the concentration of transmitter substances (% with respect to basal release) at the dentate gyrus during low frequency electrical stimulation of the basolateral amygdala. Ach, acetylcholine; NE, norepinephrine; and 5-HT, serotonine. Other transmitters or metabolites measured showed no significant changes under this condition.

Table 1
Baseline concentration (average of three consecutive samples) of neurotransmitters and related substances in the dentate gyrus of young and aged rats

Substance (units)	Young (mean ± SE)	Aged (mean ± SE)
Acetylcholine (nM)	0.34 ± 0.04	0.81 ± 0.39
Choline (nM)	389.57 ± 55.24* ($t = 2.00$)	209.12 ± 55.51
Dopamine (pg/ml)	791. 48 ± 390.33	576.18 ± 199.24
DOPAC (nM) (pg/ml)	11390.28 ± 3426.65	13944.79 ± 3271.82
HVA (pg/ml)	3777.00 ± 553.64	3059.52 ± 838.17
Norepinephrine (pg/mL)	459.10 ± 125. 67	365.42 ± 136.76
5-HT (pg/mL)	359.12 ± 144.74	188.31 ± 70.46
5-HIAA (pg/mL)	9461.05 ± 520.26* ($t = 3.26$)	6149.04 ± 980.43
Aspartate (nM)	129.55 ± 15.16*($t = 2.13$)	77.97 ± 9.19
Glutamate (nM)	915.87 ± 152.54* ($t = 1.89$)	465.89 ± 45.23
Citrulline (nM)	287.23 ± 46.91	175.27 ± 63.34
Glycine (nM)	1188.19 ± 192.74* ($t = 2.85$)	318.21 ± 92.00
Taurine (nM)	3386. 96 ± 480.02	1480.00 ± 230.47
GABA (nM)	163.98 ± 63.20* ($t = 1.43$)	13.55 ± 3.32

* Significant difference between groups, $p < 0.05$, Student's t test.

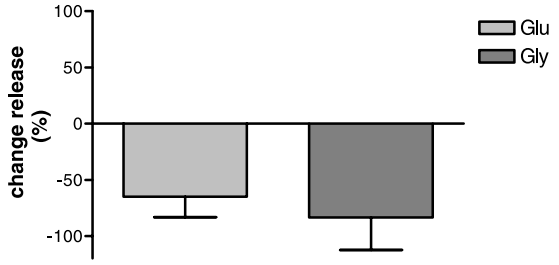

Fig. 2. Changes in the concentration of transmitter substances (% with respect to basal release) at the dentate gyrus after access to water in deprived animals. Glu, glutamate; Gly, glycine. Other transmitters or metabolites measured showed no significant changes under this condition.

The behavioral reinforcing protocol induced a different variation. As shown in Fig. 2, a significant decrease in glutamate and glycine was observed, while other transmitters and metabolites remained unaltered.

Such changes were not observed among aged rats which failed to show any significant variation in transmitter release after BLA stimulation or behavioral reinforcement.

Discussion

Baseline levels: young vs. aged

Brain aging involves not only the loss of neurons and synapses, but also a consequent reorganization in neuronal circuitries [8]. Therefore, the changes in neurotransmitter release are the overall result of the death or atrophy of specific neuronal populations, compensatory synaptic rearrangements, and also the modifications in general metabolism that accompany the aging process. In this work, we observed a decrease in the hippocampal microdialysate concentrations of several molecules related to neurotransmission in aged, cognitively impaired rats. The measurement of compounds in microdialysis samples from brain tissue provides a relative estimate of neurotransmitter levels at the synaptic cleft. However, these measurements can be also influenced by non-synaptic release of transmitters, spontaneous leakage, and inter-cellular exchange of substances for metabolic processes [51]. The latter factor could explain the decrease in the levels of several amino acids that we found in the microdialysates from aged rats, where a decrease in general metabolic activity has been described [11,45]. The reduced glutamate concentration might also be related to the dysfunctional pyramidal cell activity and impaired synaptic transmission in the aged hippocampus, where stereological studies have ruled out major cell loss [17]. Early studies have shown that both glutamate and GABA levels are decreased in Alzheimer's disease brain [20]. This mirrors our results in aged, cognitively impaired rats, a plausible animal model of Alzheimer's disease. A more recent and detailed analysis of the hippocampus of Alzheimer's patients has revealed impairment in glutamate–glutamine cycle and a tissue redistribution of glutamine synthetase, a glutamate consuming enzyme [42]. In addition, the recently reported age-related decrease in rat hippocampal glutamate decarboxylase isoform 67 [49] could be responsible for the reduced GABA release in the present experiments.

The fact that we failed to find a decrease in the dialysate concentration of ACh in the aged-cognitively impaired rats of the present experiment, while on the contrary, a non-significant tendency to increased levels was apparent, is surprising given the recognized cholinergic dysfunction in the aged brain [35] Basal dialysate acetylcholine levels have been found decreased in the dorsal hippocampus of aged rats [57]. Possible differences in the neurochemical changes during aging among different hippocampal subfields may account for this discrepancy. However, the recent findings of unimpaired or up-regulated cholinergic markers in the brains of mild Alzheimer's disease patients, which challenge and adds complexity to the traditional cholinergic hypothesis [56] are more compelling.

In addition, our finding of decreased extracellular 5-HIAA levels in the hippocampus of aged rats is consistent with previous reports of impaired 5-HT neurotransmission and metabolism in aged animals [12,41,55]. We failed to observe a corresponding decrease in 5-HT concentrations, perhaps due to the higher turnover of extracellular neurotransmitters compared with neurotransmitter metabolites. Serotonergic activity in hippocampus has been correlated with cognitive performance in rats [50], while neurodegenerative changes in the nucleus raphae dorsalis have been described in Alzheimer's brains [59]. These results underscore a role for serotonergic deficit in age-related brain functional impairment.

BLA induced modifications in young adult rats

The low frequency stimulation of the BLA induced an increase in ACh release and a reduction of NA and 5-HT release in the dentate gyrus of young animals. Previous results have shown that the reinforcing effect on E-LTP of stimulating the amygdala can be blocked by atropine (a muscarinic receptor blocking agent) and propanolol (a β-1 receptor antagonist) [22]. However, it must be considered that, those drugs were applied in the ventricle, thus away from the dentate gyrus (the presumed structure of action) where we have now directly measured transmitter release via microdialysis.

Present results support a direct involvement of cholinergic mechanisms in the reinforcement of E-LTP at the dentate gyrus by stimulation of the amygdala, while the actions of NA and 5-HT appear to be, more likely,

indirect, perhaps on other structures projecting to the dentate gyrus. It must be kept in mind that the stimulation pattern used in this study (0.1 Hz during 45 min) was different from the one used in the mentioned experiments, in which a high frequency was used (3 trains of 15 impulses at 200 Hz). Considering the complex interactions modulating transmitter release in the hippocampus, due to presynaptic inhibition or facilitation [58] the differences in stimulation patterns might lead to differences in the release of neurotransmitters in both conditions.

An increase in NA release at the dentate gyrus after induction of LTP at the dentate gyrus has been reported previously using microdialysis sampling methods [16]. The modulating role of NA from the locus coeruleus on electrophysiological and behavioral processes is well documented (for a review see [10]). Similar effects on LTP have been described for the median raphe-serotonergic inputs [44]. Our results do not disclose a role for those systems on reinforcing mechanisms of LTP at the dentate gyrus, but suggest an indirect action.

BR induced modifications in young adult rats

Reinforcement of an E-LTP into a L-LTP can be obtained by applying behavioral stimuli with a strong motivational value [29,46,47]. Electrophysiologically this effect is quite similar to the reinforcement induced by stimulating the amygdala. In both cases, a longer-lasting LTP, without modifications in amplitude, was obtained. Similar time windows have also been described for both reinforcing paradigms. Moreover, we have demonstrated that the amygdala is a key structure for behavioral reinforcement of LTP [3].

Based on those analogies we had expected to obtain a similar change in the pattern of neurotransmitter release after both, electrical stimulation of the amygdala and behavioral reinforcement. The results have shown, however, completely different neurochemical modifications. No change was observed in ACh, NE or 5-HT release after behavioral reinforcement, but a significant reduction in glutamate and glycine transmission.

This suggests that despite the mentioned analogies, the mechanism of action of behavioral reinforcement might be different to that observed for the amygdala stimulation. A decisive involvement of hypothalamus in the behavioral paradigm seemed likely. Changes in dopamine and acetylcholine release have been measured in the lateral hypothalamus associated with drinking after deprivation [40].

However, we cannot exclude the possibility that the modulatory action of behavioral reinforcement ocurrs at time points different to that which we have measured here. Further experiments are required to clear these apparent contradictions.

BLA and BR induced modifications in aged, cognitively impaired rats

Aged rats with cognitive impairments showed deficient mechanisms of behavioral and amygdala-stimulation reinforcement of LTP [2,9]. Our results showed that none of the reinforcement protocols induced any change in transmitter release. This might simply be an expression of the reduced basal levels of many transmitters among aged rats caused by the deafferentation of this structure with aging, or may reflect an additional functional defect of those systems that reduce their ability to react in physiological adaptive responses. A similar lack of neurochemical reactivity in aged animals has been observed in other brain regions [18].

LTP impairments with age are mostly related with its maintenance, while the induction mechanisms are better preserved [6]. The impaired neurochemical response demonstrated by aged rats in both situations might be functionally related to the lack of reinforcing effect. Again, it is also possible that we just did not investigate the time window in which the modulatory action takes place.

The growing knowledge on the functional impairments associated with aging and the characterization of their neurochemical correlates might be useful in the development of rational drugs to alleviate such deficits.

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Capítulo 9: El reforzamiento conductual de la potenciación sináptica duradera se afecta por el envejecimiento

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El proceso de envejecimiento, tanto en animales como en humanos afecta las capacidades de memoria en todas sus formas y esto se asocia a deficiencias en los mecanismos de neuroplasticidad. También es conocido que el envejecimiento altera el balance afectivo asociándose frecuentemente con ansiedad y depresión.

Nuestro trabajo ha demostrado que los procesos de neuroplasticidad son modulados por factores afectivos mediante mecanismos que involucran a estructuras de las que se conoce, sufren despoblación neuronal severa, como la amígdala y la región septal. También hemos mostrado que sistemas de neurotransmisión noradrenérgica y colinérgica son mediadores importantes de estos procesos. Ambos sistemas se encuentran afectados como consecuencia del envejecimiento.

Nuestra hipótesis para este trabajo y el que sigue fue: *los mecanismos de reforzamiento de procesos neuroplásticos por factores afectivos se encuentran deteriorados como resultado del envejecimiento.*

En este trabajo exploramos esa hipótesis en el modelo de reforzamiento conductual y en el que sigue lo hicimos mediante estimulación de la amígdala.

Los resultados de este trabajo muestran que, en efecto, en los animales viejos con deficiencias cognitivas los mecanismos de reforzamiento conductual no funcionan de manera adecuada. La causa de esta inoperancia pudiera estar en el deterioro funcional que sufren los sistemas colinérgico y noradrenérgico que demostramos en el estudio de microdiálisis.

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Letter to Neuroscience

BEHAVIORAL REINFORCEMENT OF LONG-TERM POTENTIATION IS IMPAIRED IN AGED RATS WITH COGNITIVE DEFICIENCIES

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Key words: long-term potentiation, aging, reinforcement, dentate gyrus, learning, cognitive impairment.

Behavioral stimuli with emotional/motivational content can reinforce long-term potentiation in the dentate gyrus, if presented within a distinct time window. A similar effect can be obtained by stimulating the basolateral amygdala, a limbic structure related to emotions. We have previously shown that aging impairs amygdala–hippocampus interactions during long-term potentiation. In this report we show that behavioral reinforcement of long-term potentiation is also impaired in aged rats with cognitive deficits. While among young water-deprived animals drinking 15 min after induction of long-term potentiation leads to a significant prolongation of potentiation, cognitively impaired aged rats are devoid of such reinforcing effects. In contrast, a slight but statistically significant depression develops after drinking in this group of animals. We suggest that an impaired mechanism of emotional/motivational reinforcement of synaptic plasticity might be functionally related to the cognitive deficits shown by aged animals.

Since its discovery, long-term potentiation (LTP) has been the most popular cellular model of learning and memory formation (Bliss and Collingridge, 1993; Krug et al., 1990). Various reports have shown that LTP is negatively influenced by aging, similarly to memory (Barnes, 1979; Bergado et al., 1997a,b).

In the hippocampus and the dentate gyrus, LTP is normally induced by tetanic stimulation of glutamatergic afferents (Collingridge, 1992). However, a role of heterosynaptic, non-glutamatergic afferents on late phases of LTP is gaining increased recognition (Bergado Rosado and Almaguer Melian, 2000; Frey and Morris, 1997; Matthies, 1998). Heterosynaptic influences of this kind may mediate the recently described behavioral reinforcement of LTP (Seidenbecher et al., 1997). In this study, behavioral stimuli, with strong emotional/motivational content, applied within a defined time window after the induction of a protein synthesis-independent short-lasting early-LTP (EARLY-LTP <4 h) were able to prolong it into a protein synthesis-dependent late-LTP (LATE-LTP >4 h).

In a recent study we have shown that the stimulation of the amygdala can affect LTP in the dentate gyrus in a similar reinforcing manner (Frey et al., 2001). The amygdala is considered as an important neurological substrate of emotions (LeDoux, 1993) and is known to enhance memory consolidation (McGaugh et al., 1996), suggesting that the similarities between both of these types of reinforcements, i.e. behavioral and amygdala-dependent, might be causal, not casual.

The amygdala–hippocampus interactions on synaptic plasticity are likely mediated via septo-hippocampal afferents as the lesion of the fimbria-fornix abolishes the effects of amygdala stimulation on LATE-LTP (Jas et al., 2000). We have recently studied amygdala–hippocampus interactions in aged rats and found that aging severely impairs such interactions (Almaguer et al., 2001). It appears, therefore, interesting to study whether aging affects the behavioral reinforcement of LTP.

Early stages of LTP were similar between young and aged, cognitively impaired rats (see Experimental Procedures; Fig. 1A). Both groups reach a similar level of potentiation 5 min after tetanization, and the *U*-test showed no significant difference between groups at any time point for the population spike amplitude (PSA). Notably similar results were obtained for the field excitatory postsynaptic potential (EPSP) measured in a single series with a relatively large EPSP component (level

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Abbreviations: EARLY-LTP, protein synthesis-independent LTP (<4 h); EPSP, excitatory postsynaptic potential; LATE-LTP, protein synthesis-dependent LTP (>4 h); LTP, long-term potentiation; PSA, population spike amplitude.

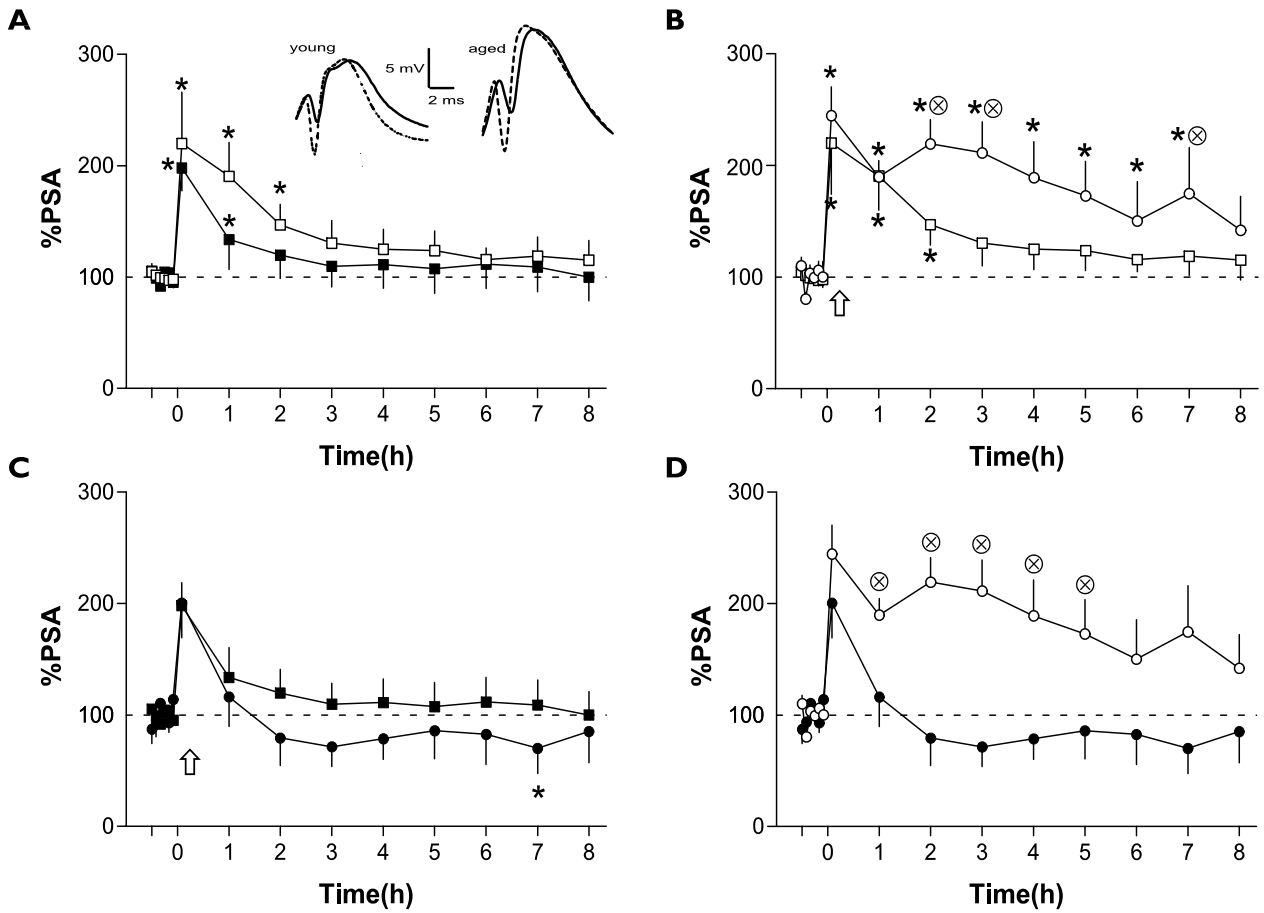

Fig. 1. Time course of LTP, expressed as the relative magnitude of the PSA (%PSA) to baseline value. Each time point is represented by the mean PSA and the S.E.M. Symbols: open squares, control LTP from young animals ($n = 6$); filled squares, control LTP from old animals ($n = 9$); open circles, behavioral reinforcement from young animals ($n = 5$); filled circles, behavioral reinforcement from old animals ($n = 6$). The arrows indicate the time at which access to water was allowed in the behavioral reinforcement groups. *Statistically significant differences to baseline values before tetanization within the same group (Wilcoxon test for paired samples, $P < 0.05$). ⊗Statistically significant differences between groups (U-test, $P < 0.05$). (A) Control LTP in young and aged animals. No significant differences were found between groups, however among aged rats, LTP seemed to decay faster, according to the Wilcoxon test it remained increased only for the first hour after induction. The inserts show analog traces of recordings taken from a representative young animal (left) before (continuous line) and 5 min after LTP-induction (broken line). The right insert represents traces from an aged cognitively impaired rat, respectively. The amplitude was measured between the first positive and the subsequent negative peak. (B) Behavioral reinforcement of LTP. In young animals, drinking after water-deprivation prolongs LTP, which remained significant up to 7 h after induction. (C) Behavioral reinforcement was absent in old animals, instead a slight depression appears after allowing access to water. (D) Comparative results of behavioral reinforcement between young and old animals. The initial level of LTP does not differ between groups, however significantly higher levels were found among young animals during the subsequent hours (U-test).

of tetanized field EPSP measured as percentage change of the slope 5 min after tetanization: 119.07% in young rats and 118.04% in aged, cognitively impaired rats). The main difference seemed to be the decay rate of LTP. While among the young rats the Wilcoxon test identified a significant difference in the increase of the PSA until 2 h after LTP-induction; aged rats retain LTP only for the first hour (field EPSP as above but 1 h after LTP-induction: 117.21% in young versus 107.91% in aged rats).

Comparing young and aged animals, there were no detectable differences in the input/output characteristics except a slight, statistically non-significant smaller PSA in aged rats.

Allowing young water-deprived rats to drink 15 min after tetanization, does not change the level of synaptic potentiation, but produces a significant lengthening of LTP (Fig. 1B) up to 7 h after tetanization (Wilcoxon test). The U-test identified statistically significant differences with control LTP at 2, 3 and 7 h (similar results were obtained for the slope of the field EPSP: e.g. % of potentiation in young rats versus motivationally treated young rats: 117.21% versus 121.93% at 2 h and 110.19% versus 100.00% after 7 h). Drinking alone did not influence field potentials in the dentate gyrus as shown by previous results (Seidenbecher et al., 1997).

Such reinforcing effect of a behavioral–motivational stimulus on LTP was absent among aged, cognitively impaired animals (Fig. 1C). The U-test confirmed no significant differences between control LTP and behaviorally reinforced LTP at any time point (examples for the slope of the field EPSP in aged control rats versus motivationally treated aged rats: 99.49% versus 96.86%

Table 1. Results of the behavioral test to select cognitively impaired aged rats

Group	*N*	Latency (s)			Crossings		
		mean	S.D.	S.E.M.	mean	S.D.	S.E.M.
Young controls	10	23.04	4.38	1.46	8.5	3.24	1.03
Aged impaired	12	45.27[a]	5.27	1.46	1.42[a]	1.38	0.39

N: number of animals per group. Values of latency resulting from averaging the data over all trials. Crossings are the group average of the number of times the animals crossed over the area where the platform used to be located.
[a]Statistically significant differences between groups (*U*-test, $P < 0.05$; for details see Experimental procedures).

after 2 h and 92.49% versus 84.06% after 7 h). On the contrary, the behavioral stimulus, resulted in a slight tendency towards depression. While control LTP in aged rats is statistically significant up to 1 h after its induction, behaviorally reinforced animals showed only a significant increase 5 min after induction (Wilcoxon test). For the rest of the observation period, behaviorally reinforced animals remained under the baseline level and that reduction was significant at 7 h (Wilcoxon test).

According to this, behavioral reinforcement of LTP was different between young and aged, cognitively impaired rats. As shown in Fig. 1D, significant differences were found from 1 to 5 h after tetanization between those groups (*U*-test).

The results in young rats confirm previous studies reporting an effect of behaviorally relevant stimuli on LTP (Seidenbecher et al., 1995, 1997). Both positive and mild negative reinforcers were able to prolong LTP, while water deprivation itself was shown to have no effect. We have used the same tetanization paradigm which normally induces only an EARLY-LTP, lasting less than 4 h. If this potentiation was associated with drinking 15 min after tetanization, the EARLY-LTP was transformed into a prolonged potentiation. As mentioned previously, the stimulation of the basolateral amygdala within a comparable time window, produces an almost identical result, i.e. an extension of the time span of LTP (Frey et al., 2001). This effect of the amygdala is protein synthesis-dependent and likely to be mediated by heterosynaptic, noradrenergic and cholinergic, afferents (Frey et al., 2001) reaching the hippocampus via the fimbria-fornix (Jas et al., 2000). These results suggest that limbic structures like the amygdala and probably the septum, are parts of the behavioral reinforcement system.

It is well known that aging is very often characterized by cognitive impairments. Impaired synaptic plasticity has been suggested as one of the causes for the age-dependent decline in memory (Barnes, 1993; deToledo-Morrell et al., 1988). While impaired synaptic plasticity is likely to be multicausal, our results suggest that an altered emotional/motivational modulation of late stages of LTP might importantly contribute to that functional deficit.

In previous experiments we have shown that amygdala–hippocampus interactions in synaptic plasticity are impaired in old animals with cognitive impairments (Almaguer et al., 2001). In the present report we extend this observations to show that also behavioral reinforcement of LTP is impaired in a similar way by the aging process. The behavioral stimulus not only failed to prolong LTP, but seemed to have some slight but long-lasting depressive effect. While the mechanisms of such an effect are unclear, it seems interesting to mention that, in previous control experiments, we have found that stimulation of the amygdala alone induces a slight, but significant long-lasting depression of the PSA (Frey et al., 2001), similar in magnitude and time course to the depression observed in aged rats after behavioral reinforcement.

Emotional alterations are also a major consequence of aging in rodents and humans (Miyagawa et al., 1998; Davidson and Irwin, 1999). We suggest that this age-dependent emotional/motivational impairment might be causally related to cognitive dysfunction because it impairs the reinforcement of ongoing changes in synaptic efficacy.

Structural and functional alterations within the amygdala have been reported as a consequence of aging (Aggleton, 1993; Hyman et al., 1990) and might contribute to the impaired emotional/motivational influence on the late stages of LTP observed in the present study. In addition, the septal cholinergic projection to the hippocampus is reduced during aging (Baxter et al., 1999; Taylor and Griffith, 1993). This modulatory input plays an important role on cognition (Bartus, 2000; Blokland, 1995) and synaptic plasticity (Bergado et al., 1996, 1997a; Buzsáki and Gage, 1989) and is probably involved in the amygdala–hippocampal interactions in LATE-LTP (Jas et al., 2000). We hypothesize that the observed lack of behavioral reinforcement among aged rats with cognitive impairments is due to a dysfunctional amygdalo-septal influence on the dentate gyrus, resulting in the prevention of the modulation of molecular cascades leading to protein synthesis and the reinforcement of EARLY-LTP into LATE-LTP.

These results open new ways to understand the physiological basis of geriatric memory dysfunction and stress the relevance of emotional/motivational factors and their impaired influence on late phases of hippocampal LTP in the chain of alterations that lead to cognitive impairments. Such an approach could also be useful for designing new therapeutic interventions to ameliorate one of the most widespread and invalidating consequences of aging.

EXPERIMENTAL PROCEDURES

Aged Sprague–Dawley male rats (24–27 months at the begin-

ning of the experiments) and a group of young control animals (8 weeks old at the beginning of the experiments) were used. Animals were kept in translucent cages (five animals/cage) with free access to water (except for the 24-h period preceding behavioral reinforcement experiments) and food. Efforts were made to minimize pain or discomfort to the animals, under observance of the Cuban Regulations for the Use of Laboratory Animals.

Aged rats were classified as cognitively impaired according to their results in the Morris water maze. In this behavioral test rats have to learn to find a submerged platform using extra maze cues. Eight trials were performed each day during four consecutive days (60 s maximal search time per trial, 30 s rest at the platform between trials). On day 5 only four trials were performed. The fifth trial on that day was a probe trial in which the platform was removed and the number of crossings over the location, where originally the platform was placed, were counted. The criteria to consider a rat as cognitively impaired was an average latency over the 5 days of training (eight trials per day) exceeding in two standard deviations the average latency of young controls. Since the total number of aged rats without cognitive impairment was too low for a separate group to study, only the group with cognitively impaired animals were used (Table 1). A control test with visible platform revealed no difference between aged and young animals excluding sensory or motor impairments as cause of the longer latencies.

After the end of training the animals were stereotactically implanted, under chloral hydrate narcosis (420 mg/kg), with one recording electrode at the dentate gyrus (coordinates: anterioposterior = −3.8 mm; mediolateral = 2.0 mm; dorsoventral = −3.5 mm, from bregma) and one stimulating electrode at the perforant pathway (coordinates: anterioposterior = −7.5 mm; mediolateral = 4.0 mm; dorsoventral = −4.5mm, from bregma). In aged animals the recording electrodes had to be inserted slightly deeper than in young animals. Since the implantation was performed under electrophysiological control the final, optimal positioning for the electrodes was evaluated by electrophysiological means. Miniature screws were fixed on the skull and served as reference electrode and earth, respectively. The whole assembly was secured with dental cement. Animals were allowed 1 week recovery from the operation before starting the LTP studies.

Before the electrophysiological experiments, animals were habituated to the recording chamber for at least 4 h. An input/output curve was then constructed and the intensity required for inducing a 40% maximal population spike determined and used for further test recording and induction of LTP.

On the following day a baseline was established, recording every 5 min for 30 min, before inducing LTP with three trains of 15 impulses at 200 Hz. In control LTP experiments, test recordings were performed every 15 min up to 8 h after induction. For behavioral reinforcement experiments, rats were water-deprived for 24 h before inducing LTP with an identical protocol. Fifteen minutes after LTP-induction rats were allowed to drink freely up to the end of the recording period. All deprived animals, young or aged, drank abundantly when the water pipet was placed at the experimental set.

The PSA relative to the baseline average value (expressed as percentage of baseline; % PSA) was used as an indirect measure of synaptic efficacy. In a few experiments with a larger field EPSP we have checked whether this component showed similar time courses as the PS. This was always the case suggesting that the recorded and analyzed PS is not just a measure of changes in excitability. Where possible, i.e. in a few series with a large EPSP component, data of the time course of the field EPSP are given from single experiments.

The Wilcoxon paired samples test was used to confirm significant increases above baseline within groups. For between-groups comparisons the Mann–Whitney *U*-test was used. In both cases a two-tailed significance level of $P < 0.05$ was used.

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Capítulo 10: El envejecimiento afecta las interacciones entre hipocampo y amígdala implicados en el reforzamiento de la potenciación sináptica duradera

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En este artículo abordamos la misma hipótesis anterior pero empleando en este caso la estimulación de la amígdala como agente de reforzamiento.

Los resultados demostraron que en los animales viejos con deficiencias cognitivas tampoco esta forma de reforzamiento fue efectiva.

En estos animales se exploraron también formas de plasticidad sináptica de corta duración mediante la técnica de pulsos pareados. No se encontraron diferencias entre animales jóvenes y viejos lo que excluye que deficiencias de los mecanismos básicos de transmisión sináptica sean la causa de la falta de reforzamiento.

En conjunto estos dos trabajos demuestran que los mecanismos de reforzamiento de procesos neuroplásticos por factores afectivos se encuentran afectados en animales viejos que muestran ya deficiencias cognitivas.

Teniendo en cuenta la frecuencia con que deficiencias cognitivas y alteraciones afectivas concurren en individuos envejecidos, estos resultados sugieren que existe una relación disfuncional entre las esferas cognoscitiva y afectiva que hace más grave el deterioro.

Este postulado puede ser importante para entender más cabalmente el deterioro funcional que acompaña al envejecimiento normal o patológico, sobre todo en enfermedades que afectan severamente la esfera cognoscitiva como las demencias.

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Aging impairs amygdala-hippocampus interactions involved in hippocampal LTP

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Abstract

Aging impairs amygdala-hippocampus interactions involved in hippocampal LTP. NEUROBIOL. AGING. We have recently shown that the stimulation of the basolateral nucleus of the amygdala (BLA) is able to prolong early-LTP (<4h) into late-LTP (>4h) in the dentate gyrus. To study whether aging affects this interaction, aged (24–27 months) rats were used, classified as cognitively impaired (I), or non-impaired (N) by means of their results in the Morris water maze. Paired pulses (30–90 ms interval) showed no differences among age groups. Among young controls, the early-LTP induced in the dentate gyrus by stimulation of the perforant path (PP) was prolonged in a late-LTP when the BLA was stimulated 15 min later. In aged-impaired rats the stimulation of the PP induced a reduced LTP, decaying to baseline in less than 2 h. BLA stimulation was without effect. Aged non-impaired rats showed an early-LTP identical to that of young animals; however, stimulation of the BLA showed no effect. These results suggest that deficient synaptic plasticity and memory functions in aged animals might be caused, in part by impaired mechanisms of heterosynaptic reinforcement.

Keywords: Aging; Long-term potentiation; Hippocampus; Amygdala; Rat; Cognition; Emotion

1. Introduction

Aging can affect learning and memory, as well as forms of neuronal plasticity, like long-term potentiation (LTP) suggesting that impaired plasticity might be involved in deteriorating processes of memory formation during aging [5,8].

Recent results from our laboratories have shown that the amygdala, a limbic structure considered as a neurological substrate of emotions [27], contributes to the induction of late-LTP. Our cellular studies revealed that stimulation of the basolateral nucleus of the amygdala within a distinct time window is able to reinforce a transient early-LTP (which has, under normal conditions, a duration of less than 4h and is protein synthesis-independent) into the long lasting, protein synthesis-dependent late-LTP in the dentate gyrus. This effect was blocked by cholinergic receptor antagonists [18] and could be abolished by lesioning the fimbria-fornix [25], the major cholinergic input to the hippocampus.

A reduced or dysfunctional cholinergic input to the hippocampus is considered as one of the main factors causing age-related cognitive and plasticity deficits [11,34]. If this input mediates the amygdala effects on hippocampal LTP, it can be hypothesized that aging might impair amygdala-hippocampal interactions in synaptic plasticity.

2. Materials and methods

To this purpose, we have studied LTP at perforant path (PP)-granule cells synapses in aged male Sprague Dawley rats (24–27 months old at the beginning of the experiments) previously classified as cognitively impaired (I) or not impaired (N) animals according to their performance in a spatial memory task (Fig. 1). The behavioral test for classifying aged rats was performed in the Morris water maze. Rats have to learn to find a submerged platform to escape from water. Escape latencies were measured in each of 8 trials during 5 consecutive daily training sessions. The data

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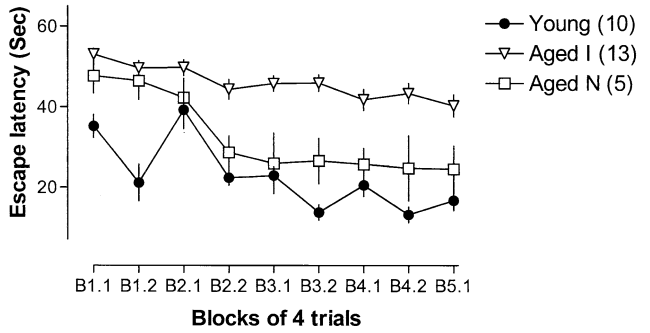


Fig. 1. Results of the behavioral test. Average (mean ± SEM) latencies to find the platform and escape from water are presented for each of the animal groups, grouped in blocks of 4 trials (2 blocks for each training day). Open squares: young animals ($n = 10$); open triangles: aged rats without cognitive impairment ($n = 5$); filled circles: aged rats cognitively impaired ($n = 13$). For further details see Materials and Methods.

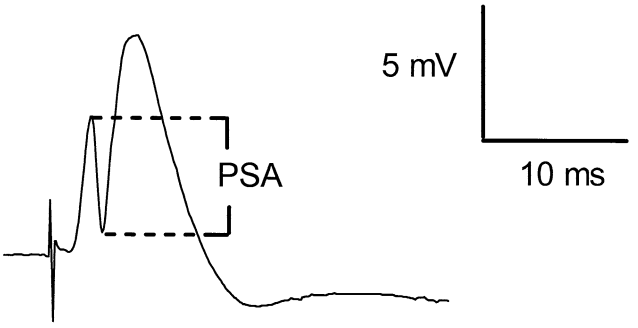


Fig. 2. Representative field potential evoked in the dentate gyrus by stimulation of the perforant pathway taken from an aged, cognitively impaired rat. PSA: population spike amplitude.

of 4 trials were averaged and presented as blocks (B1.1, B1.2, B2.1. . .); the first number indicates the day of training. Aged rats were considered as cognitively impaired (aged I) if their mean average latency over the 5 days exceeds by 2 standard deviations that of young controls (2 months old animals of the same sex and strain), otherwise they were considered as not impaired (aged N). In previous studies we have confirmed that these differences are not caused by sensorimotor disturbances of the aged rats [15].

One week after behavioral testing a monopolar recording electrode was implanted (under chloral hydrate narcosis, 420 mg/kg), at the hilar region of the dentate gyrus (AP: -3.8 mm, ML: 2.0 mm, DV:-3.5 mm), and bipolar stimulating electrodes were positioned at the perforant path (AP: -7.5 mm, ML: 4.0 mm, DV: 3.9 mm) and the basolateral amygdala (BLA)(AP: -2.4 mm, ML: 5.0 mm, DV: -8.5 mm), respectively (all coordinates to bregma, with bregma and lambda at the same level). The electrodes were connected to a socket and the assembly was fixed to the skull with dental cement (Paladur, Wehrheim, Germany). All electrodes were made from 125 μ m epoxy-isolated stainless steel.

After 7–10 days recovery, but one day previous to the first experiment, the rats were habituated to the recording chamber for at least 4 h. On that day an I/O curve was recorded and from it the intensity needed to obtain a 40% max. population spike was determined and used for test recordings, paired pulse studies and tetanization, i.e. LTP induction.

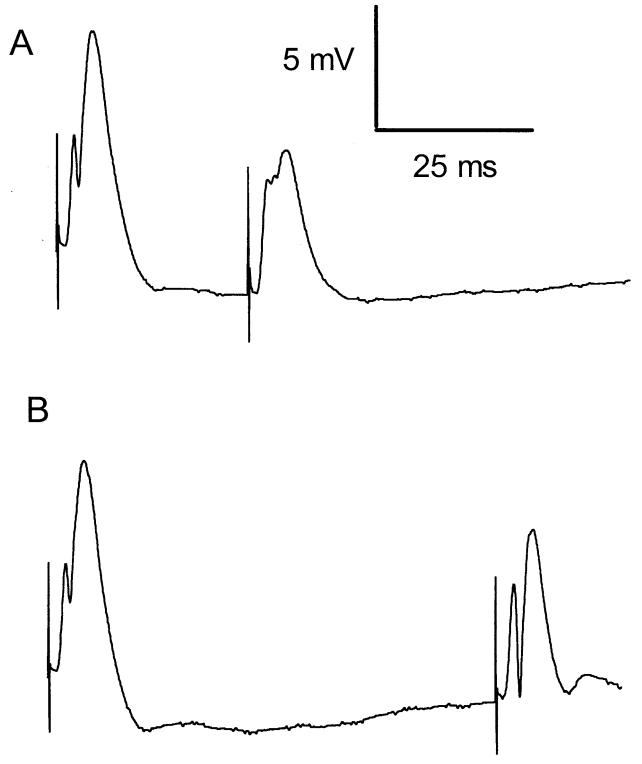
To evaluate short-term synaptic interactions paired-pulse stimuli were delivered to the PP with interstimulus intervals ranging from 30 to 90 ms. Five consecutive impulses at 0.01 Hz were averaged on line with each interval. The relative amplitude of the second population spike (P2) to the first one (P1) was calculated and expressed as percent (%P2/P1).

Long-term synaptic plasticity studies were started at the following day. A baseline (5 consecutive impulses at 0.01Hz per time point were averaged on line every five minutes) was recorded for 30 min before tetanizing the PP with 3 trains of 15 impulses each (0.1 ms square pulses) at 200 Hz. Five minutes after this first tetanization a record was made to asses the initial level of LTP. Further test recordings were taken every 15 min up to 8 h after tetanization. The population spike amplitude (PSA) was measured (Fig. 2) and its relative value to pre-tetanization baseline values was calculated (%PSA). One week later, after recovery of the baseline values, the same animals were again tetanized using the same protocol. Fifteen minutes after tetanizing the PP, the BLA was stimulated using an equal paradigm. The %PSA was calculated, as the measure of the change in synaptic efficacy, during the following 8 h period.

The animals were randomly assigned to one of the electrophysiological paradigms (i.e. paired pulses, control LTP or BLA stimulation) and some of them were used again for a different protocol under the following conditions: at least seven days delay between studies and a complete recovery of baseline values.

After the end of the study the location of the electrodes was histologically confirmed. Although all animals showed an apparently correct placement of the electrodes according to histology, 2 animals from the aged-impaired group were retired from the BLA-stimulation group because they consistently failed to produce an evoked potential at the dentate gyrus after stimulation of the BLA. This response consists of a 1–2 mV monophasic wave with a latency of 20 ms. Other animals, young or aged included in that study showed this potential, and no age-related differences were observed.

Statistical analysis included the Wilcoxon test for paired samples for within group comparisons relative to baseline values. Between groups comparisons were carried out using the Kruskal-Wallis test for comparing whole curves (i.e. all points in one curve, after LTP induction were compared to all points in the second curve), followed by the Mann-Whitney U test to establish significant differences at individual time points. In every test a two-tailed, $P < 0.05$ significance level was used.



Fig, 3. Representative examples of recordings after paired pulses stimulation of the perforant pathway taken from an aged, cognitively impaired rat. A) 30 ms interstimulus interval. Notice the strong depression of the population spike on the second potential. B) 70 ms interstimulus interval. The second population spike is facilitated.

3. Results

The paired pulse study (Fig. 3) showed the typical relationship of inhibition with short stimulus interval (30 ms) and facilitation at longer intervals (70 and 90 ms). All groups behaved similarly in this study (Fig. 4). Aged rats, whether cognitively impaired or not, showed a similar pattern of inhibition and facilitation as young controls. No significant differences between groups were found at any interstimulus interval (*U* test). The apparent greater variability among aged rats could be a consequence of smaller number of animals per group.

As shown in Fig. 5A, early-LTP induced in the dentate gyrus by stimulation of the PP was reinforced into late-LTP (lasting for more than 4 h) when the BLA was stimulated 15 min after tetanization of the PP. The tetanization of the PP alone induced a significant increase of the population spike that declined to baseline level after two hours (Wilcoxon test). After the sequenced stimulation of the PP and the BLA 15 min later, the increase in synaptic efficacy remained significantly increased during the 8 h recording period (Wilcoxon test). The Kruskal-Wallis showed significant differences between both curves, while the *U* test confirmed significant differences 4 to 7 h after tetanization.

In aged rats with cognitive impairment (Fig. 5B) the stimulation of the PP induced an LTP whose initial value, 5 min after induction, does not differ significantly from that of young animals (*U* test). However, it seemed to decay faster. Statistically significant increases of the PSA were found only during the first hour after tetanization (Wilcoxon test). When the whole curve was compared to that of young animals the Kruskal-Wallis test showed significant differences, strengthening the suggestion of an accelerated decay rate of LTP in the group of aged-impaired rats. Interestingly, the concurrent stimulation of the amygdala does not change the level of synaptic potentiation or its decay rate among these animals. Again, an increase of synaptic efficacy lasting only one hour (Wilcoxon test) was observed. The Kruskal-Wallis test showed no difference to the control LTP curve of the same group, but it differed significantly from that of young animals after BLA stimulation. Significant differences between these two groups were demonstrated at 1 and 4 to 7 h after tetanization (*U* test). This suggests that aging impairs the reinforcing effect of the amygdala on hippocampal LTP.

On the other hand, aged rats without cognitive impairment showed a control LTP that does not differ from that of the young rats (Fig. 5C). The initial level and duration of synaptic potentiation appeared also similar to that of the young animals, i.e. a statistically significant LTP up to 2 h after its induction was observed (Wilcoxon test). The Kruskal-Wallis test showed no differences between this curve and the one corresponding to control LTP of the young rats, but significant differences to aged impaired animals. However, among these animals the stimulation of the amygdala apparently failed to prolong LTP. The period of increased synaptic efficacy remained two hours (Wilcoxon test), the same as in control LTP. The Kruskal-Wallis test found no significant differences between the BLA and control LTP curves among these animals. This finding may suggests that reinforcing effects of BLA stimulation might be failing also in these animals, even before cognitive impairments appear. However, this should be taken with caution because of the small number of animals in this group and the fact that no significant differences were found when

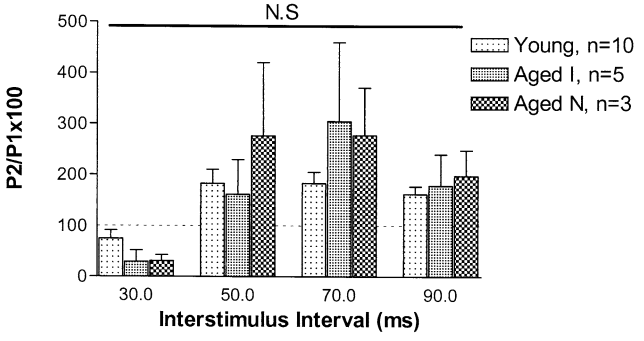


Fig. 4. Results of the paired pulse study in the different age groups. The values of the relative amplitude of the population spike amplitude in the second potential with respect to the first potential (P2/P1 × 100) are shown (mean ± SEM) for the different groups. N.S.: no significant differences between groups were found with the Mann-Whitney's U-test.

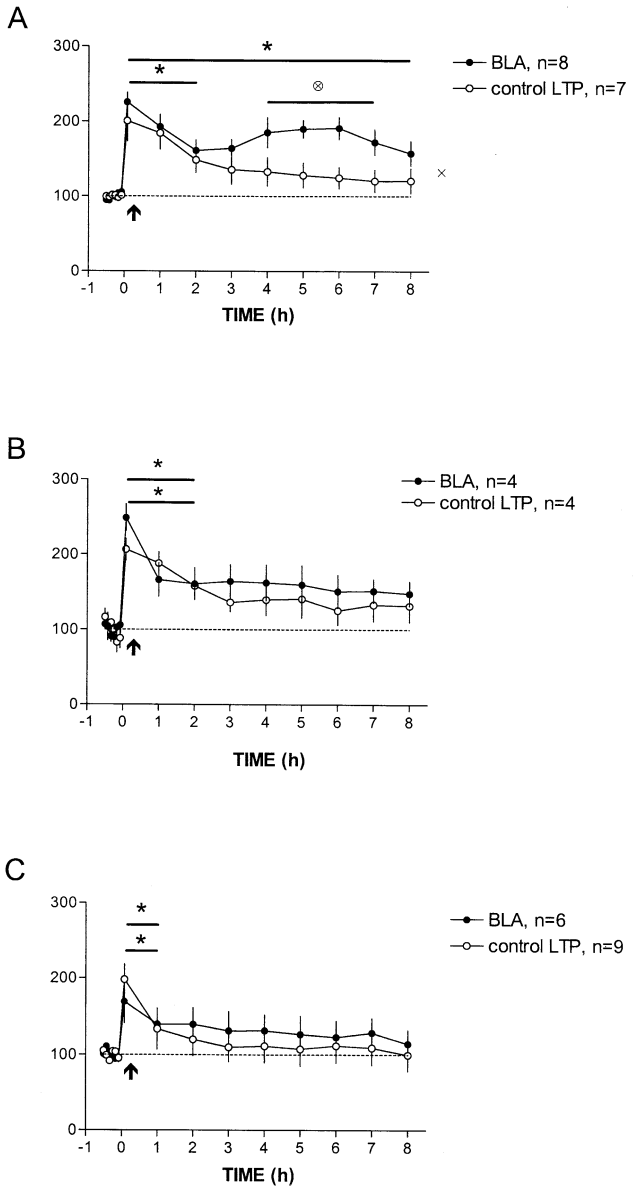


Fig. 5. Amygdala-hippocampus interactions in LTP. The values (mean ± SEM) of the population spike amplitude, relative to the baseline values (%PSA) are presented. Open symbols: control LTP after tetanization of the perforant path (PP) only; filled symbols: BLA, tetanization of the PP followed by tetanization of the BLA 15 min later. A) Young animals, B) aged rats without cognitive impairment, C) aged rats with cognitive impairments. * significant differences to own baseline ($P < 0.05$ Wilcoxon test for paired samples). The horizontal line below the asterisk indicates how long significant differences were obtained for each curve (inferior line: control LTP, superior line: BLA). X significant differences between curves ($P < 0.05$, Kruskal-Wallis test). ⊗, significant differences between groups at the time points indicated ($P < 0.05$, Mann-Whitney's U test). LTP induction by PP tetanization was performed at time 0. The arrow indicate the time of tetanization the BLA.

compared (Kruskal-Wallis) with the curve of young animals after BLA stimulation. In general terms, aged animals without cognitive impairments behave intermediate between young animals and impaired old rats.

4. Discussion

Changes in the excitatory post-synaptic potential (EPSP) are considered by some authors to be a more reliable indicator of modifications in synaptic efficacy. We have, in fact observed a general tendency of the EPSP slope to increase after LTP induction in the present study, however, we have used the population spike amplitude to this purpose instead of the EPSP for several reasons. The recording electrodes were positioned at the hilar region of the dentate gyrus, which is the usual location for chronically implanted electrodes. This is, however, distant from the optimal point to evaluate EPSP's. The size of the granular cell layer in the dentate gyrus makes EPSP-recordings in the intact animal quite unreliable, if compared with the CA1-region. In the hilar region each EPSP is masked by the dipole of the overlaying population spike. Calculating the EPSP from these potentials in freely moving animals is inappropriate because it varies strongly with the behavioral state of the animal. Finally, many LTP studies in slices, both at the CA1 and the dentate gyrus, as well as in vivo studies in narcotized animals in which behavioral influences are absent, have shown that both, the EPSP and the population spike potentiation strongly and positively correlate. We have tested this correlation over a wide range of frequencies to induce LTP and found always a positive correspondence of the EPSP and the population spike potentiation, except for very high frequencies (400 Hz) [28]. For these reasons we considered the PSA as a reliable indicator of changes in synaptic efficacy under our experimental conditions.

The reinforcing effect of activation of the BLA on LTP in the dentate gyrus in young rats is in good agreement with our previous findings [18] and those of other [2,24]. Early-LTP can be reinforced into a late-LTP by heterosynaptic modulatory inputs. As we have shown in those earlier experiments, this process depends on protein synthesis and is blocked by cholinergic and noradrenergic receptor antagonists. Those transmitter systems are potent activators of protein kinases, like PKC and PKA, known to be part of molecular cascades that regulate gene expression and protein synthesis [19], and therefore, essential for late-LTP.

LTP duration appears to be reduced in aged-impaired, but not in aged-not-impaired rats. Comparable results were described in classic LTP studies [4,5,12] and using the model of calcium-induced LTP [13]. The reasons for such different synaptic plastic response are unclear and might involve differences in calcium homeostasis [16,17] or downstream molecular cascades, particularly important for late-LTP. The results of our paired pulse study disclose differences in retroactive inhibition or short-term facilitation as cause of the reduced plasticity shown in LTP experiments.

The reinforcing effect of BLA prolonging LTP, however, was absent in aged rats with cognitive impairment, and likely also in not impaired aged animals. A reduced emotional reactivity has been demonstrated in both, impaired and not-impaired aged rats [30]. The amygdala is consid-

ered to be a key element of an emotional-motivational system [26], which contributes to memory reinforcement in the hippocampus within defined time windows [29,32]. It is tempting to speculate that impairments in emotional reinforcement of LTP occur before cognitive deficits, and that aged non-impaired rats are using some compensatory mechanisms, i.e. an increased excitability of granule cells [31] to overcome the impaired emotional reinforcement. This, however, requires a closer evaluation of cognition and emotion, using a combination of memory tasks, particularly considering the stressful properties attributed to the water maze.

In addition to the results presented here, we had recently shown that septo-hippocampal projections might be involved in the reinforcing effect of the amygdala on LTP [25]. Interestingly, a cooperative interaction of the amygdala and the septum during different learning tasks has been recently described [23]. The fact that the amygdala activates the hippocampus via cholinergic afferents [14] supports the idea that the amygdala-septal-hippocampal projection might be responsible for the reinforcing effect of BLA stimulation on LTP. Such would also be in line with the so called 'cholinergic hypothesis of geriatric memory dysfunction' [6] and the abundant literature demonstrating a link between aging, cholinergic deafferentation to the hippocampus, and cognitive decline [22,33]. Other subcortical inputs arising from brain stem nuclei provide the hippocampus with aminergic innervation. This systems are also able to influence late phases or LTP [21] and are also affected by aging [3] and should be considered along with cholinergic mechanisms to interpret our present data.

Our results suggest that beyond the intrinsic impact that this loss of afferents might produce on hippocampal plasticity [9,10], it could also impair the mechanisms of heterosynaptic modulation of late phases of LTP, like those arising from the amygdala. Both effects could cooperate in causing the cognitive impairments observed in these animals.

This heterosynaptic, associative mechanism may be impaired in aged rats due to neuronal loss and atrophy within the amygdala itself [1], at the septal relay level [11], or both.

The need of a heterosynaptic modulation of late-LTP is becoming increasingly accepted [7] and stresses the relevance of activating cellular cascades, which modulate protein synthesis and an adequate delivery mechanism of this proteins to the activated synapses for late-LTP to occur. The synaptic tagging hypothesis [20] offers a plausible explanation for such mechanisms. We suggest that the failure of reinforcement observed among aged animals could be mainly due to an inadequate or insufficient activation of the molecular cascades, through modulatory inputs, which lead to the synthesis of the plasticity-related proteins. Whether the setting of molecular 'tags' at the activated synapses to capture the newly synthesized proteins is also deficient in aged rats with cognitive impairments should be addressed in future experiments.

The results presented here point to the existence of functional links between two major negative consequences of aging: reduced emotional/motivational reactivity and impaired cognition. The further study of these processes will open new ways to understand the complex phenomenon of aging and its impact on brain function.

Acknowledgments

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Capítulo 11: La estimulación post-entrenamiento de la amygdala mejora en aprendizaje especial en ratas con lesión de fimbria-fornix

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Los efectos descritos hasta ahora se han obtenido estudiando solo una forma de neuroplasticidad (plasticidad sináptica del tipo LTP) en una estructura cerebral (el giro dentado del hipocampo) y en animales sanos. Si los mecanismos propuestos para explicar estos efectos estuvieran restringidos solo a esas condiciones, su estudio sería de poco valor teórico y casi ninguno práctico.

En los dos trabajos que se presentan en el final de este documento hemos intentado extender la exploración de estos mecanismos, en un intento de obtener evidencias de su universalidad.

El primero de ellos explora los efectos de la estimulación de la amígdala en animales lesionados, y estudia una forma más general de expresión de la neuroplasticidad: la memoria espacial. La hipótesis que lo guía se puede expresar como:

Si el reforzamiento de procesos neuroplásticos es un fenómeno universal, entonces debe expresarse en otras formas de plasticidad e incluso en animales lesionados.

Utilizamos ratas con lesión de la fimbria fornix que fueron entrenadas en un modelo de memoria espacial utilizando el laberinto acuático diseñado por Morris. Las ratas fueron lesionadas una semana antes y en ese momento se les implantó un electrodo de estimulación en la región basolateral de la amígdala.

La lesión de fimbria fornix provoca un severo déficit en la adquisición de pruebas de este tipo, lo cual se comprueba en todos los grupos controles con lesión. La estimulación diaria de la amígdala, 15 minutos después de concluido el entrenamiento de cada animal, mejoró su aprendizaje, a pesar de la lesión que se les había provocado. Estos animales, a

partir del 3er día de entrenamiento se diferencian de los restantes grupos lesionados y se aproximan al comportamiento de los animales sanos empleados como control.

Este resultado muestra que el reforzamiento de la neuroplasticidad por estimulación de la amígdala no es exclusivo de la LTP y que puede darse también en portadores de lesiones cerebrales.

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Post-training stimulation of the basolateral amygdala improves spatial learning in rats with lesion of the fimbria-fornix

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Abstract. *Purpose:* To evaluate the capacity of amygdala stimulation to improve neural plasticity in animals bearing lesions of the fimbria-fornix (FF) system. *Methods:* The animals were lesioned under narcosis (chloral hydrate, 420 mg/kg ip.) using a bilateral transection of the FF procedure. During the same surgery some animals were implanted with an electrode in the right basolateral amygdala (BLA) to allow the electrical stimulation of this structure. Training was carried out one week after surgery using a Morris water maze. Animals were trained in four consecutive days (8 trials/day) in the non-visible platform condition except in the fourth day in which only 4 trials were performed followed by a probe trial in which the escape platform was removed. On day 5 of training 8 trials with visible platform were performed. After each of the first 3 training days one group of animals received trains of electrical stimulation to the BLA, while control groups were not stimulated. A group of non-lesioned animals served as control. The location of the electrode was confirmed histologically after the end of the experiments. *Results:* The learning capacity of the lesioned animals was improved by the electrical stimulation of the amygdala. The latency to find the submerged platform within this group approaches that of the non lesioned animals in the course of training (2-way ANOVA with repeated measures), while other lesioned animals continued to show severely impaired learning abilities. *Conclusions:* This is the first evidence that stimulating the BLA can positively influence the learning abilities of lesioned animals. Further experiments should contribute to improve the stimulation paradigms to make it more effective, if possible.

1. Introduction

Plasticity is a common property of the Nervous System that can be expressed in several forms, from subtle functional changes to massive growth of neurites, or even neurons (at least in certain regions of the adult brain) [13].

Long-term potentiation (LTP) is one form of plasticity characterized by a lasting change in synaptic efficacy that is induced after tetanic stimulation of specific afferents [16]. According to its duration and the mechanisms involved two phases can be distinguished in LTP [41, 60]. An initial early-LTP (E-LTP) lasting less than 4 hours is followed by a protein synthesis-dependent late-LTP (L-LTP) lasting more than 4 hours [29,30].

We have recently shown that an E-LTP in the dentate gyrus, induced by a weak tetanus to the perforant pathway, can be reinforced and converted into a L-LTP by the stimulation of the basolateral amygdala within a 30 minutes time window [28]. The effect is protein synthesis dependent, suggesting a real conversion of the phenomenon into an L-LTP. This suggests that the

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amygdala activity is able to stimulate the cellular cascades involved in L-LTP in other brain regions.

However, LTP as induced in the laboratory is a localized form of neural plasticity restricted mainly to the stimulated synaptic population. We were, therefore, interested in testing whether amygdala stimulation might also be able to support more widespread forms of neural plasticity.

The amygdala is able to modulate memory storage in other brain regions, like the hippocampus [11, 39,47,52,54] providing thus a functional link between affective and mnesic processes [2,46], and suggesting that neural plasticity might be influenced by emotional/motivational factors. Memory formation is perhaps the most widespread and common form of expression of neural plasticity. Memory may depend on plastic mechanisms at systems and cellular level. LTP is considered a cellular mechanism of memory [49], and the fact that both, memory and LTP can be reinforced by amygdala-depending emotional factors seems to reflect a functional relationship and not merely a coincidence.

The amygdala is a key structure in emotional behavior, motivation and emotional memory [43,44] in rodents and humans [3,21,71]. An impaired amygdala function is likely to be involved in several psychiatric/neurological conditions affecting mood and memory, like Alzheimer's disease, autism and depression [4, 12,35,38,65]. However, all animal the studies linking amygdala, memory and synaptic plasticity have been performed in healthy animals, but it is not known whether similar actions can be demonstrated in animals bearing brain lesions.

The fimbria-fornix (FF) fiber system is an important projection system to the hippocampus. The FF provides the hippocampal formation with aminergic terminals arising in brain stem nuclei, as well as a strong cholinergic projection from the basal forebrain [69]. It has been extensively documented that lesions of the FF provoke a severe and long lasting impairment in spatial learning [5,17,19,22,31,68]. It have been previously shown that such impairment shows no spontaneous recovery over several months [10,20].

Such a lesion model appears, therefore, suitable to study whether the stimulation of the amygdala is capable of improving memory in lesioned animals.

Based on these antecedents we have designed a first experiment to test the hypothesis that the stimulation of the basolateral nucleus of the amygdala can be beneficial for improving the learning abilities impaired by a specific brain lesion. The results that will be presented provide preliminary evidences that this is indeed possible.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 250–300 at the time of surgery were used. The animals were obtained from professional breeders (CENPALAB, Havana) and housed in translucent plastic cages (5 animals per cage) under controlled environmental conditions (temperature, humidity, light-dark cycle) with free access to water and food (Rat Chow, CENPALAB, Havana) throughout the experiment.

2.2. Surgery

For surgery, the animals were anaesthetized with chloral hydrate (420 mg/kg) and mounted in a stereotactic frame. Hair and skin of the scalp were removed and the points for trepanation were marked on the skull. The procedure for FF lesion has been described elsewhere [37]. Briefly, a bilateral window was opened at coordinates: anterior-posterior (AP) $= -1.4$ mm; medial-lateral (ML) from 0.5 to 5.2 mm (all coordinates from bregma). A reduced no. 11 blade was then lowered at AP $= -1.4$ mm; ML $= 0.8$ mm and dorsoventral (DV) -5.0 mm tilted 15° from the vertical. The blade was slowly moved 3.1 mm laterally and, after 1 min, extracted from the skull.

A stainless steel electrode was positioned at the coordinates AP $= -2.8$ mm; ML $= 5.0$ mm; DV $=$ 8.5 mm, aiming the basolateral part of the amygdaline nuclear complex (BLA). The electrode was connected to a female socket and fixed to the skull with dental cement. A miniscrew attached to the frontal bone served as return electrode and to anchoring the implant to the skull.

2.3. Training

One week after surgery the animals were trained in the Morris water maze for 5 consecutive days. During the first 4 days the escape platform remained submerged in a definite place in the NE quadrant of the circular pool. In the 5th day, the platform were moved to the SW quadrant and made visible by lowering the water level and applying a dark blue ribbon to its border. Eight trials were performed each day starting randomly from 4 different points. The time spent by the animal to find the platform and escape from water (escape latency) was measured. The animals were allowed to search for the platform during 60 sec in each trial. After that

time, they were gently transferred to the platform. A resting period of 30 sec on the platform was allowed between trials. In the 4th day only 4 ordinary trials were performed. The results of 4 consecutive trials were averaged and presented as blocks, numbered for the day and block (e.g. Block 3.1 corresponds to the first 4 trials of day 3).

A fifth trial with removed platform was performed (probe trial) at the end of the day 4 training session, and the number of crossing over the place where the platform used to be located were counted.

2.4. Experimental groups

According to surgery and the treatment received after training several groups of animals were formed. One group of lesioned animals (FFBLA) received electrical stimulation of the amygdala 15 minutes after ending the behavioral training (3×15 impulses at 200 Hz, 400 μA). A second group of lesioned animals bearing also the electrodes in the amygdala were handled as the former and connected to the stimulation device, but no stimulus was applied (FFCE). A different group of lesioned and implanted animals returned to their cages after training and were not handled neither stimulated (FFCI). A group of lesioned animals without electrodes (FF) and a non-lesioned control group, both without post-training handling were also included.

2.5. Statistics

A two way ANOVA (Group and Block) with repeated measures was used to evaluate results of the training, both in the non-visible and visible platform conditions. For the probe trial a one-way ANOVA (Group) was performed. A post hoc analysis using the Duncan's test was performed when the ANOVA resulted significant to assess between groups differences. A p value inferior to 0.05 was considered significant.

2.6. Histology

After the end of training the animals were transcardially perfused with formalin under narcosis. The brains were removed and processed (Cresyl violet) for microscopic confirmation of the electrode location, and with acetylcholine esterase histochemistry to confirm the efficay of the FF-lesion. Only those animals showing a correct electrode placement were included in the final evaluation (see Fig. 4).

3. Results

Figure 1 shows the results of training in the water maze with non-visible platform for the different experimental groups. There is a significant time effect in the reduction of the latencies as a consequence of learning (ANOVA, $F_{6,51} = 22.93$, $p < 0.05$). This is, however, different among the experimental groups. The ANOVA for the group factor showed significant differences between groups ($F_{4,51} = 6.33$, $p < 0.05$). The *post hoc* Duncan's test showed that there were no significant differences between groups for the first block of 4 trials (B1.1). However, during the last four trials of the first day (B1.2) and the second training day (B2.1 and B2.2) all lesioned groups, including those with stimulation of the amygdala, differed significantly from the non-lesioned controls. This shows that lesioned animals are impaired in their spatial learning abilities. After two days of training and electrical stimulation of the amygdala a different pattern appears. Starting in the first block of trials from day 3 (B3.1) and through the remaining trials, the group of animals receiving post-trial stimulation of the amygdala does not differ any more from the non-lesioned controls. They do not differ either from the rest of the lesioned groups, but it should be stressed that all those groups of animals bearing lesion, with the only exception of the BLA stimulated animals, do significantly differ from control until the end of the training period.

The results of the probe trial (Fig. 2) showed that the number of crossings was significantly different between groups ($F_{4,51} = 14.48$, $p < 0.05$). The control animals crossed the area of the previous location of the platform significantly more times than any lesioned group. The group that received stimulation of the amygdala (FFBLA) showed a significantly higher number of crossings than the sham-stimulated group (FFCE).

The last two blocks of trials with visible platform performed at day 5 (Fig. 3) showed no differences between groups (Two way ANOVA with repeated measures, $F_{4,50} = 1.31$, $p > 0.05$).

4. Discussion

The absence of differences in the visible platform condition is an indicator that lesioned animals suffer no major visual or motor impairments that could negatively influence their escape latencies. This is relevant because in the non-visible platform condition the an-

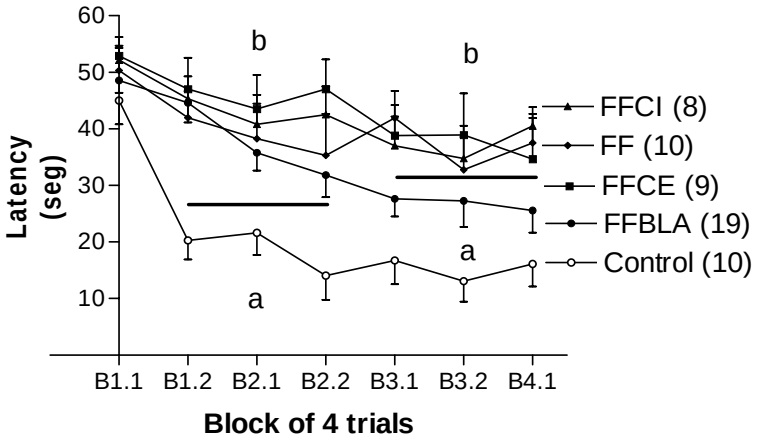

Fig. 1. Results of the training in the Morris water maze (non-visible platform). The mean values (± standard error of the mean) of the escape latencies for blocks of 4 trials are given for each experimental group (see legend). In parenthesis are the final numbers of animals in each group. The horizontal lines separate different groups according to the *post hoc* comparison with the Duncan's test ($p < 0.05$). No difference between groups was found for block B1.1. For blocks B1.2 to B2.2 two different groups were discriminated by the Duncan's test. The control animals (a) differed significantly from all other groups (b). From block B3.1 to the end of training controls animals and animals with BLA stimulation do not differ between each other and were separated together (a) from the rest of the experimental groups, above the line (b).

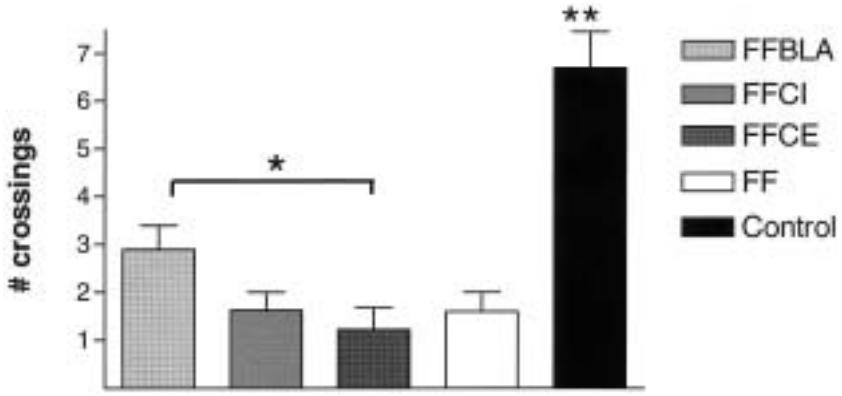

Fig. 2. Average number of crossings (mean ± SEM) of the experimental groups in the probe trial. The control animals showed significantly more crossings than any other group. The group with BLA stimulation (FFBLA) showed significantly more crossings over the area of former platform location than the sham-stimulated animals (FFCE). ($p < 0.05$ post hoc Duncan's test).

imals have to use visual information from extra maze cues to solve the problem and escape from water. The differences observed in the non-visible platform condition and in the probe trials are, therefore, attributable to an impaired spatial learning ability of the lesioned animals.

Several studies have proven that FF-lesioned animals showed learning impairments in similar tests [7, 20,45]. These impairments showed no spontaneous recovery over long time periods [10] and have been attributed [58,68] to the cholinergic denervation at the hippocampus provoked by the lesion. While the probable mediation of other modulatory influences provided to the hippocampus by the FF (i.e. dopaminergic, noradrenergic, serotoninergic) can not be disclosed, the cholinergic hypothesis of memory dysfunction have inspired a great deal of experimental research showing that the restoration of cholinergic inputs via trophic factors or transplants can benefit learning abilities impaired by FF lesion [14,18,23,24,26,42,67].

Other approaches, however, using repeated training [36] or self stimulation [73] suggest that improvements on learning abilities in FF-lesioned animals can also be induced by non-cholinergic mechanisms.

Our results show that stimulating the BLA 15 minutes after ending each day training session have a positive influence on the learning abilities. This is expressed mainly, in the fact that the behavior of the ani-

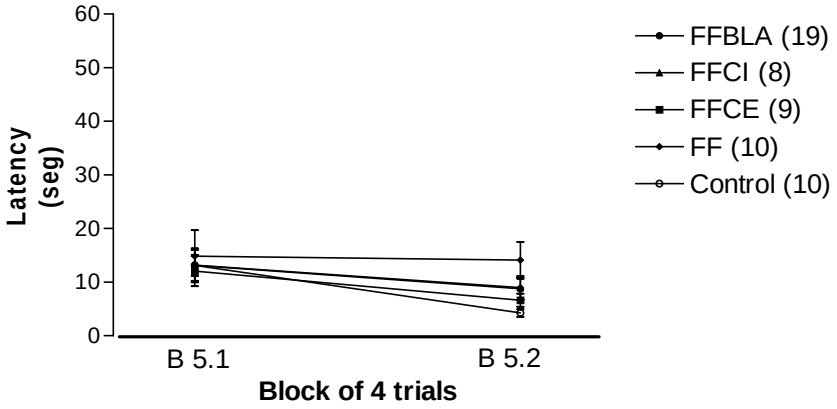

Fig. 3. Results of the training in the Morris water maze (visible platform). The mean values (± standard error of the mean) of the escape latencies for blocks of 4 trials are given for each experimental group (see legend). In parenthesis are the final numbers of animals in each group. No significant differences among groups were found (two-way ANOVA).

mals in the FFBLA group progressively tend to be similar to that showed by control, non-lesioned animals. In the probe trial, these animals, although still showing fewer crossings than the control group, have a better retention of the platform location when compared to other of the lesioned groups (FFCE).

The inclusion in the experimental design of lesioned groups bearing electrodes in the amygdala (FFCI) and sham-stimulated (FFCE) disclose any effect of the presence of the electrode at the BLA or the post-training handling of the animals as causal factors for the improvement observed in the FFBLA group, and strongly suggests that the behavioral recovery showed by these animals is due to the electrical activation of the BLA region of the amygdala.

The ability of the amygdala to improve memory by interactions with other brain structures have been extensively documented [15,34,50,51,55]. The mechanisms for the memory-improving effect of amygdala stimulation might involve its reinforcing effect on LTP-like processes in the hippocampus [1,6,8]. LTP-reinforcement by amygdala stimulation is protein synthesis-dependent [28]. Based on pharmacological studies we have hypothetized that the synthesis of plasticity-related proteins might be activated by modulatory afferents (i.e. cholinergic and/or noradrenergic) [28].

We have previously shown that the lesion paradigm used in the present study leads to a severe cholinergic denervation of the dorsal hippocampus which showed no recovery over a period of 3 months [9]. It is therefore unlikely, that the improvement shown by the animals under BLA stimulation might be caused by a re-growth of cholinergic terminals to this structure. An alternative explanation may arise from the BLA projection to the entorhinal cortex which is the main glutamatergic-excitatory input to the hippocampal formation [40,70]. These projections are reciprocal and may mediate functional interactions between both structures leading to memory consolidation [62,64,66,72]. Our results show that also in animals bearing lesions of the FF, the activation of the amygdala is still able to exert a reinforcing action on memory consolidation.

As previously mentioned, the amygdala is considered a central structure for emotions and motivationally guided behavior [48,53]. There is strong evidence supporting the view that amygdala activation, in a learning context, might represent a functional link between the affective status and memory consolidation [2,61]. This does not mean, however, that the amygdala is the only structure whose activity is able to prolog LTP, enhance memory consolidation or promote memory recovery in lesioned animals. The amygdala is part of a widely distributed network involving other cortical (i.e. the prefrontal cortex) or subcortical structures, whose activation might also contribute to recovery. In line with this, we have previously shown that the stimulation of the septum [27] or the locus coeruleus (Frey S., unpublished results) are also able to reinforce LTP at the hippocampus in healthy animals. Of course, a role for the septum under our experimental conditions is difficult to conceive because the lesion interrupted its main projection to the hippocampal formation.

It remains to be established whether other patterns of amygdala stimulation (different from the ones we used here) can be also effective, or even more effective. This is particularly important regarding the timing of the stimuli (internal frequency within a train, number

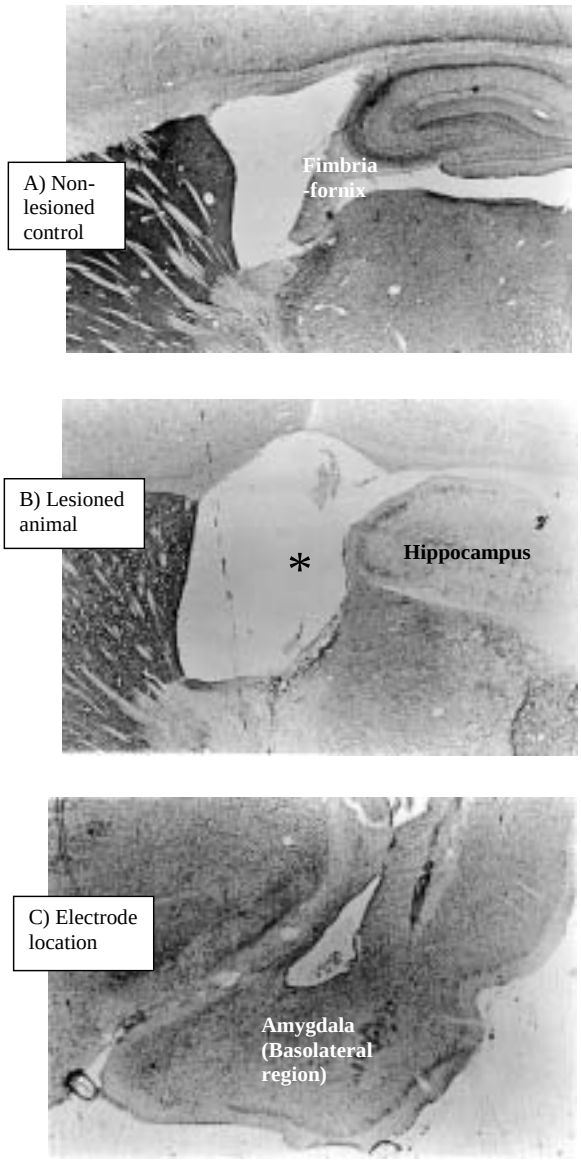

Fig. 4. Histology. A) Low magnification (5x) microphotograph showing the fimbria-fornix system (FF) and the hippocampal formation from a control non-lesioned rat. Notice the intense staining of acetylcholine esterase (ACE) in all hippocampal subfields. B) Low magnification (5x) microphotograph showing the absence of the FF (asterisk) and the severe reduction of ACE staining in the hippocampus. C) Low magnification (5x) microphotograph of the temporal region showing the basolateral amygdala and the electrode track.

of trains, intertrain interval) as well as whether there exist a restricted time window for the effect as is the case with LTP-reinforcement [28].

Further experiments should also contribute to clarify the mechanisms involved in the improving effect shown here. For instance, whether there is an activation of growth factors. Considering the universality of the mechanisms of neural plasticity [13] it is likely that other forms of plasticity might also be modulated by amygdala-dependent emotional factors. This can also be true for plastic re-arrangements mediating recovery of brain function after trauma or degeneration.

Depression is a common sequel of brain lesion [33, 59] affecting the speed and completeness of recovery [25,57]. A new form for treating depression have emerged in recent years using transcranial magnetic stimulation (TMS) of the dorsolateral prefrontal cortex [32,63]. As there is a link from the prefrontal cortex to the amygdala [56] is likely that both, the improving effect of depressive symptoms and the reinforcement of neural plastic processes may share common mechanisms. Such would open perspectives for the use of TMS in neurological patients to treat their depression and simultaneously, enhance the plastic mechanisms stimulated by the rehabilitation therapy.

The results presented here constitute a first evidence that amygdala stimulation might be a useful tool to stimulate neural plasticity in the lesioned Central Nervous System, and could be useful for future developments in the treatment of chronic neurological diseases. New experiments are required to clarify the possible mechanisms of such effects and to establish the most effective stimulation paradigms to reach that goal.

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Capítulo 12: La estimulación de la amygdala mejora la adquisición de una habilidad motora.

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Este trabajo continúa y expande la hipótesis de universalidad de los mecanismos de reforzamiento afectivo de procesos neuroplásticos que, para este, pudiéramos expresar de la siguiente forma: *Si el reforzamiento de procesos neuroplásticos es un fenómeno universal, entonces debe expresarse en otras formas de plasticidad e incluso en otras regiones del encéfalo*.

La forma de neuroplasticidad seleccionada fue la memoria motora que se acompaña de cambios en los mapas motores corticales correspondientes al hemicuerpo y grupo muscular involucrado.

Para su realización empleamos animales sanos a los que se implantó un electrodo de estimulación en la amígdala basolateral. Los animales fueron entrenados en días sucesivos para la adquisición de una habilidad motora hasta alcanzar un criterio previamente definido. El grupo experimental recibió, 15 minutos después de cada sesión de entrenamiento, estimulación eléctrica de la amígdala basolateral, mientras que grupos controles fueron implantados y manipulados de la misma forma pero sin estimulación. Los resultados mostraron que la estimulación de la amígdala redujo el número de sesiones de entrenamiento requeridas para alcanzar el criterio. El entrenamiento por su parte, produjo en todos los animales entrenados una expansión del área cortical de representación de la extremidad anterior. La estimulación de la amígdala no modificó la naturaleza, ni la extensión del cambio cortical; solo hizo que ocurriera más rápido.

Estos resultados están en concordancia con la hipótesis y refuerzan la convicción de que los mecanismos de reforzamiento de procesos neuroplásticos constituyen un mecanismo universal.

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Stimulation of the basolateral amygdala improves the acquisition of a motor skill

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Abstract. *Purpose*: We have previously shown that the stimulation of limbic structures related to affective life such as the amygdale can improve and reinforce neural plastic processes related to hippocampus-dependent forms of explicit memory, as spatial memory and LTP. We now assessed whether this effect is restricted to the mentioned structure and memory type, or represents a more general form of modulatory influence.
Methods: Young, male Sprague Dawley rats were implanted stereotactically with one electrode in the basolateral amygdala (BLA) and trained to acquire a motor skill using their right anterior limb. A group of animals received 3 trains of 15 impulses at the BLA 15 minutes after each daily training session. A second group of implanted animals was handled in the same way, but not stimulated, while a third group was not implanted. After reaching the training criterion the left motor cortex was mapped by the observation of the movements induced by stimuli applied in discrete points of the cortex.
Results: Cortical representation of the anterior limb was increased in all trained animals, showing that the motor cortex is involved in the acquisition of the new skill. Animals receiving stimulation of the BLA showed similar cortical changes, but learned faster than non-stimulated controls.
Conclusions: Reinforcement of neural plasticity by the activation of the amygdala is not restricted to hippocampus-dependent explicit memory, but it might represent a universal mechanism to modulate plasticity.

1. Introduction

Plasticity is one of the most interesting and potentially useful properties of the nervous system and has attracted the interest of researchers and clinicians in the last decades. A large number of studies have shed light on the different forms, mechanisms, and expressions of neural plasticity, as well as their involvement in development, learning, and recovery after different types of trauma [4].

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The role played by emotional and motivational factors on learning was recognized, and applied -mostly on empirical terms- since long ago by teachers and trainers. The mechanisms of affective influences on learning have been the subject of a series of studies which have identified the key role played by the amygdala in reinforcing different forms of explicit learning [25, 26]. However, little is known about the potential influences of this limbic structure on other forms of plastic mechanisms.

In collaboration with the group of J. Frey in Germany, we have carried out a series of experiments that demonstrated a positive reinforcing role of the amygdala – in particular of its basolateral region (BLA)- on Long-Term Potentiation (LTP) a widely used model of synaptic plasticity. We have shown that the BLA is

involved in reinforcement of LTP by motivational stimuli [3], and that the stimulation of the BLA can effectively substitute the behavioral stimulus to reinforce LTP [11]. This effects seemed to depend on the activation of noradrenergic and cholinergic afferents, and demands the synthesis of proteins, suggesting the mediation of molecular cascades regulating gene expression and macromolecular metabolism [5,11].

In an effort to extend this observations, we have recently shown that post-training stimulation of the BLA can improve the acquisition of spatial memory in fimbria-fornix lesioned rats [2]. This type of memory is hippocampus dependent, as well as the effects on LTP mentioned above.

Therefore, we were now interested in studying whether plastic processes occurring in other structures, like the cerebral cortex, and related to other forms of memory (i.e. implicit memory) like the acquisition of a motor skill, can also be influenced by the stimulation of the amygdala.

2. Materials and methods

2.1. Animals

Young (8 weeks) male Sprague Dawley rats, obtained from the Cuban Center for the Production of Laboratory Animals (CENPALAB) were used. The animals were caged in translucent plastic cages (5 animals per cage) under controlled room conditions (temperature, humidity, light: dark cycle) and with free access to water throughout the experiment. In the groups of animals submitted to training for skilled grasping the diet was restricted to 85% the daily requirements during the training period. All animal handling and experimental procedures were conducted under strict observance of the Cuban Regulations for the Use of Laboratory Animals.

2.2. Surgery

Two groups of animals were operated to implant a stimulating electrode into their right basolateral amygdala. The right BLA was selected to be stimulated in order to avoid damaging the left cortex, in the vicinity of the motor region, that may induce unspecific changes in the motor mapping. Under deep chloral hydrate narcosis (420 mg/kg) the animals were mounted in a stereotactic frame (David Kopf, Saint Louis). The scalp was opened and the skull exposed to drill a hole for inserting a stainless steel (0.125 mm, Teflon coated) electrode aimed to the basolateral region of the amygdala (coordinates AP: -3.5 mm, ML: 5.0 mm, DV: -7.6 mm from bregma). A miniscrew with a soldered copper wire was screwed on the right frontal bone, to function as returning electrode during stimulation. Both electrodes were connected to a bipolar female socket and attached to the skull with dental cement.

2.3. Motor training

The animals were trained for skilled grasping movements with their right anterior limb using the staircase model introduced by Montoya and coworkers [29] (see Fig. 1A). The food-restricted animal was placed in the cage, upon a central platform surrounded left and right by a 7 steps staircase. In a central depression in each step 2 small pieces of food were placed, only in the right stair. The animals were allowed to pick up and eat all the pieces they can grasp and carry to their mouth using their right anterior limb during a period of 15 minutes every day. The construction of the cage prevented the animals to use their left limb or turning their body to improve reaching and grasping. Once an animal was able to collect all pieces up to level 6 during 3 consecutive days training was stopped and the motor cortex was mapped. The number of days required by each animal to reach this criterion was counted and considered an indicator of its motor learning ability.

2.4. Amygdala stimulation and experimental groups

In one group of animals (BLA, $n = 10$) the basolateral region of the amygdala was stimulated 15 minutes after finishing each training session. The stimulus consisted of 3 trains of 15 impulses (0.1 ms pulse duration) at 200 Hz and 400 μA intensity. A second group of implanted animals (sham, $n = 8$) was also connected to the stimulus apparatus at the same time, but no stimulus was applied. A third group of control animals (trained, $n = 8$) was simply returned to the home cage at the end of training. Finally, a group of naïve, non-trained and non-stimulated animals ($n = 19$) was used only for mapping the motor cortex. The number of animals given in parentheses is the final amount of rats included in the statistical analysis for each group.

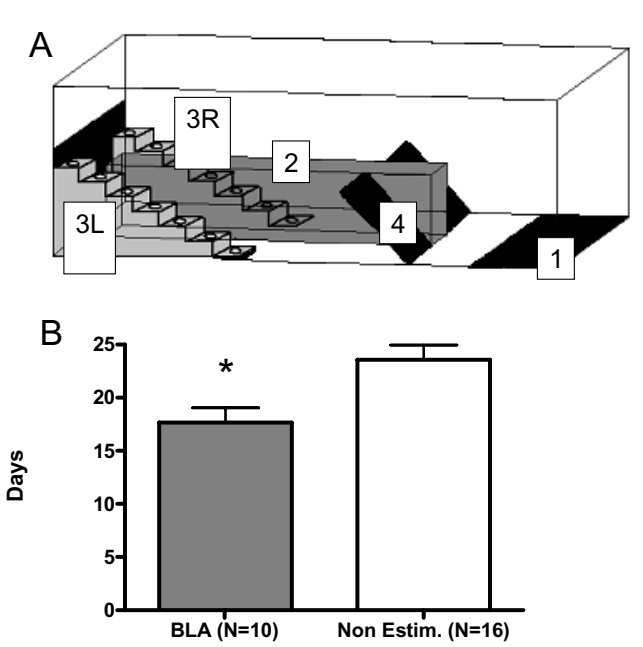

Fig. 1. A) Drawing of the staircase device used to train the animals. 1: opening to enter the rat; 2: central platform; 3: lateral staircases (Left and Right); 4: blocker of foot motion. Only the right staircase was baited. B) Results of training to develop a motor skill for the different groups (mean $\pm$ SEM). * $P < 0.05$, Student's t test.

2.5. Mapping the motor cortex

After reaching the learning criteria, the motor cortex of the animals was mapped and compared to a group of non-trained animals. The procedure was performed under ketamine narcosis (7 mg/kg) supplemented with diazepam and atropine. A window was opened on the left hemisphere (AP: 3.5 to -1 mm, ML: 0.0 to 5.0 mm, all coordinates from bregma) to expose the motor cortex. The dura was carefully removed and a 0.5 MΩ stainless steel monopolar microelectrode (World Precision Instruments, Sarasota, USA) was lowered to -1.5 mm from the cortical surface at different points with 0.5 mm interval in each direction (AP or ML) starting at AP: 3.5 mm, ML: 1.5 mm and finishing at AP: -0.5 mm, ML: 5.0 mm. In cases in which a motor response was obtained in any of the border points the stimulation area was increased in 0.5 mm in the required direction until a no-response border surrounded the whole area. The stimulus applied at each point consisted of 2 trains of 9 impulses (0.2 ms pulse duration, 3 ms interpulse interval and 400 μA intensity) separated by a 1.2 sec intertrain interval. The response in any of the following contralateral body parts was determined by direct observation of one experienced observer, and annotated in the protocol using the following code numbers: 0- no response, 1- vibrissae, 2- anterior limb, 3- neck, 4- shoulder, 5- trunk, and 6- posterior limb. The total area of the cortex controlling the movement of each body part was then calculated by multiplying the number of response points for each part by 0.25 mm^2. In case that the stimulation of one point was followed by a motor response in two body regions, an area of 0.125 mm^{2}was added to each.

2.6. Histology

After finishing the motor mapping, and still under narcosis the animals were transcardially perfused with formaline, the brains removed and prepared for histological observation to ascertain the location of the implanted electrode. Only animals showing a correct location of the electrode at the BLA were considered for further analysis. One animal intended for the BLA-stimulated group, and two for the sham-stimulated group were rejected by this reason.

2.7. Statistics

One way and two ways ANOVAs were performed to ascertain significant differences among groups. A Student's t-test was performed, post hoc to compare between groups. In every case differences were considered significant only if $P < 0.05$.

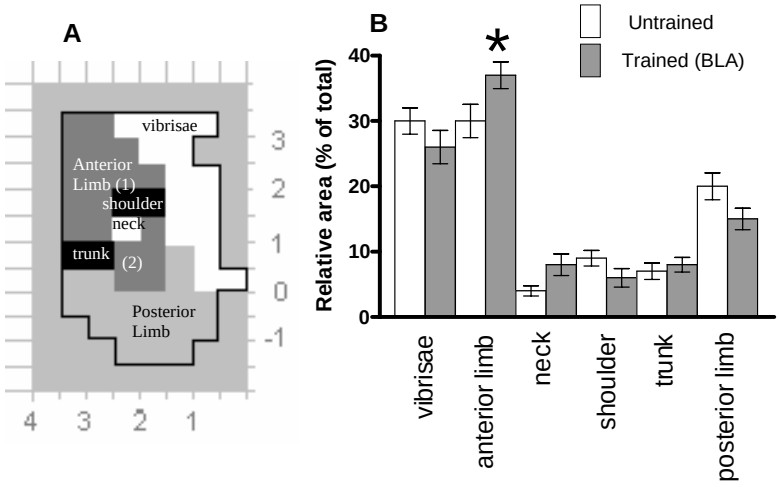

Fig. 2. A) A representative example of the motor map on the left cortical surface from non-trained animals. The areas representing the different body parts are named and identified using different tones. The values in the scale are in mm, the value 0 representing the position of the reference bregma. B) Changes in the relative area of the different body parts as a result of training. Shaded columns: trained animals (mean $\pm$ SEM). Non-shaded columns: non-trained animals (mean $\pm$ SEM). * Significant difference due to training (ANOVA 2-way).

3. Results

Analyzing the results of the motor training, the univariate ANOVA showed that the stimulation of the BLA significantly improved the acquisition of the motor task ($F_{2,23} = 6.377,\ p < 0.05$). The t-test showed no significant differences between the groups of animals in which the BLA was not stimulated, the trained and sham groups ($t = 1.630113,\ p = 0.125362$). When these two groups were pooled together and compared to the BLA stimulated animals, the t-test showed significant differences ($t = 2.918267,\ p = 0.007530$, see Fig. 1B).

The results of motor cortex mapping are shown on Fig. 2. At the left side (Fig. 2A) a representative motor map, obtained from naïve, non-trained animals, is shown. The total area of the motor cortex was about 10 mm^2. As can be seen, the area for the vibrissae, as well as the anterior and posterior limbs, occupies the greater portions of the motor cortex. The right part of the Fig. 2(B) shows the relative distribution of the mapped body regions in non-trained animals compared to that of trained animals receiving BLA stimulation. All the groups of trained animals showed similar changes, irrespective of post-training handling or stimulation. As can be noticed, the area representing the anterior limb was increased after training. The 2-way ANOVA showed that this increase is statistically significant ($F_{1,41} = 5.18,\ p < 0.05$) but caused only by training. The stimulation of the BLA did not influence the final modification in the motor cortical map, except for the fact that it occurred faster.

Our results confirm that cortical maps can be modified by experience, as an expression of cortical plasticity, and show that the mechanisms involved in such changes can be modulated by the stimulation of limbic sub cortical structures like the BLA.

4. Discussion

The primary motor cortex is the main cortical output to the lower motor neurons in the spinal cord or the brain stem and it plays a pivotal role in the performance of skilled movements [30]. Within motor cortex there is a topographic representation of the different body parts, known as motor homunculus in humans, or motor map in the rat. Although such maps are hardwired, they maintain the ability to change [7,20] in response to different challenges like lesions [14] or training [32].

Previous reports have shown that the area representing the anterior limb within the rat primary motor cortex expands after learning specific skilled movement, but not after non-specific general exercise [21] or force training [32]. These results are in line with those reported in the present article. The training method applied to the animals lead to an expansion of the cortical area initiating movements of the anterior limb. We may, therefore, assume that the measured expansion is functionally related to the acquired motor ability.

The mechanisms involved in such changes might be several and may be different in the different stages of acquisition and consolidation of the learned skill [8]. Synaptic plasticity, in form of a LTP-like enhancement of connectivity between cortical neurons, has been demonstrated within the motor cortex [34]. This type of cortical LTP shows mechanistic similarities with hippocampal LTP, as its dependence on NMDA receptors activation [12], and has been related to the initial phases of skilled movement acquisition [28]. Interestingly, similar changes have been demonstrated in humans [39]. Continued motor training may lead to longer-lasting plastic changes within the motor cortex, like an increased synaptogenesis [22] during late phases of acquisition, which may finally be expressed in form of a modified motor map. All those mechanisms depend on protein synthesis [20] as it has been shown for neural plasticity in other brain regions [4].

Neural plasticity processes in the cortex may be influenced by the activity of modulatory subcortical systems. A relevant influence of basal forebrain cholinergic inputs on cortical plasticity, in motor [9], sensory [31], and association areas [38] have been experimentally documented. Our results show that the stimulation of the BLA can also exert a modulatory influence on motor cortical plasticity, facilitating the acquisition of a motor skill that correlates with changes in the motor cortex map.

We have previously shown that the stimulation of the BLA prolongs LTP in the dentate gyrus of the hippocampus [11]. This reinforcing effect is protein synthesis dependent and is likely mediated by cholinergic and noradrenergic inputs [11]. The modulating afferents seemed to reach the hippocampal formation via the basal forebrain-fornical projection [18] likely activated by the BLA stimulation [19].

A similar mechanism might mediate the facilitating effect of BLA stimulation on the acquisition of a motor skill. The motor cortex receives cholinergic and noradrenergic projections [23,27] from the basal forebrain [13] and the locus coeruleus [6,37]. These modulating systems can activate neuronal metabolic cascades [15,17] leading to an enhanced and/or modified synthesis of the proteins required to consolidate synaptic plasticity and to promote synaptogenesis.

Alternative pathways might include the prefrontal cortex which have been recently shown to influence the acquisition of motor skills [10], or the insular cortex [36].

As mentioned in the Methods section, we have stimulated the BLA contralateral to the hemisphere involved in the motor skill acquisition to avoid damaging a near cortical region that might have caused plastic changes not related to training. The BLA projection to cortical regions is bilateral [1], but even if the contralateral projection is weaker, our results showing that stimulating the BLA improves the acquisition of a motor skill gain in value.

The amygdala is part of a complex brain system responsible for the evaluation of affective (emotion, motivation) status of the organism [24,35]. Accumulating evidence indicate that affective factors can influence neural plasticity, not restricted to memory, but also the cellular and subcellular mechanisms involved in plasticity [1,3,16,33,35]. Our results confirm and expand that observation showing that the activation of the BLA can influence also cortical plasticity.

Our results may be generalized to suggest that the modulatory influence of the amygdala on neural plasticity might represent a universal property of the brain, not just restricted to particular structures or processes. Understanding the interactions between affective factors and neural plasticity appears, therefore, to be not only of great theoretical interest, but potentially relevant to practical use in different fields, from educational fields to restorative neurology as well.

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DISCUSIÓN GENERAL

Como elemento central de esta discusión reproducimos a continuación el texto del último trabajo realizado en esta serie.

Este experimento estuvo diseñado con el propósito de precisar el papel de los sistemas de neurotransmisión noradrenérgico y colinérgico, teniendo en cuenta las estructuras que les dan origen.

Para evitar la falta de especificidad que provoca la inyección intraventricular, se empleó la aplicación tópica de las sustancias en cada una de las estructuras seleccionadas.

Los resultados mostraron que el locus coeruleus es un mediador de los efectos reforzadores de la estimulación de la amígdala. La activación del locus se realiza mediante una proyección colinérgica. Así mismo, la proyección noradrenérgica del locus a la región septal y al giro dentado son parte imprescindible del sistema neural de reforzamiento. De esta estructura nace una proyección colinérgica que alcanza al giro dentado del hipocampo mediante la fimbria-fornix y que resulta necesaria para que el efecto reforzador se manifieste. También por la vía fimbria-fornix llegan al giro dentado fibras noradrenérgicas directas.

Basados en esos resultados se propone un mecanismo neural que se ilustra en la figura 7 del trabajo. En él se propone que el reforzamiento de la LTP en el giro dentado por factores afectivos requiere la activación secuencial y ordenada de la amígdala, la cuál por medio de una proyección colinérgica activa al locus coeruleus. La proyección noradrenérgica que nace de esta estructura tiene un efecto estabilizador sobre fases intermedias de la LTP inducida en el giro dentado y, por otra parte, activa la proyección

colinérgica del septo medial al giro dentado que se necesita para reforzar las fases tardías del proceso neuroplástico.

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Cholinergic afferents to the locus coeruleus and noradrenergic afferents to the medial septum mediate LTP-reinforcement in the dentate gyrus by stimulation of the amygdala

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Abstract

Transient long-term potentiation (E-LTP) can be transformed into a long-lasting LTP (L-LTP) in the dentate gyrus (DG) by behavioral stimuli with high motivational content. Previous research from our group has identified several brain structures, such as the basolateral amygdala (BLA), the locus coeruleus (LC), the medial septum (MS) and transmitters as noradrenaline (NA) and acetylcholine (ACh) that are involved in these processes. Here we have investigated the functional interplay among brain structures and systems which result in the conversion of a E-LTP into a L-LTP (reinforcement) by stimulation of the BLA (BLA-R). We used topical application of specific drugs into DG, and other targets, while following the time course of LTP induced by stimulation of the perforant pathway (PP) to study their specific contribution to BLA-R. One injection cannula, a recording electrode in the DG and stimulating electrodes in the PP and the BLA were stereotactically implanted one week before electrophysiological experiments. Topical application of atropine or propranolol into the DG blocked BLA-R in both cases, but the effect of propranolol occurred earlier, suggesting a role of NA within the DG during an intermediate stage of LTP maintenance. The injection of lidocaine into the LC abolished BLA-R indicating that the LC is part of the functional neural reinforcing system. The effect on the LC is mediated by cholinergic afferents because application of atropine into the LC produced the same effect. Injection of lidocaine inactivating the MS also abolished BLA-R. This effect was mediated by noradrenergic afferents (probably from the LC) because the application of propranolol into the MS prevented BLA-R. These findings suggest a functional loop for BLA-R involving cholinergic afferents to the LC, a noradrenergic projection from the LC to the DG and the MS, and finally, the cholinergic projection from the MS to the DG.

Keywords: Early-LTP; Late-LTP; Hippocampus; Reinforcement; Memory formation; Learning; Memory system

1. Introduction

Long-term potentiation (LTP) is a form of neural plasticity that represents a common cellular mechanism for many forms of learning (Matthies, 1989), and is gaining recognition as a putative mechanism for functional recovery after brain insult (Carmichael, 2003).

The importance of affective behavioral factors (motivation, emotions) for the consolidation of memory is well established on empirical basis, and supported by extensive animal research. Affective modulation of memory is mediated by limbic structures such as the amygdala (McIntyre, Power, Roozendaal, & McGaugh, 2003; Richter-Levin, 2004). Similar modulatory influences affect the acquisition of motor skills (Bergado, Rojas, Capdevila,

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Gonzalez, & Almaguer-Melian, 2006) and the recovery of mental functions in stroke patients (Dam, 2001; Narushima, Chan, Kosier, & Robinson, 2003) as well as memory formation in animal models created by the use of brain lesion (Almaguer-Melian et al., 2005). Thus, affective modulation can be described as an universal mechanism, not restricted to a particular form of plasticity process or brain locus.

LTP can also be modulated by affective factors. One example of this is the transformation of an early-LTP (E-LTP) into a late-LTP (L-LTP) lasting more than 4 hours, by the action of a behavioral act with a high motivational value (Seidenbecher, Reymann, & Balschun, 1997; Straube, Korz, & Frey, 2003; Uzakov, Frey, & Korz, 2005). This 'behavioral reinforcement' (BR) of LTP is mediated by protein synthesis (Bergado, Almaguer-Melian, Kostenko, Frey, & Frey, 2003) and depends on BLA function as demonstrated in experiments using animals with temporal or permanent inactivation of this limbic structure (Almaguer-Melian, Martínez-Martí, Frey, & Bergado, 2003). In addition, stimulating the BLA within a specific effective time window, before or after the induction of LTP using a weak tetanus (BLA-R, see Methods) mimics the effects of behavioral reinforcement (Frey, Bergado-Rosado, Seidenbecher, Pape, & Frey, 2001). In other words, a reinforcing effect can also be obtained by stimulation of the BLA (BLA-R).The reinforcing effect of the BLA on LTP in the dentate gyrus (DG) seems to involve noradrenergic and cholinergic afferent innervation (Frey et al., 2001). This suggests the participation of the locus coeruleus (LC) and the medial septum (MS) in these processes as the main sources of noradrenergic and cholinergic innervation to the DG (Vizi & Kiss, 1998). Indeed, we recently found that LTP in the DG can be reinforced by stimulation of the MS (Frey, Bergado, & Frey, 2003) and the LC (Frey, unpublished results).

However, in prior pharmacological studies the antagonists were applied intracerebroventricularly (icv), thus the question arises of where in the brain the mentioned pharmacological effects actually take place. Here we use an approach of topical antagonist application into the structures of interest: the DG, the BLA, the LC , and the MS. The results suggest the existence of a functional loop for BLA-R involving cholinergic afferents to the LC, a noradrenergic projection from the LC to the DG and the MS, and finally, the cholinergic projection from the MS to the DG.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 250–300 g (8 weeks old) at the beginning of the study were used. After surgery the animals were housed individually in plastic translucent boxes with free access to food and water and controlled room conditions (12 h light/dark cycle, temperature and humidity) during the entire experiments. For all experiments with animals ethical approval was sought prior to the studies according to the German requirements for the use of experimental animals.

2.2. Surgery and implants

Rats were anaesthetized using nembutal (40 mg/kg, i.p.) and mounted in a stereotactic frame (David Kopf, Saint Louis, USA). The standard preparation included the implantation of one stainless steel monopolar recording electrode in the DG (0.125 mm diameter, coordinates: anteroposterior AP = −4.0 mm, mediolateral ML = 2.3 mm from bregma and dorsoventral DV ≈2.5 mm from the dural surface, with lambda and bregma at the same high). A bipolar stainless steel electrode was placed in the medial perforant path (PP) (AP = −7.5 mm, ML = 4.1 mm from bregma, DV −3.0 mm from the dural surface) and a monopolar electrode into the BLA (AP = −3.5 mm, ML = 5.0 mm from bregma, DV = −7.6 mm from the dural surface). The position in the DV axis of the electrodes in the DG and the PP was adjusted under observation of the evoked potentials to optimize its location.

Guide cannuli for the topical injection of substances were also placed in one of the following locations: LC (AP = −10.3 mm, ML = 1.1 mm, DV = − 5.7 mm, bregma 3 mm lower than lambda); MS (AP = 0.7 mm, ML = 1.8 mm, DV = −6.6 mm, bregma and lambda at the same high, tilted 15° in lateral direction); the BLA or the DG (same coordinates as given for the electrodes). DV coordinates were selected so that the guide cannula was positioned with its tip approximately 1 mm above the target. In the animals with cannuli implanted into the BLA or the DG the electrode was attached to it, protruding 0.5 mm the bevel edge to form a so called chemitrode. The guide cannuli were made of 0.5 mm (external diameter) stainless steel tubes cut to the appropriate length according to the target. A 0.3 mm stainless steel "stiletto" was placed into the cannula to prevent the outflow of cerebrospinal fluid.

All implants were placed at the right hemisphere. Miniscrews were attached at the skull in the right frontal bone, left parietal bone and the left parieto–occipital junction, to serve as ground, indifferent electrode and mechanical anchorage. A silver wire with the tip thermically rounded was placed over the right parietal dura to serve as returning electrode for stimulating the BLA. Electrodes were then connected to flexible rubber female sockets and fixed to the skull with dental cement.

2.3. Electrophysiology

2.3.1. Habituation and I/O curves

A week after surgery the animals were tested and habituated to the recording chamber for no less than 4 h. An input–output was obtained stimulating the PP (100–800 μA) to determine the stimulus intensity to be used in each animal (40% maximal population spike). There were no differences in the average stimulus intensity and baseline values between the experimental groups.

2.3.2. General design

The experiments were performed on the following day. The general experimental design included the recording of a baseline for 1 h (12 recordings with 5 min interval) followed by the induction of an early-LTP by a weak tetanus (see below) to the PP. Ten minutes after tetanus substances were applied topically into the LC, the MS, the BLA or the DG. The BLA was stimulated 15 min after LTP induction (i.e. 5 min after administration of substances) with an identical pattern of weak tetanus. The time course of LTP was followed up for 8 h with recordings every 15 min. A further recording was made 24 h after tetanization (see Fig. 1e).

2.3.3. Recording of evoked potentials at the dentate gyrus

Each record consisted of 5 averaged waveforms evoked by square pulses (0.1 ms) at 0.1 Hz (Isolated Pulse Stimulator, A-M System, USA) to the PP. Signals were filtered between 1 and 5000 Hz using an AB 621 G Biolelectric Amplifier (Nihon Kohden, Japan) and fed to a 1401 Plus A/D converter (CED, Cambridge, UK), and processed by a special software (Intracell, Institute for Neurobiology, Magdeburg, Germany). The amplitude of the population spike (PSA) was measured in mV (from the most positive point reached by the first deflection to the subsequent most negative point, see Fig. 1e—measurement between the two markers

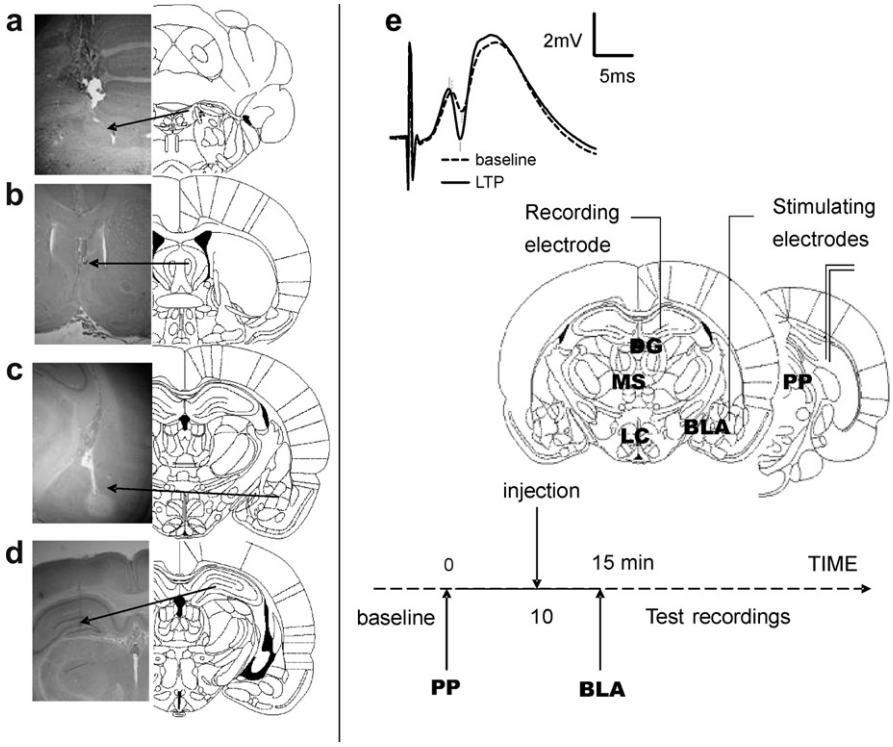

Fig. 1. (a–d) Schematic summary of the results of the histological study, showing the location of the cannuli at the LC (a) the MS (b) the BLA (c) and the DG (d). A representative microphotograph and a schematic representation of the electrode location, and the approximate cannula locations are shown. (e) The inset above, shows representative evoked potential before (broken line) and after the induction of LTP (filled line) recorded at the DG. The population spike amplitude (PSA) was measured between the markers shown. Below, a summarized view of the experimental design is presented as an example with recording electrodes positioned in the dentate gyrus and stimulating electrodes in the PP as well as in the BLA.

per analog trace). The PSA was averaged in the last 6 baseline recordings to determine pre-tetanus baseline value. The PSA of the post-tetanus recordings for each animal were compared to these values and expressed in percent changes. We used the PSA as measure of LTP instead of the EPSP for several reasons. Working in vivo, in freely moving animals, and using a single recording electrode it is difficult to asses simultaneously the EPSP and the PSA. On the other hand, the PSA represents the discharge of action potentials by the postsynaptic population, which is functionally the more relevant outcome of synaptic function.

2.3.4. LTP induction

A weak tetanus protocol was used to induce LTP at the PP-DG synapses and to stimulate the amygdala. It consisted of 3 trains of 15 impulses each at 200 Hz (square biphasic pulses 0.2 ms). Intertrain interval was 10 s. The same stimulus pattern was used for stimulation of the BLA using a constant current of 400 μA.

2.3.5. Drugs and application

The substances used in this experiment were: lidocaine (Lidocainhydrochlorid 2%, Hexal, Holzkirchen, BRD) a local anesthetic, to produce the temporary inactivation of the target region. Propranolol ((*S*)-(−)-propranolol hydrochloride, Sigma–Aldrich, Saint Louis, USA) to block locally the β-adrenergic receptors, and atropine (atropine sulfate, salt, Sigma–Aldrich, Saint Louis, USA) to block locally the muscarinic acetylcholine receptors, all dissolved in saline solution. The injection cannuli were made of stainless steel tubes (0.3 mm external diameter) cut to an appropriate length to protrude 1 mm from the bottom of the guide cannula. The injector was carefully introduced in to the implanted guide cannula, and 1 μl of each substance or vehicle was injected using a Hamilton microsyringe (CR-700-20, Hamilton Co., Reno, USA) in 2 min (≈0.05 μl every 5 s). The cannula was left in place for 1 min before slowly retracting it. The volumes injected contained 6.76 nmol propranolol or 1 nmol atropine, respectively.

In a small group of animals ($n = 3$) we have tested the injection procedure in the dentate gyrus. The injection of 1 μl saline in the dentate gyrus does not change the form or amplitude of the evoked potentials recorded at the tip of the nearby located recording electrode (data not shown). The injection of 1 μl lidocaine provoked a transient reduction of the evoked potentials of nearly 30% the PSA, which returned to normal values in about 20 min. Apparently, no mechanic disruption was caused by the injection despite the vicinity between the cannula and the electrode, however they were close enough to allow a direct influence of the injected substance on the recorded cell population.

The effectiveness of the used drug concentrations were documented in previous papers (Almaguer-Melian et al., 2003, 2005; Frey et al., 2001, 2003).

2.3.6. Experimental groups

Several experimental groups were randomly formed, according to the stimulation protocol, the substance applied and the place of injection.

Injections into the DG, 10 min after LTP induction (5 min before BLA stimulation) were done using atropine ($n = 9$), propranolol ($n = 10$), or saline ($n = 10$). To asses a possible direct effect of both substances on LTP or the evoked potentials, the following controls were also carried out: Injection of atropine ($n = 7$), propranolol ($n = 8$), or saline ($n = 10$) after LTP induction without stimulating the BLA; as well as injection of atropine ($n = 8$), propranolol ($n = 8$), or saline ($n = 8$) without LTP induction or BLA stimulation.

Similarly, 10 min after LTP induction one of these substances was applied into the LC: lidocaine ($n = 8$), atropine ($n = 8$), or propranolol ($n = 8$). For injection into the MS: lidocaine ($n = 7$), atropine ($n = 7$), or

propranolol ($n = 8$). For the injection into the BLA: atropine ($n = 9$), or propranolol ($n = 9$). A group of 9 animals injected in these structures with saline solution served as common control group, as there were no differences in their initial level and time course of potentiation (LC $n = 4$, MS $n = 3$ and BLA $n = 2$, Fig. 2a—inset). In all groups (with the exception of those assessing substance effects on baseline or simple LTP) the BLA was stimulated 5 min after injection, i.e. 15 min after LTP induction.

2.3.7. Behavioral control and time of the experiments

All electrophysiological (recordings or stimulation of the PP or the BLA) and injection procedures were performed in awake and freely moving animals. The behavior of the animals during, and in the period after each procedure was carefully observed by an experienced behavior analyst. No signs of behavioral modification were observed in any of these conditions. To minimize and standardize circadian influences all the experiments were carried out at approximately the same time of the day. Tetanization of the PP to induce LTP (time 0) was carried out always between 10 and 10:30 a.m.

2.3.8. Statistics

The percent values of the PSA were compared between treatments (groups) using a two-way ANOVA with TREATMENT and TIME (repeated measures) as factors in independent analysis for each injection site. Significant differences between groups at specific time points were ascertain using the post hoc test of Tukey (honest significant difference, HSD) when the ANOVA showed significant differences between groups (TREATMENT) or the interaction. In every case a $p < .05$ was considered significant.

2.3.9. Histology

At the end of the experiment the animals were transcardially perfused with formaline under nembutal narcosis. The brain was extracted and post-fixed. Frozen brains were cut into 40 μm thick slices containing the regions of interest and stained with toluidin blue. The location of the cannula was estimated by direct inspection of the local damage in the surrounding tissue. Animals showing a misplaced cannula or electrode were rejected and not considered in the final group analysis.

3. Results

Histological analyses allowed us to select only those animals showing a correct placement of the cannuli. Fig. 1a–d shows a constructed summary for the cannula location in each of the targets. The LC is a small, deep region of the brain stem. For this reason the number of rejections for this structure was higher than for any other structure, where rejections were required only exceptionally.

The direct administration of the antagonists into the DG showed significant effects on the reinforcing effects of

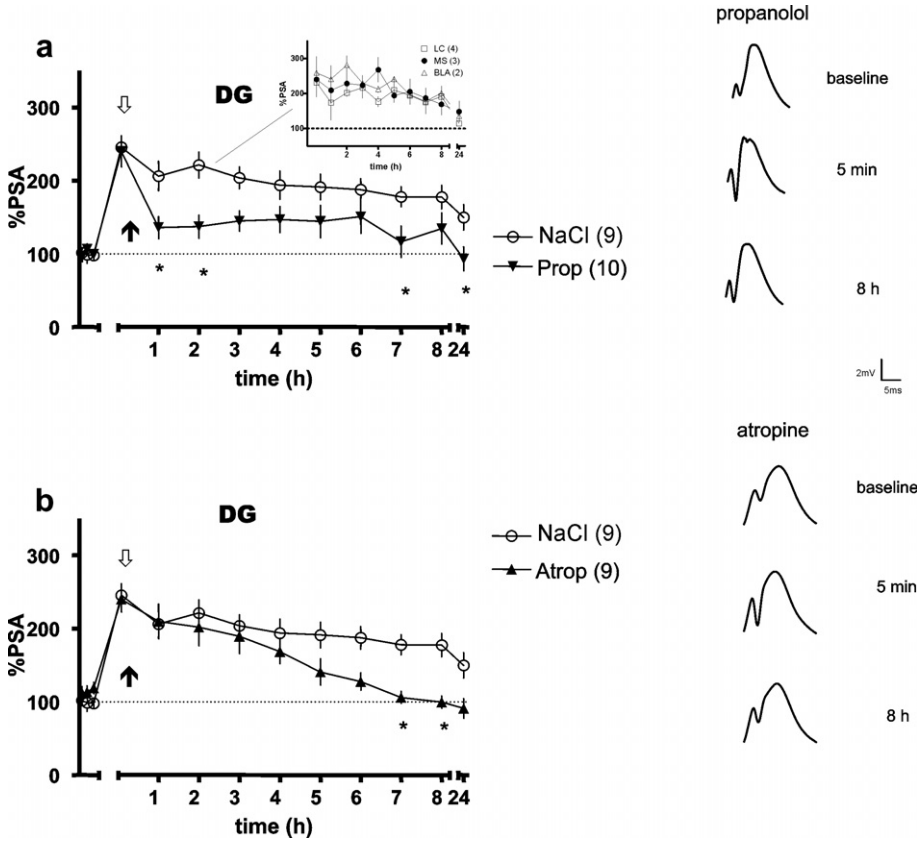

Fig. 2. Effects of topical application of drugs into the DG on LTP reinforcement by stimulation of the BLA—(a) after propranolol injection into the BLA (inset represents the time course of the averaged control group provided in the graphs which consists of 9 animals injected in structures of interest with saline solution ($n = 4$ LC, $n = 3$ MS and $n = 2$ BLA), and (b) after atropine. Mean values (±SEM) of the PSA (% PSA, relative to baseline) for the experimental groups are shown (the numbers in parentheses indicates the number of animals in each groups). LTP was induced in the DG by stimulation of the PP at $t = 0$, and reinforced by stimulation of the BLA 15 min later (black arrow). Substances were topically applied into the DG between both stimuli ($t = 10$ min, open arrow). Asterisks indicate significant differences between groups in the post hoc test (Tukey HSD, $p < .05$). Representative traces from one animal in selected groups, before (baseline) and after E-LTP induction (5 min and 8 h) are shown in the right part of the figure.

BLA stimulation (Fig. 2). Two-way ANOVA showed TREATMENT as a significant factor ($F_{2,27} = 3.79097$). Atropine produced a delayed decay of LTP, which starts about 4–5 h and reached significance 7–8 h after LTP induction (Fig. 2b). Propranolol also showed a negative influence on BLA-R (Fig. 2a), but the effect was much earlier and apparently biphasic. The level of potentiation after BLA-R in the propranolol group was significantly reduced 1–2 h after induction, and at 7–24 h.

When these drugs were applied to non-tetanized animals, atropine showed no significant effect. Propranolol, however induced a mild, but significant depression of the PP-DG evoked potentials (Fig. 3a). When propranolol or atropine were applied to animals in which E-LTP was induced without BLA stimulation, none of them showed significant effects on LTP time course (Fig. 3b).

The administration of the same drugs at the BLA itself produced more moderate effects. No significant treatment effect was statistically confirmed ($F_{2,24} = 1.79602$). However the interaction time × treatment was significant ($F_{18,216} = 2.00483$). The post hoc Tukey test detected significant differences only 4 h after atropine application (see Fig. 4).

Fig. 5 shows the results of pharmacological manipulation in the LC after LTP induction and before BLA stimulation. The two-way ANOVA showed TREATMENT as a significant factor ($F_{3,30} = 7.01437$). Results of the post hoc comparison of the drug/time interaction are shown in detail in graphs a, b, and c, all compared to the control group treated with saline (NaCl). The injection of lidocaine (Fig. 5a) abolished the reinforcing effect on DG-LTP of stimulating the amygdala. Significant differences between groups were seen starting at 2 h. The administration of atropine has a similar blocking effect as lidocaine (Fig. 5b), while propranolol produced no significant effect (Fig. 5c).

When substances were applied to the MS (Fig. 6) the factor TREATMENT also was significant (two-way ANOVA, $F_{3,28} = 15.17692$). Inactivation of this region by topical application of lidocaine caused a blockade of the reinforcing effect of BLA stimulation on DG-LTP (Fig. 5a) showing a decay of the potentiation that significantly differs from the saline controls (NaCl). The topical administration of propranolol in this structure caused a very fast and sustained decay of LTP (Fig. 5c). Atropine, on the other hand, produced no significant effect (Fig. 5b).

4. Discussion

Our results indicate that both, topically applied propranolol as well as atropine in the DG blocked the reinforcing effect of DG-LTP by stimulating the amygdala (BLA-R). These results are, in general terms, similar to those previously reported by our group showing that the

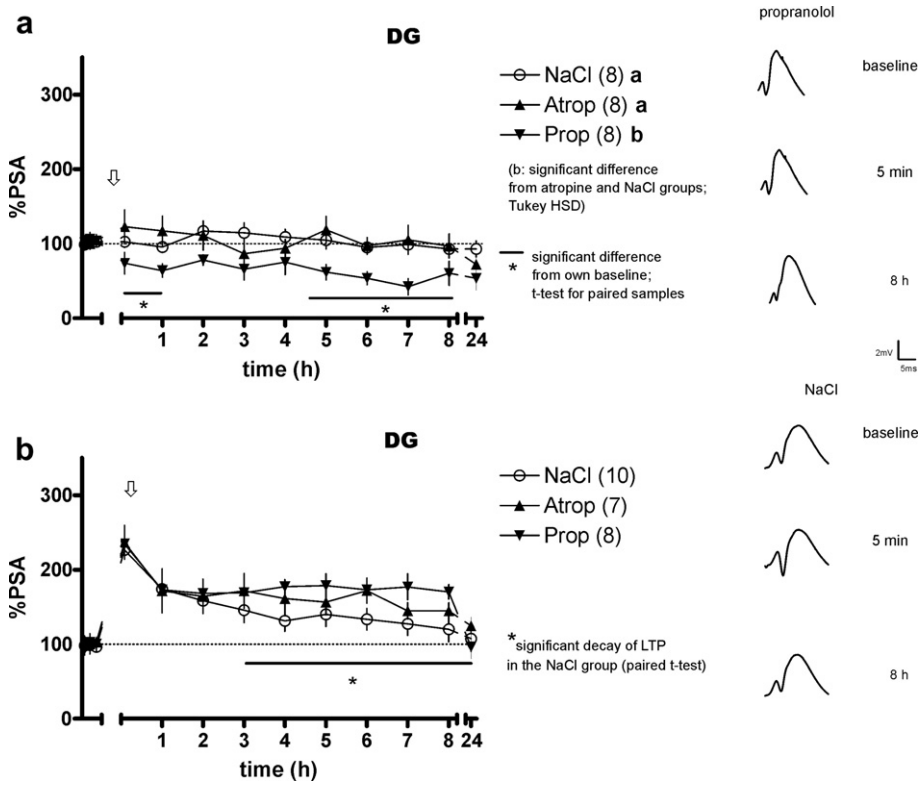

Fig. 3. Effects of topical application of drugs into the DG on baseline evoked potential (a) and on DG-LTP induced by PP stimulation (b). Mean values (±SEM) of the population spike amplitude (% PSA, relative to baseline) for the experimental groups are shown (the numbers in parentheses indicate the number of animals in each groups). Asterisk indicates significant differences between groups in the post hoc test (Tukey HSD, $p < .05$). Representative traces from one animal in selected groups, before (baseline) and after E-LTP induction (5 min and 8 h) are shown in the right part of the figure.

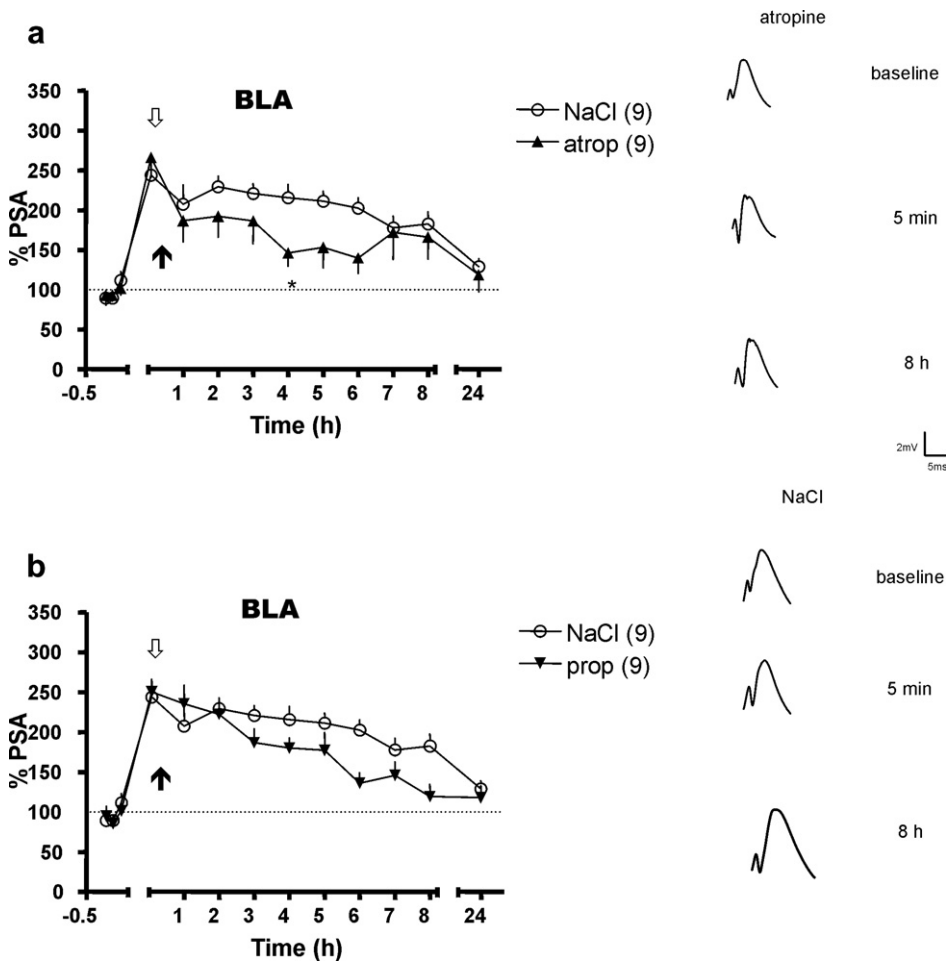

Fig. 4. Effects of topical application of drugs into the BLA on BLA-R. Mean values (±SEM) of the PSA (% PSA, relative to baseline) for the experimental groups are shown in (a) after atropine, and (b) propranolol application (the numbers in parentheses indicates the number of animals in each groups). LTP was induced in the DG by stimulation of the PP at $t = 0$, and reinforced by stimulation of the BLA 15 min later (black arrow). Substances were topically applied into the BLA between both stimuli ($t = 10$ min, open arrow). The asterisk under the line indicates significant differences between groups in the post hoc test (Tukey HSD, $p < .05$). Representative traces from one animal in selected groups, before (baseline) and after E-LTP induction (5 min and 8 h) are shown in the right part of the figure.

intraventricular administration of propranolol or atropine blocked the reinforcing effect of BLA-stimulation on E-LTP in the DG (Frey et al., 2001) and suggest that the antagonisms of both substances are due, at least in part, to a direct action within the DG. However, in the case of propranolol a much earlier effect is seen when the substance is applied directly into the DG. The different time points at which noradrenergic or cholinergic innervation of the DG interfere with BLA-R suggest that NA is also required to sustain an intermediate stage of LTP—linking E-LTP and L-LTP—while acetylcholine apparently acts on mechanisms involved only at later stages. This might also explain our previous results on the effect of the direct application of NA and ACh agonists on LTP reinforcement, showing that epinephrine was able to mimic BLA-R, while a muscarinic agonist (oxotremorine) was unable to produce a similar effect (Almaguer-Melian et al., 2005). The lack of effect of the muscarinic agonist may depend on the need of a previous activation of the noradrenergic projection to the DG. These results suggest the participation of the LC and the MS (the origin of NA and ACh innervation to the DG) in BLA-R which is supported by the results of directly applying substances in both targets.

A functional LC seems to be necessary for BLA-R, as the inactivation of the LC with lidocaine abolishes the reinforcement of DG-LTP. This effect is mediated by cholinergic afferents because atropine at the LC provokes a similar blockade. Noradrenergic afferents do not seem to be involved considering the lack of an effect of propranolol. However, the participation of other afferents to the LC cannot be ruled out. Similar caution is required regarding the specificity of the effects when substances are applied to the LC. Considering the small size of this structure and the volume injected, the possibility of partial diffusion outside the target can not be excluded. However, in this case the substance would reach first the periLC region which contains the peripheral dendrites of the LC neurons (Luppi, Aston-Jones, Akaoka, Chouvet, & Jouvet, 1995) which is functionally a receptive zone for the LC.

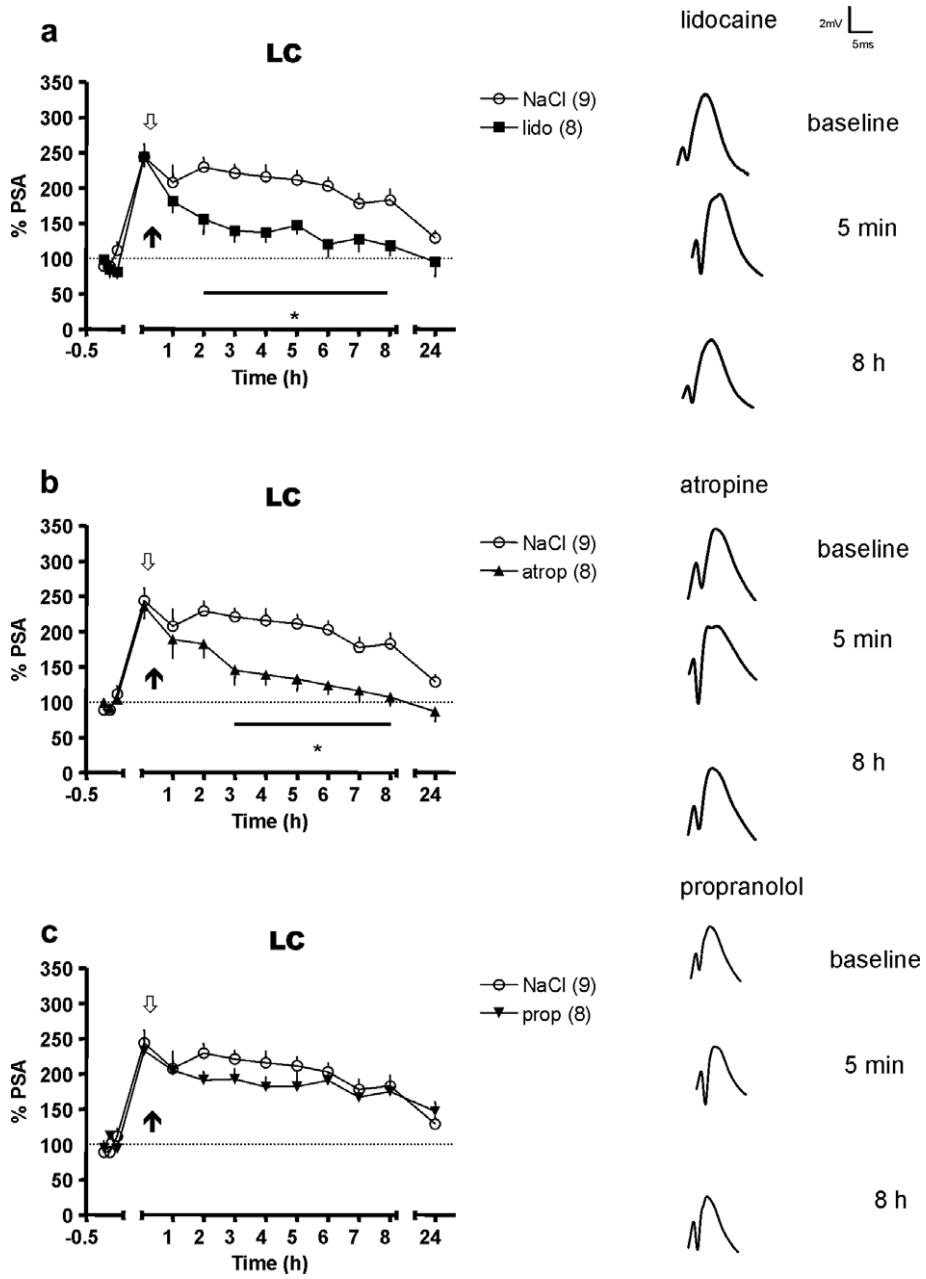

Fig. 5. Effects of topical application of drugs into the LC on BLA-R. Mean values (±SEM) of the PSA (% PSA, relative to baseline) for the experimental groups are shown in (a) after lidocaine, (b) atropine, and (c) propranolol application (the numbers in parentheses indicate the number of animals in each group). LTP was induced in the DG by stimulation of the PP at $t = 0$, and reinforced by stimulation of the basolateral amygdala 15 min later (black arrow). Substances were topically applied into the LC between both stimuli ($t = 10$ min, open arrow). The asterisk under the line indicates significant differences between groups in the post hoc test (Tukey HSD, $p < .05$). Representative traces from one animal in selected groups, before (baseline) and after E-LTP induction (5 min and 8 h) are shown in the right part of the figure.

The LC is the main source of noradrenergic innervation to the forebrain and the hippocampal formation (Berridge & Waterhouse, 2003) but does not receive noradrenergic projections itself. This may explain the lack of an effect of propranolol applied to this structure on BLA-R. The LC receives afferents from the central amygdala mainly at the 'periLC'-region, but this projection does not seem to involve cholinergic fibers (Luppi et al., 1995). Cholinergic fibers to the LC have been described from neighbor brain stem nuclei, like the nucleus pedunculopontinus or the laterodorsal tegmental nucleus which form dendro-dendritic contacts within the LC and the 'periLC'-region (Luppi et al., 1995). Apparently, the strong blocking effect of disconnecting the LC with lidocaine, or the topical administration of cholinergic antagonists on BLA-induced DG-LTP reinforcement, is meditated by an indirect projection from the BLA to one of these putative regions from which cholinergic projection to the LC arises. The functional implications of the cholinergic projection on the activity of the LC are not well characterized yet, but our results indicate an activating, modulatory effect.

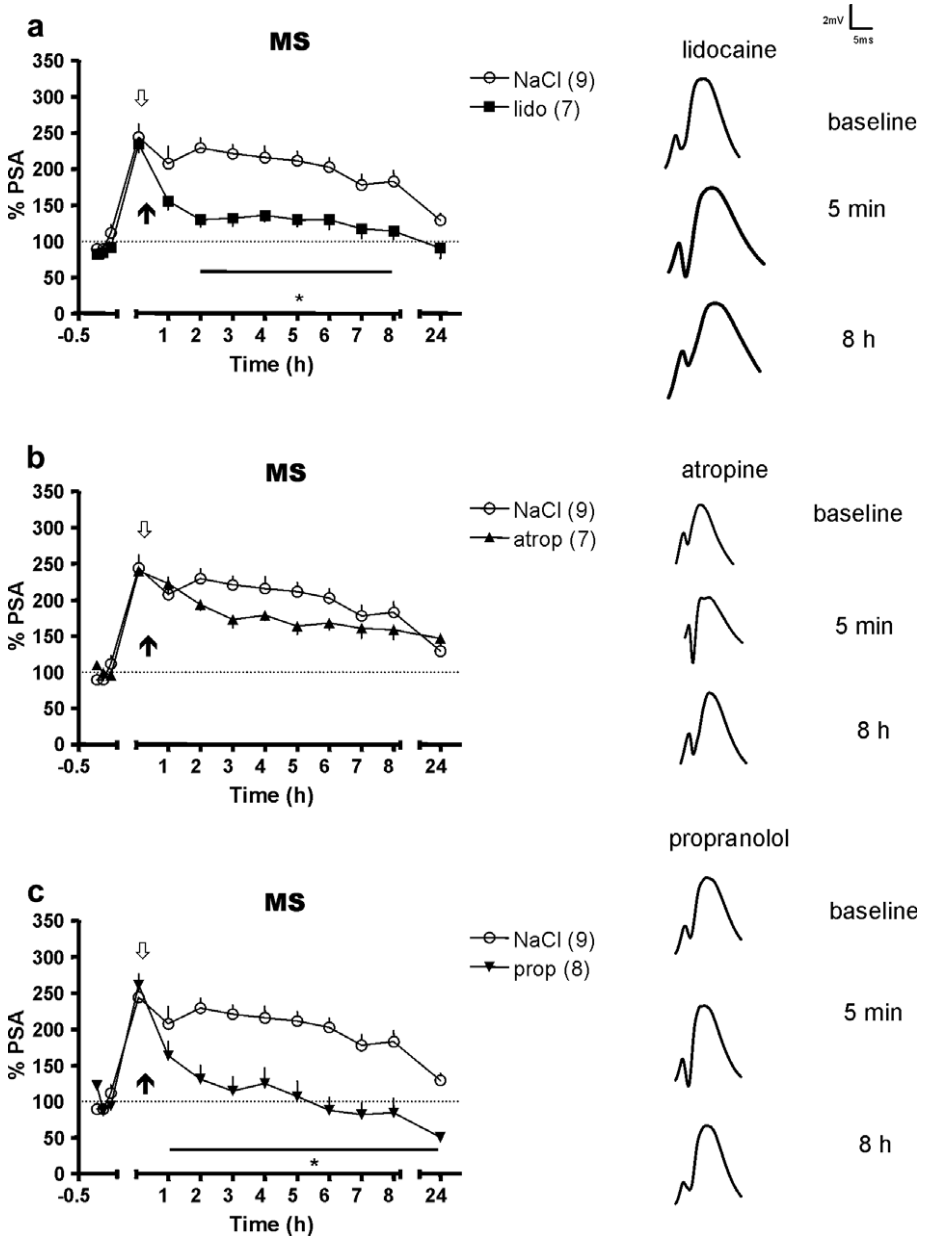

Fig. 6. Effects of topical application of drugs into the MS on BLA-R. Mean values (±SEM) of the PSA (% PSA, relative to baseline) for the experimental groups are shown in (a) after lidocaine, (b) atropine, and (c) propranolol application (the numbers in parentheses indicates the number of animals in each groups). LTP was induced in the DG by stimulation of the PP at $t = 0$, and reinforced by stimulation of the BLA 15 min later (black arrow). Drugs were topically applied into the MS between both stimuli ($t = 10$ minutes, open arrow). The asterisk under the line indicates significant differences between groups in the post hoc test (Tukey HSD, $p < .05$). Representative traces from one animal in selected groups, before (baseline) and after E-LTP induction (5 min and 8 h) are shown in the right part of the figure.

Similarly, a functional MS at the time of BLA stimulation, appears to be necessary to obtain BLA-R; if the results after topical inactivation with lidocaine are considered. However, on this structure, the effect seems to be mediated by noradrenergic afferents, and not by cholinergic fibers, because propranolol at this site completely abolished BLA-R, while atropine was without effect. The LC sends a strong noradrenergic projection to the MS which forms synapses with cholinergic neurons in the basal forebrain (Zaborszky, Cullinan, & Luine, 1993) which project to the cortex and the hippocampal formation.

The topical application of drugs at the BLA showed only moderate effects. This might be a consequence of the direct electrical stimulation of that region which may overcome any postsynaptic blockade due to the one drug or the other. However, the late effect observed in the atropine treated group, suggests that some tonic BLA activity (probably initiated by cholinergic afferents) might be required during the poststimulus period.

Our results can be interpreted within a functional frame in which the activation of the BLA reinforces LTP in the DG by an indirect cholinergic activation of the LC, which

sends a direct noradrenergic projection to the DG, important to sustain the potentiated status in the transition between E-LTP and L-LTP. Additionally, the LC projection to the MS activates the cholinergic projection to the hippocampal formation whose activity seems responsible for L-LTP consolidation. There could be a direct activation of the MS via a putative excitatory projection from the BLA.

Regarding the cholinergic system, it has been extensively documented that the activation of the septal cholinergic projection to the hippocampal formation plays a critical role in memory consolidation (Balse et al.,1999; Blokland, 1995; Gasbarri, Introini-Collison, Packard, Pacitti, & McGaugh, 1993; Izquierdo et al., 1993; McGaugh & Cahill, 1997; Ramirez Lugo, Miranda, Escobar, & Bermudez-Rattoni, 2003). This cholinergic projection to the hippocampus and the DG modulates also LTP (Abe, Nakata, Mizutani, & Saito, 1994; Adams, Winterer, & Muller, 2004; Auerbach & Segal, 1994; Bergado, Moreno, & Nuñez, 1996; Blitzer, Gil, & Landau, 1990; Jerusalinsky, Kornisiuk, & Izquierdo, 1997; Leung, Shen, Rajakumar, & Ma, 2003; Markevich, Scorsa, Dawe, & Stephenson, 1997; Shinoe, Matsui, Taketo, & Manabe, 2005).

The MS, and its cholinergic projection play a pacemaker role in the induction of a rhythmic and synchronic neural activity characterized in EEG recordings as theta rhythm (Bland, Oddie, & Colom, 1999; Monmaur, Ayadi, & Breton, 1993; Vinogradova, Brazhnik, Stafekhina, & Kichigina, 1995; Vinogradova, Kitchigina, & Zenchenko, 1998). Hippocampal theta activity have been related to a variety of important functional states like REM sleep, exploration and attentive wakefulness (Bassant, Apartis, Jazat-Poindessous, Wiley, & Lamour, 1995; Bland & Bland, 1986; Bland, Seto, Sinclair, & Fraser, 1984; Vinogradova et al., 1995, 1998), as well as spatial memory (O'Keefe, 1993).

The aminergic-cholinergic synapses may be part of an extended neural system (summarized in Fig. 7) involved in the affective modulation of plasticity (alternative or complementary routes can also be implied: e.g. there is a direct—probably excitatory—input from the BLA to the basal forebrain (Zaborszky et al., 1993).

On the other hand, a noradrenergic projection from the LC reaches directly the DG, where it inhibits feed-forward inhibitory interneurons (Brown, Walling, Milway, & Harley, 2005) and may be responsible of some forms of potentiation described in the DG after LC activation (Kitchigina, Vankov, Harley, & Sara, 1997; Klukowski & Harley, 1994; Walling & Harley, 2004) or noradrenalin administration (Dahl & Sarvey, 1989; Izumi & Zorumski, 1999). This disinhibitory action of the noradrenergic projection may be related to the very rapid depotentiation that we have observed after propranolol injection in the DG in animals with a combined LTP/BLA-R protocol. Such an effect can also explain the depression in the evoked potentials at the DG after local propranolol injection.

Such a neural system would also make sense in physiological conditions. Sensory information is distributed from

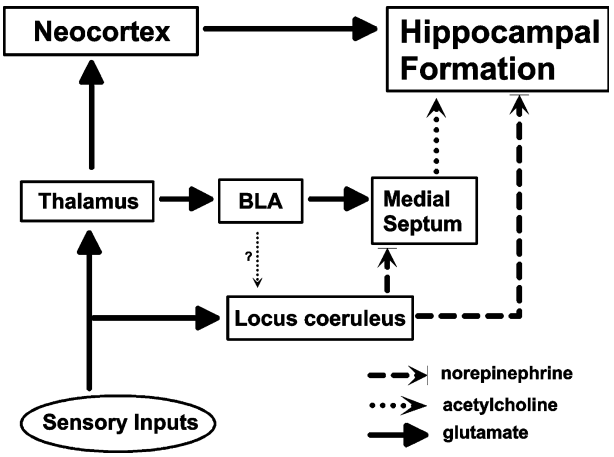

Fig. 7. Functional schemata of the putative neural system and neurotransmitters involved in the reinforcing effect of stimulating the BLA on LTP at the dentate gyrus. Explanation in the text.

the thalamus to the cortex and the amygdala. From higher order cortical fields the signal reaches the entorhinal cortex from where it is transferred to the DG and stored temporarily by mechanisms of short-term synaptic plasticity (i.e., E-LTP). The same information is processed in parallel by the limbic system (represented by the BLA) that via the neural system previously described, reinforces the storing mechanisms (modulation) by converting the E-LTP into a L-LTP. Note that parts of this system can be activated independently, under different circumstances. For instance, a strong sensory signal could activate the LC, as a part of an attentional system (Berridge & Waterhouse, 2003), which could have a similar effect on the plastic changes at synapses relevant for memory storage. We are aware that the circuit components presented here are likely to be incomplete. It has been described that the hippocampal theta-driving system includes other nuclei like the nucleus raphe in the brain stem, and the supramammillary nucleus of the hypothalamus (Borhegyi & Freund, 1998; Vertes, 2005; Vertes & Kocsis, 1997). Our own unpublished work suggests that the supramammillary nucleus is part of the reinforcing system in the DG, i.e. stimulation of this structure is also able to reinforce early- into late-LTP in the DG if it was stimulated 15 min after early-LTP-induction.

Finally, the reinforcing effects of affective factors and structures on neural plasticity processes may not be restricted to LTP in the dentate gyrus. There are reports that demonstrate an essential role for the cholinergic projection from the basal forebrain in cortical plasticity in sensory (Kilgard & Merzenich, 1998; Mercado, Bao, Orduna, Gluck, & Merzenich, 2001) and motor areas (Conner, Culberson, Packowski, Chiba, & Tuszynsky, 2003). We have recently demonstrated that stimulation of the BLA accelerates the plastic changes at the motor cortex, involved in the acquisition of a motor skill (Bergado et al., 2006). These might imply that the neural system proposed to explain the reinforcement of LTP at the dentate gyrus, could act on other forms of plasticity and

widespread cortical areas. Further studies are required to confirm the putative system advanced here; as well as to complete the list of structures and transmitters involved in the affective modulation of neural plasticity.

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CONSIDERACIONES FINALES

El esquema que se propone al final del artículo sirve también para interpretar las deficiencias detectadas en los estudios con animales viejos. El deterioro funcional del sistema cerebral colinérgico y noradrenérgico han sido ampliamente documentados en la literatura científica. Estos sistemas se encuentran en el centro mismo de la interacción entre neuroplasticidad y afectividad y su deterioro con la edad, conduciría a un mayor deterioro de la memoria al dificultar el reforzamiento de los procesos neuroplásticos que le sirven de base.

El principio de universalidad que estamos proponiendo tiene un cierto carácter axiomático, en tanto que resulta imposible diseñar un experimento que lo niegue o confirme más allá de toda duda. Para confirmar que el reforzamiento de procesos neuroplásticos inducido por la estimulación de la amígdala, tiene verdaderamente un carácter universal habría que realizar experimentos veritativos explorando todos los tipos de procesos neuroplásticos, en todas las estructuras cerebrales, en todas las especies animales. Tal tarea está más allá de la capacidad de cualquier grupo y dista mucho del nivel de evidencia aquí presentado. Pero el hecho de que en los dos estudios realizados para comprobar la universalidad hayan ofrecido evidencias positivas es importante porque bastaría con solo una evidencia negativa para negarlo. En tanto la evidencia acumulada afiance la certeza, o que aparezcan resultados que la limiten, proponemos la tesis de que *los factores afectivos modulan los procesos de neuroplasticidad en todas sus formas de expresión, en todas las regiones del encéfalo y en todas las especies con un cerebro desarrollado. Los mecanismos se ajustan al mecanismo común de control celular mediante el cuál se regula el metabolismo macromolecular neuronal y los procesos de*

crecimiento o cambios funcionales que de ellos dependen. En los mamíferos los sistemas colinérgico de origen septal y noradrenérgico de origen en el locus coeruleus, activados por la amígdala forman parte fundamental del mecanismo fisiológico de modulación afectiva de procesos de neuroplasticidad.

De este principio se derivan algunos corolarios que pueden tener utilidad práctica como guía de futuras investigaciones:

- Los agentes que estimulan, modulan o refuerzan un proceso neuroplástico (p. ej. la memoria) tienen, con gran probabilidad, el mismo efecto sobre otras formas de neuroplasticidad (p.ej. el crecimiento neurítico).
- Los agentes que estimulan, modulan o refuerzan un proceso neuroplástico en una etapa del desarrollo tienen, con gran probabilidad, el mismo efecto en otras etapas del desarrollo.
- Los agentes que estimulan, modulan o refuerzan un proceso neuroplástico en una especie animal tienen, con gran probabilidad, el mismo efecto en otras especies.

Reiteramos que estos corolarios son solo una guía para la formulación de hipótesis experimentalmente verificables. Evidentemente, cualquier intento de aplicación concreta de algún presunto agente modulador que pueda inferirse basado en estos corolarios pasa, sin excepciones, por la inaplazable comprobación experimental previa.

En la actualidad las propiedades neuroplásticas del Sistema Nervioso constituyen la mejor herramienta de que dispone la Neurología Restaurativa para lograr detener o enlentecer, recuperar o compensar funciones nerviosas afectadas por enfermedad o trauma. Comprender sus relaciones con los factores afectivos y los mecanismos de que depende para utilizarlos sabiamente en el logro de mejores resultados y tratamientos es

una aspiración. Haber contribuido en algo a ese noble empeño es nuestra mejor y más valiosa recompensa.

CONCLUSIONES

1. El reforzamiento conductual actúa como evento modulador de la plasticidad sináptica mediante la activación de los mecanismos celulares de biosíntesis que aportan las proteínas requeridas por el proceso neuroplástico para su consolidación. Esta acción moduladora depende de la actividad de sistemas de neurotransmisión noradrenérgica y colinérgica.
2. La amígdala es parte del sistema neural implicado en el reforzamiento de procesos neuroplásticos por factores afectivos.
3. La proyección septo-hipocampal y la vía fimbria-fornix son mediadores necesarios en el reforzamiento de procesos neuroplásticos por factores afectivos.
4. Es posible mimetizar el efecto de reforzamiento afectivo mediante la administración sistémica de agonistas de la neurotransmisión noradrenérgica en dosis adecuadas.
5. El envejecimiento afecta la capacidad de reforzamiento de procesos neuroplásticos por factores afectivos que pudiera estar causada por la reducción en la capacidad de movilizar los neurotransmisores requeridos.
6. Los mecanismos de reforzamiento de procesos de neuroplasticidad por factores afectivos no son exclusivos de una forma particular de expresión de la neuroplasticidad, ni de una región específica del cerebro, ni del sujeto sano y parecen representar un mecanismo y universal mediante el cuál el valor afectivo de una experiencia modula el desarrollo y persistencia de fenómenos neuroplásticos inducidos por esa misma experiencia.

RECOMENDACIONES

1. El sistema neural propuesto para explicar la acción moduladora de los factores afectivos sobre los procesos de neuroplasticidad se basa, fundamentalmente en las evidencias obtenidas por nuestro grupo de trabajo en los experiemnetos que se recogen en este documento. Sin embargo, evidencias de otros autores sugieren que otras estructuras pudieran participar también en estos mecanismos. Entre ellas se destacan dos: el núcleo *accumbens septi* y el núcleo del rafe medial que están siendo estudiadas en experimentos en curso o concluidos recientemente.
2. El carácter universal que atribuimos a los mecanismos propuestos justifican la búsqueda de equivalentes funcionales en seres humanos y la posible extensión de estos resultados al tratamiento neurorestaurativo en uso en nuestra institución. Un trabajo, concluido recientemente, muestra que es posible lograr activación de la amígdala mediante técnicas de estimulación acupuntural. Eso posibilita el diseño de experimentos en humanos sanos para obtener evidencias del efecto modulador de la estimulación de la amígdala sobre procesos de neuroplasticidad y, de comprobarse, el diseño de ensayos terapéuticos para verificar su efecto en pacientes con afecciones neurológicas crónicas.

DIVULGACIÓN DE LOS RESULTADOS RECOGIDOS EN LA TESIS

Eventos científicos

Los resultados recogidos en el presente documento de tesis han sido presentados en **eventos científicos** nacionales e internacionales celebrados en Cuba y en el extranjero. Entre ellos mencionamos los siguientes:

Sexto Congreso Latinoamericano Neuropsicología. Varadero, Octubre 17-20 de 1999.

-Plasticidad sináptica y envejecimiento.

-Sistema Límbico y memoria. Nuevas evidencias a la luz de la plasticidad sináptica.

USA-CUBA Neuromeeting. La Habana, Octubre 20-23 de 1999.

-Amygdala-hippocampal interaction in synaptic plasticity.

Xth Magdeburg International Neurobiological Symposium: Mechanisms of Learning and Memory. Magdeburgo 16-19 Septiembre 2000

- Aging impairs amygdala-hippocampus interactions in LTP.

2nd Experimental Neuroscience Meeting. La Habana Marzo 2001

-Heterosynaptic interactions on late phases of long-term potentiation in the hippocampus: Mechanisms and impairments by aging. Presentación oral

Joint Meeting 18th Biennial Meeting of the International Society for Neurochemistry and 32nd Annual meeting of the American Society for Neurochemistry, Buenos Aires 26-31 de Agosto, 2001

-Aging impairs amygdala-hippocampus interactions involved in hippocampal LTP. Poster

Annual Meeting of the Society for Neuroscience (USA). San Diego. Noviembre 2001

- Heterosynaptic modulation of late phases of long-term potentiation in the hippocampus by limbic structures and their impairment by aging. Bergado, J.; Almaguer, W.; Frey, S*.; Frey, J.U*. Poster

Curso de Conferencias de la IBRO, (Organizado por la Visiting Lecture Team de IBRO), La Habana, Junio 20-28 de 2001

- Synaptic plasticity, aging and memory.

6to Congreso Mundial de la IBRO, Praga, Julio 10-15, 2003

- Effects of aging on limbic plasticity and affective modulation of LTP. Almaguer, W., Jas, J., Ravelo, A., Alvaré, J.A., Bergado, J., Frey, S., Frey, J.U.

I Simposium Internacional de Rehabilitación Neurológica: Neurorehabilitación 2003

- Influencia de los factores emocionales y motivacionales sobre los procesos neuroplásticos. Bergado, J.A. Conferencia

Cuba US Workshop on Biological Psychiatry. La Habana 15-20 de enero 2004

- Motivation cognition and synaptic plasticity. Conferencia

IV Foro Iberoamericano sobre Demencias y Enfermedad de Alzheimer, La Habana 25 de marzo 2004

- Plasticidad sináptica y envejecimiento. Nuevas bases para entender la interacción entre afectividad y memoria

XXXIX Congreso Nacional de la Asociación Colombiana de Ciencias Biológicas. Ibagué 11-15 octubre de 2004

- Memoria, motivación y plasticidad sináptica. Bergado, J.A; Almaguer-Melían, W.; Frey, S.; Frey, J.U. (Conferencia)

XI Magdeburg International Neurobiological Symposium, Magdeburgo 28 Mayo al 1ro de Junio, 2005

- Modulation of cellular memory consolidation in young and aged animals. Conferencia Invitada

9th International Conference on Cognitive Neurosciences (ICON). La Habana, Sept. 6-9, 2005

- Affective modulation of synaptic plasticity. Implications for cognitive functions. Conferencia.

Publicaciones

Aunque aparecen en el cuerpo de la tesis, relaciono a continuación y en el orden de su aparición los **artículos científicos** que recogen los resultados que aquí se presentan:

- Jas, J., Almaguer, W., Bergado, J.: **Lesioning the fimbria-fornix impairs basolateral amygdala induced reinforcement of LTP in the dentate gyrus** Brain Res., 861: 186-189, 2000. (Factor de impacto FI: 2.296, Citado:

- Bergado, J., Almaguer, W.: **Mecanismos celulares de neuroplasticidad** Rev. Neurol. 31 (11) 1074-1095, 2000. (FI: 0.391, citado 9 veces)

- Frey, S., Bergado Rosado, J.; Seidenbecher, T., Pape, H-C, and Frey, J.U.: **Reinforcement of early LTP in dentate gyrus by specific stimulation of the basolateral amygdala: Heterosynaptic induction mechanisms of late LTP** J. Neurosci., 21, 3697-3703, 2001.(FI: 7.706, citado 83 veces)

- Bergado, J.A., Almaguer, W., Ravelo, J., Rosillo, J.C., Frey, J.U. **Behavioral reinforcement of long-term potentiation is impaired in aged rats with cognitive deficiencies** Neuroscience, 108(1):1-5,2001 (FI: 3.41, citado 6 veces)

- Almaguer, W., Estupiñán, B., Frey, J.U, Bergado, J.A. **Aging impairs amygdala-hippocampus interactions involved in hippocampal LTP** Neurobiol. Aging, 23(2):319-324, 2002. (FI: 5.312, citado 11 veces)

- Bergado, J.A.; Almaguer-Melián, W.: **Aging and synaptic plasticity: a review** Neural Plast. 9 (4): 217-232, 2002. (FI: , citado 4 veces)

- Frey, S.; Bergado,J.A.; Frey, J.U. **Modulation of late phases of long-term potentiation in rat dentate gyrus by stimulation of the medial septum** Neuroscience, 118: 1055-1062, 2003 (FI: 3.41, citado 10 veces)

- Almaguer-Melian, W.; Martínez-Martí, L.; Frey, J.U.; Bergado, J.A. **The amygdala is part of the behavioral reinforcement system of long-term potentiation in rat hippocampus.** Neuroscience, 119 (2): 319-322, 2003 (FI: 3.41, citado 14 veces)

- Bergado, J.A; Almaguer-Melian, W.; Kostenko, S.; Frey, S.; Frey, J.U: **Behavioral reinforcement of long-term potentiation in rat dentate gyrus in vivo is protein synthesis-dependent [rapid communication]**. Neurosci. Lett., 351 (1) 56-58, 2003 (FI: 1.891, citado 8 veces)

- William Almaguer-Melian, Yeneissy Rojas-Reyes, Armando Alvare, Juan C. Rosillo, Julietta U. Frey and Jorge A. Bergado: **Long-term potentiation in the dentate gyrus in freely moving rats is reinforced by intraventricular application of norepinephrine, but not oxotremorine**. Neurobiol. Learn. Mem., 83: 72-78, 2005. (FI: 4.091, citado 9 veces)

- William Almaguer, Vladimir Capdevila, Magaly Ramírez, Araceli Vallejo, Juan C. Rosillo and Jorge A. Bergado: **Post-training stimulation of the basolateral amygdala improves spatial learning in rats with lesion of the fimbria-fornix**. Restor. Neurol. Neurosci., 23: 43-50, 2005. (FI: 1.825, citado 1 vez)

- W. Almaguer-Melian, R. Cruz-Aguado, C. de la Riva, K.M. Kendrick, J.U. Frey, J. Bergado: **Effect of LTP-reinforcing paradigms on neurotransmitter release in the dentate gyrus of young and aged rats**. Biochem. Biophys. Res. Comm., 327: 877-883, 2005. (FI: 3.0, citado 5 veces)

- Almaguer-Melian, W.; Rosillo, J.C.; Frey, J.U.; Bergado, J.A.: **Subcortical deafferentation impairs behavioral reinforcement of LTP in the dentate gyrus of freely moving rats.** Neuroscience, 2006, 138: 1083-1088 (FI: 3.41, citado 1 vez)

- Jorge A. Bergado, Yeneissy Rojas, Vladimir Capdevila, Odalys Gonzalez, William Almaguer-Melian: **The stimulation of the basolateral amygdala improves the acquisition of a motor skill.** Restor. Neurol. Neurosci. 2006, 24(2):115-121 (FI: 1.825)

- Jorge A. Bergado, Sabine Frey, Jeffrey López, William Almaguer and Julietta U. Frey**: Cholinergic afferents to the locus coeruleus and noradrenergic afferents to the medial septum mediate LTP-reinforcement in the dentate gyrus by stimulation of the amygdala** 2007 Neurobiol. Learn. Mem., 88: 331-341. (FI: 4.091)

Premios

Los trabajos que recoge la presente tesis han recibido las distinciones que a continuación se relacionan:

· Premio Anual de Salud 2000, MINSAP. Premio a nivel Nacional. Autor Principal del Trabajo: **Efecto de la desaferentación subcortical hipocampal sobre el reforzamiento de la LTP en el giro dentado por estimulación de la amígdala basolateral**

· Premio Anual de Salud 2003, MINSAP. Premio a nivel Nacional. Autor Principal del Trabajo: **El envejecimiento afecta la interacción amígdala-hipocampo implicada en la LTP hipocampal**.

· Premio Anual de Salud 2004, MINSAP. Premio al Nivel Nacional. Autor Principal del Trabajo: **Estudio sobre la participacion de la amigdala en los procesos de reforzamiento conductual de la potenciación a largo plazo**

· Premio Anual de Salud 2005. La Tesis de Doctorado, de la cuál fui TUTOR: **Mecanismos de consolidación motivacional de la plasticidad sináptica duradera. Efectos del envejecimiento cerebral** de William Almaguer Melian recibió una de las tres MENCIONES que otorga el jurado del como Mejor Tesis Doctoral.

· Premio Anual de la Academia de Ciencias de Cuba 2005 por el trabajo: **Estudio de los mecanismos de reforzamiento de procesos neuroplásticos por factores afectivos**. Autor Principal

· Premio Anual de Salud 2006, MINSAP. Mención al Nivel Central al trabajo **Efecto de la administración de norepinefrina y oxotremorina sobre el mantenimiento de la potanciación sináptica de larga duración**. Coautor

· Premio Anual de Salud 2007, MINSAP: Premio al Nivel Central al trabajo **Universalidad de los efectos de la estimulación de la amígdala basolateral sobre los procesos de neuroplasticidad.** Autor