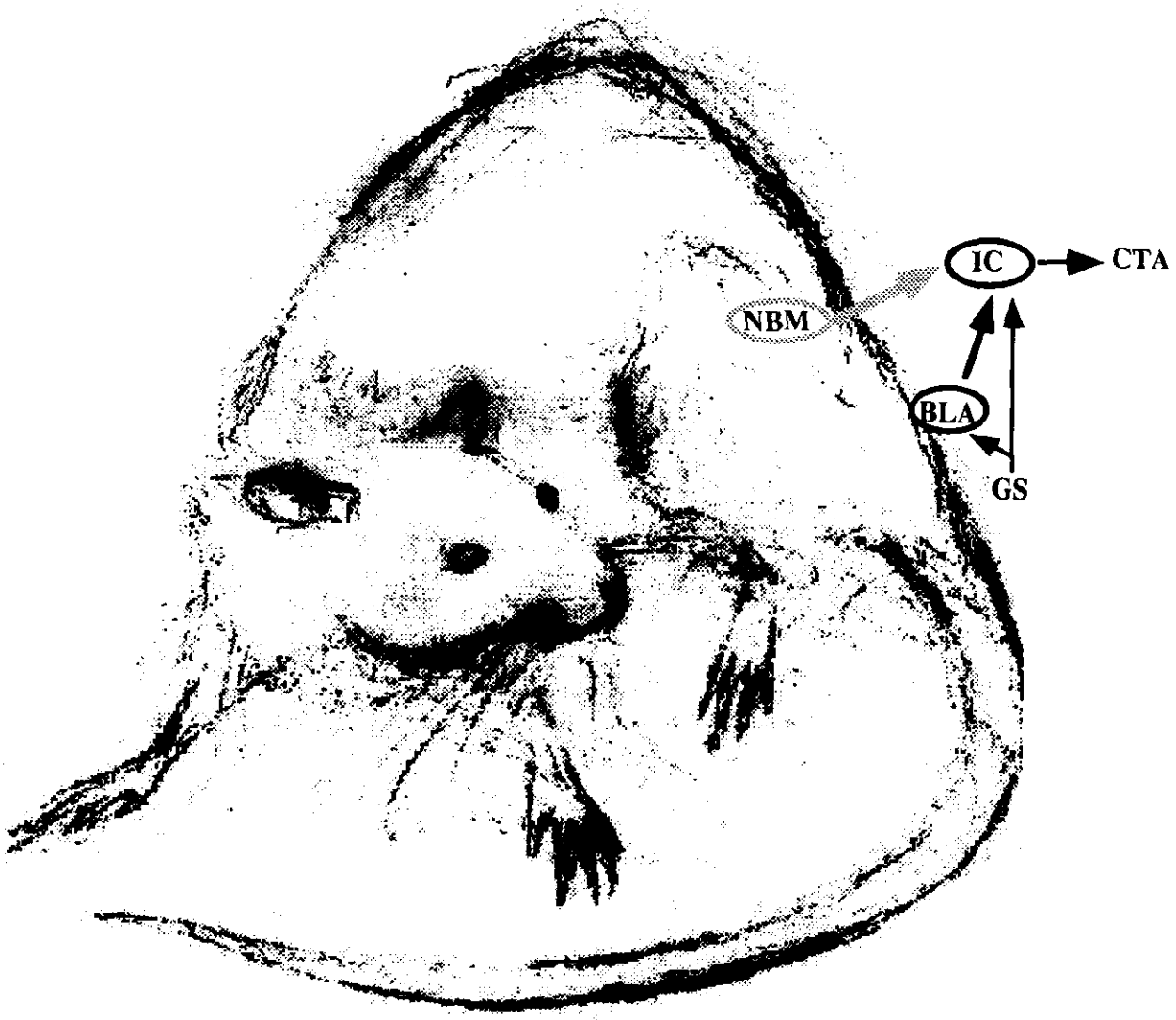
EL SISTEMA BASAL DE PROYECCION COLINERGICA:

MODULACION DE FUNCIONES ASOCIATIVAS



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EL SISTEMA BASAL DE PROYECCION COLINERGICA: MODULACION DE FUNCIONES ASOCIATIVAS

TESIS

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Resumen

La región basal del cerebro anterior provee de modo exclusivo, una amplia y difusa inervación colinérgica hacia la corteza, la amígdala y el hipocampo. Numerosos estudios han demostrado una participación de este sistema de proyección en procesos de aprendizaje y memoria. Las fuertes deficiencias de aprendizaje observadas tras lesiones excitotóxicas practicadas en la región basal se han atribuido clásicamente a la resultante deplesión de acetil colina en las correspondientes áreas de proyección. Esta clase de evidencias han dado lugar a lo que se conoce como *la hipótesis colinérgica de la regulación cortical de la memoria*. La aparición relativamente reciente de una inmunotoxina específica dirigida contra el receptor de NGF de baja afinidad p75, que destruye selectivamente a las neuronas colinérgicas de la proyección basalo cortical y septohipocampal, ha dado lugar al cuestionemiento de la hipótesis colinérgica en su formulación clásica: a pesar de la drástica deaferentación observada como resultado de inyecciones intraparenquimales de esta inmunotoxina (192IgG-saporina), el sistema de lesión no reproduce las deficiencias conductuales normalmente observadas tras la aplicación de lesiones generalizadas en la misma región. En el presente documento se lleva a cabo un detallado análisis de la actividad colinérgica de la corteza tras inyecciones intrabasales de 192IgG-saporina por un lado y la excitotoxina N-metil- D-asptartato (NMDA) por el otro. Mediante un estudio de microdiálisis in vivo se muestra que la inmunotoxina resulta en una completa deaferentación colinérgica cortical mientras que las lesiones inducidas con NMDA sólo dan lugar a una reducción marginal en la actividad cortical de este neurotransmisor. A nivel conductual, sin embargo, solo las lesiones excitotóxicas (NMDA) resultan en una fuerte deficiencia en el aprendizaje de un modelo de condicionamiento mediado por la corteza, el condicionamiento aversivo a los sabores (CAS), mientras que, a pesar de la masiva reducción colinérgica resultante, las lesiones inmunotóxicas carecen de efecto sobre este condicionamiento. Estos datos demuestran, que el efecto conductual observado tras la destrucción inespecífica de la región basal, no puede ser el resultado exclusivo de una disfunción colinérgica cortical, revelándose de este modo, la existencia de otro u otros sistema de modulación originado en la propia región basal.

En un segundo estudio se explora la interacción entre la región basal y la amígdala por un lado y la proyección basalo cortical por el otro. Nuevamente se hace uso de una estrategia comparativa entre lesiones específicas inmunotóxicas y lesiones inespecíficas excitotóxicas en la region basal. En un análisis bioquímico, se observó que la lesión inmunotóxica dió lugar a una fuerte deaferentación colinérgica de la corteza dejando intactas las proyecciones hacia la amígdala mientras que la lesión de NMDA resultó en una fuerte deaferentación de la amígdala y una pobre deaferentación cortical. Con base en los efectos conductuales descritos arriba, se podría concluir que la modulación ejercida por la región basal, tiene lugar a través de la proyección basalo amigdalina y no mediante la proyección basalocortical. Sin embargo, las lesiones excitotóxicas de la amígdala carecen de efectos adversos sobre el CAS. Observamos no obstante, que la combinación de lesiones de la amígdala basolateral, con lesiones inmunotóxicas en la región basal resultan en una fuerte disfunción en el desempeño de la tarea de condicionamiento, siendo que ninguna de éstas, por sí sola tienen efecto sobre la tarea. El efecto de esta doble lesión resulta comparable con la simple lesión excitotóxica de la región basal, sugiriendo que las deficiencias de aprendizaje asociadas con lesiones inespecíficas de los núcleos basales son el resultado de la disfunción simultanea de la interacción basalo cortical y basalo amigdalina. Se propone un modelo según el cual la modulación de procesos asociativos originada en la región basal del cerebro anterior se lleva a cabo de modo redundante mediante dos vías alternativas: la vía ascendente basalo cortical y la interacción basalo amigdalina.

Abstract

The cholinergic basal forebrain provides afferent cholinergic input to the cortical mantle, amygdala and hippocampus. Mnemonic deficits resulting from excitotoxic lesion of the basal forebrain have been classically attributed to the resulting depletion of cortical acetylcholine activity. In spite of the strong cholinergic depletion following injections into the basal forebrain of a newly introduced immunotoxin (192IgG-saporin), no detectable deficit can be found in the acquisition of several well known learning tasks, including conditioned taste aversion. Conversely, N-methyl-D-aspartate (NMDA)-induced lesions of the basal forebrain strongly impair taste aversion learning.

In a first study, we have performed a detailed analysis of the cholinergic status of the insular cortex (IC) following local injections of either 192IgG-saporin (192IgG-sap) or N-methyl-D-aspartate (NMDA) directly into the nucleus basalis magnocellularis (NBM). By means of *in vivo* microdialysis we show that the immunotoxin lesion results in an almost complete lack of extracellular acetylcholine release, whereas NMDA-induced lesions result in a marginal reduction in cortical cholinergic activity. Choline-acetyltransferase activity in the IC further confirmed this differential pattern of cortical deafferentation. Surprisingly, however, only NMDA-induced lesions showed a strong disruptive effect upon taste aversion learning whereas no detectable deficits could be found following 192IgG-sap lesions. By combining intrabasal injections of 192IgG-sap with acute pre-training infusions of the cholinergic antagonist scopolamine into the IC, a strong disruption of taste aversion was attained. These results imply that residual cholinergic activity, following 192IgGsaporin lesions, might be still critical for normal cortical mediation of memory processing. They also support the role of basal forebrain in mediating learning and memory processes, and demonstrate that mnemonic deficits resulting from excitotoxic lesions of the basal forebrain are not the sole result of cortical acetylcholine activity hypofunction.

In a second study we show that 192IgG-saporin produces an efficient and selective cholinergic deafferentation of the rat neocortex but not the amygdala. Furthermore, a stronger relationship between severity of memory impairment following NMDA lesions and basoamygdaloid cholinergic deafferentation was found. Subsecuently, we show that combining NMDA-induced lesions into the basolateral amygdala with 192IgG-saporin injections into the basal forebrain result in a strong disruption of taste aversion learning, whereas none of these treatments were by themselves capable of producing any detectable impairment in this learning task. The double lesion effect was only paralleled by simple NMDA lesions into the basal forebrain, suggesting that the learning deficits associated to excitotoxic lesions of the basal forebrain is the result of the simultaneous destruction of the corticopetal and basoamygdaloid interaction. A model is proposed according to which the modulation of learning processes exerted by the basal forebrain can be redundantly carried out by both the basal cortical or basoamygdaloid pathway.

Indice

Introducción

Modulación basal de la función cortical de memoria.

En la región adyacente a la zona ventral del globus pálidus existe un grupo de grandes células cuya fuerte actividad de colin acetiltransferasa (ChAT) y acetil colinesterasa (ChE) les identifica como neuronas colinérgicas (Abdulla, Abu-Bakra, Calamicini, Stephenson y Sinden, 1995). A esta zona se le conoce, en su conjunto, como la región basal del cerebro anterior, el cual incluye a la región septal, los brazos vertical y horizontal de la banda de broca, sustancia innominata y nucleus basalis magnocelularis (NBM), considerado, este último, como la estructura homóloga al núcleo basal de Meynert en humanos (Everitt et al., 1987).

Se ha establecido con bastante claridad que la totalidad de la inervación colinérgica hacia la neocorteza y otras regiones del cerebro, procede de los somas colinérgicos de la región basal del cerebro anterior (Hebb, Krnjevi y Silver, 1963; Everitt, Sirkia, Roberts, Jones y Robbins, 1988; Bigl, Woolf y Butcher, 1982; Wainer y Mesulam, 1990; Butcher, Oh, Woolf, Edwards y Roghani, 1992; Bronzetti et al., 1993) (figura I). Este sistema de proyección colinérgica ha atraído fuertemente la atención en la última década, debido a que alteraciones anatómicas dc estas vías durante el envejecimiento se correlacionan fuertemente con alteraciones en funciones asociativas en un gran conjunto de modelos conductuales de aprendizaje y memoria (Perry et al., 1978; Collerton, 1986; Abdulla et al., 1995), implicando la posible participación del sistema basal colinérgico en la regulación de dichas funciones (para una revisión ver Sinden, 1995; Ammasauri, 1993; Hasselmo, 1995; Ikegami, 1994).

Sistema colinérgico y funciones asociativas

La correlación mencionada entre la disfunción colinérgica y las alteraciones anatómicas asociadas al envejecimiento dieron lugar a lo que se conoce como la "hipótesis colinérgica de las disfunciones geriátricas de memoria" dos de cuyos conceptos más importantes

podrían formularse del siguiente modo (Bartus, Dean, Beer y Lippa, 1982):

a) El sistema colinérgico de la región basal provee una función esencial relacionada con procesos cognitivos en particular aprendizaje y memoria.

b) Las deficiencias en aprendizaje y memoria durante el envejecimiento son atribuibles, al menos en parte, a un decaimiento en la integridad funcional del sistema colinérgico de la región basal.

En su formulación inicial esta hipótesis fue propuesta en el contexto del envejecimiento normal; sin embargo, con base en evidencias clínicas y neuroanatómicas, se ha extendido para incluir demencias seniles de tipo Alzheimer, un desorden caracterizado por un deterioro progresivo de las funciones cognitivas, las cuales se correlacionan con la pérdida de marcadores colinérgicos en la corteza y el hipocampo, así como la pérdida y/o atrofia de las propias neuronas colinérgicas de la región basal (Dunnet, Whishaw, Jones y Bunch, 1987; Dunnet y Fibiger, 1993).

Durante las últimas décadas, y de modo independiente, se ha acumulado una gran cantidad de evidencia, procedente tanto de estudios en humanos como de modelos animales, que sugiere, fuera de condiciones de disfunción patológica, una participación más general de los sistemas colinérgicos centrales en los procesos superiores de aprendizaje y memoria (Berman, Crosland Jenden y Altman, 1988; Connor, Langlais y Thal, 1991; Blockland ,1996; Wenk, Stoeher, Mobley, Gurney y Morris, 1996). Una de las primeras y más extensas líneas de evidencia provino de estudios basados en manipulaciones farmacológicas del sistema colinérgico en los cuales se han mostrado alteraciones en las funciones normales de memoria (Nabeshima, 1993). Compuestos anticolinérgicos como la escopolamina y la atropina, administrados sistémicamente, interfieren con el aprendizaje y la memoria de una gran variedad de pruebas conductuales. Por otro lado, agonistas colinérgicos como la oxotremorina o bien inhibidores reversibles de la acetil colinesterasa (enzima de degradación de acetilcolina) tales como la fisostigmina, se ha mostrado que facilitan dichas funciones y revierten deficiencias de memoria inducidas experimentalmente (Nabeshima, 1993). Hasta aquí esta evidencia sólo sugiere que, en efecto, algún o algunos sistemas colinérgicos en el cerebro juegan un papel importante en algunas formas de aprendizaje y memoria.

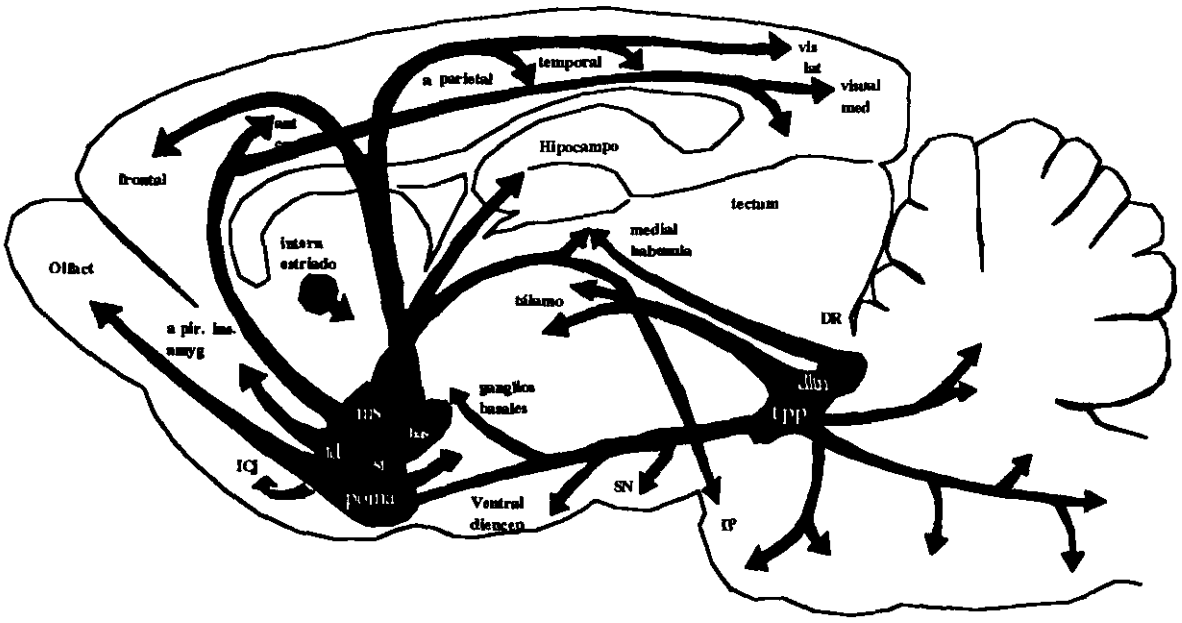

Fig I. Representación esquemática del plano horizontal de los principales sistemas colinérgicos en el cerebro de mamíferos (modificado de Woolf, 1991). Como se observa en la figura, las neuronas colinérgicas centrales muestran dos esquemas organizacionales fundamentales: (a) circuito de células locales, ejemplificadas por las interneuronas del núcleo caudado-putamen, nucleus acumbens, tubérculo olfatorio y el complejo de islotes de Calleja ***(ICj)*** *y (b) neuronas de proyección (i.e. aquellas que conectan a dos o más regiones diferentes). De las proyecciones de neuronas colinérgicas que se interconectan con estructuras centrales, se han descrito dos complejos principales: (a) el complejo colinérgico del cerebro anterior compuesto por neuronas positivas a ChAT en el núcleo septal medial* ***(ms)****, el núcleo de la banda diagonal* ***(td)****, substancia innominata* ***(si)****, campo preóptico magnocelular* ***(poma)****, el núcleo basalis* ***(bas)*** ***así como*** *proyecciones a todo el telencéfalo no estriado y (b) el complejo colinérgico pontomesencefalotegmental compuesto por células inmunoreactivas a ChAT en el núcleo tegmental pendunculopontino* ***(tpp)*** *y laterodorsal* ***(dltn)****, proyectando ascendentemente al tálamo y a otros sitios diencefálicos y descendentemente a la formación reticular pontina y medular y núcleo profundo cerebelar y vestibular. No se muestran en este esquema las neuronas simpáticas, parasimpáticas y otras eferentes colinérgicas de los nervios craniales 3-12 y las neuronas a y g-motoras autónomas de la médula espinal. Otras abreviaturas: amígdala* ***(amyg)****, corteza anterior cingulada* ***(ant cg)****, núcleo del nervio craneal dorsal* ***(CrN)****, diencéfalo* ***(diencep)****, núcleo rafé dorsal* ***(DR)*** *corteza entorinal* ***(ento)*** *corteza frontal* ***(frontal)****, corteza insular* ***(ins)****, locus coeruleus* ***(LC)****, núcleo lateral reticular* ***(LR)****, bulbo olfatorio* ***(olfact)****, corteza piriforme* ***(pir)****, núcleo pontino* ***(PN)****, corteza perirhinal* ***(pr)****, corteza parietal* ***(par)****, substancia nigra* ***(SN)****, núcleo espinal del nervio craneal 5* ***(SpV)****, corteza temporal* ***(temporal)****, corteza visual lateral* ***(vis lat)****, corteza visual media* ***(visual med)****.*

Sin embargo, las deficiencias observadas tras el bloqueo sistémico, y generalizado de la transmisión colinérgica central no permite un acuerdo sobre los mecanismos anátomo funcionales subyacentes. En realidad cualquier debate basado en este tipo de datos carece de fundamento debido a que parten de un virtual "vacío anatómico". Siendo que prácticamente todo

el eje neural se halla inervado por neuronas colinérgicas y que los receptores a este neurotransmisor se distribuyen a todo lo largo y ancho del sistema nervioso (Woolf, 1991, ver figura I), queda claro que los mecanismos fisiológicos mediados por acetilcolina se hallan involucrados en un gran número de funciones nerviosas. De este modo, si bien no hay duda alguna de que los agentes anticolinérgicos afectan un amplio espectro de conductas aprendidas, las características de la anatomía colinérgica central implicarían que cualquier intento de dar una explicación neuroanatómica sobre la base de tratamientos farmacológicos sistémicos carecería de justificación.

La convergencia entre los estudios farmacológicos mencionados y la hipótesis colinérgica de las disfunciones geriátricas se verificó gracias a estudios subsecuentes basados en lesiones excitotóxicas en los núcleos colinérgicos de la región basal (Green, Halpern y Niel, 1970; Everitt et al., 1987; Arendt et al., 1989), manipulaciones farmacológicas localizadas, así como experimentos de transplantes de tejido nervioso fetal procedentes del cerebro basal (Sinden, Hodges y Gray, 1995). Todo ello, contribuyó a aumentar la evidencia en favor de una participación del sistema basal de proyección en las funciones de aprendizaje y memoria en sujetos normales, con lo cual se consiguió asignar una identidad anatómica al elusivo sistema colinérgico implícito en los enfoques farmacológicos sistémicos.

Desde un punto de vista anatomo-funcional se ha dividido tradicionalmente al sistema basal de proyección colinérgica en dos grandes ramas: El sistema de proyección septo-hipocampal y el sistema basalo-cortical (Wainer y Mesulam, 1990; Frotscher y Naumann, 1992; Mallet, Beninger, Flesher, Jhamandas y Boegman, 1995). Las lesiones excitotóxicas de la región septal (que proyecta hacia el hipocampo) o bien de la región basal, incluyendo los brazos verticales y horizontales de la banda diagonal, sustancia innominata, ansa lenticularis y nucelus basalis magnocelularis (que proyectan hacia la neocorteza) interfieren de manera independiente con las funciones de aprendizaje mediadas, respectivamente, por el hipocampo y la corteza (Sangsttock et al., 1992; Sinden et al., 1995). Tanto a un nivel anatómico grueso (la amplia inervación de numerosas regiones) como a niveles estructurales finos (múltiples contactos sinápticos involucrando varios tipos celulares), el sistema basal de proyección colinérgica parecería estar organizado de manera difusa.

Evidencias en favor de un modo difuso de acción incluyen registros electrofisiológicos poblacionales en los que se ha observado que la región basal controla la generación de ritmos de actividad cortical e hipocampal asociados con niveles de alertamiento conductual (ver Sinden et al., 1995). Dado que este tipo de ritmos pueden registrarse sincrónicamente en regiones extensas de la corteza o el hipocampo, se ha sugerido que el

sistema de proyección colinérgica podría jugar un papel de tipo modulatorio sobre las funciones corticales e hipocampales respectivamete, más que hallarse involucrado en la transmisión discreta de información neural (Hasselmo y Barkai, 1995).

La convergencia entre los estudios de correlación de disfunción colinérgica en condiciones patológicas (envejecimiento y demencias tipo Alzheimer), los bloqueos farmacológicos tanto sistémicos como localizados, así como deficiencias de aprendizaje tras lesiones experimentales en la región basal, llevaron en conjunto a postular que el sistema basal estaba involucrado en la modulación de funciones asociativas mediadas respectivamente por el hipocampo y la corteza a través de las correspondientes proyecciones colinérgicas ascendentes (para una revision ver Everitt, 1997). En el resto del presente documento nos referiremos a esta presunción como a "la hipótesis colinérgica de la modulación de la memoria", o simplemente "hipótesis colinérgica".

Crítica a la hipótesis colinérgica.

La atribución de las disfunciones asociativas, en condiciones patológicas, como es envejecimiento y demencias tipo Alzheimer , a la degeneración de las neuronas colinérgicas magnocelulares de la región basal, llevó a realizar lesiones en animales en esta región como modelos experimentales de estas forma de demencia (Olton, y Wenck, 1987; para una revision ver Sinden, 1995). Este tipo de estudios experimentales tiene como base el uso de inyecciones de excitotoxinas en la región basal y el subsecuente estudio de sus efectos conductuales. De este modo, las inyecciones locales de ácido iboténico, N-metil-d-aspartato (NMDA) o ácido quiscuálico tanto en la región septal como en las inmediaciones de las sustancia innominata y NBM dan lugar a disfunciones en una gran variedad de pruebas experimentales de aprendizaje y memoria (Sinden, 1995; Everitt et.al., 1997; Wenk, 1997). Sin embargo el estudio detallado de los mecanismos y eficiencias relativas (a nivel conductual y bioquímico) asociadas a las lesiones, pronto reveló que las diferentes excitotoxinas tienen efectos diferenciales sobre diversas subpoblaciones de neuronas en la región basal (Dunnet et.al, 1987; Everitt, Robins, Evenden, Marston, Jones y Sirkia, 1987). Debido a la heterogeneidad celular (y probablemente funcional) de esta zona, la interpretación de estos estudios en términos estrictamente colinérgicos se hace difícil.

Gracias a la aparición de excitotoxinas más potentes y selectivas para las neuronas de proyección colinérgica, como AMPA (ácido alfa-amino-3- hidroxi -5- metil- 4 -isoxasol propiónico) que dan como resultado una menor destrucción de neuronas no colinérgicas y una reducción más potente en la inervación colinérgica hacia la corteza, se ha podido mostrar que las simples lesiones de la región basal no proveen de un modelo confiable de alteración de la modulación colinérgica sobre funciones mnémicas de la

corteza y el hipocampo (Everitt y Robins, 1997). Esto es, a pesar del efecto profundo y relativamente específico en los marcadores colinérgicos neocorticales, las lesiones de AMPA en la región basal no producen efecto alguno sobre el desempeño en las pruebas clásicas de aprendizaje y memoria (Everitt y Robins, 1997).

Mediante comparaciones sistemáticas entre distintas excitotoxinas y sus efectos conductuales pronto se observó que muchos de los déficits de aprendizaje y memoria tradicionalmente atribuidos al sistema de proyección cortical se debían, no a la destrucción de neuronas colinérgicas en el NBM, sino a la destrucción de proyecciones hacia el estriado originadas en la corteza que pasan por la región ventral del globus palidus (Everit y col, 1987, Everitt y Robins, 1997). Por otra parte, un estudio llevado a cabo por el grupo de Boegman (1994), documentó una clara falta de correlación entre las disfunciónes colinérgicas en la neocorteza y los defectos de aprendizaje tras lesiones inducidas con diversas excitotoxinas como AMPA, iboténico, kainato, n-metil-d-astpartato y quiscuálico en la región basal (Mallet, Beninger, Flescher, Jhamandas y Boegman, 1995). En la misma línea, se observó una mayor correlación entre los efectos conductuales y la deaferentación colinérgica, no en la corteza, sino en los núcleos amigdalinos, particularmente el núcleo basolateral. Esto es, aquellas excitotoxinas que tenían mayor efecto sobre el desempeño en pruebas de memoria, dan lugar a una mayor destrucción de marcadores colinérgicos en la amígdala (Beninger, Kuhnemann, Ingles, Jhmandas y Boegman, 1994).

Estudios estructurales de la región basal, revelan una clara heterogeneidad en la población neuronal de esta zona. Adicionalmente, la interpretación tradicional de los efectos de lesiones en la región basal como un resultado exclusivo de la comunicación basal cortical es injusta toda vez que esta región provee de proyecciones colinérgicas y *no colinérgicas* no sólo hacia la corteza en general, sino también hacia el estriado, amígdala e incluso hacia algunos núcleos talámicos (Everitt y Robin, 1997).

Con base en las evidencias descritas en este apartado, se ha cuestionado la validez de la hipótesis colinérgica en su formulación tradicional. Sin embargo, en ausencia de un método adecuado y específico de lesión colinérgica, resultaba difícil avanzar en la resolución de esta controversia.

Destrucción especifica del sistema de proyección colinérgico basalo cortical

La relativamente reciente, introducción de un método de lesión basado en el uso de una inmunotoxina selectiva para las neuronas colinérgicas de la región basal, ha permitido profundizar más en el esclarecimiento del significado funcional de este sistema de proyección.

Describamos a continuación el fundamento de este método de lesión:

Se ha establecido que infusiones directas del anticuerpo monoclonal IgGMab 192 dirigido contra el receptor de baja afinidad de NGF (p75) en los ventrículos laterales, resultan en su internalización (Thomas, Book, Schweitzer, 1991), transporte retrógrado y acumulación específica y bilateral en neuronas de la región basal del cerebro anterior (región septal, banda diagonal, núcleo preóptico, substancia inominata y núcleo basal magnocelular). Estas neuronas muestran la morfología y localización característica del sistema colinérgico magnocelular de la región basal, lo cual es consistente con la expresión de receptores para NGF por parte de las neuronas colinérgicas de la región basal y su capacidad de respuesta al mismo factor (Schweitzer, J. B., 1989,. Schweitzer, J. B., 1987). Con base en estas observaciones se propuso la utilización de anticuerpos convenientemente seleccionados con objeto de alterar funcionalmente a las células de la región basal.

Infusiones sistémicas de un conjugado del anticuerpo 192IgG y una potente toxina de origen vegetal, la saporina, resultan en la destrucción selectiva de neuronas simpáticas posganglionares y neuronas sensoriales (sensibles al factor de crecimiento neuronal). Por otro lado, las infusiones intraventriculares del complejo dan lugar a la destrucción de células colinérgicas de la región basal (Wiley, Oeltman, Lappi, 1991).

Tratamientos intraparenquimales e intraventriculares con el complejo 192IgG-saporina dan lugar a una extensa deaferentación colinérgica *exclusivamente* de las principales áreas de proyección del sistema basal: hipocampo y corteza. Este tratamiento, por otro lado, no produce efecto alguno sobre las aferentes dopaminérgicas y noradrenérgicas a las propias áreas de proyección o en la actividad de colín-acetil transferasa en otras regiones del cerebro (Heckers, Othake, Wiley, Lappi, Geula, Mesulam, 1994).

Tratamientos intraparenquimales e intraventriculares con el complejo 192IgG-saporina resultan en la desaparición específica de células inmunoreactivas a la expresión de p75, exclusivamente presentes en la región basal del cerebro anterior (Singh, Schweitzer, 1995). Al mismo tiempo, este tratamiento conduce a un incremento en la síntesis y liberación de NGF en las áreas de proyección de la región basal (Yu, Wiley, Perez-Polo, 1996., Holley, Wiley, Lappi, Sarter, 1994).

El tratamiento con 192-IgG saporina no produce efectos sobre las neuronas colinérgicas y no colinérgicas *negativas* a la expresión del receptor de NGF presentes en cualquier otra región del cerebro incluida la propia región basal (Waitte, Wardlow, Chen, Lappi, Wiley, Thal, 1994). La inmunoreactividad de colin-acetil transferasa en interneuronas septales,

interneuronas del estriado, en la habénula y el tallo no resultan afectadas por el tratamiento.

Este método ha sido propuesto como una herramienta eficiente de lesión específica del sistema colinérgico basal. La comparación entre los efectos bioquímicos resultantes de lesiones inducidas mediante la inmunotoxina y las excitotoxinas tradicionales, mostró que la 192 IgG-saporina resulta en una dramática deaferentación de la corteza y el hipocampo siendo esto significativamente más eficiente que ninguna otra neurotoxina hasta el momento utilizada.

Siendo que es justamente este sistema de aferencia cortical el objeto de atención en el presente trabajo, consideramos que representa una metodología adecuada estudiar el papel del sistema de proyección colinérgico en la regulación de las funciones corticales de aprendizaje y memoria.

El problema.

Como ya se mencionó, lesiones generalizadas de la región basal dan lugar a impedimentos asociativos en numerosas pruebas conductuales (Sinden et al., 1995; Wenk, 1997). Sin embargo, a pesar de la deaferentación masiva de la corteza y el hipocampo obtenida como resultado de inyecciones intrabasales de 192IgG-saporina, no se logro detectar ninguna deficiencia asociativa en las mismas pruebas de aprendizaje (Berger-Sweeney et al., 1994; Torres et al., 1994; Wenk et al., 1994; Baxter et al., 1995; Waite and Thal, 1996; Wenk et al., 1996).

Estos resultados han contribuido de modo muy importante, gracias a la validación del sistema experimental utilizado, al cuestionamiento de la hipótesis colinérgica, al menos en su formulación clásica.

Una alternativa es simplemente abandonarla. Sin embargo, es necesario reconocer que el conjunto completo de evidencia disponible da lugar a una controversia en ningún modo resuelta. En efecto, si bien la crítica en contra de la interpretación colinérgica de las lesiones excitotóxicas de la región basal es perfectamente sostenible, sigue siendo cierto que la región basal de algún modo se haya involucrada en las funciones de aprendizaje y memoria a través de mecanismos aún no esclarecidos.

Por otra parte, el componente colinérgico en la modulación que ejerce la región basal aún no se puede descartar definitivamente. En efecto, si bien la deaferentación selectiva de la inervación colinérgica hacia el hipocampo carece de efectos sobre pruebas de aprendizaje espacial (mediadas por esta última estructura), la infusión directa de agentes anticolinérgicos, en la región dorsal del hipocampo, producen claros déficits de aprendizaje (ver Riekinen y Riekinen, 1991). En el mismo sentido Naor y Dudai (1996) han reportado que las infusiones directas de diferentes antagonistas colinérgicos directamente en la corteza insular impiden el aprendizaje del condicionamiento aversivo a los sabores (mediado por dicha región cortical), observación congruente

con el hecho de que las lesiones excitotóxicas del NBM también interrumpen el desempeño de esta tarea (Lopez-Garcia, Fernández-Ruiz, Escobar y Bermúdez-Rattoni, 1993). Por último, los transplantes corticales de tejido nervioso fetal procedentes de la región basal, revierten las deficiencias en el aprendizaje inducidos previamente mediante lesiones de los núcleos colinérgicos basales (Sinden, Hodges y Gray, 1995). En este sentido, la sola implantación cortical de fibroblastos genéticamente modificados para liberar acetilcolina es suficiente para revertir del mismo modo las deficiencias de aprendizaje inducidas mediante lesiones excitotóxicas de la región basal (Winkler, Suhr, Gage, Thal y Fisher, 1995)

Lo anterior en conjunto nos lleva a plantear la siguiente serie de preguntas:

¿Participa la región basal en la modulación de funciones de aprendizaje y memoria?

Si lo anterior es el caso, ¿Esta modulación se ejerce específicamente a través de la proyección colinérgica hacia la corteza?

¿Existe algún otro sistema, colinérgico o no colinérgico, de modulación asociativa originado en la región basal?

En el presente trabajo, se hace el intento de esclarecer la naturaleza de la modulación originada en el sistema basal de proyección colinérgica, sobre las funciones superiores de aprendizaje y memoria.

El Modelo

La corteza insular (CI) representa un modelo con múltiples ventajas para el estudio de funciones asociativas. Esta región en las ratas se define como un área que se extiende desde la corteza frontal lateral hacia la corteza perirhinal en la dirección rostro caudal y del límite ventral de la corteza somatosensorial hacia la corteza piriforme en la dirección dorsoventral (Saper, 1982). La corteza insular (áreas 13 y 14 de Krieg en ratas) ha sido considerada como una corteza visceral, dado que recibe información gustativa y visceral del tálamo y se sabe que se halla involucrada en reacciones viscerales y de tensión (Van-der-Kooy, Koda, McGinty, Gerfen, y Bloom, 1984).

Entre las conexiones de la corteza insular que podrían ser importantes en los procesos de memoria se encuentran los del sistema límbico, la amígdala, los núcleos dorsomediales del tálamo y la corteza prefrontal. La corteza insular y el núcleo central de la amígdala están interconectados funcional y recíprocamente (Braun et.al., 1982) . Se ha sugerido que las conexiones corticales hacia la amígdala, incluyendo las de la corteza insular, transmiten información de tipo cognitivo que a continuación se integra con procesos emocionales y motivacionales (Braun et. al., 1982). Recientemente, se ha reportado que la corteza insular está implicada, tambien, en procesos cognitivos en humanos (Gahem, Mellet, Crivello, Tzourio, Mazoyer, Berthoz y Denis, 1997).

Los primeros experimentos relativos al papel de la región insular en procesos asociativos

tuvieron a principios de los años 70. En esos estudios se relacionó a la corteza insular con el aprendizaje (adquisición) y la retención de un modelo conductual conocido como "condicionamiento aversivo a los sabores" (CAS). El CAS es un modelo conductual simple pero robusto en el que los animales adquieren aversión a un determinado sabor cuando éste es seguido de un malestar gástrico (Garcia, 1990). Las lesiones experimentales de la corteza insular producidas antes o después de la adquisición del CAS interfieren con este aprendizaje (Kiefer y Braun, 1979, Braun, Lasiter y Kiefer, 1982). Dado que se ha probado que estas lesiones no producen deficiencia alguna en la discriminación de sabores o en la sensibilidad gastrointestinal, parece claro que las lesiones de la región insular impiden la representación mnemónica de los sabores y/o de sus consecuencias gastrointestinales (Kiefer y Brown, 1979; Yamamoto, Matsuo, Kawamura, 1980; Braun, Lasiter y Kiefer, 1982). Dicho en otras palabras, la corteza insular al parecer está involucrada en los aspectos exclusivamente asociativos del CAS. Si bien en un principio los estudios en esta región se enfocaron en la representación mnemónica (engrama) de las asociaciones entre estímulos viscerales y estímulos gustativos ahora es claro que esa región podría procesar un espectro más amplio de condicionamientos conductuales, ya que las lesiones tanto reversibles como irreversibles de la corteza insular producen severos impedimentos en la adquisición de otros dos modelos de aprendizaje aversivo de tipo espacial conocidos como prevención pasiva y laberinto de agua (Bermúdez-Rattoni y McGaugh, 1991; Bermúdez-Rattoni, Yamamoto y Bures, 1998).

En particular, el CAS representa un modelo muy apropiado debido a que las estructuras neurales involucradas en este condicionamiento se encuentran actualmente bien definidas (figura II). La información gustativa accede al sistema nervioso a través de los pares cranianos séptimo noveno y décimo los cuales proyectan directamente al núcleo del tracto solitario. Neuronas secundarias procedentes del tracto solitario hacen a continuación sinapsis en el área parebraquial del puente. Las fibras procedentes de esta última estructura proyectan a su vez hacia el núcleo postero ventromedial del tálamo, la amígdala central y el hipotalamo lateral. La corteza insular a su vez recibe las proyecciones gustativas desde el tálamo y como se ilustra en la figura II existe comunicación bilateral entre esta estructura cortical y el complejo amigdalino. La relación existente entre la región basal y el CAS se estableció previamente en nuestro laboratorio cuando se encontró que las lesiones bilaterales con ácido quisquálico en el NBM evitan la adquisición del condicionamiento aversivo a los sabores (Lopez-Garcia et.al., 1993). En lo que respecta a la actividad colinérgica específicamente en la corteza insular y su relación con el CAS, resultados obtenidos mediante microdiálisis *in*

vivo en nuestro laboratorio, muestran una significativa activación colinérgica en la corteza insular como respuesta a la presentación de un estímulo gustativo (Miranda y Bermudez-Rattoni, en prensa).

Con base en lo anterior, se puede ver que el CAS y su relación con la corteza insular representa un modelo adecuado para el estudio del papel fisiológico del sistema de proyección basalo cortical en sus respectivos aspectos bioquímicos y conductuales.

Regulación basalo cortical vs regulación colinérgica

En el documento que a continuación se transcribe (*Brain Research*, 1999), se enfoca el problema planteado aquí de tal forma que no se da por sentada, en modo alguno, la equivalencia entre la regulación ejercida por la región basal en su conjunto y la posible regulación específicamente colinérgica *originada* en la misma región.

Haciendo uso del modelo anatómico y conductual descrito en el apartado anterior, llevamos a cabo una comparación directa entre los efectos de dos estrategias de lesión. Tomando como punto de partida el hecho de que las lesiones excitotóxicas en el NBM interfieren con el aprendizaje del CAS, indujimos lesiones inespecíficas de la región basal mediante la infusión directa de NMDA y comparamos los efectos bioquímicos y conductuales de esta manipulación con la destrucción selectiva de las neuronas colinérgicas de proyección hacia la corteza en general y la corteza insular en particular.

Mediante microdiálisis *in vivo* y ensayos enzimáticos de actividad de colin acetiltransferasa (la enzima de síntesis de acetilcolina), monitoreamos el estatus colinérgico de la corteza insular, resultante de cada una de las estrategias de lesión, y lo relacionamos a sus correspondientes efectos en aprendizaje.

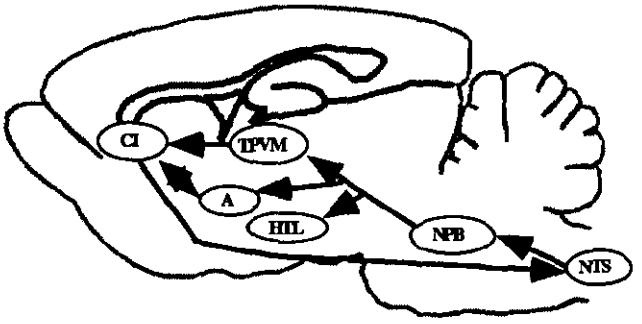

Figura II Esquema del corte sagital de un cerebro de rata en la que se muestran algunas de las principales conexiones de la corteza insular con otros núcleos del cerebro en su relación con el CAS: (NTS) núcleo del tracto solitario, (NPB) Núcleo parabraquial del puente, (TPVM) núcleo talámico postero- ventromedial, (HTL) Hipotálamo lateral, (A) amígdala, (CI) corteza insular.

Differential effects of 192IgG-saporin and NMDA-induced lesions into the basal forebrain on cholinergic activity and taste aversion memory formation.

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Mnemonic deficits resulting from excitotoxic lesion of the basal forebrain have been classically attributed to the resulting depletion of cortical acetylcholine activity. In this study, we have performed a detailed analysis of the cholinergic status of the insular cortex (IC) following local injections of either 192IgG-saporin (192IgG-sap) or N-methyl-D-aspartate (NMDA) directly into the nucleus basalis magnocellularis (NBM). By means of *in vivo* microdialysis we show that the immunotoxin lesion results in an almost complete lack of extracellular acetylcholine release, whereas NMDA-induced lesions result in a marginal reduction in cortical cholinergic activity. Choline-acetyltransferase activity in the IC further confirmed this differential pattern of cortical deafferentation. Surprisingly, however, only NMDA-induced lesions showed a strong disruptive effect upon taste aversion learning whereas no detectable deficits could be found following 192IgG-sap lesions. By combining intrabasal injections of 192IgG-sap with acute pre-training infusions of the cholinergic antagonist scopolamine into the IC, a strong disruption of taste aversion was attained. These results imply that residual cholinergic activity, following 192IgGsaporin lesions, might be still critical for normal cortical mediation of memory processing. They also support the role of basal forebrain in mediating learning and memory processes, and demonstrate that mnemonic deficits resulting from excitotoxic lesions of the basal forebrain are not the sole result of cortical acetylcholine activity hypofunction.

Theme: Neural basis of Behaviour
Topic: Learning and memory: systems and functions.
Keywords: Acetylcholine, ChAT, microdialysis, muscarinic antagonist, NBM.

The cholinergic basal forebrain complex provides widespread, topologically organised afferent cholinergic innervation to many brain regions including the whole cortical mantle, hippocampus and amygdala [7]. A great number of studies have implicated the involvement of basal forebrain cholinergic neurons in the mediation of learning and

memory processes in experimental animals [11, 12, 13, 17, 23]. Learning deficits associated with lesions of nucleus basalis magnocellularis (NBM) produced by injections of excitatory aminoacid agonists have been demonstrated in a great variety of tasks [24, 27]. Mnemonic deficits associated to these lesions have been classically attributed to the resulting depletion of cortical acetylcholine activity. Nevertheless, many researchers have reported that the magnitude of decrease in cortical choline acetyltransferase (ChAT) following excitotoxic lesions of the NBM is unrelated to the degree of cognitive impairment [4, 10, 21].

Recently, depletion of the nerve growth factor (NGF) receptor-bearing cholinergic neurons in the rat basal forebrain, with a corresponding selective loss of cholinergic innervation in the related cortical regions has been obtained following intracerebral injections of a newly introduced immunotoxin, 192IgG-saporin [8, 30]. Investigations carried out using this highly efficient immunotoxin have repeatedly failed to reproduce the kind of memory deficits normally found as a result of the less selective excitotoxic lesions, thus questioning the concept of a direct role for cortical acetylcholine (ACh) input in memory formation [3, 5, 25, 26, 28, 29]. Here we use a well known cortically mediated learning paradigm, conditioned taste aversion (CTA), in order to directly address the issue of whether the ascending cholinergic input is critically involved in the cortex mediation of memory formation. The CTA is a very robust and widely used model for the study of learning and memory processes [6]. In this behavioural model, an animal acquires aversion to a novel taste when it is followed by digestive malaise. The anatomical substrates responsible for CTA learning have been well established [6, 18], being the insular cortex (IC) the only cortical locus directly involved in acquisition and retention of CTA learning [1, 9, 18, 19, 31]. Excitotoxic lesions of the nucleus basalis magnocelularis (NBM) as well as cholinergic antagonists injections directed to the IC disrupt CTA learning implying the involvement of the ascending cholinergic pathway in this learning paradigm [20, 22].

This study comprises two experiments. In the first one we determined directly whether the extracellular ACh release in the IC is actually affected by intraparenchymal injections of either 192IgG-saporin or NMDA into the NBM. Therefore, we compare the effects of both toxins by means of *in vivo* microdialysis as a reliable and appropriate method for assessing the cholinergic status of the IC. In the second one, we compare the behavioural effects of both lesioning strategies as a valuable approach for assessing the specific involvement of the basal cortical projection in CTA learning. Subsequently, by combining intrabasal injections of 192IgG-sap with acute pre-training infusions of the cholinergic antagonist scopolamine into the IC we confirm the critical involvement of cholinergic activity in the cortical meditation of memory formation.

Seventy male Wistar rats weighing 250-300 g were used in this study. They were individually caged and kept in a 12 hr light/dark cycle. Three independent groups were used for the microdialysis study. Animals received unilateral injections of either 192IgG-saporin (n=5), NMDA (n=4) or vehicle (n=5) directly into the NBM according to the following procedure: Animals were anaesthetised with sodium pentobarbital (65mg/kg) and placed in a head holder. Lesions were made by stereotaxic infusion of the toxin via a 30-gauge stainless steel cannula connected via teflon tubing to a 10µl glass microsyringe mounted in a microdrive pump. The stereotaxic co-ordinates used for intrabasal lesions were anteroposterior: -1.0 mm from bregma; lateral: 2.5mm; and dorsoventral: -8.0 mm. A total volume of 0.8 µl of a solution of either 0.2µg/µl of 192IgG-saporin (Chemicon, Tamecula. Ca.) or NMDA (10µg/µl, Sigma) in phosphate buffered saline was unilaterally infused at a constant rate of 0.5µl/min. The injector was left in place for 3min to allow proper diffusion. The contralateral hemisphere in each animal

remained intact as a control. After that, animals were bilaterally implanted with microdialysis intracerebral guide cannulae (CMA12, bioanalytical systems; BAS) directed to the IC. The tips of the cannulae were aimed to 2 mm above the IC (AP=+2; ML+-5.5; DV=-4.0; from bregma). They were attached firmly to the skull with dental acrylic cement and anchored with two stainless steel screws on the skull. Dialysis procedure started two weeks after surgery by connecting the microdialysis probe (BAS CMA 12; diameter 0.5mm; length, 3mm) to the intracerebral guide via microdialysis tubing (BAS FEP; 0.66mm outer diameter, 0.12mm inner diameter) to a microinfusion pump (Carnegie Medicin). Ringer's solution containing neostigmine bromide (sigma 10μM) was continuously perfused at a rate of 2μl/min. The first 60 min sampling was discarded, and then samples were collected every 15 min. (i.e., 30 μl /sample) and immediately frozen. Six samples were obtained for each analysed hemisphere. In the third sample, KCl (56mM) was added to the perfusion medium, whereas the NaCl concentration was lowered to 82mM to maintain physiological osmolarity.

The remaining 56 animals were divided into the following 7 groups: Groups NMDA (n=10) and Veh (n=8) received bilateral injections of NMDA and vehicle respectively. IgG-S, (n=10); IgG-S/veh, (n=7); and IgG-S/sc, (n=7) received bilateral microinjections of 192IgG-sap directly into the NBM. The last two groups (IgG-S/veh and IgG-S/sc) as well as an additional non lesioned group (Sc, n=4) were implanted bilaterally with 15 mm 23 gauge stainless steel cannulae. Finally, ten animals remained unoperated during the whole procedure as an intact control group (CTR). For this study we used the same co-ordinates, toxin dose, injection, implantation and surgical procedures described above. Two weeks after the surgery all the rats were left without drinking water for the following 24 hours. After that, they were given water every 12 hours for 15 min (in their home cages) and consumption was measured. When they reached an asymptotic level of consumption, they received an acquisition trial. The presentation of a novel taste was done by adding saccharin in the water (1g/l). Fifteen minutes later, a malaise-inducing drug (lithium chloride, 0.15 M) was administered i.p. Subsequent drinking trials were performed with water only. After three drinking trials the subjects were presented with the saccharin flavoured water for the second time and their consumption was used as a measure of strength of aversion. The three cannulated groups (IgG-S/veh, IgG-S/sc and sc) received bilateral microinjections of 0.5μl per hemisphere of either PBS (IgG-S/veh) or scopolamine chloride (IgG-S/sc and sc) directly into the insular cortex 20 min before starting the above described training trial. Injections were made through the intracerebral cannulae using dental needles (30 gauge which protrude 2 mm from the tip of the guide cannula) attached to a microinfusion pump (Carnegie Medicin). Either scopolamine (sigma, 60μg/μl dissolved in PBS) or vehicle alone was infused with a flux rate of 0.2μl/min during a 3min period. Intracortical infusions were given to hand restrained conscious animals. Data obtained in the conditioned taste aversion task were analysed by analysis of variance (ANOVA) and with post-hoc Fisher test where appropriate.

One day following the behavioural study randomly sampled animals from the groups that received 192IgG-saporin (n=7) or NMDA (n=7)-induced lesions as well as a sample of control animals (n= 8) were sacrificed by decapitation. Insular cortex and dorsal striatum tissue (as a non NBM-dependent control) samples were dissected under a stereoscopic microscope and stored at -70°C prior to analysis of ChAT activity. Each sample was sonicated in 1 ml of 25mM phosphate buffer containing 0.5% triton X-100 and maintained in ice to avoid over heating. The homogenate was centrifuged at 20000g for 15 min. ChAT activity in 200μl of the supernatant was assayed by adding 50μl of a substrate solution containing 10mM choline chloride, 0.2mM Neostigmine, 20mM EDTA and 0.4mM acetyl coenzyme-A in 0.1M sodium phosphate buffer, pH 7.4, and

incubated for 5 min at 37°C. Reaction was stopped by adding 10µl of 1M perchloric acid on ice and 1ml of distilled water. The mixture was passed through a 30 000MW ultraspin filter (Cole-Parmer, IL) and stored at -70°C prior to chromatographic analysis.

Samples obtained from both the enzymatic and microdialysis studies were assayed for ACh levels using high performance liquid chromatography (HPLC) with electrochemical detection, using a mobile phase, pH 8.5, containing 50 mM sodium phosphate buffer and 0.5% Kathon reagent (BAS) microbicide. All samples were injected on a polymeric reversed phase column (BAS ACh-choline assay kit), ACh and choline were then converted into hydrogen peroxide and betaine in a postcolumn reactor containing immobilised acetylcholinesterase and choline oxidase (BAS). A choline oxidase/catalase reactor (BAS) was added in order to avoid choline detection in the substrate solution. The hydrogen peroxide was detected electrochemically with a platinum electrode set at 500mV (vs Ag /AgCl). The detection limit, defined as the amount of ACh producing a peak twice the area of the basal noise, was approximately 0.1pmol. Following both the behavioural and microdialysis study, animals were transcardially perfused with a 4% solution of paraformaldehyde in phosphate buffer and transferred to a 30% buffered sucrose solution and stored until they were cut. Coronal sections (40mm thick) were mounted and processed for acetylcholinesterase histochemistry according to a modified protocol from Paxinos and Watson [23]. Briefly, sections were incubated overnight in 50 mM sodium acetate buffer, pH 5.0, 4 mM copper sulfate, 16 mM glicine, 4 mM acetylthiocholine iodide, and 0.1 mM ethopropazine. After overnight incubation the slides were immersed into a developing solution (1% sodium sulfide, pH 7.5) for 10 min.

Figure 1 shows the extracellular release of ACh in the IC following high potassium depolarisation. Paired t-tests were used for comparisons between extracellular release in the insular cortex ipsilateral to the injection site and the contralateral normal hemisphere. The addition of 56 mM KCl to the perfusion fluid provoked an almost immediate overall increase in the ACh levels in both the vehicle treated hemisphere and its corresponding control side. In contrast the group that received unilateral microinjections of 192IgG-saporin into the NBM showed a dramatic reduction of cortical cholinergic release even after high potassium stimulation compared with the control hemisphere (mean reduction: 97±1.6%, $p < 0.01$). Although intrabasal NMDA microinjections showed a definite trend, cortical extracellular release did not reach statistical significance in ACh reduction (mean reduction: 28±1.8 %; $t = 1.41$, $p = 0.25$). ANOVA analysis showed no differences between control levels of ACh release among groups ($F_{2,11} = 0.193$, $p > 0.05$).

Subsequent histochemical staining for acetyl cholinesterase activity confirmed the relative extent of cortical cholinergic depletion due to the unilateral injections of either 192IgG-saporin or NMDA into the NBM (see Fig. 2). As reported, a marked decrease in the cholinesterase staining was evident in the 192IgG-sap treated animals as compared with the control treatment. NMDA induced lesions showed a mild though appreciable reduction in the cholinergic marker.

Having established the differential degree of deafferentation obtained after each lesioning technique, we explored the relative effects of either toxin upon CTA learning. Simple ANOVA was done on the test day consumption volume for all treated groups during the taste aversion trial. No differences were found in the baseline water intake among groups ($F_{6,42} = 0.921$). As shown in figure 3A and B, during the test presentation of saccharin solution (the novel gustatory stimulus), significant differences were found among groups ($F_{6,42} = 16.68$, $p < 0.01$). A *Post hoc* pairwise Fisher test showed that intrabasal NMDA-induced lesions resulted in a significant disruption in the acquisition of taste aversion, as indicated by the increased saccharine consumption when compared with

the control and vehicle groups (p's<0.01). Surprisingly, no disruption of taste aversion was detected in the 192IgG-Saporin treated group when compared with the control and vehicle groups. In contrast, consistent with previous reports [22], pre-training microinjections of the cholinergic antagonist scopolamine directly into the insular cortex resulted in a strong learning impairment when compared with the control group (p< 0.01). Moreover, scopolamine but not vehicle injections prior to CTA training in animals that previously received intrabasal 192IgG-sap lesions, also induced a strong learning deficit when compared with the control group (p< 0.01). This results suggest that the normal learning performance after intrabasal 192IgG-sap lesions is being carried out throughout residual levels of cortical ACh.

Comparisons of ChAT activity in the IC following either 192IgG-sap or NMDA-induced lesions are shown in Table 1. Simple ANOVA revealed statistical differences among groups ($F_{2,19}$= 20.92, p<0.01). Subsequent *Post hoc* pairwise Fisher tests showed that both NMDA and 192IgG-Saporin induced lesions displayed markedly reduced ChAT activity in the insular cortex relative to the control group (55.3% and 85.7% reduction, respectively; p's<0.01 against the control group). However, the strongest reduction in enzymatic activity was apparent in the immunotoxin lesioned group resulting in significant differences between both lesioned groups (p<0.05), implying that, at the dose used, 192IgG-sap treatments result in a significantly stronger cholinergic deaferentation of the cortex as compared with that resulting from intrabasal microinjections of NMDA.

In these experiments we show that NMDA injections into the NBM result in a strong disruption of CTA learning, whereas 192IgG-saporin-induced lesions into the NBM do not. The existing discrepancy between both kind of lesions can be explained in two ways: First, cholinergic neurons in the NBM are intermingled with other neurons in the basal forebrain, including GABAergic neurons of the dorsal and ventral pallidum and noncholinergic magnocellular corticopetal neurons. The use of excitotoxins, specially those acting through NMDA receptors such as NMDA and ibotenic acid can also destroy noncholinergic pallidal and other neurons in the substantia innominata [13,14], whereas there seems to be little doubt that for basal cholinergic neurons, which are enriched with the low-affinity nerve growth factor receptor, 192IgG-saporin is a powerful and selective lesioning tool. Second, those excitotoxins previously reported to produce the greatest mnemonic deficits, when injected into the NBM, also produced the largest decreases in the basolateral amygdaloid ChAT [4, 21]. As for the immunotoxin, virtually all of the cholinergic cells within the NBM are vulnerable to this compound, with the exception being those cholinergic neurons that send an efferent projection to the basolateral amygdala [15, 16]. Here we have found an inverse relationship between cortical ACh activity and the ability to acquire CTA. In the present conditions, intrabasal lesions by 192IgG-sap resulted in a strong reduction in both cortical ACh release and ChAT activity (97% and 85% respectively). To our knowledge this is the first assessment of the actual cortical ACh release following these immunotoxic lesions. In contrast, NMDA-induced lesions into the basal forebrain resulted in a mild 25% reduction in extracellular ACh release and 55% reduction in ChAT activity. Consistent with previous reports [2], this result suggests that ChAT activity does not necessarily reflect the actual neurotransmitter availability. Being that only NMDA-induced lesions disrupted CTA learning, this result directly implies that learning deficits obtained after NMDA-induced lesions into the basal forebrain can not be the sole result of cortical cholinergic hypofunction, since a much stronger and selective reduction in cortical ACh does not result in aversion learning impairments. Our results are in agreement with an increasing number of studies demonstrating that intraparenchymal injections of 192IgG-sap, in spite of producing an efficient cholinergic deafferentation, consistently fail to reproduce

the kind of memory deficits normally found as a result of the less selective excitotoxic lesions [3, 5, 25, 26, 28, 29]. It is to be stressed, however, that this result does not rule out the possible critical involvement of the basal-cortical cholinergic projection in memory processing. In fact, by combining intrabasal injections of 192IgG-sap with acute pre-training infusions of the cholinergic antagonist scopolamine into the IC, a strong disruption of taste aversion was observed. This result is consistent with those reported by Naor et al. (1996) according to which acute microinjections of the muscarinic antagonists scopolamine, atropine and AF DX-116 directly into the insular cortex disrupted CTA learning. Taken together these data suggest that cholinergic neurotransmission into the cortex plays a critical role in memory processing and that residual ACh levels after 192IgG-saporin lesions might be still sufficient for memory processing.

The results of this study extend previous work which investigated the effect of 192IgG-saporin and excitatory neurotoxins treatments into the NBM, and provide support to the notion that the cholinergic basal forebrain modulation plays a critical role in aversive conditionings and further provide a framework that may help to solve the well documented discrepancies concerning the effects of different neurotoxins in basal forebrain lesion studies.

Table 1.
Choline acetyltransferase activity

Group	Anatomical region	
	Insular cortex	Dorsal striatum
Intact control	123.25 ±17.51	480.19±78.7
NMDA	55.09 ±6.45** ♦	533.41±62.0
192IgG-saporin	17.63 ±4.25**	455.26±49.32

Values shown represent mean enzyme activity ±SEM (*pmol/min/mg protein*).
**p<0.01 relative to control group values; ♦ p<0.05 relative to 192IgG-saporin lesioned group.

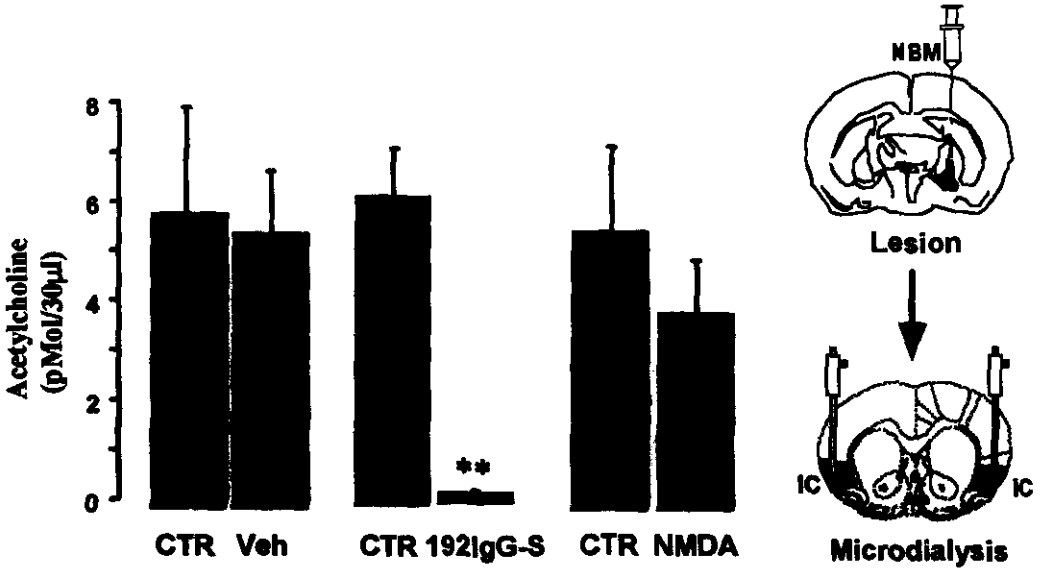

Figure 1 : Mean values (±SEM) of extracellular ACh release in the insular cortex during the microdialysis procedure following high potassium stimulation. As represented in the drawing, subjects received unilateral injections of either vehicle (Veh), 192IgG-sap (IgG-S) or NMDA directly into the NBM. For each group, values of ACh release in the insular cortex ipsilateral to the injection site (black bars) were compared with the contralateral intact hemisphere (CTR: gray bars). Note the lack of acetylcholine release in the immunotoxin treated hemisphere after the addition of KCl as compared with its own control hemisphere. **p<0.01 compared with the control hemisphere.

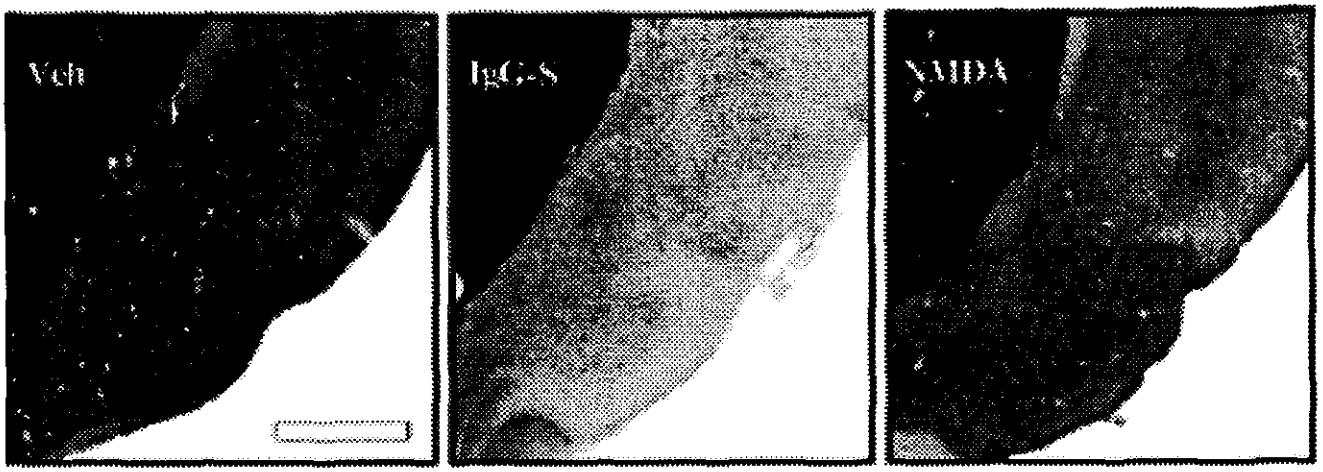

Figure 2: Photomicrographs of AChE histochemistry of coronal sections taken from each of the above described groups showing the cortical region ipsilateral to the injection side. Note the reduction of cortical AChE staining in both lesioned brains as compared to the control vehicle-iniected brain. Scale bar: 1mm.

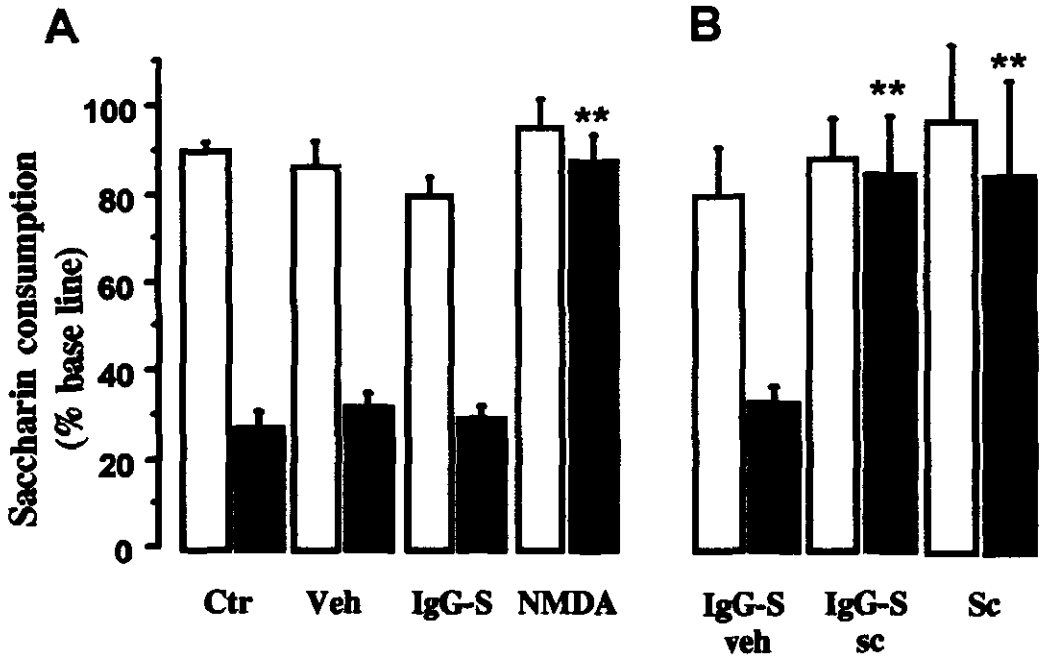

Figure 3. A: Effects of intrabasal vehicle, 192IgG-saporin, or NMDA microinjections on conditioned taste aversion. Mean (±SEM) percentage of baseline consumption during the acquisition trial (empty bars) and retention test trial (black bars). CTR, intact control; Veh, Vehicle injected control; IgG-S 192IgG-saporin lesioned group; NMDA, NMDA-lesioned group. B: Intracortical injections of vehicle (IgG-S/veh) and scopolamine (IgG-S/sc) prior to CTA training, in animals that previously received intrabasal 192IgG-sap lesions, an additional group received only pre-training intracortical scopolamine injections (Sc). As can be seen the cholinergic antagonist induced a strong learning deficit when compared with the control group. **p<0.01 compared with the intact control during the test trial

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Una alternativa modulatoria

Las lesiones excitotóxicas de los núcleos colinérgicos basales afectan la adquisición de prácticamente todos los modelos de condicionamiento actualmente estudiados, incluido el condicionamiento aversivo a sabores. Sin embargo, la destrucción específica y selectiva de las células colinérgicas de proyección hacia la corteza, no parece tener efecto alguno sobre la habilidad de aprender en estos modelos (Wenk et.al., 1994; Berger-Sweeney et.al., 1994; Torres et.al., 1994; Baxter et.al., 1995; Waite y Thal, 1996; Wenk et.al., 1996). En el trabajo anterior hemos encontrado una discrepancia similar con respecto al CAS.

Hemos observado, tambien, una relación inversa entre la habilidad de los animales para adquirir un condicionamiento aversivo y el grado de deaferentación colinérgica del dominio cortical involucrado. Esto es, la lesion que mayor efecto tiene sobre el desempeño conductual (NMDA) es al mismo tiempo la que menor efecto muestra tiene sobre la actividad colinérgica en la corteza insular, sucediendo lo contrario en el caso de la lesión inmunotóxica. Este simple hecho, implica por necesidad que la deficiencia cognitiva obtenida como resultado de la lesión excitotóxica en el NBM, *no puede ser* el producto de una disfunción en la comunicación colinérgica entre la región basal y la corteza insular, revelando por lo tanto la existencia de otro sistema de modulación originado en la propia región basal.

Con respecto a la participación de la inervación colinérgica, la ausencia de efecto tras la destrucción selectiva de esta proyección pareciera implicar que su participación es nula.

Sin embargo, la combinación de infusiones directas del antagonista colinérgico escopolamina en la corteza insular y lesiones inmunotóxicas en el NBM, dieron como resultado nuevamente una fuerte disfunción cognitiva.

Este último resultado sólo admite dos posibles interpretaciones:

1.- El anticolinérgico podría estar interfiriendo inespecíficamente con la comunicación de otros sistemas de neurotransmisión, sistemas cuya participación podría ser crítica en el desempeño de la prueba de memoria.

2.- El anticolinérgico podría estar abatiendo niveles residuales de actividad colinérgica en la corteza, garantizando de ese modo la interrupción de las funciones asociativas normales.

El grupo de Dudai (Naor y Dudai 1996) ha llevado a cabo bloqueos con

anticolinérgicos en la corteza insular, mostrando una subsecuente disfunción asociativa. En el estudio citado, los autores utilizaron con los mismos resultados, tres antagonistas muscarínicos distintos: escopolamina, atropina y AF-DX. Si concedemos que la participación colinérgica es nula entonces debemos suponer que los tres anticolinérgicos muestran *todos*, efectos inespecíficos, haciendo de la hipótesis 1 una suposición considerablemente más improbable (aunque no imposible). Lo cual nos lleva a la hipótesis 2.

De acuerdo con ésta, y a la luz de los resultados mostrados habrá que suponer que la función cortical de memoria se ve gravemente comprometida *sólo* cuando los niveles de acetilcolina extracelular descienden por debajo de un nivel de concentración mínimo. Nivel que por lo visto, es capaz de alcanzar valores extraordinariamente bajos.

De todo lo anterior se desprenden las siguientes tres conclusiones:

i.- Las funciones asociativas mediadas por la corteza en general pueden llevarse a cabo a pesar de una reducción casi completa en los niveles de acetilcolina cortical.

ii.- No obstante lo anterior, las funciones asociativas corticales parecieran ser críticamente dependientes de los niveles residuales de actividad colinérgica.

iii.- Existe un sistema de modulación originado en la propia región basal, físicamente distinto a la proyección colinérgica basalocortical, cuya participación es necesaria para las funciones asociativas.

Con la intención de avanzar en el esclarecimiento de la tercer conclusión, partimos de lo siguiente:

Los núcleos colinérgicos de la región basal proyectan extensamente hacia la corteza, el hipocampo, los núcleos amigdalinos y marginalmente hacia el estriado (Bigl, Woolf y Butcher, 1982). Todas estas áreas son estructuras fuertemente involucradas en procesos mnemónicos. En el caso de la amígdala, una alta densidad de terminales inmunoreactivas a colin acetiltransferasa puede observarse en el núcleo basolateral y el amígdalohipocampal. Una densidad media en el núcleo lateral, el cortical y el basal accesorio mientras que se detecta una baja densidad en los núcleos medial y central (Heckers y Mesulam, 1994). Si bien estas proyecciones provienen en su totalidad de la región basal, tanto el núcleo medial como el central y el basolateral (este último con la mayor densidad de fibras colinérgicas) muestran una muy baja densidad de fibras inmunoreactivas al receptor de baja afinidad para el factor de crecimiento neuronal, siendo que, por otro lado, los restantes núcleos amigdalinos con media o baja densidad de fibras colinérgicas muestran una alta densidad de fibras positivas al receptor de NGF (Heckers et al., 1994). ¿Implica esto que algunas neuronas colinérgicas de la región basal carecen de este receptor? En efecto, se

ha observado que, a pesar de la destrucción específica de neuronas colinérgicas, el tratamiento intraventricular con la inmunotoxina 192IgG-saporina deja intacta una subpoblación de células inmunoreactivas a colin acetiltransferasa en las inmediaciones del complejo sustancia innominata-nucleus basalis. El mismo tratamiento da como resultado una deaferentación colinérgica en los núcleos amigdalinos, proporcional a su inmunoreactividad al receptor de NGF dejando intactas las fibras colinérgicas en el núcleo basolateral y parcialmente disminuídas en el medial. En resumen, Heckers y colaboradores (1994) describieron una drástica deaferentación colinérgica hacia la corteza y el hipocampo pero no en la amígdala. Esto, como resultado del tratamiento intraventricular con la inmunotoxina.

Ahora bien, las pruebas conductuales normalmente utilizadas, todas sensibles a lesiones excitotóxicas en el complejo colinérgico basal son en la mayoría de los casos igualmente sensibles a lesiones excitotóxicas en la amígdala. Para el condicionamiento aversivo a sabores las tres áreas de convergencia de informacion gustativa y visceral y potenciales sitios de asociación son el núcleo parabraquial del puente, la corteza insular y la amígdala central y basolateral (Bermúdez-Rattoni, Yamamoto, 1998). Por otra parte, existe una extensa comunicación bidireccional entre la amígdala basolateral (ABL) y la corteza insular y, por último: lesiones excitotóxicas con ácido iboténico en el núcleo basolateral producen un marcado déficit en la adquisición de este condicionamiento (Yamamoto, 1993). Hay que aclarar que para el CAS, a diferencia del resto de las conductas estudiadas, los efectos de lesiones en la amígdala no están a la fecha tan claros y en el mejor de los casos parecieran depender fuertemente del método de lesión utilizado. Esto es, si bien las lesiones con AMPA, iboténico y NMDA en la amígdala afectan igualmente la adquisición de cualquier conducta espacial, se ha reportado que sólo las lesiones con iboténico y específicamente en el núcleo basolateral, producen déficits en el CAS. Las lesiones con NMDA en el núcleo basolateral (llevadas a cabo en el laboratorio en numerosas ocasiones) carecen de efecto sobre este condicionamiento a pesar de los claros efectos histológicos observables (Dunn y Everitt, 1988; Bermúdez-Rattoni y McGaugh, 1991; ver también el siguiente manuscrito) .

Por otro lado, dado que las lesiones excitotóxicas destruyen somas neuronales en forma inespecífica es claro que éstas, al llevarse a cabo en la región basal, no distinguen entre los somas inmunoreactivos al receptor de NGF y los que no lo son. En este sentido y como ya se indicó, aquellas excitotoxinas que mayor efecto tienen sobre el desempeño en pruebas de memoria, dan lugar a una mayor destrucción de marcadores colinérgicos, no en la corteza sino en la

amígdala (Beninger, Kuhnemann, Ingles, Jhmandas y Boegman, 1994).

Dado que la proyección basalo amigdalina en particular no puede ser destruida por el sistema 192IgG-saporina y siendo por otro lado que los núcleos amigdalinos juegan un papel central en los procesos asociativos, bien pudiera ser la modulación que la región basal ejerce sobre la amígdala el factor que explicaría la discrepancia que intentamos ahora resolver.

Es posible entonces, que los efectos conductuales observados como resultado de lesiones inespecíficas se deban entonces a la destrucción *simultánea* de las proyecciones colinérgicas hacia la amígdala y la corteza insular.

En el documento que a continuación se transcribe (*The Journal of Neuroscience*, en prensa), se explora la interacción entre la región basal, la corteza insular y la amígdala basolateral con la intención de dilucidar la contribución relativa de ambos sistemas de proyección (NBM-CI y NBM-ABL respectivamente) en la modulación de las funciones asociativas. Nuevamente se toma ventaja de los efectos diferenciales de los dos métodos de lesión anteriormente utilizados, con objeto de establecer una conexión entre la deaferentación relativa de ambas estructuras, la corteza y la amígdala y el consiguiente desempeño asociativo.

Redundant basal forebrain modulation in taste aversion memory formation.

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The cholinergic basal forebrain provides afferent cholinergic input to the cortical mantle, amygdala and hippocampus. Mnemonic deficits resulting from excitotoxic lesion of the basal forebrain have been classically attributed to the resulting depletion of cortical acetylcholine activity. In spite of the strong cholinergic depletion following injections into the basal forebrain of a newly introduced immunotoxin (192IgG-saporin), no detectable deficit can be found in the acquisition of several well known learning tasks, including conditioned taste aversion. Conversely, N-methyl-D-aspartate (NMDA)-induced lesions of the basal forebrain strongly impair taste aversion learning. In this study we show that 192IgG-saporin produces an efficient and selective cholinergic deafferentation of the rat neocortex but not the amygdala. Furthermore, a stronger relationship between severity of memory impairment following NMDA lesions and basoamygdaloid cholinergic deafferentation was found. Therefore, in a second experiment, we show that combining NMDA-induced lesions into the basolateral amygdala with 192IgG-saporin injections into the basal forebrain result in a strong disruption of taste aversion learning, whereas none of these treatments were by themselves capable of producing any detectable impairment in this learning task. The double lesion effect was only paralleled by simple NMDA lesions into the basal forebrain, suggesting that the learning deficits associated to excitotoxic lesions of the basal forebrain is the result of the simultaneous destruction of the corticopetal and basoamygdaloid interaction. A model is proposed according to which the modulation of learning processes exerted by the basal forebrain can be redundantly carried out by both the basal cortical or basoamygdaloid pathway.

Key words: Conditioned Taste Aversion; Learning; Cholinergic basal forebrain; ChAT

The cholinergic basal forebrain complex including the nucleus basalis magnocellularis (NBM) provides widespread, topologically organized afferent cholinergic innervation to many brain regions including the whole cortical mantle, hippocampus and amygdala (Bigl et al., 1982). Many studies have implicated these cholinergic neurons in the mediation of learning and memory processes (Hepler et al., 1985; Etherington et al., 1987; Everitt et al., 1987; Dunnett and Fibiger, 1993; Sinden et al., 1995). Lesions of the NBM using injections of excitatory aminoacid agonists have been associated with

learning deficits in a great variety of tasks (Sinden et al., 1995; Wenk, 1997). Mnemonic deficits resulting from this type of lesions have been classically attributed to the depletion of cortical acetylcholine activity. However, those excitotoxins previously reported to produce the greatest mnemonic deficits also produce the largest decreases in amygdaloid choline acetyltransferase (ChAT) (Dunnet et al., 1987; Beninger et al., 1994; Mallet et al., 1995).

It has been demonstrated that the novel immunotoxin, 192IgG-saporin, injected into the NBM induces a selective loss of cortically projecting cholinergic nerve growth factor receptor-positive neurons. However this treatment spares basolateral amygdaloid (BLA) projecting fibers (Heckers and Mesulam, 1994). Interestingly, in spite of the massive reduction of cortical cholinergic input (Wiley, 1992; Book et al., 1994), this immunotoxin has repeatedly failed to reproduce the kind of memory deficits normally found as a result of less selective excitotoxic lesions (Berger-Sweeney et al., 1994; Torres et al., 1994; Wenk et al., 1994; Baxter et al., 1995; Waite and Thal, 1996; Wenk et al., 1996). Taken together these data support the view that learning deficits associated to excitotoxic lesions are the result of basoamygdaloid deafferentation rather than the destruction of basal cortical cholinergic projection. This interpretation, however, challenges the extensive evidence supporting a direct role for the cortical cholinergic input in memory formation.

Here we use a cortically mediated learning paradigm, conditioned taste aversion (CTA), in order to study the modulation exerted by the basal forebrain on memory formation. CTA is a very robust model for the study of learning and memory processes (Bermúdez-Rattoni and Yamamoto, 1998). In this model, an animal acquires an aversion to a novel taste when it is followed by digestive malaise. It has been shown that bilateral lesions of the insular cortex (IC) disrupt acquisition and retention of CTA (Kiefer and Brown, 1979; Yamamoto et al., 1980; Aggleton et al., 1981;; Braun et al., 1982; Kiefer, 1985; Bermúdez-Rattoni et al., 1998). In addition, the participation of the basolateral and central amygdaloid nucleus in associative processing of gustatory stimuli has been described (Bermúdez-Rattoni and Yamamoto, 1998). The amygdaloid complex receives gustatory, visceral and olfactory afferents (Norgren, 1974). Furthermore, the insular cortex and amygdaloid system are reciprocally and functionally interconnected (Kapp et al., 1985; Lasiter and Glanzman, 1985; Escobar et al., 1997).

Excitotoxic lesions of the NBM disrupt the ability to acquire taste aversion learning, implying the involvement of the basal cholinergic projecting system in memory processing of CTA (Lopez-García et al., 1993). However it remains to be tested whether the behavioural effects of this kind of lesion are exclusively due to the basal-cortical cholinergic component rather than to the basoamygdaloid pathway. The CTA thus offers an appropriate model for the analysis of the possible functional role of the ascending cholinergic pathway in cortically mediated learning mechanisms.

The following experiments were designed to reveal the relative contribution of both the basal cortical and basoamygdaloid projection in the mediation of taste aversion learning. In the first experiment, we used comparisons between 192IgG-saporin and the less specific NMDA-induced lesions directly into the NBM as a valuable tool for assessing the possible differential involvement of both ascending pathways. In the second experiment, by means of a combined lesion strategy we further inspected the possible interplay between the NBM-amygdala and NBM-cortex interaction on this behavioural paradigm.

EXPERIMENT 1

In this experiment we explore the possible differential contributions of either the basal cortical or basoamygdaloid projection in the mediation of taste aversion learning. To this end, we relate the behavioral effects with the extent of cholinergic deafferentation in both the insular cortex and amygdala following either bilateral injections of 192IgG-saporin or NMDA directly into the NBM.

Materials and methods

Subjects: Forty-nine male Wistar rats weighing 250-300 g were used in this experiment. They were individually caged and kept in a 12 hr light/dark cycle. All behavioral and biochemical manipulations were carried out in the light cycle phase.

Surgical procedure: Animals were anesthetized with sodium pentobarbital (65mg/kg) and placed in a head holder. Lesions were made by stereotaxic infusion of the toxin via a 30-gauge stainless steel cannula connected via teflon tubing to a 10µl glass microsyringe mounted in a microdrive pump. The stereotaxic coordinates used were anteroposterior, -0.8 mm from bregma; lateral, +/-2.5mm; and dorsoventral -7.5 mm. A total volume of 0.8 µl of a solution of either 0.2µg/µl of 192IgG-saporin (Chemicon, Tamecula. Ca.) or NMDA (10µg/µl, Sigma) in phosphate buffered saline was bilaterally infused at a constant rate of 0.5µl/min. The injector was left in place for 3min to allow proper diffusion.

Following the aforementioned surgical procedure, 18 animals (N-I) received bilateral microinjections of 192IgG-saporin directly into the NBM. Another group of 15 animals (N-E) received bilateral intra NBM injections of NMDA. An additional group of 16 animals remained unoperated during the whole procedure as an intact control group (CTR).

CTA Procedure: Two weeks after the surgery all the rats were prevented to drink and left without water for the following 24 hours. After that, they were given water in their home cages every 12 hours for 15 min and consumption was measured. When they reached an asymptotic consumption level, they received an acquisition trial. The presentation of a novel taste was done by adding saccharin to the water (1g/l). Fifteen minutes later, a malaise-inducing drug (LiCl, 0.15 M, 7.5ml/kg) was administered i.p. Subsequent drinking trials were performed with water only. After three drinking trials the subjects were presented with the saccharin flavored water for the second time and their consumption was used as a measure of strength of aversion.

Enzymatic assay: One day following the behavioral study randomly sampled animals obtained from each group were sacrificed by decapitation (N-I, n=10; N-E, n=7, and CTR, n=8). Samples of insular cortex, amygdaloid complex were disected under a stereoscopic microscope and stored at -70°C prior to analysis of ChAT activity. Dorsal striatum tissue samples were included as a non basal dependent cholinergic control. Each sample was sonicated in 1 ml of 25mM phosphate buffer containing 0.5% triton X-100 and maintained on ice to avoid over heating. The homogenate was centrifugated at 20000g for 15 min. ChAT activity in 200µl of the supernatant was assayed by adding 50µl of a substrate solution containing 10mM choline chloride, 0.2mM Neostigmine, 20mM EDTA and 0.4mM acetyl coenzime-A in 0.1M sodium phosphate buffer, pH 7.4, and incubated for 5 min at 37°C. Reaction was stopped by adding 10µl of 1M perchloric acid on ice and 1ml of distiled water. The mixture was passed through a 30000MW ultraspin filter (Cole-Parmer, IL) and stored at -70°C prior to chromatographic analysis.

Analysis of ACh levels: Samples were assayed for ACh levels using high performance liquid chromatography (HPLC) with electrochemical detection, using a mobile phase, pH 8.5, containing 50 mM sodium phosphate buffer and 0.5% Kathon reagent (BAS) microbicide. All samples were injected on a polymeric reversed phase column (BAS ACh-choline assay kit), ACh and choline were then converted into hydrogen peroxide and betaine in a postcolumn reactor containing immobilized acetylcholinesterase and choline oxidase (BAS). A choline oxidase/catalase reactor (BAS) was added in order to avoid choline detection in the substrate solution. The hydrogen peroxide was detected electrochemically with a platinum electrode set at 500mV (vs. Ag /AgCl). The detection limit, defined as the amount of ACh producing a peak twice the basal noise, was approximately 0.2 pmol.

Histology: Immediately after the behavioral study, five animals were randomly selected for each of the N-I , N-E and CTR

groups respectively. Animals were perfused and 40μm sections were obtained. Acetylcholinesterase histochemistry was then carried out according to a protocol modified from Paxinos and Watson (1982). Briefly, sections were incubated overnight in 50 mM sodium acetate buffer, pH 5.0, 4 mM copper sulfate, 16 mM glicine, 4 mM acetylthiocholine iodide, and 0.1 mM ethopropazine. After incubation the slides were immersed into a developing solution (1% sodium sulfide, pH 7.5) for 10 min. Following cholinesterase histochemistry, in order to obtain a quantitative estimation of the effects of intrabasal NMDA or 192IgG-saporin-induced lesions upon AChE-positive fibers in the basolateral amygdala (BLA), computer images of the basolateral amygdala were directly acquired using a DDC camera coupled to a light microscope. After the automatic segmentation of the BLA, mean color density of this nucleus was obtained using the standard 256 levels gray density scale. Six sections throughout the BLA nucleus were bilaterally analyzed per brain. The average value of mean gray level among sections was determined for each brain.

The basal forebrain cholinergic cells have been shown to stain intensely for acetylcholinesterase (AChE), and show a particularly rapid recovery of enzyme activity following systemic administration of the irreversible inhibitor di-isopropylfluorophosphate (DFP). This procedure easily reveals the cholinergic somata of the basal forebrain and their proximal processes to an appreciable extent. Therefore, 3 animals from each of the N-I, N-E and CTR groups respectively were subjected to this analysis. DFP-cholinesterase pharmacohistochemical regime was carried out according to the protocol of Bigl et. al. (1982). Briefly, animals were injected intramuscularly with 1.8mg/kg DFP (Calbiochem,inc.; La Jolla, CA) 2 hours prior to perfusion. Once the brains were obtained and cut, mounted sections were subjected to the normal acetylcholinesterase histochemestry described above .

Results

CTA

Simple ANOVA was done on the test day consumption volume for all groups. No differences were found in the baseline water intake among groups ($F_{2,46}= 0.325$, $p>0.05$). Mean base line water intake was 15.08±0.64, 15.74±0.85 and 14.95± 1.13 for each of the CTR, N-I and N-E groups respectivelly . As can be seen in figure 1, during the acquisition trial, no differences in saccharine consumption (the novel gustatory stimulus) were found among groups ($F_{2,46}= 0.24$, $p>0.05$). During the test presentation of saccharine solution significant differences were found among groups ($F_{2,46}=43.94$, $p<0.01$). A *post hoc* pairwise Fisher test showed that only the NMDA-induced lesions resulted in a significant disruption in the acquisition of taste aversion as indicated by the increased saccharine consumption when compared with the control and immunotoxin-lesioned groups (p's<0.01). The 192IgG-Saporin-treated group showed no disruption of taste aversion as compared with the intact control group. The behavioral difference observed after either treatment further implies a neurotoxin-specific effect.

ChAT activity

Simple ANOVAs were used for comparisons of ChAT activity among groups and *post hoc* pairwise Fisher test where appropriate. As can be seen in figure 2 both NMDA and 192IgG-saporin induced lesions displayed markedly reduced ChAT activity in the insular cortex relative to the control group ($F_{2,22}= 18.46$, $p<0.01$). However, the strongest reduction in enzimatic activity was apparent in the immunotoxin-lesioned group. Subsequent *post hoc* tests showed statistically significant differences between the intact control and both lesioned groups (p's<0.01). Additionally, significant differences were found between

both treated groups ($p<0.05$) implying that, at the used dose, 192IgG-saporin treatments result in a significantly stronger cholinergic deafferentation of the cortex as compared with that resulting from intrabasal microinjections of NMDA. As shown in figure 2 an inverse pattern of ChAT activity was apparent in the amygdala. Significant differences among groups were found ($F_{2,22}=6.36$, $p<0.01$). The corresponding *post hoc* tests showed that only the NMDA treated group resulted in a significant reduction in the amygdaloid ChAT activity as compared with the intact control ($p< 0.01$), whereas no significant reduction in ChAT activity in the amygdala was detected in the immunotoxin treated group. No effect was found in the dorsal striatum following either treatment ($F_{2,22}= 0.38$, $p>0.05$).

Confirmation of the lesions

The location and extent of the cholinergic lesions were confirmed in all experimental conditions used by means of cholinesterase and the DFP cholinesterase pharmaco histochemical regime. Figure 3 shows the cholinesterase fiber staining in the cortex for intact controls (3A), as well as for both 192IgG-saporin (3B) and NMDA-induced intrabasal lesions (3C). Note the relative difference in AChE staining in both lesioned groups when compared with the control staining. As can be seen in figure 4A, B, and C both immunotoxic lesions and NMDA induced lesions into the NBM resulted in a strong reduction of AChE positive somata in the basal forebrain as compared with the control staining. Although the above described enzymatic assay of ChAT activity was restricted to a tissue sample of the amygdaloid complex it can be argued, given the close proximity of the amygdaloid nuclei, that the cholinergic effects do not necessarily reflect the biochemical status of the basolateral nucleus itself. Therefore, in figure 4D, E and F we compared the effects of intrabasal NMDA-induced excitotoxic lesions and 192IgG-saporin injections on cholinerterase fiber staining in the BLA. Subsequent color density analysis showed a strong significant difference between the BLA staining in the NMDA lesioned animals when compared with the control group ($F_{2,12}= 8.83$, $p< 0.01$, see Table 1). In contrast, 192IgG-saporin lesions into the NBM did not produce any detectable effect in the cholinergic marker when compared with the control group. These data confirm previous findings regarding the effects of intrabasal excitotoxic and immunotoxic lesions upon the NBM-BLA pathway (Heckers & Mesulam, 1994; Heckers et al., 1994; Mallet et al., 1995).

EXPERIMENT 2

As shown in the previous experiments, in spite of an even strong cholinergic deafferentation of the IC, immunotoxic lesions into the NBM do not affect CTA learning. Conversely, excitotoxic lesions of the NBM impair taste aversion learning. Similar discrepant results with respect to both excitotoxic and immunotoxic lesions have been repeatedly found in other learning tasks as spatial water maze, radial maze, inhibitory avoidance, etc. (see Wenk et al., 1994; Berger-Sweeney et al., 1994; Torres et al., 1994; Baxter et al., 1995; Waite and Thal, 1996; Wenk et al., 1996). Here, we have found a better correlation between severity of memory impairment and simultaneous deafferentation of the cortex and amygdala. These results are consistent with previous findings according to which those excitotoxins reported to produce the greatest mnemonic deficits, also produced the largest decreases in amygdaloid ChAT (Beninger et al., 1994; Mallet et al., 1995). Additionally, in agreement with Heckers and Mesulam, (1994); and Heckers et al., (1994) we have found that 192IgG-saporin lesions produce an efficient and selective deafferentation of the rat neocortex but selectively spares an important population of basolateral amygdala projecting fibers. Taken together, these data might support the view of a primary role for the basoamygdaloid interaction on the regulation of memory formation exerted by the basal forebrain. Should this be the case, one would expect BLA lesions to disrupt CTA learning. However, excitotoxic lesions applied into the BLA do not disrupt taste aversion learning (Dunn and Everitt, 1988;

Bermúdez-Rattoni and McGaugh, 1991). A possible solution for this paradox is that the modulation of learning processes exerted by the basal forebrain might be simultaneously carried out by both the basal-cortical and basoamygdaloid pathway.

To test this hypothesis, we assessed the effects of i) NMDA-induced lesions into the NBM, ii) 192IgG-saporin injections into the NBM, or iii) combined NMDA lesions into the BLA with 192IgG-saporin injections into the NBM, using the experimental groups described in Table 2.

Materials and methods

Subjects: Forty eight male rats weighting 250-300 g were used in this experiment. They were kept and maintained as described in Experiment 1.

One group of animals received bilateral injections of 192IgG-saporin into the NBM following the same procedure and coordinates indicated in Experiment 1 (N-I; n=8). Another group of animals (A-E; n=8) received NMDA-induced lesions into the BLA using the following stereotaxic coordinates from bregma: anteroposterior -1.8 mm; lateral, +/- 4.7mm, and dorsoventral -8.3 mm. A total volume of 0.8µl, of NMDA (10µg/µl,) was bilaterally infused at a constant rate of 0.5µl/min. A third group (N-I/A-E; n=8) received both intra amygdala NMDA-induced lesions and intrabasal 192IgG-saporin microinjections in the same dose and coordinates indicated in the previous experiment. The fourth group of animals (N-E; n=8) received bilateral intra NBM injections of NMDA. Another group (N-V/A-V; n=7) received bilateral vehicle injections (0.8µl of phosphate buffered saline) in both BLA and NBM. An additional group remained unoperated as an intact control group (CTR; n=9). Following a postoperative period of two weeks all the animals were subjected to taste aversion training according to the same procedure described in the previous experiment.

Histology. Brains were processed for standard acetylcholinesterase histochemistry and the DFP cholinesterase pharmaco histochemical regime (not shown), as described in the previous experiment. Immunohistochemistry for p75 NGF receptor detection was carried out using the standard avidin biotin ABC procedure (Hsu, Raine & Fanger, 1981). Anti-p75 monoclonal antibodies (1:500) were obtained from Boerhinger Manheim (Germany).

Results:

CTA

Simple ANOVA was done on the test day consumption volume for all groups. No differences were found in the baseline water intake among groups ($F_{5,42}$= 0.57, p>0.05). Mean base line water intake was 14.94±0.69, 15.94±1.23, 14.87± 0.61, 17.25± 1.0, 17.55± 1.21 and 17.47± 0.52 for each of the CTR, N-I, A-E, N-I/A-E, N-E and N-V/A-V groups respectively . Again, during the acquisition trial no differences in saccharine consumption were found among groups ($F_{5,42}$= 0.66, p>0.05). As shown in Figure 5, during the test presentation of the saccharin solution, significant differences were found among the six groups ($F_{5,42}$= 19.368, p< 0.01). A *post hoc* pairwise Fisher test showed that NMDA induced lesions into the NBM (N-E) as well as the combined NMDA lesions into the BLA with 192IgG-saporin injections into the basal forebrain (N-I/A-E) had a significant disruption in the acquisition of taste aversion as indicated by the increased saccharine consumption when compared with the control and vehicle treated groups on the retention trial (p's<0.01). Neither the amygdala lesioned group (A-E), the group that only received immunotoxin lesions into the NBM (N-I), nor the vehicle treated group (N-V/A-V) showed any impairment in the ability to acquire CTA as compared with the intact control group.

Confirmation of the lesions

All intrabasal NMDA and 192IgG-saporin induced lesions were again verified by means of DFP Cholinesterase pharmaco histochemical regime (in order to avoid repetition these fotomicrographs are not shown). The specific destruction of the low affinity NGF receptor-positive cholinergic cells in the basal forebrain, due to 192IgG-

saporin microinjections, was verified by means of p75 immunohistochemistry. Samples are shown in Fig.6A and D. The correct placement of NMDA lesions into the NBM and BLA was verified by means of cholinesterase histochemistry (Fig. 6 B,C and E, F).

DISCUSSION

In the present experiments, we started from the fact that NMDA injections into the NBM result in a strong disruption of CTA learning, whereas 192IgG-saporin lesions into the NBM do not. The existing discrepancy between both kinds of lesions can be explained in two ways. First, cholinergic neurons in the NBM are interspersed with other neurons in the basal forebrain, including GABAergic neurons of the dorsal and ventral pallidum and noncholinergic magnocellular corticopetal neurons. The use of excitotoxins, specially those acting through NMDA receptors such as NMDA and ibotenic acid, can also destroy noncholinergic pallidal and other neurons in the substantia innominata, whereas there seems to be little doubt that 192IgG-saporin is a powerful and selective lesioning tool for basal cholinergic neurons, which are enriched in the low-affinity nerve growth factor receptor. Second, different excitotoxins when injected into the NBM, produce differential effects on cholinergic projections to the cortex and amygdala (Boegman et al., 1992). That is, those excitotoxins previously reported to produce the greatest mnemonic deficits, also produced the largest decreases in the basolateral amygdaloid ChAT (Beninger et al., 1994; Mallet et al., 1995). In contrast, virtually all of the cortically projecting cholinergic cells within the NBM are vulnerable to the immunotoxin, with the exception being those cholinergic neurons that send an efferent projection to the basolateral amygdala (Heckers and Mesulam, 1994; Heckers et al., 1994).

Following intrabasal microinjections of 192IgG-saporin, no detectable disruption in taste aversion learning was found in the acquisition and performance of this well known cortically mediated learning paradigm. Previous results from our laboratory (Gutiérrez et al., in press) demonstrate that IC mediated associative processes involved in taste aversion learning can be carried out in spite of up to 86% reduction in mean ChAT activity and at least 97% reduction in extracelular acetylcholine release as assesed by intracortical *in vivo* microdialysis. This result is consistent with previous reports according to which, in spite of the massive reduction of cortical cholinergic input, intraparenchimal treatments carried out using this immunotoxin have repeatedly failed to reproduce the kind of memory deficits normally found as a result of the less selective excitotoxic lesions (Berger-Sweeney et al., 1994; Torres et al., 1994; Wenk et al., 1994; Baxter et al., 1995; Waite and Thal, 1996; Wenk et al., 1996; Dornan et al., 1997). In our present study, subsequent monitoring of cholinergic markers in the basolateral amygdala confirms the sparing of amygdaloid projecting fibers following the 192IgG-saporin treatment. In contrast, NMDA-induced lesion into to basal forebrain do affect cholinergic projections to both the cortex and basolateral amygdala. However, consistent with previous findings comparing several excitotoxins versus 192IgG-saporin (Waite and Thal, 1996), NMDA microinjections into the basalforebrain resulted in significantly less cholinergic loss in the cortex than that obtained by the administration of 192IgG-saporin. This result directly implies that learning deficits obtained after NMDA-induced lesions into the basal forebrain can not be the sole result of cortical cholinergic hypofunction, since a much stronger and selective reduction in cortical acetylcholine does not result in aversion learning deficits. Taken together, these results prompted us to hypothesize an additional involvement of the basoamygdaloid projection in the regulation of memory processes exerted by the basal forebrain.

There seems to be little doubt concerning the involvement of the amygdaloid complex (particularly the basolateral and central nucleus) in associative processing of gustatory stimuli (Bermúdez-Rattoni and Yamamoto., 1998). However, NMDA and ibotenic acid-induced lesions in

the BLA alone, in spite of the extensive destruction of this nucleus, fail to produce any detectable deficit in CTA acquisition and retention (Bermúdez-Rattoni and McGaugh, 1991; Chambers, 1990; Dunn and Everitt, 1988). Given the differential effects of 192IgG-saporin and NMDA-induced lesions into the basal forebrain upon CTA acquisition, we suggest that the cholinergic modulation of learning processes exerted by the basal forebrain might be redundantly carried out by both the basal cortical and the basoamygdaloid pathway. Should this be the case, one would expect the combined deafferentation of the cortex and the excitotoxic ablation of BLA (hence the disruption of the putative NBM-BLA function) to result in the same learning deficits normally found as a result of the less selective excitotoxic destruction of the NBM.

By combining NMDA lesions into the basolateral amygdala with 192IgG-saporin injections into the basal forebrain we have found a strong disruption of taste aversion learning, even when none of these treatments were by themselves capable of producing any detectable impairment in this learning task. This behavioral deficit was in fact only paralleled by the effect of intrabasal NMDA microinjections. The cooperative effects of both lesions suggest the possibility of a redundant scheme according to which the modulatory function exerted by the basal forebrain can be, to a given extent, facultatively carried out by both the NBM-amygdala and NBM-cortex pathway. Relevant cellular mechanisms have been documented to take place in both the insular cortex and amygdala during the early processing of a novel gustatory stimulus including cholinergic-dependent thyrosine phosphorylation of NMDA receptors (Rosenblum et al., 1995; Rosemblum et al., 1997), cAMP-mediated gene transcription (Dudai, 1987; Lamprecht et al., 1997), and PKC activity (Yasoshima and Yamamoto, 1997). Interestingly, combined ibotenic acid-induced lesions aimed at the basolateral amygdala and insular cortex have stronger disruptive effects on CTA learning than either treatment alone (Yamamoto et al., 1990; Yamamoto, 1993). These data further support the concept of a complementary and/or redundant role for both structures in the processing of gustatory information. Moreover, the fact that NMDA-induced lesions of the basal forebrain result in a strong learning disruption implies that the basal system is also involved in this CTA processing circuit. Since the insular cortex is ultimately needed for the taste aversion conditioning to be learned and expressed, the NBM-meditated associative processing of the gustative stimulus should at the end reach this cortical area.

Figure 7 shows a model of the proposed modulation exerted by the basal forebrain during taste aversion processing. The only two structures functionally linked to the cholinergic basal forebrain so far known to be involved in taste aversion conditioning are the amygdaloid complex and the insular cortex (Bermúdez-Rattoni and Yamamoto, 1998). Therefore, the disruptive effects of the basal excitotoxic lesion can be explained in terms of both ascending pathways. Taking into account that even a strong cortical deafferentation does not *per se* destroy the cortical function, and being on the other hand that the NMDA-induced lesion into the NBM also reduces cholinergic markers in the basolateral amygdala, our combined lesion study suggests that both ascending pathways are, to some extent, redundantly participating in CTA memory processing. It remains, however, to be directly tested whether the basal forebrain-amygdala interaction is in fact being carried out through the basoamygdaloid cholinergic projection fibers, rather than through an alternative indirect and/or non cholinergic pathway. A proper answer to these questions demands a detailed characterization of the basal forebrain-amygdala communication pathways, whether or not cholinergic.

It should be pointed out that these data do not necessarily imply a roughly equivalent role for the basal forebrain cholinergic modulation upon cortex and amygdala functions. Differences in the relative weight of both ascending projections are to be expected. Indeed, direct infusions of several muscarinic antagonists into the insular cortex have been shown to disrupt taste

aversion learning (Naor and Dudai, 1996), suggesting that the sole blockade of the cortical cholinergic input can by itself disrupt the cortical learning function. The fact that an almost complete cortical cholinergic depletion does not result in detectable learning deficits suggests that some residual cortical cholinergic activity is still sufficient for mnemonic function. In agreement with this, recent findings in our laboratory show that intracortical infusion of scopolamine in animals that previously received intrabasal microinjections of 192IgG-saporin result in a strong disruption of taste aversion learning. Taken together this data suggest a highly critical role for the basal cortical cholinergic pathway. On the other hand, the ablation of the BLA nucleus (and the corresponding disruption of the NBM-BLA-IC circuit) does not by itself disrupt aversion learning, thereby implying a less critical role for this pathway. According to our model, the involvement of the NBM-BLA interaction becomes evident as soon as *both* the basal cortical and basoamygdaloid pathways are compromised. In fact both ascending pathways might be participating in different processes cooperatively involved in learning mechanisms. It has been suggested that the NBM-amygdala projections have a role in retention of affective aspects of conditioning processes whereas the basal cortical projection might be contributing to attentional processes (Everitt & Robins, 1997). In this regard, recent findings have shown that although classical learning paradigms (i.e., radial maze, water maze, and taste aversion learning) are not sensitive to even a strong basal-cortical deafferentation (following intraparenchimal 192IgG-saporin treatments), more delicate attention-sensitive tasks show a contrasting critical dependence upon intact cortical cholinergic function (Chiba et al., 1995; Baxter et al., 1995). In the present study, we show evidence for an additional functional overlapping attained by both ascending projections, since functional deficits associated to experimentally induced cortical cholinergic hypofunction can be seemingly overcome by the NBM-BLA-cortex circuit and viceversa.

The results of this study extend previous work which investigated the effect of 192IgG-saporin and excitatory neurotoxins treatments into the NBM, and provide support to the notion that the basal forebrain-amygdala interaction participates in aversive conditionings. They further provide a framework that may help to solve the well documented discrepancies regarding the effects of different neurotoxins in basal forebrain lesion studies. Moreover, they suggest that the regulation exerted by the basal forebrain can be redundantly and additively carried out by both the basal cortical and the basoamygdaloid projection systems in adult normal rat.

Table 1. Color density analysis of AChE positive fibers in the BLA

CTR	N-I	N-E
198.08 ±9.75	196.304 ±5.67	155.93 ±8.11**

CTR: intact control group; N-I: intrabasal 192IgG-saporin-lesioned group: N-E: intrabasal NMDA-induced lesioned group. Values are mean gray scale levels ± standard error of mean; **p<0.01.

Table 2. Experimental groups used in the behavioral study of Experiment 2.

	Group					
	CTR (n=9)	N-I (n=8)	A-E (n=8)	N-I/ A-E (n=8)	N-E (n=8)	N-V / A-V (n=7)
NBM	—	192-IgG Saporin	—	192-IgG Saporin	NMDA	Vehicle
Amygdala	—	—	NMDA	NMDA	—	Vehicle

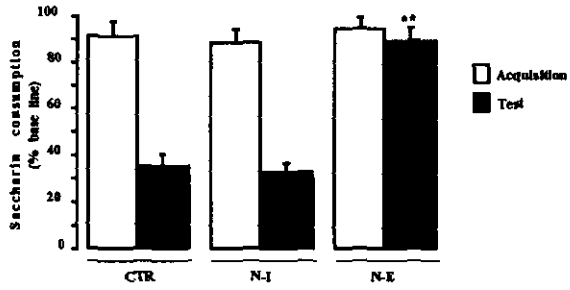

Figure 1. Effects of intrabasal 192IgG-saporin, or NMDA microinjections on taste aversion retention test. Aversion is expressed as mean (±SEM) percentage of baseline consumption during the retention trial. *CTR*, intact control; *N-I*, 192IgG-saporin lesioned group; *N-E*, NMDA-induced lesioned group. ** $p<0.01$ versus intact control. Comparisons between saccharin consumption values during the acquisition trial expressed as men (±SEM) percentage of baseline consumption are also shown

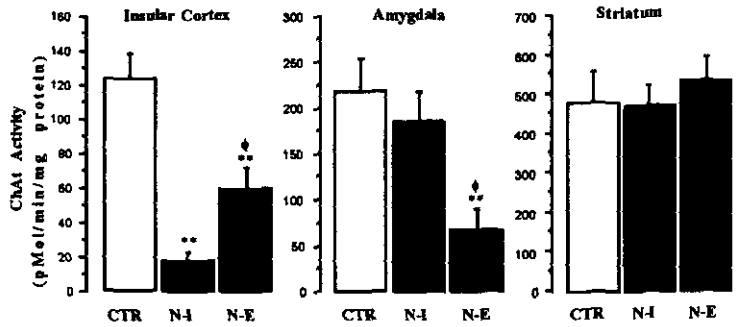

Figure 2. ChAT activity analysis in the isular cortex, amygdala and dorsal striatum for each of the groups used in experiment 1. *CTR*, intact control; *N-I*, 192IgG-saporin lesioned group; *N-E*, NMDA-induced lesioned group. Activity is expressed as mean pmol of acetylcholine formed/min/mg protein ±SEM. ** $p< 0.01$ against the intact control group. ϕ $p< 0.05$ against the immunotoxin lesioned group (only comparisons between both lesioned groups are shown, see results).

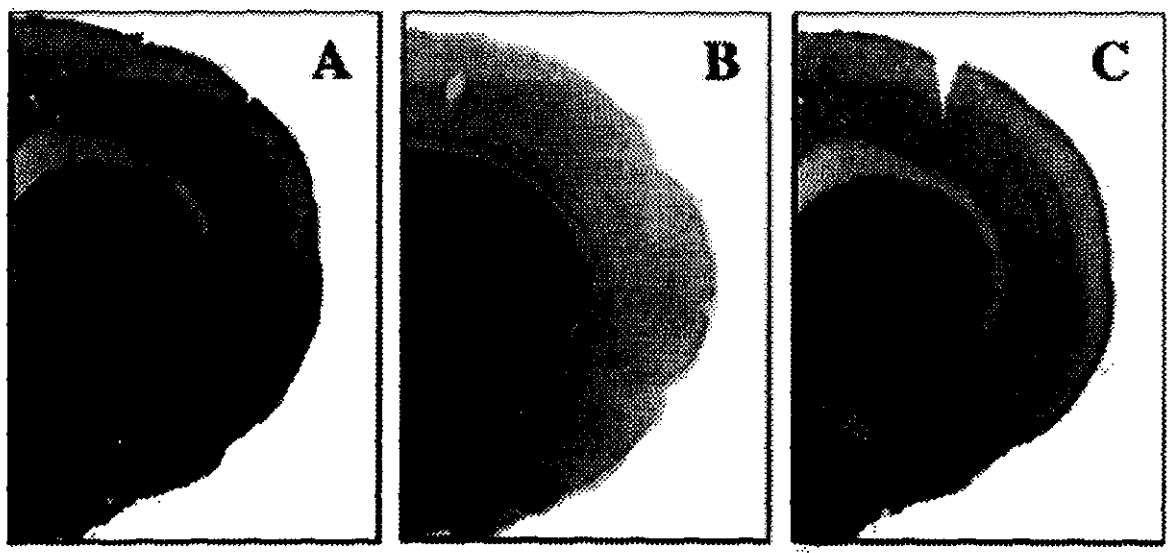

Figure 3. Photomicrographs of AChE histochemistry of coronal sections taken from a control brain (A), an immunolesioned brain (192IgG-saporin, B), and NMDA-lesioned brain (C). Note the reduction of cortical AChE staining in both lesioned brains as compared to the control brain.

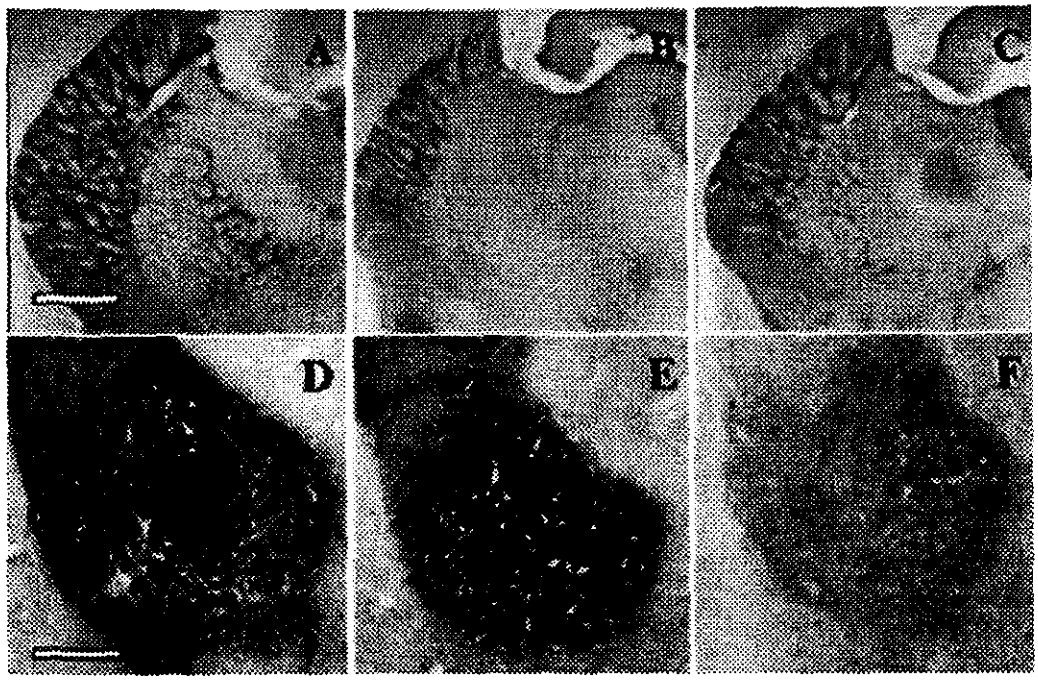

Figure 4. A-C photomicrographs taken at the level of the anterior nucleus basalis following DFP-Cholinesterase pharmacohistochemical regime. AChE-positive somata in the basal forebrain in a control brain (A), 192IgG-saporin treated brain (B) and NMDA-induced intrabasal lesion (C). Scale bar, 1mm. D-F photomicrographs taken at the level of the basolateral amygdaloid nucleus showing cholinesterase fiber staining in either control (D), intrabasal 192IgG-saporin treated (E) and intrabasal NMDA-induced lesioned brains. Scale bar, 200μm.

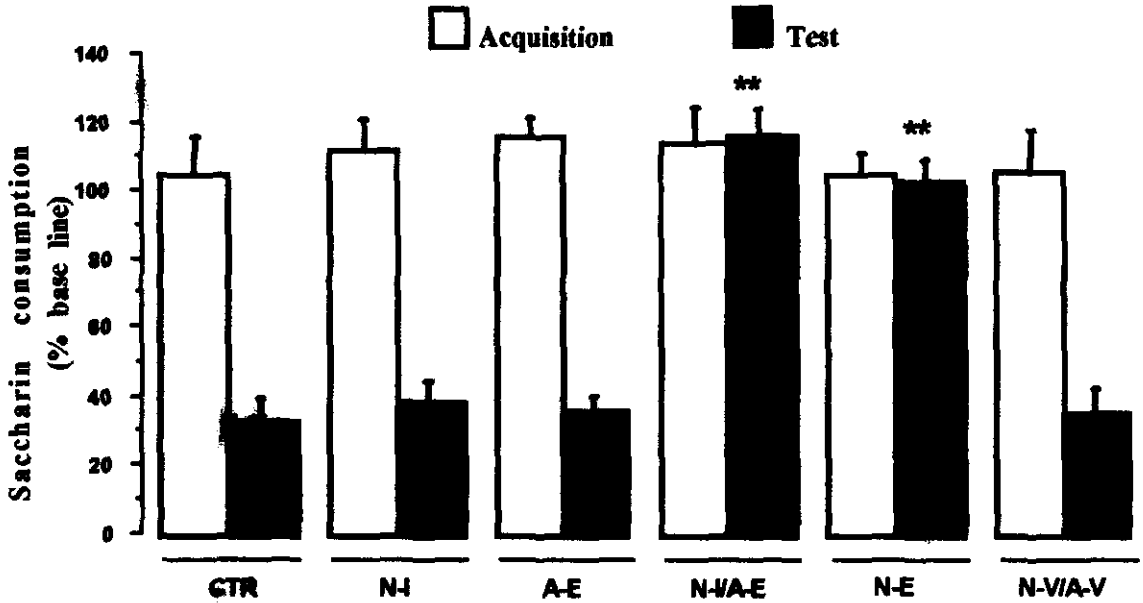

Figure 5. Effects of intrabasal 192IgG-saporin, intraamygdala NMDA-induced lesions and combined intrabasal immunotoxin and amygdala excitotoxic lesions on CTA retention test (see table 2). *CTR*, intact control; *N-I*, 192IgG-saporin lesioned group; *N-E*, NMDA lesioned group; *N-I/A-E*, Combination of intrabasal immunotoxic lesion with intraamygdala NMDA induced lesion; *N-V/A-V*, combined vehicle administration (see methods).** $p<0.01$ versus intact control.

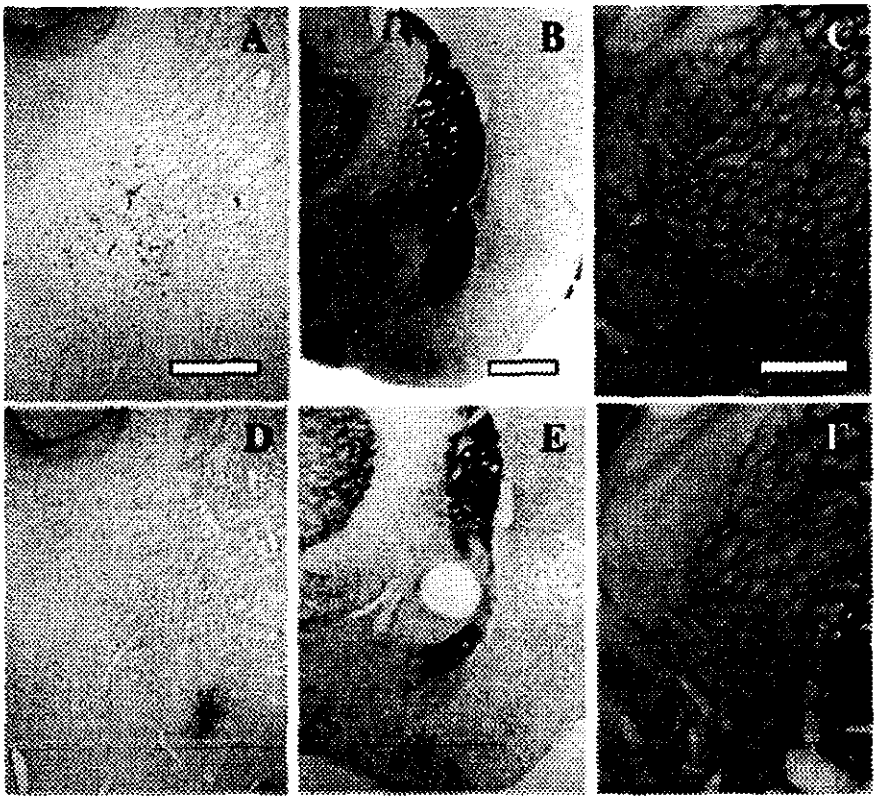

Figure 6. Confirmation of the location and extent of the lesions used in Experiment 2. *A and D*, p75 immunostaining of NBM magnocellular neurons in a control and immunolesioned brain respectively. Scale bar, 600μm. *B and E*, AChE histochemistry at the level of the basolateral amygdala in a control and NMDA-induced intraamgydala lesion respectively. Scale bar, 1mm. *C and F*, AChE histochemistry in the basal forebrain in a control and NMDA-induced intrabasal lesion respectively. Scale bar, 600μm.

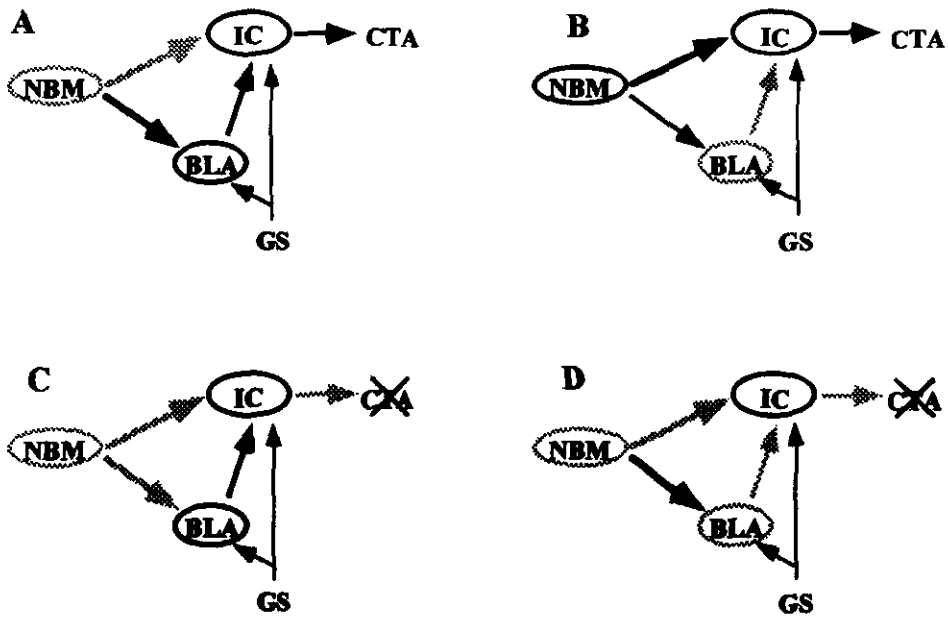

Figure 7. Schematic representation of the proposed model of the modulation of learning processes exerted by the basal forebrain (NBM), according to which this regulation can be redundantly carried out by both the basal cortical and basoamygdaloid interaction. BLA, IC and GS represent the basolateral amygdala, insular cortex and gustative stimulus respectively. A) Effective modulation of taste aversion learning (CTA) after a strong reduction of basal-cortical cholinergic input. B) CTA is still acquired following BLA excitotoxic lesions and the subsequent disruption of the NBM-BLA-IC circuit. C) Defective basal forebrain modulation of CTA learning due to excitotoxic lesioning of the NBM and subsequent simultaneous (though partial) deafferentation of both the cortex and BLA. D) Combination of A and B conditions resulting in the functional inactivation of both modulatory pathways

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Conclusión

El sistema basal ejerce una indiscutible influencia sobre los procesos asociativos que tienen lugar en sus áreas de proyección (Sinden, Hodges y Gray, 1995). Sin embargo hasta ahora se desconoce la naturaleza precisa de dicha modulación. En acuerdo con este y previos estudios, la destrucción generalizada de los núcleos de la región basal resulta en una consistente y notoria disfunción de aprendizaje en numerosos modelos conductuales (Sinden et al., 1995; Wenk, 1997). Sin embargo la inactivación específica de la proyección colinérgica basalo-cortical en ningún caso da lugar a los mismos déficits de aprendizaje (Berger-Sweeney et al., 1994; Torres et al., 1994; Wenk et al., 1994; Baxter et al., 1995; Waite and Thal, 1996; Wenk et al., 1996).

En lo que respecta al modelo de condicionamiento aquí utilizado, la importancia de la proyección colinérgica basalo cortical se volvió a hacer evidente, tan pronto se verificó una inactivación simultánea de la amígdala basolateral. Siendo que cada manipulación por sí sola, carece de efectos adversos en la habilidad de los animales de adquirir el condicionamiento aversivo, el resultado descrito sugiere un esquema redundante según el cual *cierto componente crítico* de la modulación ejercida por la región basal tiene lugar a través tanto de la interacción NBM-CI como de la interacción NBM-ABL (ver figura 7, pag 37).

Sin embargo, es conveniente en este punto detenerse un momento en las implicaciones teóricas de esta conclusión.

Tomando en cuenta la masiva reducción colinérgica obtenida como resultado de las lesiones inmunotóxicas, podría en primera instancia sugerirse –a la luz de los datos aquí mostrados- que la proyección NBM-ABL es sencillamente capaz, por sí sola, de substituir *en su totalidad* la función de la proyección basalo cortical. Sin embargo, si se considera la enorme extensión de la proyección colinérgica hacia la corteza en general y se compara con la reducidas dimensiones de la proyección basalo amigdalina (sin mencionar las dimensiones del propio blanco), la presunción de una simple duplicación funcional por parte de ambos sistemas se vuelve considerablemente improbable. A ello habría que añadir el costo evolutivo de tan espúrea redundancia: ¿Cómo habría de

conservarse a lo largo de la evolución una función trivialmente duplicada?

Sin embargo, los resultados obtenidos al combinar la lesión inmunotóxica con la inactivación reversible de receptores muscarínicos en la corteza insular, directamente implican la participación necesaria y suficiente de niveles residuales de acetilcolina en los mecanismos corticales involucrados en la adquisición del condicionamiento aversivo (esto, siempre y cuando cierta función amigdalina permanezca intacta). Si convenimos en esto, la conclusión más conservadora sería precisamente que existe cierta sobreposición funcional, entre la proyección basalo cortical y la interacción basaloamigdalina. Al menos con respecto a cierta proporción de actividad colinérgica modulatoria

Partiendo, por lo tanto, del prejuicio de que la modulación colinérgica no se encuentra simplemente y por completo duplicada (más adelante se discute la evidencia que justamente apoya este prejuicio), la siguiente conclusión más simple que se obtiene del presente estudio es que la mayor parte o al menos una proporción considerable del componente colinérgico sencillamente participa en otras funciones corticales que no forman parte de procesos mnemónicos *per se*.

En efecto, la deaferentación colinérgica casi total de la corteza carece de efectos sobre cualquier prueba conductual de aprendizaje y memoria hasta ahora ensayada. Y, a menos que supongamos que toda esta actividad se halla en simple exceso, habrá que buscar evidencia de su significado funcional en algún otro lado.

Papel del sistema colinérgico

Una serie de estudios llevados a cabo en el laboratorio muestran que la activación colinérgica en la corteza insular se da como resultado de la presentación de un estímulo gustativo novedoso. Esta activación es decreciente durante subsecuentes presentaciones del mismo estímulo y ausente durante la expresión del recuerdo (Miranda y Bermúdez-Rattoni, 1999). Este efecto coincide con estudios previamente reportados que muestran la misma clase de activación colinérgica en la corteza frontal de la rata durante estimulación manual del animal (Rosenbland y Nilsson, 1993). En otros reportes, el monitoreo de la liberación de acetilcolina en la corteza visual y auditiva de la rata muestra por su parte una clara activación exclusivamente como resultado de la presentación de estímulos auditivos y visuales novedosos o previamente asociados a intensos estímulos aversivos (condicionamiento de miedo, Bakin y Weinberger, 1996). En contraste con ello, ninguna respuesta colinérgica se pudo observar tras la presentación de los mismos estímulos cuando han sido previamente

habituados (Bakin y Weinberger, 1996). Todo ello apunta fuertemente hacia la idea de que el sistema de proyección colinérgico, de alguna manera modula la actividad cortical durante cierto procesamiento de estímulos *novedosos* o en algún sentido conductualmente relevantes (asociados, por ejemplo a intensos estimulos aversivos).

Si bien la activación colinérgica observada en los estudios mencionados, sólo muestra una correlación con las características de novedad o importancia de un estímulo recientemente presentado, esta clase de estudio representa un buen punto de inicio, ya que se hace improbable suponer que esta actividad sea sólo un subproducto e inocuo de procesos verdaderamente importantes llevándose a cabo en algún otro lado del cerebro; sobre todo tomando en cuenta la masiva extensión del sistema de proyección colinérgico basalo cortical.

Recientemente, en un interesante estudio se ha observado que la estimulación eléctrica del NBM simultáneamente con la presentación de un determinado estímulo auditivo, da lugar a una reorganización del área de representación auditiva en favor de la frecuencia utilizada. Esto es, se verifica una expansion del área cortical de representación de la frecuencia en cuestión. Impresionantemente, esta expansión en el área de representación de la frecuencia "señalizada", se impide mediante la destrucción específica de la proyección colinérgica basalo cortical (Kilgard y Merzenich, 1998). En la misma línea, la eliminación de la mayoría de las vibrisas de la rata da lugar a la misma clase de reorganización en la representación somatotópica de las vibrisas restantes (Zhu y Waite, 1998). Una vez más esta reorganización se impide mediante la destrucción selectiva de las neuronas de proyección colinérgica hacia la corteza. Con base en esta clase de estudios se ha propuesto que la acetilcolina cortical podría estar involucrada en procesos relacionados con la detección o asignación preferencial de recursos de procesamiento en favor de estímulos sensoriales conductualmente relevantes (novedosos o asociados a estumulos aversivos o reforzantes).

Everitt (1997) ha mostrado mediante lesiones con AMPA (que destruye preferencialmente a las neuronas de proyección hacia la corteza), que la disfunción colinérgica en la corteza resulta en marcados déficits en pruebas de atención visual, dejando intactas las funciones mnémicas *per se*. En la misma línea el grupo de Gallagher ha mostrado que las lesiones selectivas del sistema de proyección colinérgica hacia la corteza, da lugar a considerables dificultades en la habilidad para condicionar subsecuentemente estímulos que previamente fueron asociados (sin mayor dificultad) a contingencias conductualmente

relevantes (en el sentido sugerido arriba., ver Baxter, Holland y Gallagher, 1997).

Todo lo anterior en su conjunto pareciera apuntar hacia la idea de que la actividad colinérgica cortical está en gran medida involucrada en la modulación de la eficiencia cortical del procesamiento de estímulos conductualmente relevantes, *salientes o novedosos*.

Finalmente es interesante en este punto contrastar la información aquí expuesta con los datos presente en la literatura sobre otros importantes sistemas de proyección cortical. Destacan entre estos los sistemas de proyección dopaminérgica y serotoninérgica. Con respecto a ellos, se ha propuesto que ambos neurotransmisores además del sistema colinérgico representan neuromoduladores de funciones integrativas llevadas a cabo en la corteza (Hasselmo 1995). Se ha documentado, por ejemplo, la modulación, por dopamina, de receptores de NMDA en la corteza prefrontal, durante pruebas de alternancia diferida (Verma & Moghaddam 1996). En particular la modulación dopaminérgica en tareas de memoria de trabajo mediadas específicamente por recepores tipo D1 en la corteza prefrontal (Muller, Von Cramon & Pollman, 1998; Seamans, Floresco & Phillips, 1998). Mediante pruebas de microdiálisis *in vivo* se ha demostrado que la liberación extracelular de dopamina en la corteza prefrontal en primates se verifica específicamente durante tareas de alternancia diferida, no siendo el caso en pruebas controles de conducta guiada por estimulación sensorial (Watanabe, Kodama & Hikosaka, 1997).

Con respecto a la posible modulación serotoninérgica, se ha observado que ésta y la dopamina podrían interactuar jugando papeles antagónicos en la corteza en pruebas de memoria de trabajo (Luciana, Collins & Depue, 1998). En la misma línea se ha reportado que la excesiva liberación de serotonina cortical da lugar a impedimentos en pruebas de memoria, dependientes normalmente de una adecuada liberación dopaminérgica (Santucci, Knott & Haroutunian, 1996).

Los paralelismos observados entre estos sistemas apuntan en la dirección de una compleja interacción modulatoria de funciónes corticales llevada a cabo por estos tres sistemas de proyección: colinérgico, dopaminérgico y serotoninérgico (Hasselmo, 1995). En este sentido, atrae la atención el hecho de que se ha documentado efectivamente que la administración de agonistas dopaminérgicos tipo D1 promueven la liberación cortical de acetilcolina mejorando el desempeño en pruebas de memoria (Steele, Hodges, Levesque, Locke & Sandage, 1996). Sin embargo, es necesario reconocer que la naturaleza precisa de la modulación ejercida por estos sistemas de proyección, así como su particular participación en las funciones asociativas

mediadas por la corteza en general representan problemas aún no resueltos.

Los resultados aquí mostrados por una parte arguyen en favor de una función no mnémica para una determinada proporción de la actividad colinérgica cortical. Por otro lado, revelan la existencia de un importante componente colinérgico involucrado (redundantemente) en funciones necesarias para los mecanismos asociativos *per se*.

Sin embargo, hacen falta estudios subsecuentes para determinar la naturaleza precisa de la interacción NBM-amígdala-corteza, y su particular participación en los mecanismos cerebrales de aprendizaje y memoria.

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