UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

INSTITUTO DE FISIOLOGIA CELULAR

EFECTOS DEL FACTOR DE CRECIMIENTO NEURONAL SOBRE LA RECUPERACION CONDUCTUAL MEDIADA POR TRANSPLANTES NEOCORTICALES

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INDICE

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RESUMEN DE LA TESIS DOCTORAL:

EFECTOS DEL FACTOR DE CRECIMIENTO NEURONAL SOBRE LA RECUPERACION CONDUCTUAL MEDIADA POR TRANSPLANTES NEOCORTICALES

PRESENTADA POR:

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RESUMEN

En nuestro laboratorio se ha demostrado que transplantes homotópicos fetales de corteza insular (CI) producen recuperación en la capacidad de adquirir el Condicionamiento Aversivo a los Sabores (CAS), en ratas que previamente la habían perdido debido a lesiones de la CI. Existen evidencias en el sentido de que la CI presenta actividad colinérgica considerable, así como de que el bloqueo farmacológico de la transmisión de acetilcolina (ACh), provoca trastornos en la adquisición del CAS. Algunas observaciones efectuadas en nuestro laboratorio señalan que los transplantes homotópicos neocorticales que promueven la recuperación del aprendizaje, liberan ACh, en tanto que en los transplantes heterotópicos que no inducen recuperación funcional no se registra liberación del citado neurotransmisor. La serie de investigaciones que forman parte del presente trabajo tiene como objeto el conocer la participación de las moléculas tróficas involucradas en la promoción de la sobrevivencia, integración y capacidades funcionales de los transplantes de CI, como parte de los procesos de regeneración neuronal. En particular el factor de crecimiento neuronal (FCN), ha sido ampliamente estudiado en relación al restablecimiento de las neuronas colinérgicas del sistema nervioso central, las cuales están involucradas en los procesos de aprendizaje y memoria. En la primera etapa de esta serie de estudios, efectuamos un análisis conductual y citoarquitectónico, siguiendo el curso temporal (15, 30, 45 y 60 días) de desarrollo de los transplantes, en ausencia de FCN, mostrando que la recuperación conductual comienza a manifestarse a partir de los 30 días de desarrollo post-transplante, alcanzando su mejor expresión hacia los 60 días. En otra fase del presente proyecto, se demostró la participación de la proyección colinérgica entre el nucleo basalis magnocellularis y la CI, en la integración neural de la información gustativa. Los estudios efectuados en nuestro laboratorio combinando implantes fetales de CI con el FCN, mostraron que la asociación de ambos elementos produce una aceleración de los procesos de recuperación de la capacidad de aprender que se manifiesta a partir de los 15 días de desarrollo post-transplante, en

ratas previamente lesionadas en la CI. Posteriormente, llevamos a cabo el análisis *in vivo* de la actividad colinérgica de transplantes homo (CI) y heterotópicos (corteza occipital), encontrando diferencias significativas entre ambos. El análisis de la conducta de estos grupos mostró que los transplantes heterotópicos a diferencia de los homotópicos, no produjeron recuperación conductual. Esto sugiere que la especificidad de los tejidos transplantados es un factor importante para el restablecimiento tanto de la actividad colinérgica de los implantes como de las manifestaciones conductuales que subyacen a la integración de los mismos.

Finalmente, nuestros hallazgos muestran también que la actividad colinérgica (medida *in vivo* e *in vitro*) a diferencia de la GABAérgica, juega un importante papel en la recuperación conductual mediada por los transplantes de CI en combinación con el FCN.

ABSTRACT

We have shown that insular cortex (IC) grafts induce recovery on the ability to acquire a conditioned taste aversion (CTA) on previously IC lesioned animals. We have demonstrated that choline acetyltransferase (ChAT) activity, in the IC is higher, than in other cortical areas, and that IC-grafts acetylcholine (ACh) release is correlated with the recovery of CTA. In the present work we evaluated the role of the nerve growth factor (NGF) in the behavioral recovery induced by IC grafts. NGF promotes survival and growth of cholinergic neurons in the central nervous system, which are involved in learning and memory processes. In the first part of the present work we demonstrate that in absence of NGF, the behavioral recovery starts 30 days after grafting and reaches its best expression at 60 days when the best signs of reconnectivity and maturity of the grafts were found. In other part of this project we showed that the nucleus basalis magnocellularis is involved in the neural integration of feeding behavior and that its cholinergic projection to the IC is one of the implicated neurotransmitter systems. The behavioral data obtained when IC grafts were supplemented with NGF indicates that at 15 days post-graft the group with homotopic IC grafts in combination with NGF promotes recovery of the ability to acquire, the CTA. This indicates that the administration of NGF in the cortical graft significantly affects the functional recovery observed at 15 days post-graft.

Our biochemical analyses showed that cholinergic activity in the IC-grafts plus NGF was similar to that in the IC of intact control animals, whereas in the IC grafts plus vehicle it was considerably reduced. Furthermore studies using *in vivo*, assays of ChAT and ACh levels have demonstrated that IC but not heterotopic grafts with NGF reestablish cholinergic activity.

In addition, measurement of glutamate decarboxylase (GAD) activity, at 15 days post-graft, in contrast to ChAT, did not show any significant differences between groups. These results suggest that

GABA mediated neurotransmission does not play an important role in graft-promoted mediated behavioral recovery.

The present findings suggest that NGF when associated simultaneously with homotopic IC-grafts accelerates recovery of learning abilities in IC-lesioned rats and the reestablishment of ChAT activity in the grafts at 15 days post-graft.

RESUMEN

En nuestro laboratorio se ha demostrado que transplantes homotópicos fetales de corteza insular (CI) producen recuperación en la capacidad de adquirir el Condicionamiento Aversivo a los Sabores (CAS), en ratas que previamente la habían perdido debido a lesiones de la CI (Bermúdez-Rattoni y col, 1987; Escobar y col, 1989). Existen evidencias en el sentido de que la CI presenta considerable actividad colinérgica, así como de que el bloqueo farmacológico de la transmisión de acetilcolina (ACh), provoca trastornos en la adquisición del CAS (Woolf y Butcher, 1982; Bermúdez-Rattoni y col, 1983; López-García y col, 1990a). Algunas observaciones efectuadas en nuestro laboratorio señalan que los transplantes homotópicos neocorticales que promueven la recuperación del aprendizaje, liberan ACh, en tanto que en los transplantes heterotópicos que no inducen recuperación funcional no se registra liberación del citado neurotransmisor (López-García, 1990b). La serie de investigaciones que forman parte del presente trabajo tiene como objeto el conocer la participación de las moléculas tróficas involucradas en la promoción de la sobrevivencia, integración y capacidades funcionales de los transplantes de CI, como parte de los procesos de regeneración neuronal. En particular el factor de crecimiento neuronal (FCN), ha sido ampliamente estudiado en relación al restablecimiento de las neuronas colinérgicas del sistema nervioso central, las cuales están involucradas en los procesos de aprendizaje y memoria (Korsching y col, 1985; Gage y col, 1986; Barde y col, 1987; Barde, 1989). En la primera etapa de esta serie de estudios, efectuamos un análisis conductual y citoarquitectónico, siguiendo el curso temporal (15, 30, 45 y 60 días) de desarrollo de los transplantes, en ausencia de FCN, mostrando que la recuperación conductual comienza a manifestarse a partir de los 30 días de desarrollo post-transplante, alcanzando su mejor expresión hacia los 60 días. En otra fase del presente proyecto, se demostró la participación de la proyección colinérgica entre el nucleo basalis magnocellularis y la CI, en la integración neural de la información gustativa. Los estudios efectuados en nuestro laboratorio combinando implantes fetales de CI con el FCN, mostraron que la

asociación de ambos elementos produce una aceleración de los procesos de recuperación de la capacidad de aprender que se manifiesta a partir de los 15 días de desarrollo post-transplante, en ratas previamente lesionadas en la CI. Posteriormente, llevamos a cabo el análisis *in vivo* de la actividad colinérgica de transplantes homo (CI) y heterotópicos (corteza occipital), encontrando diferencias significativas entre ambos. El análisis de la conducta de estos grupos mostró que los transplantes heterotópicos a diferencia de los homotópicos, no produjeron recuperación conductual. Esto sugiere que la especificidad de los tejidos transplantados es un factor importante para el restablecimiento tanto de la actividad colinérgica de los implantes como de las manifestaciones conductuales que subyacen a la integración de los mismos.

Finalmente, nuestros hallazgos muestran también que la actividad colinérgica (medida *in vivo* e *in vitro*) a diferencia de la GABAérgica, juega un importante papel en la recuperación conductual mediada por los transplantes de CI en combinación con el FCN.

INTRODUCCION

I. LOS TRANSPLANTES DE TEJIDO NERVIOSO FETAL Y LA PLASTICIDAD DEL SNC

Hoy en día, se sabe que el Sistema Nervioso Central (SNC) es capáz de generar procesos que promueven la recuperación de funciones después de haber sido dañado. En respuesta a la denervación por ejemplo, numerosas fibras presentan rebrote axonal (sprouting) y forman nuevas sinapsis que reemplazan a las perdidas. En algunos casos, tales procesos de reconexión pueden participar en la recuperación funcional (Cotman y col, 1981). Sin embargo en casos de daño severo, la recuperación no ocurre a menos que se utilicen los implantes de tejido cerebral fetal (Bjorklund y Stenevi, 1984; Gash y col, 1985; Collier y col, 1988).

Son varios los mecanismos posibles mediante los cuales los implantes estimulan la recuperación funcional. Por ejemplo, los implantes pueden restablecer la circuitería interrumpida tras una lesión, o incrementar la disponibilidad de un neurotransmisor para facilitar la comunicación neuronal. Por otra parte, los transplantes pueden estimular la vascularización, eliminar substancias tóxicas o promover la sobrevivencia y el crecimiento neuronales a trávés de interacciones tróficas entre el huésped y el transplante (Cotman y Kesslak, 1988).

Entre las variables más importantes que deben considerarse para transplantar tejido nervioso se encuentran: la edad del donador, el sitio en el que se coloca el implante y la edad del huésped. El transplantar tejido cerebral fetal al SNC adulto conlleva la interacción

del SNC del huésped con el transplante, la reacción de los transplantes fetales ante la pérdida de su ambiente normal, aunque su crecimiento y diferenciación son restaurados posteriormente y, el establecimiento de la comunicación entre el transplante y el huésped a través del intercambio de conexiones recíprocas. A este respecto, Gibbs y Cotman (1987) sugieren en su trabajo sobre la sobrevivencia y el crecimiento de los transplantes, que el introducir un lapso entre la lesión y el transplante incrementa la probabilidad de sobrevivencia y un adecuado desarrollo de los implantes y, que generalmente las fibras formadas en los transplantes compiten selectivamente con las proyecciones nativas del huésped durante el proceso de reinervación.

I.1 LOS FACTORES TROFICOS Y LA INTEGRACION DE LOS TRANSPLANTES.

El estado actual de las investigaciones en torno al papel que juegan los neurotransmisores y moléculas tróficas, en la promoción de la sobrevivencia neuronal, así como de la guía y crecimiento de axones y dendritas, no solo durante las primeras etapas de desarrollo, sino también en el individuo adulto como parte de los procesos de regeneración neuronal, permite pensar que tales factores intervienen de manera decisiva, en la sobrevivencia y adecuada integración de los transplantes de tejido nervioso fetal, los cuales a su vez son los responsables (como lo demuestran numerosos paradigmas conductuales) del restablecimiento de las funciones cognitivas y motoras de los sujetos transplantados.

A este respecto, se sabe que los tejidos cerebrales contienen factores tróficos que son activos *in vitro*, como promotores de sobrevivencia celular, crecimiento de neuritas y diferenciación tanto en neuronas de SNC como de sistema nervioso periférico (SNP). Nieto-Sampedro y Cotman (1986) sugieren que ciertos factores neurotróficos específicos, incrementan su disponibilidad tras una lesión, contribuyendo en consecuencia, a la sobrevivencia y crecimiento de los transplantes. Así, aparentemente el cerebro responde al daño, produciendo factores tróficos que incrementan la sobrevivencia celular y promueven el crecimiento de neuritas.

Aunados a los factores que sistemáticamente se hallan implicados en la sobrevivencia, mantenimiento y diferenciación neuronal, tales como el FCN (factor de crecimiento neuronal), el FGF (factor de crecimiento fibroblástico), el BDNF (factor trófico derivado del cerebro), el EGF (factor de crecimiento epidérmico), etc., en los últimos años se ha venido desarrollando la tendencia cada vez mayor, hacia el estudio de los neurotransmisores no sólo como moléculas implicadas en la transmisión de información, sino como importantes partícipes en la definición de la estructura de los circuitos en los cuales participan. Recientes hallazgos han demostrado el potencial de los neurotransmisores como reguladores de la motilidad del cono de crecimiento axónico durante el desarrollo (Haydon y col, 1984; Goldberg y Kater, 1985), la estructura sináptica en el cerebro adulto (Desmond y Levy, 1983; Chang y Greenough, 1984) y la neurodegeneración (Coyle y col, 1983; Maragos y col, 1987). La investigación en torno al papel que juegan los neurotransmisores en la regulación de la forma neuronal, se ha encaminado hacia el análisis

de las alteraciones morfológicas que pueden ocasionar los neurotransmisores liberados por los axones aferentes en neuronas en desarrollo (Mattson y col, 1988).

En la literatura que sobre transplantes se ha generado en los últimos años, resulta relativamente frecuente el encontrar que los fenómenos de reconexión implante-huésped son adjudicados en buena medida a la presencia y actividad de factores tróficos (Nieto-Sampedro y Cotman, 1986; Dekker y col, 1992; Dekker y Thal, 1993).

Las investigaciones en torno a transplantes de tejido nervioso fetal han registrado avances significativos, particularmente en las últimas dos décadas, ampliando consecuentemente el panorama acerca de los diversos fenómenos que conforman la plasticidad del SNC de los seres vivos. Hoy en día, la combinación de la técnica de transplantes de tejido nervioso fetal con técnicas electrofisiológicas, histoquímicas, bioquímicas, de cultivo y más recientemente con la ingeniería genética, ha generado importantes aportaciones al estudio de la recuperación funcional, la reconectividad, la neurobiología del desarrollo, así como de las interacciones tróficas entre el transplante y el huésped.

En el ámbito de la co-participación de los transplantes y la ingeniería genética por ejemplo, se han transplantado fibroblastos genéticamente modificados, para producir FCN y promover así, la sobrevivencia y crecimiento de neuronas colinérgicas en la fimbria-fórnix previamente lesionada, permitiendo el restablecimiento de las proyecciones septohipocampales (Olson y col, 1990; Stromberg y col, 1990). Jinnah y col. (1989) reportaron que transplantes de fibroblastos genéticamente modificados para producir L-DOPA,

fueron implantados exitosamente en el núcleo caudado de ratas, restableciendo las deficiencias motoras originadas por la lesión previa de la vía nigro estriatal. Freed y col. (1989) demostraron que la transferencia genética mediada por retrovirus en ciertas líneas celulares, puede ser usada para producir tirosina-hidroxilasa (TH), enzima que participa en la biosíntesis de las catecolaminas, con características altamente semejantes a las de la enzima natural.

Estos experimentos sugieren que las líneas celulares producidas mediante recombinación genética tienen un gran potencial para producir efectos funcionales una vez que han sido transplantadas al interior del SNC (Freed y col, 1989; Stromberg y col, 1990; Piccardo y col, 1992; Maysinger y col,1992a; b; c;). De manera que el conocimiento acerca de la participación de numerosos factores tróficos y neurotransmisores en el desarrollo y funcionamiento del SNC, puede ser empleado exitosamente para generar nuevas estrategias terapéuticas encaminadas a prevenir o retardar el avance de enfermedades neurodegenerativas.

II. LA CITOARQUITECTURA NEURONAL INFLUENCIADA POR LOS MENSAJEROS NEURALES Y SU RELACION CON LOS PROCESOS DE APRENDIZAJE Y MEMORIA

Numerosos datos indican que el cerebro adulto no es estructuralmente estático, sino que se encuentra en un estado de continuo flujo citoarquitectónico (Purves y col, 1986). En este sentido, los cambios estructurales pueden ser un componente necesario de las funciones cerebrales adaptativas, tales como el aprendizaje y la memoria (Bailey y Chen, 1983). Varios investigadores han observado

cambios en la citoarquitectura neuronal, mediados por neurotransmisores, que han sido correlacionados con procesos de aprendizaje y memoria. En *Aplysia* se ha mostrado que la activación serotoninérgica de receptores presinápticos en neuronas sensoriales contribuye a la facilitación del "gill withrawal response" o respuesta de escape, que constituye un modelo simplificado para el aprendizaje y la memoria (Kandel y Hawkins, 1992). En el hipocampo han sido observados cambios en la estructura sináptica de las dendritas de las neuronas piramidales, correlacionados con la fortaleza de las sinapsis, tras la producción de la potenciación a largo plazo (LTP). Los resultados demuestran que el glutamato está involucrado en el LTP y sugieren que este neurotransmisor juega un importante papel en los cambios morfológicos asociados con el aprendizaje y la memoria (Arai y Lynch, 1992; Bliss y Collingridge, 1993).

II.1 LOS TRANSPLANTES Y LA RECUPERACION FUNCIONAL.

Tradicionalmente se había considerado que la capacidad regenerativa del sistema nervioso de mamíferos era nula a diferencia de la encontrada en aves y en anfibios (Cowan y col, 1965). Ranson (1903), Saltikow (1905) y Altobelli (1914) efectuaron algunos de los primeros trabajos de transplantes de tejido nervioso en mamíferos. Más tarde el mismo Ranson (1914) y Le Gros Clark (1940), demostraron que tejido nervioso tomado de fetos de ratas no sólo sobrevivía durante periodos largos de tiempo, sino que además desarrollaba neuronas totalmente diferenciadas. Recientemente la técnica de transplantes de tejido cerebral fetal ha sido empleada como una herramienta efectiva para disminuír las deficiencias

funcionales y conductuales producidas por lesiones mecánicas o químicas (Kesslak y col, 1986; Woodruf y col, 1987; Isacson y col, 1988; Yirmiya y col, 1988; Kolb, 1992; Hodges y col, 1992; Dunnett y col, 1992; Will y col, 1992). Sin embargo la interrogante acerca de como actúan los transplantes para promover la recuperación funcional, permanece aún sin resolver. En este sentido, la participación de los factores tróficos en la recuperación inducida por transplantes ha cobrado auge en los últimos años (Stein, 1988; Nieto-Sampedro, 1988; Maysinger y col, 1992b; Piccardo y col, 1992). Entre los factores implicados en la citada recuperación destaca el FCN, que ha sido sistemáticamente relacionado con el restablecimiento de las vías colinérgicas involucradas en los procesos de aprendizaje y memoria (Varón y col, 1989; Gage, 1990; Hefti, 1990). El papel del FCN en los procesos de aprendizaje y memoria se trata más ampliamente en el trabajo I de la presente tesis.

II.2 EL CONDICIONAMIENTO AVERSIVO A LOS SABORES Y LA CORTEZA INSULAR.

En nuestro laboratorio se ha utilizado el modelo conductual de Condicionamiento Aversivo a los Sabores (CAS), originalmente propuesto por John García en la década de los 60's, para evaluar la recuperación de los transplantes de tejido cerebral en ratas previamente lesionadas. El CAS constituye un modelo sólido para el estudio de la ontogenia y filogenia de los procesos gustativos. Además, ha contribuído a mejorar el entendimiento de los procesos anatómicos y/o funcionales del SNC, que integran la información gustativa. En este modelo, los animales normales adquieren aversión

a un estímulo gustativo cuando éste es seguido de una irritación gástrica (García y col, 1985). Entre las estructuras cerebrales superiores que se han asociado al CAS, se encuentra la corteza insular (CI) (Braun y col, 1972; Norgren y Wolf, 1975). En mamíferos las aferencias gustativas que ascienden a través de los nervios VII, IX y X, establecen relevos de primer orden en los tercios rostrales del núcleo del tracto solitario. Asimismo, este núcleo recibe aferencias viscerales provenientes de las ramas hepáticas del nervio vago que son sensibles a la irritación gástrica; del área postrema, la cual detecta la presencia de toxinas en la circuitería periférica, así como del sistema vestibular, que presenta sensibilidad a la náusea producida por movimiento. El relevo gustativo de segundo orden, constituído por neuronas con capacidad de respuesta ante estímulos gustativos, se ha localizado en el área parabraquial del puente o área gustativa pontina, la cual emite proyecciones hacia el núcleo posteromedial ventral del tálamo (PMV). Las neuronas del núcleo PMV envían proyecciones gustativas hacia la corteza somatosensorial ventral e insular anterior en la rata (áreas 13 y 14 de la neocorteza de acuerdo a Krieg), denotada como corteza insular gustativa (Braun y col, 1982).

Asimismo, se ha reportado la existencia de proyecciones recíprocas directas entre la CI y el área pontina del gusto (Lasiter y col, 1982), y la CI y el núcleo del tracto solitario (Krushel y van der Kooy, 1988). Por su parte Saper (1982) y Shipley (1982) demostraron que las neuronas de la CI proyectan axones hacia el complejo parabraquial y la región caudal del núcleo del tracto solitario. Un año después, en 1983, Lasiter y Glanzman demostraron

que las aferencias pontinas que reciben tanto el núcleo ventromedial talámico como la CI tienen su origen en las mismas neuronas, es decir, colaterales axónicas del área gustativa del puente proyectan tanto hacia PMV como a CI.

El papel de cada una de estas vías neurales en la sensibilidad gustativa, se ha inferido a través de métodos conductuales, electrofisiológicos y neuroanatómicos, lo que ha hecho posible el concluír con alguna seguridad que mientras las zonas talámicas (PMV) y parabraquial pontina, se hallan involucradas en la percepción del gusto, la corteza insular se relaciona fundamentalmente con la modulación conductual gustativa (Lasiter y col, 1982).

En nuestro laboratorio se ha demostrado que transplantes homotópicos fetales de corteza insular, producen recuperación en la capacidad de adquirir el CAS, después de dos meses, en ratas que previamente la habían perdido debido a la lesión de la CI (Bermúdez-Rattoni y col, 1987). Posteriormente, tratando de conocer el grado de integración neuroanatómica de los transplantes y el papel que juega esta integración en la recuperación funcional, mostramos que los transplantes homotópicos de CI pero no los heterotópicos de tejido tectal, podían restablecer las funciones cognitivas y la conectividad con el tálamo y la amígdala del tejido huésped, con quienes la corteza insular mantiene conexiones normalmente (Escobar y col, 1989).

Más tarde, con el ánimo de comprender los procesos temporales que subyacen a la recuperación funcional y anatómica observadas, efectuamos un análisis conductual y citoarquitectónico, siguiendo el curso temporal (15, 30, 45 y 60) días de desarrollo de los

transplantes, mostrando que la recuperación conductual comienza a manifestarse a partir de los 30 días de desarrollo post-transplante, al tiempo que aparecen los primeros indicios de reconectividad, vascularización y maduréz estructural de nuestros transplantes, alcanzando su mejor expresión hacia los 60 días. Estos resultados sugieren que la maduréz morfológica y la reconectividad entre el transplante y el huésped, son necesarias para la adecuada expresión de la recuperación conductual del CAS, en sujetos previamente lesionados en la CI.

Trabajos efectuados por Woolf y Butcher (1982) y Bermúdez-Rattoni y col. (1983), señalan que la acetilcolina (ACh) juega un papel importante en el CAS. Existen evidencias, en el sentido de que la CI presenta considerable actividad colinérgica, así como de que el bloqueo farmacológico de la transmisión colinérgica provoca disturbios en la adquisición del CAS (López-García y col, 1990a). Algunas observaciones efectuadas en nuestro laboratorio, señalan que los transplantes homotópicos de CI que promueven la recuperación del aprendizaje en el CAS, liberan ACh, en tanto que en los transplantes heterotópicos de corteza occipital que no inducen recuperación funcional no se registra liberación del citado neurotransmisor. Lo cual sugiere la participación colinérgica en la recuperación conductual mediada por transplantes de corteza insular (López y col, 1990b).

Considerando los antecedentes conductuales, anatómicos y neuroquímicos obtenidos en nuestro laboratorio, los objetivos

fundamentales abordados a lo largo de la serie de experimentos que forman parte del presente trabajo fueron:

1) Debido a que el factor de crecimiento neuronal, es un factor neurotrófico que promueve la sobrevivencia, el crecimiento y las capacidades funcionales del sistema colinérgico en el SNC, intentamos analizar la participación de este factor durante el proceso de integración de los transplantes neocorticales, así como su repercusión en la recuperación funcional mediada por los mismos.

2) Analizar la integridad neuroquímica de los transplantes neocorticales en presencia del FCN, monitoreando la expresión de algunos neurotransmisores, tales como la acetilcolina y el GABA, tanto *in vivo* como *in vitro*.

3) Analizar el grado de especificidad de la acción del FCN sobre los diferentes tejidos transplantados, homo y heterotópicos, y su repercución sobre la recuperación funcional.

4) Ampliar el marco conductual de nuestros estudios, probando paradigmas de conducta tales como la prevención pasiva.

A continuación se incluyen los artículos, que han sido publicados o enviados a publicación en revistas especializadas del extranjero o nacionales, los cuales contienen los resultados experimentales obtenidos durante el desarrollo del proyecto de investigación. El artículo de revisión enviado a la revista CIENCIA contiene la información general acerca del factor de crecimiento neuronal y su relación con el sistema nervioso central, información que fundamenta en buena medida la serie de investigaciones que se resumen en los 6 artículos restantes. Estos últimos, 4 de ellos

publicados y otros 2 enviados, se presentan en el orden temporal de su realización.

TRABAJO I

EL FACTOR DE CRECIMIENTO NEURONAL EN EL SISTEMA NERVIOSO CENTRAL

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RESUMEN

Los factores neurotróficos son proteínas que desempeñan un papel decisivo en la sobrevivencia, crecimiento y capacidades funcionales de poblaciones específicas de neuronas. Por lo que se ha abierto en los últimos años un importante campo de investigación en torno a su participación en la etiología y el tratamiento de algunas patologías humanas neurodegenerativas. En particular, el factor de crecimiento neuronal, primer polipéptido biológicamente activo al cual le fue aplicado el término de factor trófico, ha sido ampliamente estudiado en relación al restablecimiento de las neuronas colinérgicas del sistema nervioso central, las cuales están involucradas en los procesos de aprendizaje y memoria y se encuentran alteradas en enfermedades como la demencia de tipo Alzheimer.

NERVE GROWTH FACTOR IN THE CENTRAL NERVOUS SYSTEM

ABSTRACT

Neurotrophic factors are proteins controlling survival growth and functional capabilities of selected populations of neurons. So recently, an important research field around its participation in the etiology and treatment of some human neurodegenerative pathologies, has emerged. Particularly, the nerve growth factor (NGF), the first biologically active polipeptide named trophic factor, has been extensively studied in relation to the re-establishment of the central nervous system (CNS) cholinergic neurons, which are involved in learning and memory processes and are altered in the Alzheimer's disease.

INTRODUCCION

La diversidad celular en el sistema nervioso se origina a partir de la acción concertada de los procesos de proliferación celular, diferenciación, crecimiento, migración, sobrevivencia y formación de sinapsis. Entre los mensajeros involucrados en la comunicación neuronal, que da origen a estos procesos, se encuentran ciertas moléculas denominadas factores tróficos. Los factores tróficos o factores neurotróficos (FNT) son proteínas que controlan la sobrevivencia, el crecimiento y las capacidades funcionales de poblaciones específicas de neuronas (Varon, 1985).

El estudio de estos factores ha llevado a la postulación de la hipótesis neurotrófica del sistema nervioso central (SNC) (Appel, 1981; Hefti, 1983; Varon, 1985), que señala:

1) Las neuronas del SNC adulto dependen de sus FNT para su mantenimiento, función y reparación.

2) Los FNT endógenos son liberados por sus territorios de inervación (parejas post-sinápticas y glía).

3) Deficiencias en los FNT endógenos originan trastornos neuronales: disfunción, hipotrofia y degeneración.

4) La administración exógena de FNT previene y/o corrige los daños o trastornos producidos por lesiones crónicas o agudas.

5) Los FNT pueden ayudar en el tratamiento de algunas patologías humanas neurodegenerativas.

El primer polipéptido biológicamente activo al cual le fue aplicado el término de factor trófico fue el factor de crecimiento neuronal (FCN). En 1951, V. Hamburger y R. Levi-Montalcini observaron que la liberación de un factor difusible, generado por

ciertos tumores de ratón implantados quirúrgicamente en embriones de pollo, causaba crecimiento de los ganglios simpáticos y sensoriales del sistema nervioso periférico (SNP) y promovía una notable emisión de neuritas en las neuronas de estos ganglios (Levi-Montalcini y Hamburger, 1951; Levi-Montalcini, 1966). Hoy en día sabemos que el FCN también actúa sobre las células colinérgicas del SNC (Korsching y col, 1985).

La presente revisión intenta mostrar un panorama global acerca del estado actual de las investigaciones en torno al FCN, con especial énfasis en su relación con el SNC. Abordaremos los avances y las interrogantes que existen en torno a su estructura, sus mecanismos de acción y biosíntesis, así como los modelos experimentales empleados para suministrarlo al SNC.

I. UN PROMINENTE MIEMBRO DE LA FAMILIA DE LAS NEUROTROFINAS

Los factores tróficos pueden ser agrupados convenientemente en familias, tomando en consideración dos criterios: a) las poblaciones celulares sobre las que actúan, es decir, considerando sus células blanco y, b) su estructura, puesto que algunos factores tróficos presentan obvias similitudes estructurales en sus secuencias aminoacídicas (Walicke, 1989; Meakin y Shooter, 1992). Con base en estos criterios, algunos factores tróficos se han agrupado en familias, como la del factor de crecimiento fibroblástico (FCF) (Gospadorowicz, 1990; Baird y Klagsbrun; 1991), la de los factores de crecimiento semejantes a la insulina (FCI) (Roth, 1988), la del factor de crecimiento epidérmico (FCE) (Derynck, 1986) y la de las

neurotrofinas, también llamada familia del FCN (Thoenen, 1991; Meakin y Shooter, 1992).

El FCN es una proteína oligomérica formada por diferentes subunidades llamadas alfa, beta y gamma (Varon y col, 1968); solamente la subunidad beta (β-FCN) posee actividad promotora de crecimiento. La subunidad β-FCN (denominada también FCN 2.5S, debido a su coeficiente de sedimentación), es un dímero con 26.5 KDa de peso molecular y un punto isoeléctrico de 9.3. Este dímero es parte de un complejo multiunitario denominado FCN 7S (Greene y Shooter, 1980) y cada una de las dos cadenas que lo constituyen tiene 118 aminoácidos. El FCN se produce en las células blanco de las neuronas sensoriales y simpáticas del SNP; en las neuronas inervadas por las células colinérgicas del SNC, principalmente neocorteza e hipocampo; en algunas células gliales, y en la glándula submaxilar de ratón (la fuente más abundante para su purificación) (Varon y col, 1968). De manera similar, se han detectado grandes cantidades de factor de crecimiento neuronal en el veneno de algunas serpientes y en el fluído seminal de los toros. El significado funcional del factor en estos tipos celulares se desconoce (Levi-Montalcini y Calissano, 1986). Resulta conveniente señalar que las células sensibles al FCN como son las neuronas de los ganglios simpáticos y sensoriales del SNP y las neuronas colinérgicas del SNC, responden a éste de manera diferencial a lo largo de su vida. Presentando durante algunas etapas de su desarrollo una dependencia total para su sobrevivencia, mientras que en otras, esta dependencia se encuentra reducida (Levi-Montalcini y Booker, 1960; Large y col, 1986; Whittemore y col, 1986). La concentración del FCN en el sistema nervioso central *in*

vivo varía entre los 0.07 y 1.5 ng/g de peso húmedo (tabla I) (Korsching y col, 1985). Con el empleo de inmunoensayos enzimáticos e hibridación "*in situ*", Larkfors y col (1988) observaron el curso temporal de la aparición del FCN y de su ARNm en el SNC de la rata. Estos estudios demostraron la existencia de niveles detectables de FCN a partir de los 15 días de desarrollo embrionario, y que la máxima concentración se alcanzó después de la tercera semana de desarrollo post-natal (coincidiendo con la maduración de la inervación colinérgica procedente del cerebro basal anterior). En el hipocampo, los niveles de FCN y de su ARNm disminuyeron en un 40 y 50% respectivamente, durante la etapa senil, comparativamente con los valores detectados durante la edad adulta.

Investigaciones recientes sobre la estructura del FCN, empleando estudios de difracción de rayos X y análisis del espectro Raman (análisis que permite conocer las propiedades dinámicas de la estructura de una molécula), tanto en la forma cristalina como en solución, revelaron que esta molécula posee una estructura β anti-paralela con escasa o nula conformación en α-hélice. La difracción de rayos X reveló a su vez, una estructura alargada y profusamente plegada, constituída por filamentos en la conformación β (Fig 1). Estos estudios revelaron también que las dimensiones aproximadas de la molécula del FCN son 60 x 25 x 15 Å (MacDonald y col, 1991; Bradshaw y col, 1993).

A. Los receptores del FCN

De manera similar a otros polipéptidos tróficos, el FCN lleva a cabo sus acciones sobre sus células blanco a través de la interacción

con receptores de superficie (Taniuchi y col, 1986; Bradshaw y col, 1993). La primera evidencia acerca de los receptores de FCN fue obtenida por estudios de unión (binding) utilizando FCN marcado con ^{125}I (Bradshaw, 1978), mediante los cuales se demostró la existencia de dos clases de receptores denominados (de acuerdo a una antigua clasificación), receptores tipo I de alta afinidad (Kd 10^{-11}M) (Hosang y Shooter, 1985) y receptores tipo II de baja afinidad (Kd 10^{-9} M) (Sutter y col, 1979). Otras investigaciones dieron a conocer que los pesos moleculares de los receptores oscilaban entre los 130-150 KDa (Costrini y col, 1981) y 65-80 KDa (Grob y Bothwell, 1983) respectivamente.

El receptor de baja afinidad, que hoy se conoce como $p75^{NGFR}$ fue clonado a partir de células sensibles a FCN de rata (Radeke y col, 1987) y de humano (Johnson y col, 1986). Sin embargo no fue sino hasta 1991 cuando se dió un gran paso en el conocimiento de los receptores del FCN, con la identificación de un receptor, con actividad de tirosina cinasa, en las células sensibles al FCN (Kaplan y col, 1991; Klein y col, 1991). Este receptor resultó ser el producto del proto-oncogene Trk, ahora denominado TrkA. La forma celular se denomina $p140^{proto\text{-}Trk}$ (Schneider y Schweiger, 1991). La tirosina cinasa TrkA es una glicoproteína transmembranal de 140 KDa que exhibe una rápida autofosforilación como consecuencia de la unión del FCN. Estudios subsecuentes han mostrado la co-existencia de los receptores $p75^{NGFR}$ y $p140^{TrkA}$ en prácticamente todas las células sensibles al FCN, tanto de SNC como periférico (Hempstead y col, 1991; Schlessinger y Ullrich, 1992) y aún en las células PC12 (línea celular procedente de un feocromocitoma de células cromafines de la

médula adrenal de rata, que se neurogeniza en presencia de FCN) (Kaplan y col, 1991).

Se han propuesto dos modelos de los receptores del FCN. El primero afirma que el anteriormente denominado receptor de alta afinidad o tipo I, es en realidad un complejo formado por la asociación de las subunidades $p75^{NGFR}$ y $p140^{TrkA}$ (Meakin y col, 1992). El segundo modelo propone que los sitios de unión de alta afinidad para el FCN pueden formarse en ausencia del receptor $p75^{NGFR}$ (Klein y col, 1991) y que la sola presencia del receptor $p140^{TrkA}$ es suficiente para desencadenar respuestas biológicas (Weskamp y Reichardt, 1991; Ibañez y col, 1992). Es claro que la naturaleza de los sitios de unión de alta afinidad y de las interacciones entre ambas subunidades presenta aún grandes interrogantes.

B. Otros miembros de la familia

El restringido número de poblaciones celulares sensibles al FCN motivó la búsqueda de factores neurotróficos relacionados. Hoy en día, el FCN forma parte de una familia de factores con actividad trófica, denominada familia de las neurotrofinas, constituída por los siguientes miembros: FCN, factor neurotrófico derivado del cerebro (BDNF), neurotrofina-3 (NT-3), neurotrofina-4 (NT-4) y neurotrofina-5 (NT-5). La purificación y clonación molecular del BDNF (Leibrock y col, 1989), permitieron la subsecuente identificación de las tres nuevas neurotrofinas NT-3, NT-4 y NT-5 (Hohn y col, 1990; Hallbook y col 1991). Las NT-3, 4 y 5 no fueron directamente purificadas a partir de una determinada fuente tisular, sino que fueron obtenidas en forma recombinante tras su expresión en cultivos celulares. Se ha

propuesto que las NT-4 y 5 son en realidad una misma molécula (Ip y col, 1992) pero este problema permanece aún sin resolver. Las cinco neurotrofinas son capaces de promover la sobrevivencia y diferenciación de un gran número de neuronas sensoriales, pero poseen distintos efectos sobre otras poblaciones celulares. Así, mientras que el FCN es importante para la sobrevivencia de las neuronas de los ganglios simpáticos, el BDNF no ejerce efecto alguno sobre ellas (Barde, 1989). Además numerosas poblaciones neuronales que no son sensibles a la acción del FCN son blancos de la acción de otras neurotrofinas; por ejemplo los ganglios retinal y nodoso son sensibles al BDNF y no al FCN (Barde, 1989). En contraste, las neuronas colinérgicas del cerebro basal anterior son sensibles tanto al BDNF como al FCN (Chao, 1992). Las neurotrofinas presentan en la secuencia genómica que las codifica, regiones conservadas que determinan su estructura básica. Sin embargo, también presentan diferentes regiones variables que determinan su especificidad neuronal diferencial.

El ya mencionado descubrimiento de que el proto-oncogene TrkA codifica para el receptor de FCN (Kaplan y col, 1991; Klein y col, 1991), ha permitido el avance en el conocimiento de los mecanismos de transducción de las señales del FCN, al mismo tiempo que ha coadyuvado en la identificación de las neurotrofinas, como ligandos, para la denominada familia de receptores Trk, que poseen actividad de tirosina cinasas. Algunos genes estrechamente relacionados con el TrkA, tales como el TrkB (Klein y col, 1989) y el TrkC (Lamballe y col, 1991) codifican proteínas que actúan como receptores para el BDNF, NT-3, NT-4 y NT-5 (Klein y col, 1992; Ip y col, 1992). La familia de

receptores Trk presentan gran semejanza con el $p140^{TrkA}$, no solamente en la región de tirosina cinasa, sino también en la región extracelular rica en residuos de cisteína (Fig 2) (Chao, 1992). Aunado a lo anterior, el receptor del FCN de 75KDa ($p75^{NGFR}$) es reconocido por la mayoría de las neurotrofinas (Rodríguez-Tebar y col, 1990; Squinto y col, 1991) y es requerido junto con el TrkA para la formación de los sitios de unión de alta afinidad (Hempstead y col, 1991). Recientemente, se ha propuesto que las neurotrofinas comparten un receptor común de baja afinidad y que sus complejos de alta afinidad se forman tras la adición de una segunda subunidad (Rodríguez-Tébar y col, 1990; Thoenen, 1991) (Fig 3).

II. LA INTERACCION ACETILCOLINA-FCN Y LOS PROCESOS DE APRENDIZAJE Y MEMORIA

Existe amplia evidencia en el sentido de que el FCN interviene en el desarrollo, mantenimiento y regeneración del SNP (sensorial y simpático) (Thoenen y Barde, 1980). Hoy en día se sabe que diferentes niveles regionales de FCN en la periferia (áreas de inervación del SNP), determinan diferentes densidades de inervación por parte de las neuronas sensibles al FCN. La regulación de la producción de FCN en esas zonas es autónoma y en particular, independiente de la actividad neuronal (Rohrer y col, 1988) (Fig 4,A). Más recientemente, se ha demostrado que el FCN incrementa la recuperación de algunas áreas del SNC lesionadas e induce el incremento de niveles de colinacetil-trasferasa, enzima de síntesis de la acetilcolina (ACh), en el cerebro basal anterior e hipocampo, de lo cual y aunado a numerosas evidencias en este sentido se deduce su

participación en el sistema colinérgico del SNC (Korsching y col, 1985; Gage y col, 1986; Auburger y col, 1987; Barde y col, 1987).

Así, en la actualidad existen numerosos datos acerca de la existencia del factor de crecimiento neuronal en el SNC. La hibridación *in situ* ha revelado la presencia de grandes cantidades de ARNm de FCN en el hipocampo, la corteza cerebral y el bulbo olfatorio (Ayer-LeLievre y col, 1988). Comparados con los niveles de otras áreas cerebrales, los niveles endógenos de FCN en la corteza y el hipocampo son también altos (Whittmore y col, 1986). Tales áreas reciben densa inervación colinérgica procedente del núcleo basalis magnocelularis (NBM) y el área septal respectivamente. Schwab y col (1979), encontraron que el FCN marcado radiactivamente y administrado en el hipocampo o la corteza, es transportado retrógradamente hacia las células del septum, los núcleos de la banda diagonal, así como al NBM (Schwab y col, 1979).

La síntesis de FCN en las neuronas del SNC tiene lugar en los sitios de proyección de las neuronas colinérgicas sensibles al FCN del cerebro basal anterior (p. ej. el hipocampo y la corteza), y está regulada por la actividad neural. Se sabe asimismo, que la mayor regulación positiva (up-regulation) ocurre a través del sistema de transmisores glutamatérgicos, mientras que la regulación negativa (down-regulation) ocurre por mediación del sistema GABAérgico (Zafra y col, 1990) (Fig 4, B).

Numerosos estudios farmacológicos han sugerido reiteradamente el papel de la ACh en un gran número de funciones cerebrales, incluyendo el aprendizaje y la memoria (Dunnet y col, 1985). Existen neuronas receptoras a la ACh en prácticamente toda la

neocorteza; investigaciones a este respecto han mostrado que entre el 60 y el 80% de la ACh cortical, proviene de fuentes extrínsecas a la corteza (Emson y col, 1979). Entre tales estructuras extracorticales destaca el NBM del cerebro basal anterior (Rye y col, 1984), cuyo papel en numerosas tareas de aprendizaje es bien conocido (Dubois y col, 1985; Miyamoto y col, 1985; Collerton, 1986). A este respecto, investigaciones efectuadas en nuestro laboratorio mostraron que el NBM está involucrado en la integración neural de la conducta de ingesta y que su proyección colinérgica a la corteza insular es uno de los factores implicados (López-García y col, en prensa).

Springer y col (1987) mostraron que neuronas del NBM son inmunoreactivas a anticuerpos contra el receptor del FCN. Se ha probado asimismo que la presencia de implantes de tejido embrionario procedente del cerebro basal anterior, colocados en la neocorteza de ratas con lesiones del NBM, restablecen parte de la inervación colinérgica original (Dunnet y col, 1985). Estudios realizados por Dekker y col (1992) demostraron que el tratamiento con FCN, administrado a través de minibombas osmóticas en ratas con lesión en el NBM producida por ácido iboténico, mejora notablemente el aprendizaje espacial.

Otro de los patrones colinérgicos más ampliamente estudiados en el SNC es la vía septo-hipocampal. Las neuronas colinérgicas del área septal proyectan sus axones a la formación hipocampal a través de un tracto bien definido: la fimbria-fórnix. La participación de esta vía en los mecanismos del aprendizaje y la memoria es bien conocida. La administración del FCN en animales con lesiones experimentales de la vía septo-hipocampal atenúa las deficiencias conductuales

inducidas por la lesión (Hefti y col, en prensa; Lapchak, 1992). Así, tras lesionar los cuerpos celulares del área septal o la vía axonal que une al septum con el hipocampo, el FCN restaura las respuestas en múltiples tareas asociadas con la consolidación y formación de la memoria, tales como el laberinto en "T" y el laberinto radial de 8 brazos (Will y Hefti, 1985; Will y col, 1990). Los resultados experimentales acumulados hasta el presente señalan que estos efectos conductuales del FCN están mediados por las neuronas colinérgicas septo-hipocampales, reafirmando así que las neuronas colinérgicas del cerebro basal anterior que son sensibles al FCN, tanto durante el desarrollo como en la vida adulta (Thoenen y col, 1987; Araujo y col, 1990; Lapchak y Hefti, 1991; Lapchak, 1992), están involucradas en los procesos de memoria (Bartus y col, 1982; Olton y Wenk, 1987).

III. MODELOS DE INTERACCION ENTRE EL FCN Y EL SNC

El postulado que señala: " Los FNT pueden ayudar en el tratamiento de algunas patologías humanas neurodegenerativas" resume en buena medida uno de los principales enfoques de la investigación en torno a la búsqueda de modelos que permitan comprender mejor la etiología, el tratamiento y por supuesto la participación de los factores tróficos en las enfermedades degenerativas del SNC.

Debido a que el FCN es un polipéptido de 26.5 KDa, y por lo tanto no atraviesa la barrera hematoencefálica, es importante considerar los procedimientos experimentales que se han ensayado para administrar el FCN en el SNC.

1) Infusión del FCN a través de cánulas o minibombas osmóticas.
2) Administración del FCN en conjunción con un implante de tejido cerebral fetal, o bien a través de células genéticamente modificadas para producir el factor, implantados en algunas regiones del SNC.
3) La vía menos cruenta, puesto que no involucra la intervención quirúrgica concomitante a los dos procedimientos anteriores, es la administración del factor por vía sistémica, conjuntamente con algún vehículo o acarreador del mismo (en virtud de la ya citada imposibilidad del FCN para atravesar la barrera hematoencefálica), o bien la administración de algún precursor del factor de crecimiento neuronal. Esto último implica por supuesto un mayor conocimiento de los mecanismos de biosíntesis inherentes al FCN.

Es importante señalar que las vías de administración mencionadas no son excluyentes, sino que frecuentemente se usa la combinación de algunos de los procedimientos experimentales citados.

A. Minibombas osmóticas y cánulas

La infusión crónica de FCN usando minibombas osmóticas implantadas subcutáneamente fue reportada por vez primera por Stromberg y colaboradores en 1985. Las minibombas tenían la finalidad de sustentar el crecimiento de implantes de tejido cromafin, colocados en el estriado de ratas, asegurando así una pausada y continua liberación del FCN. Las minibombas osmóticas han sido utilizadas también para liberar FCN en los ventrículos cerebrales, con el fin de rescatar a las neuronas colinérgicas axotomizadas que proyectan al hipocampo y a la neocorteza (Williams y col, 1986).

La administración crónica de FCN a través de cánulas o microinyecciones aplicadas estereotáxicamente, ha probado ser útil, por lo que sigue siendo una opción vigente a pesar de sus inconvenientes técnicos. Davies y Berdsall (1992) probaron que el FCN, administrado a través de microinyecciones en el estriado, previene selectivamente la degeneración de las células colinérgicas estriatales, originada por la administración de ácido quinolínico. Efectos similares en las células colinérgicas del sistema septo-hipocampal fueron observados por Lapchak y Hefti (1992), en la neocorteza y el estriado de rata (Maysinger y col, 1992a; Altar y col, 1992), y en el cerebro basal anterior de primates (Koliatsos y col, 1991) tras la microinyección de FCN en cada una de las áreas mencionadas. La sinaptogénesis neocortical también fué estimulada por la administración intracerebroventricular del factor (Garofalo y col, 1992).

Sin embargo, la utilización de las minibombas osmóticas tiene varios inconvenientes. El principal es que tanto la minibomba como la cánula de diálisis ubicada en el interior del cerebro son fuentes potenciales de infección. Otro de los problemas radica en que las minibombas disponibles funcionan como máximo entre 2 y 4 semanas, de manera que si se requiere de liberaciones más prolongadas, se tiene que acudir a otro tipo de aproximaciones experimentales (Olson y col, 1990).

B. Los implantes de tejido cerebral fetal

Hoy en día, se sabe que el SNC es capaz de generar procesos que promueven la recuperación de funciones después de haber sido

dañado. En respuesta a la denervación por ejemplo, numerosas fibras presentan rebrote axonal (sprouting) y forman nuevas sinapsis que reemplazan a las perdidas. En algunos casos, tales procesos de reconexión son suficientes para producir la recuperación funcional (Cotman y col, 1981). Sin embargo, en casos de daño severo, la recuperación no ocurre a menos que se utilicen los implantes de tejido cerebral fetal (Bjorklund y Stenevi, 1984; Gash y col, 1985).

Son varios los mecanismos posibles mediante los cuales los implantes estimulan la recuperación funcional. Por ejemplo, los implantes pueden restablecer la circuitería interrumpida tras una lesión, o incrementar la disponibilidad de un neurotransmisor para facilitar la comunicación neuronal. Por otra parte, los implantes pueden estimular la vascularización, eliminar sustancias tóxicas o promover la sobrevivencia y el crecimiento neuronales a través de interacciones tróficas entre el huésped y el implante (Cotman y Kesslak, 1988).

En la extensa literatura que sobre implantes se ha generado en los últimos años, se encuentra frecuentemente que los fenómenos de reconexión implante-huésped son adjudicados en buena medida a la presencia y actividad de factores tróficos. Nieto-Sampedro y Cotman (1986), sugieren que ciertos factores neurotróficos específicos incrementan su disponibilidad tras una lesión, contribuyendo en consecuencia a la sobrevivencia y crecimiento de los implantes. Así, aparentemente el cerebro responde al daño, produciendo factores tróficos que incrementan la sobrevivencia celular y promueven el crecimiento de neuritas.

En el ámbito de los implantes de tejido cerebral fetal, Toniolo y col (1985), encontraron que el FCN incrementa la actividad de la colinacetil-transferasa en las neuronas colinérgicas transplantadas en el hipocampo deaferentado, pero no tiene efectos sobre los implantes colocados en el hipocampo intacto. El implante de glándula sublingual de ratón (tejido rico en FCN) en la fimbria-fórnix previamente lesionada, tiene efectos tróficos sobre las neuronas colinérgicas axotomizadas (Springer y col, 1988).

Estudios efectuados en nuestro laboratorio, combinando implantes fetales de corteza insular con el FCN, mostraron que la asociación de ambos elementos produce una recuperación rápida y estable del aprendizaje, en ratas previamente lesionadas en la corteza insular. Nuestros hallazgos muestran también que la actividad colinérgica, a diferencia de la GABAérgica, juega un importante papel en la recuperación conductual mediada por los transplantes de CI (Bermúdez-Rattoni y col, 1992; Escobar y col, en prensa).

Algunas líneas celulares como las 3T3 y 3E (derivadas de la transformación de fibroblastos de ratón) han sido modificadas genéticamente para secretar FCN. Implantes de estas células han sido empleados exitosamente como fuentes continuas de FCN, tanto en el estriado (Ernfors y col, 1989a) como en la corteza (Ernfors y col, 1989b), ejerciendo efectos positivos sobre las interneuronas colinérgicas presentes en cada una de estas zonas. Además el implante de fibroblastos genéticamente manipulados para producir FCN ha probado tener efectos protectores, sobre los trastornos neurotóxicos que originan el ácido quinolínico y quiscuálico, en

diferentes áreas del SNC (Piccardo y col, 1992; Maysinger y col, 1992b). Tanto los fibroblastos como las líneas celulares modificadas genéticamente para producir factor de crecimiento neuronal, se han implantado también, en alguna de las regiones que forman parte de la proyección septo-hipocampal, tras la lesión de la fimbria-fórnix, promoviendo la sobrevivencia y recuperación funcional de las células colinérgicas septales (Rosenberg y col, 1988; Stromberg y col, 1990).

También algunas modalidades de células gliales tales como los astrocitos, se han utilizado exitosamente, en la prevención de la degeneración y aún en la restauración de los sistemas de fibras colinérgicas dañadas tras una lesión (Cunningham y col, 1991).

Sin embargo, el empleo de células modificadas genéticamente, tiene serios problemas, como el control de la secreción de FCN y el potencial tumorogénico de los implantes de esta naturaleza (Olson y col, 1990).

Como modelo alternativo, algunos investigadores han colocado implantes de tejido cerebral fetal combinados con FCN en la cámara anterior del ojo. En el microambiente proporcionado por la cámara ocular, áreas del SNC fetal tales como la corteza, el hipocampo, el septum y la médula espinal, desarrollan características estructurales y funcionales similares a las de sus contrapartes normales (Olson y col, 1988). Este sistema permite monitorear, el tamaño de los transplantes *in vivo,* a través de la córnea del animal implantado. En estos experimentos el tratamiento es iniciado generalmente con la incubación (previa al transplante) de los fragmentos de tejido cerebral fetal en el factor trófico correspondiente. Adicionalmente, el

factor neurotrófico puede ser inyectado crónicamente en la cámara anterior del ojo, empleando diferentes dosis y tiempos.

Utilizando este modelo, Eriksdotter-Nilsson y col (1989a) mostraron que los transplantes septales crecen considerablemente mejor cuando son tratados con FCN, a diferencia de los transplantes de corteza parietal o hipocampo, cuyo crecimiento fue inhibido por el factor (Eriksdotter-Nilsson y col, 1989b).

Otra interesante modalidad en la administración de un factor neurotrófico, recientemente descrita en la literatura, es la implantación de microesferas fabricadas con polímeros biodegradables. Las microesferas han proporcionado una efectiva, prolongada y controlada liberación del FCN en la neocorteza de ratas adultas (Powel y col,1990; Maysinger y col,1992c) y en los ganglios de la raíz dorsal (Camarata y col,1992)

C. La administración sistémica y los mecanismos de biosíntesis.

La imposibilidad del factor de crecimiento neuronal para cruzar la barrera hematoencefálica, ha orientado las investigaciones hacia la búsqueda de mecanismos que permitan aumentar su disponibilidad en el SNC, a través de la inducción farmacológica del incremento en su biosíntesis y liberación en poblaciones específicas de neuronas (Mochetti, 1991).

Investigaciones a este respecto han mostrado que la producción de FCN en el cerebro puede ser aumentada a través de la estimulación de los receptores a ciertos neurotransmisores, por ejemplo los receptores β-adrenérgicos de los astrocitos (Schwartz y

Mishler, 1990), los fibroblastos (Furukawa y col, 1989) y células de la línea celular C6 (derivada de un glioma de rata) (Dal Toso, 1988). La estimulación de los receptores β-adrenérgicos en estos tipos celulares, origina un incremento en la producción y liberación del FCN, el cual está mediado por la activación de una proteína cinasa A dependiente de AMPc (De Bernardi y col, 1991; Mochetti y col, 1991).

Algunas otras moléculas implicadas en la regulación de la biosíntesis del FCN son la interleucina-1 (Carman-Krzan y col, 1991) los esteroides (Mocchetti, 1991) y, dentro del SNC, neurotransmisores como la acetilcolina (Alberch y col, 1991), el ácido glutámico y el GABA (Zafra y col, 1990; Ernfors y col, 1991). Es posible que la expresión génica del FCN en diferentes regiones cerebrales esté regulada de manera específica por la presencia de diferentes estímulos locales, como pudiera ser la activación de diferentes mecanismos de transducción de señales (Mochetti, 1991).

Es importante recordar que el FCN ejerce su actividad biológica a través de la unión con sus receptores de alta afinidad (Shooter, 1989), por lo que los estudios sobre la estimulación de la biosíntesis del FCN, deben acompañarse del análisis de la expresión de sus receptores. Esta consideración se ve respaldada por la observación de que altos niveles de FCN en el cerebro, como los que se originan tras su infusión crónica o como resultado de una lesión, incrementan la expresión génica del receptor a FCN (Brunello y col, 1990).

Cabe destacar que las acciones derivadas de la actividad biológica del FCN se inhiben por la administración de los anticuerpos contra este factor. Cohen, Levi-Montalcini y colaboradores reportaron por primera vez en 1960, la destrucción del SNP simpático, debida a

la administración exógena de anticuerpos dirigidos contra el FCN (anti-FCN) (Cohen, 1960; Levi-Montalcini y Booker, 1960). Dos décadas después (Gorin y Johnson, 1979) mostraron que la exposición prenatal al anti-FCN promueve la muerte de las células sensoriales. Efectos similares se han registrado en las células colinérgicas del SNC (Vroegog y col, 1992). Recientemente ha sido desarrollado un compuesto alcaloide denominado K-252a, que tiene la capacidad de inhibir la actividad de tirosina cinasa propia del receptor de alta afinidad del FCN (Berg y col, 1992).

Lo anteriormente expuesto pone de manifiesto que el conocimiento acerca de la biosíntesis del FCN es aún incipiente, por lo que el desarrollo de agentes farmacológicos que incrementen la disponibilidad de este factor tendrá que esperar algún tiempo. Sin embargo, el panorama no es del todo desalentador ya que Frieden y colaboradores (1993), demostraron que la conjunción del FCN con un anticuerpo para el receptor de transferrina, permite que el factor cruce eficientemente la barrera hematoencefálica tras su administración sistémica. En este estudio se utilizó el modelo de implantes intraoculares de tejido colinérgico, procedente del área septal fetal (Frieden y col, 1993). Este enfoque experimental abre nuevas e interesantes expectativas en torno a la participación de este factor en los fenómenos de sobrevivencia y diferenciación, así como en el tratamiento de algunas enfermedades neurodegenerativas.

IV. ALGUNAS PROPUESTAS SOBRE EL MECANISMO DE ACCION DEL FCN.

La unión del factor de crecimiento neuronal a su receptor de alta afinidad, ya sea el $p140^{TrkA}$ o al dímero de este receptor con el $p75^{NGFR}$ (de acuerdo a las dos hipótesis aún cuestionadas, que describimos previamente), desencadena una gran variedad de señales celulares que conducen a la diferenciación y sobrevivencia de las poblaciones celulares sensibles al FCN. Sin embargo y pese a que el FCN es el factor neurotrófico más ampliamente estudiado, el estado actual del conocimiento acerca de su mecanismo de acción presenta aún múltiples interrogantes.

Una vez que el FCN se ha unido al receptor de superficie formando un complejo, se internaliza (Calissano y Shelanski, 1980) y subsecuentemente se transporta retrógradamente a través de los axones neuronales, hasta alcanzar el soma (Fig 5) (Ferguson y col, 1991). Ya en el interior del citoplasma, el complejo FCN-receptor desencadena, una cascada de eventos bioquímicos mediados por uno o varios segundos mensajeros. Otro de los posibles efectos de la internalización del complejo FCN-receptor, es el de permitir el contacto entre el factor y algunos sitios de unión perinucleares (Marchisio y col, 1980; Levi y Alemá, 1991). Es conveniente señalar sin embargo, que los eventos desencadenados por el FCN pueden ser generados a partir de la unión del factor con sus receptores de superficie, sin que necesariamente medie la internalización del complejo (Gage y Varón, 1988; Kaplan y col, 1991; Klein y col, 1991).

Numerosos segundos mensajeros han sido postulados como partícipes en la transducción de la señal del FCN. Entre los

mensajeros propuestos se encuentran productos de la metilación o hidrólisis de fosfolípidos (Skaper y Varon, 1984; Liwah-Chan y col, 1989), el AMPc (Kalman y col, 1990), el Ca^{+2} (Nicodijevic y col, 1990) y el ácido araquidónico (Halegoua y col, 1991).

Se ha observado también que entre los efectos de la actividad biológica del FCN se encuentran en orden cronológico: la metilación e hidrólisis de fosfolípidos, el plegamiento de la membrana celular, la elevación de los niveles de AMPc, la activación de cinasas, la liberación de Ca^{+2}, la activación de la ATPasa Na+/K+, la activación de la ornitina descarboxilasa, la formación de neuritas y finalmente la activación de la colinacetil-transferasa y de la tirosina hidroxilasa (Halegoua y col, 1991). La activación de cinasas, se traduce en una intensa actividad de fosforilación de proteínas importantes para las múltiples funciones celulares (Cremins y col, 1986; Hall y col, 1988), ambos procesos son considerados como parte de las acciones de tipo inmediato más relevantes, desencadenados por el FCN (Mutoh y Guroff, 1989).

De igual manera, entre los mecanismos que se activan por la unión del FCN con su receptor debemos señalar la transcripción de los genes de respuesta temprana. Estos genes son denominados terceros mensajeros puesto que convierten los estímulos extracelulares en cambios de la programación genética (Macara, 1989; Herschman, 1989). Sin embargo, la activación de los genes tempranos como respuesta al FCN es minoritaria comparada con la activación de los sistemas de segundos mensajeros. Entre los genes activados se encuentran c-fos (Greenber y col, 1985), c-jun (Wu y col, 1989), c-myc (Greenber y col, 1985) y erg-1 (Milbrandt, 1987). Recientes

hallazgos señalan que el incremento en el ARNm del FCN, está mediado por la activación de c-fos (Hengereer y col, 1990). Otro mecanismo clave es sin duda la autofosforilación que experimentan los receptores de tipo $p140^{TrkA}$, tras su unión con el FCN (Kaplan y col, 1991 ; Klein y col, 1991).

Resumiendo, el FCN sintetizado en el retículo endoplásmico y empacado en el aparato de Golgi de las células productoras del factor, es liberado presumiblemente por un proceso de exocitosis hacia el fluído intersticial sináptico. Una vez en el espacio sináptico, el FCN interactúa con sus receptores desencadenando fundamentalmente dos tipos de acciones, unas inmediatas a través de la fosforilación de proteínas (Kaplan y col, 1991) y otras de larga duración a través de la regulación de la transcripción de genes (Fig 6) (Muller y col, 1989; Sugimoto y col, 1984; Alemán, 1991).

CONCLUSIONES

El FCN ha sido ampliamente estudiado como promotor de la sobrevivencia, crecimiento y diferenciación de las neuronas sensoriales y simpáticas del SNP. Debido a esta influencia sobre las neuronas catecolaminérgicas periféricas, se han realizado varios intentos para determinar si el FCN afecta de la misma manera a las neuronas catecolaminérgicas centrales (Bjorklund y Stenevi, 1972). Hasta la fecha, la conclusión es que el FCN no es un efectivo agente trófico para estas últimas (Konkol y col, 1978; Dreyfus y col, 1980).

Sin embargo, recientes experimentos han demostrado contundentemente la influencia del FCN sobre las neuronas colinérgicas del SNC, las cuales están estrechamente relacionadas con

los procesos de aprendizaje y memoria (Korsching y col, 1985; Gage y col, 1986; Auburger y col, 1987; Barde y col, 1987). Por lo que el conocimiento acerca de los factores tróficos y su relación con el sistema nervioso central se ha incrementado considerablemente en la última década. Pese a ello, aún existen grandes interrogantes que deberán ser aclaradas antes de que los factores tróficos sean administrados de manera generalizada, como parte de las estrategias terapéuticas empleadas en el tratamiento de enfermedades neurodegenerativas del SNC humano (Hefti, 1990; Olson y col, 1992).

En los últimos años la investigación acerca del factor de crecimiento neuronal, se ha visto estimulada por una serie de acontecimientos que resumiremos a continuación.

1) El descubrimiento de la familia de las neurotrofinas relacionadas con el FCN, es decir el BDNF y las NT-3, NT-4 y NT-5 (Hohn y col, 1990; Hallbook y col, 1991).

2) La caracterización de dos receptores para el FCN, denominados $p75^{NGFR}$ y el proto-oncogene $p140^{TrkA}$ (Bothwell, 1991), así como la de otros miembros de la familia de tirosina cinasas (Trk), denominados $p145^{TrkB}$ y $p145^{TrkC}$ (Ip y col, 1992).

3) El conocimiento de la estructura tridimensional del FCN (MacDonald y col, 1991).

4) La reirterada observación, de que el FCN es capáz de estimular la sobrevivencia y capacidades funcionales de las neuronas colinérgicas adultas del SNC, tras haber experimentado una lesión o un proceso degenerativo como producto de alguna alteración patológica.

Estas son algunas de las premisas que amplían las perspectivas de acción y enfatizan la trascendencia de los factores tróficos como

importantes partícipes de los mecanismos básicos de sobrevivencia, crecimiento y diferenciación neuronal, y como una opción terapéutica ante el problema que representan las enfermedades degenerativas del SNC.

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FIGURAS

Figura 1. Esquema que muestra la estructura alargada y plegada de la fracción activa del factor de crecimiento neuronal. En el ángulo inferior derecho se indican las dimensiones de la molécula en un plano tridimensional (McDonald y col, 1991).

Figura 2. En este esquema puede apreciarse la similitud entre los diferentes miembros de la familia de receptores Trk, tanto en las porciones ricas en cisteína como en las que poseen actividad de tirosina cinasa. El receptor p 75^{NGFR} es capáz de unirse a todas las neurotrofinas.

Figura 3. Estructuras hipotéticas de los receptores de alta afinidad de las neurotrofinas. Se ha propuesto que las neurotrofinas comparten un receptor común de baja afinidad y que sus complejos de alta afinidad se forman tras la adición de una segunda subunidad (Thoenen, 1991).

Figura 4. Comparación esquemática entre la regulación de la síntesis del FCN en el SNP y el SNC. (A) En la periferia una gran variedad de células no neuronales sintetizan FCN, en las áreas blanco de las neuronas sensoriales y simpáticas, sensibles al factor. La regulación de la producción del factor en estas zonas es autónoma e independiente de la actividad neuronal. (B) En contraste, la síntesis del FCN en las neuronas colinérgicas sensibles al FCN del cerebro basal anterior, está regulada por la actividad neuronal. Se cree que la

regulación positiva (up regulation) ocurre a través del sistema de transmisores glutamatérgicos, mientras que la regulación negativa (down regulation) ocurre por mediación del sistema GABAérgico.

Figura 5. Esquematización del transporte retrógrado que experimenta el FCN, una vez que ha interactuado con los receptores de superficie. Puede apreciarse también la producción y subsecuente transporte anterógrado de los receptores hasta alcanzar la terminal sináptica donde se pondrán en contacto con el factor (Gage y Varón, 1988).

Figura 6. Representación hipotética de algunas de las acciones desencadenadas por el FCN. Se muestra la fosforilación de proteínas subsecuente a la activación del receptor, a su vez, la fosforilación activa otros sistemas de segundos mensajeros como el de la proteína cinasa C. Algunas de las proteínas fosforiladas, pueden interactuar con el genoma celular activando algunos genes de respuesta temprana como fos, myc, jun y erg-1. La activación de otros segundos mensajeros puede ocurrir tras la unión del factor con sus receptores. Esquema modificado de Alemán (1991).

Tabla I. Niveles de FCN en algunas áreas del SNC de rata. Los datos están reportados en ng de FCN por g de peso húmedo. Datos recopilados por Korsching y col (1985).

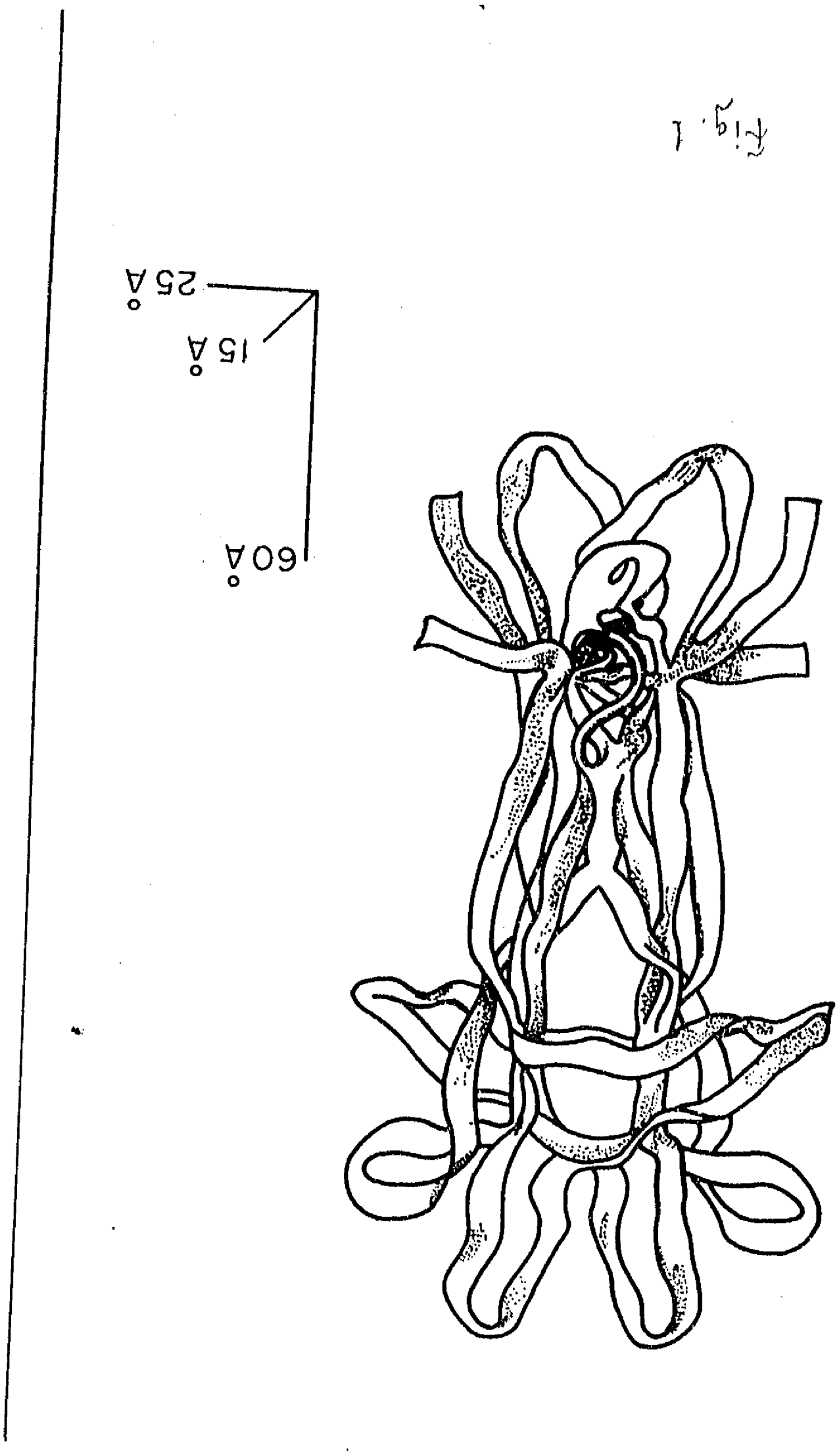


Fig. 1

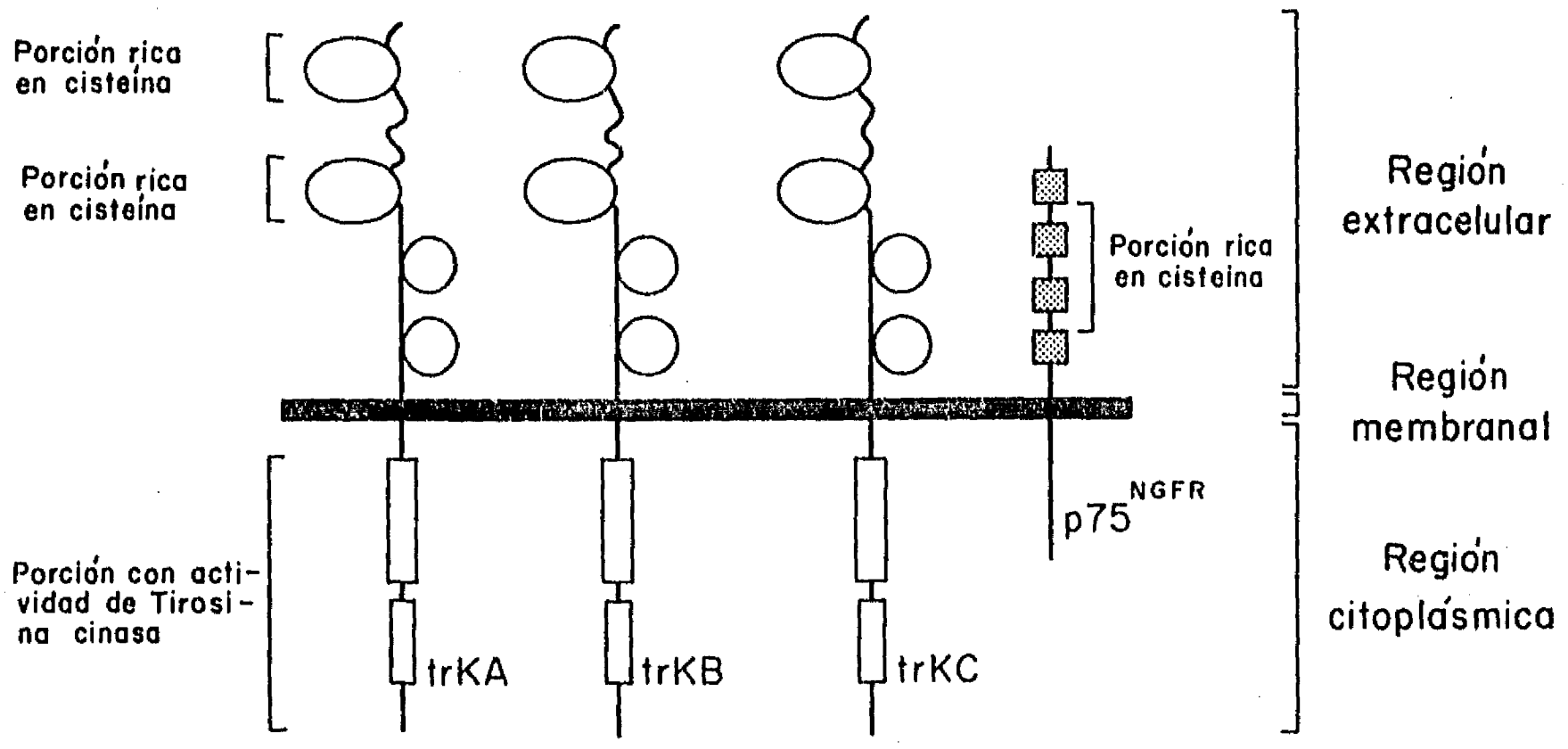

Porción rica en cisteína
Porción rica en cisteína
Porción con actividad de Tirosina cinasa
trKA
trKB
trKC
Porción rica en cisteína
p75 NGFR
Región extracelular
Región membranal
Región citoplásmica

Fig. 2

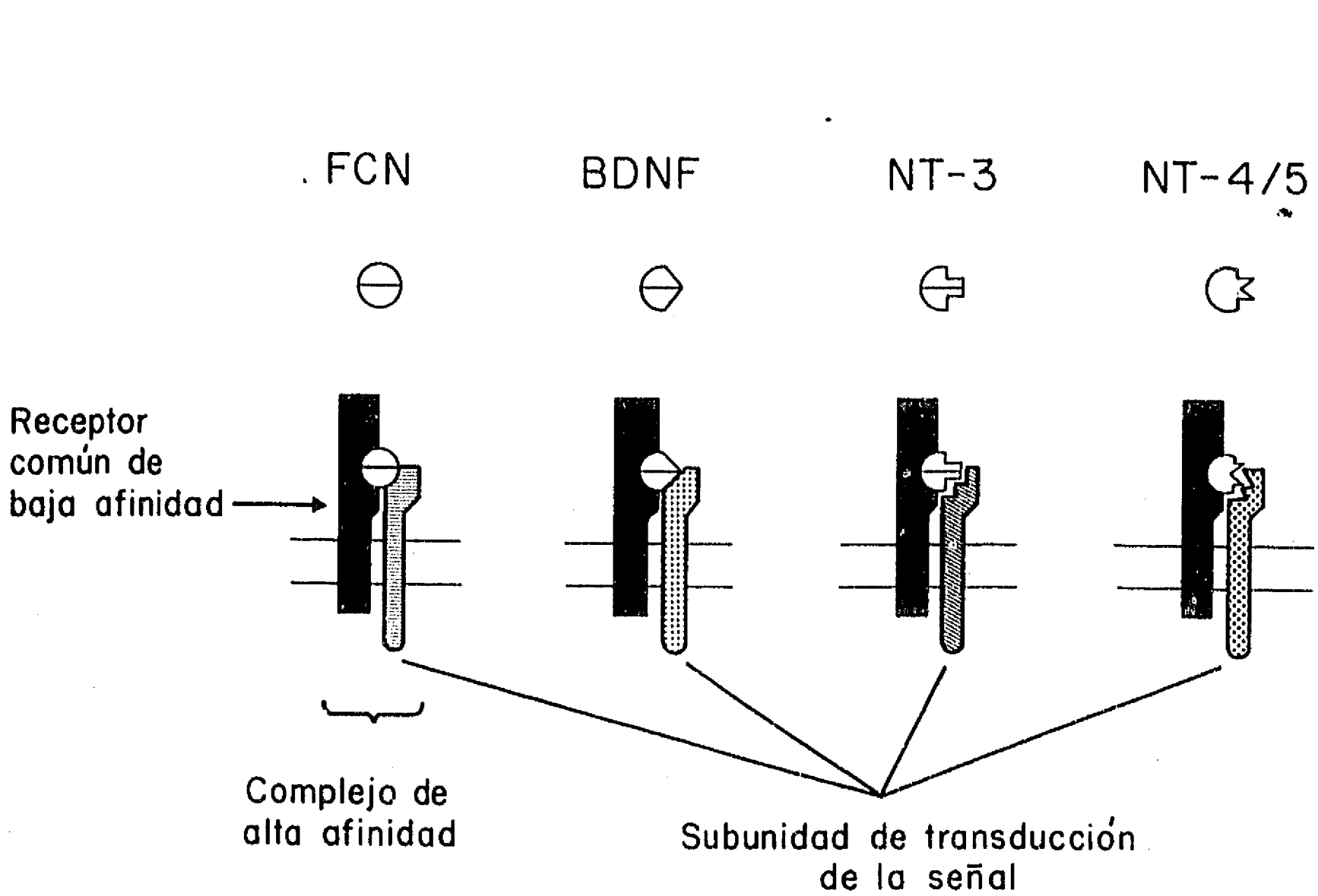
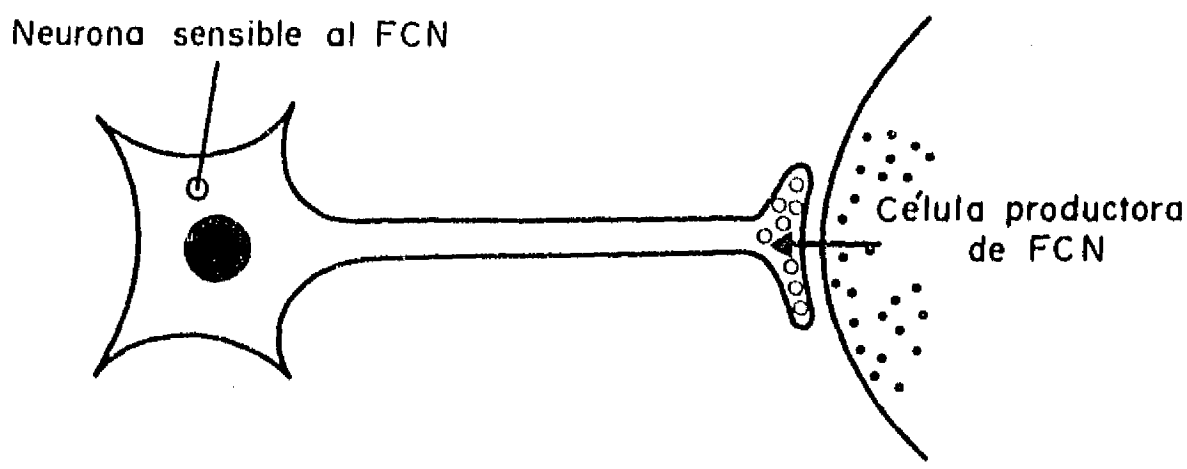

FCN
BDNF
NT-3
NT-4/5
Receptor común de baja afinidad
Complejo de alta afinidad
Subunidad de transducción de la señal

Fig. 5

SISTEMA NERVIOSO PERIFERICO

A



B

SISTEMA NERVIOSO CENTRAL

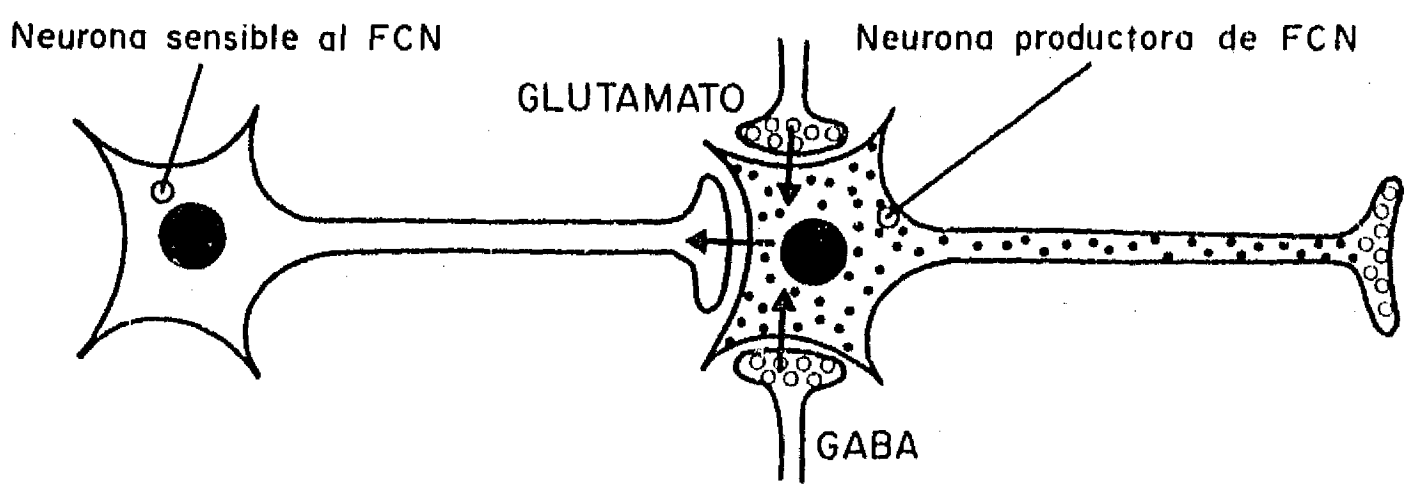


Fig. 4

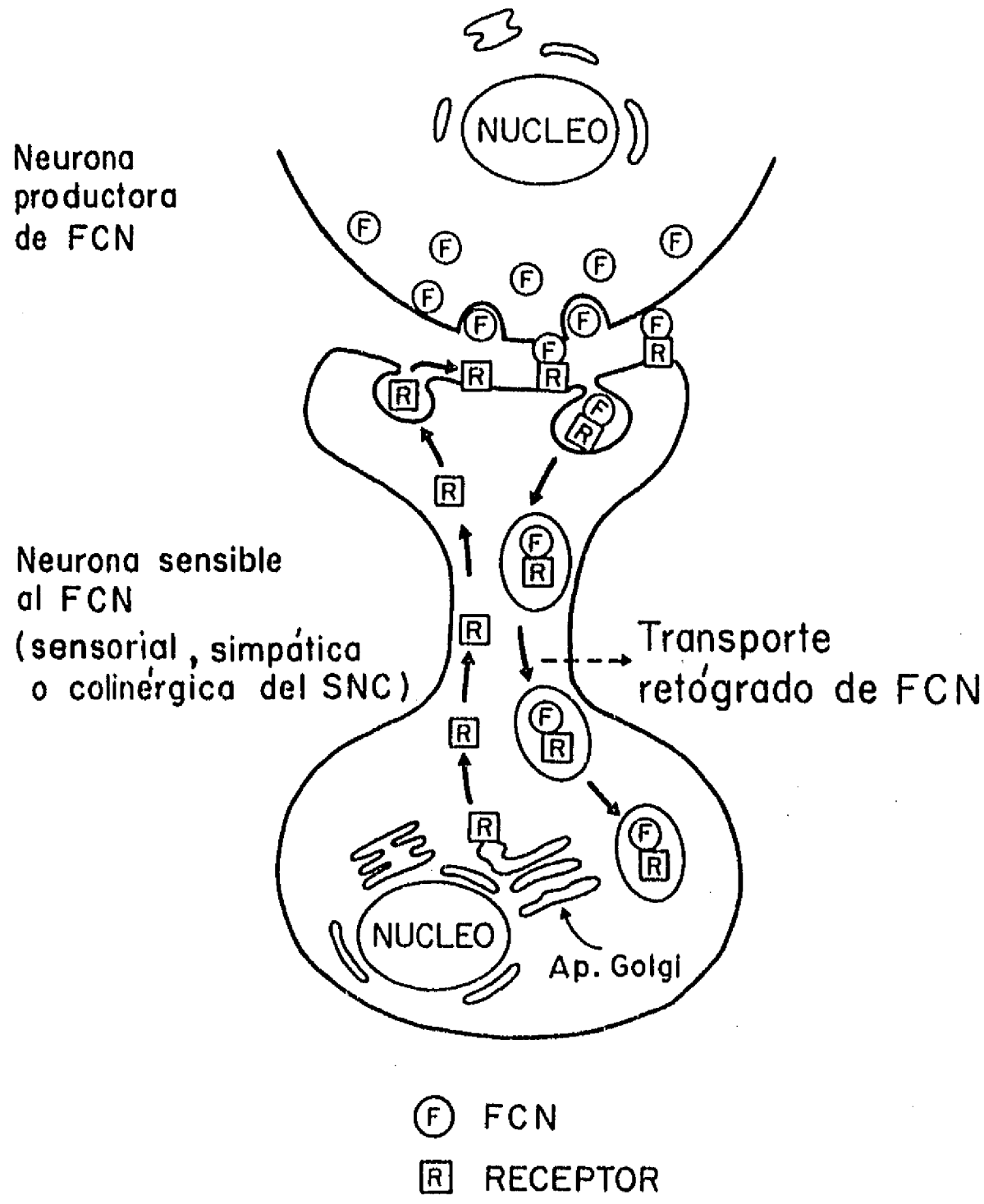

Neurona
productora
de FCN
NUCLEO
Neurona sensible
al FCN
(sensorial , simpática
o colinérgica del SNC)
Transporte
retógrado de FCN
NUCLEO
Ap. Golgi
F FCN
R RECEPTOR

Fig. 5

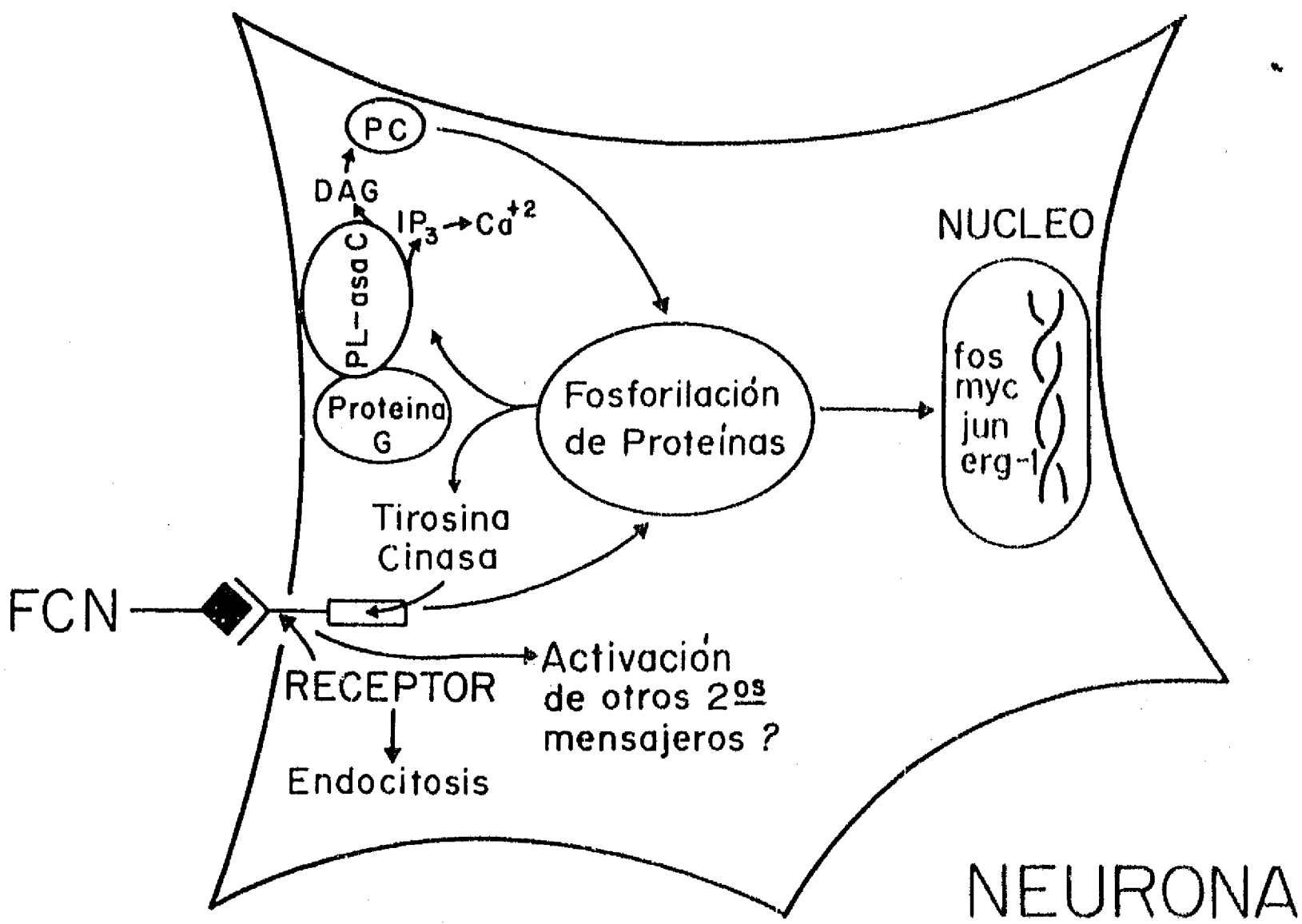

PC
DAG
IP3 → Ca+2
PL-asa C
Proteina G
Fosforilación de Proteínas
NUCLEO
fos
myc
jun
erg-1
Tirosina Cinasa
FCN
RECEPTOR
Activación de otros 2os mensajeros ?
Endocitosis
NEURONA

Fig. 6

NIVELES DE NGF EN SNC DE RATA

TEJIDO (PESO SECO)		ng NGF g^{-1}
HIPOCAMPO	REGIONES INERVADAS POR	1.41 ± 0.08
BULBO OLFATORIO	NEURONAS COLINERGICAS	0.40 ± 0.04
NEOCORTEZA	MAGNOCELULARES	0.53 ± 0.08
SEPTUM	REGIONES QUE CONTIENEN	0.51 ± 0.06
BANDA DIAGONAL DE BROCA	LOS CUERPOS CELULARES DE NEURONAS COLINERGICAS	0.71 ± 0.11
NUCLEUS BASALIS DE MEYNERT	MAGNOCELULARES	0.37 ± 0.04
CORPUS STRIATUM	REGIONES CON OTROS TIPOS	0.08 ± 0.01
RETINA	DE NEURONAS COLINERGICAS	0.07 ± 0.01
HIPOTALAMO		0.16 ± 0.02
CEREBELO	REGIONES QUE NO CONTIENEN	0.21 ± 0.03
TECTUM OPTICO	NEURONAS COLINERGICAS	0.07 ± 0.01

Tabla I

TRABAJO II

BEHAVIORAL AND NEURAL BIOLOGY 55, 179–193 (1991)

Time-Dependent Recovery of Taste Aversion Learning by Fetal Brain Transplants in Gustatory Neocortex-Lesioned Rats

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We recently showed that fetal brain transplants produced a significant recovery in the ability of gustatory neocortex-lesioned rats to learn a conditioned taste aversion. In this report we assessed the capability of gustatory neocortex fetal brain transplants to produce behavioral recovery at different times. Four groups of male Wistar rats showing disrupted taste aversions due to gustatory neocortex lesions were studied. The lesioned animals received fetal cortical grafts, obtained from 16-day-old fetuses, and were retrained in the behavioral procedure after 15, 30, 45, or 60 days postgraft. Behavioral results showed a very good functional recuperation at 60 days, slight recovery at 45 and 30 days, and a poor recovery at 15 days postgraft. Results with HRP histochemistry revealed that at 30, 45, and 60 days postgrafting there were increased connections with the ventromedial nucleus of the thalamus and with the amygdala. At 15 days postgrafting there was an absence of HRP-labeled cells. In addition, behavioral recovery was correlated with increased acetylcholinesterase activity, detected histochemically, and with morphological neuronal maturation, revealed by Golgi staining. These results suggest that morphological maturity and reconnectivity between grafts and host tissue are important for behavioral recovery in gustatory neocortex-lesioned rats.

The fetal brain transplant technique has been used recently as a very effective tool to ameliorate functional and behavioral deficits produced by either mechanical (Kesslak, Nieto-Sampedro, Globus, & Cotman, 1986; Woodruff, Braisden, Whittington, & Benson, 1987), chemical

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0163-1047/91 $3.00

(Isacson, Dunnett, & Bjorklund, 1986; Kesslak, Walencewicz, Calin, Nieto-Sampedro, & Cotman, 1988), or degenerative injuries to adult mammal brain (Collier, Gash, & Sladek, 1988. Fine, Dunnett, Bjorklund, & Iversen, 1985; Huang, Kissane, & Hawrylewicz, 1987).

Conditioned taste aversion (CTA) has been widely used as a model for the study of learning processes (Garcia, Lasiter, Bermudez-Rattoni, & Deems, 1985). In this model animals can acquire aversion to a taste cue (conditioned stimulus, CS) when it is followed by gastrointestinal illness (unconditioned stimulus, US). The anatomical pathways involved in CTA have been extensively studied (for review see Garcia et al., 1985; Kiefer, 1985). Briefly, the posterior ventromedial (VPM) and ventromedial nuclei (VM) of the thalamus receive afferents from the pontine taste area. These thalamic nuclei send fibers to both the gustatory neocortex and the amygdala (Kiefer, 1985; Lasiter & Glanzman, 1985); reciprocal connections between the amygdala and gustatory neocortex have recently been described (Escobar, Fernandez, Guevara-Aguilar, & Bermudez-Rattoni, 1989; Lasiter & Glanzman, 1985).

Lesions of the gustatory neocortex region (GN) in adult rats lead to a behavioral impairment in both acquisition and retention of conditioned taste aversions (Kiefer, 1985; Lasiter & Glanzman, 1985). It has been demonstrated that cortical fetal brain transplants induce recovery of taste aversion learning in rats with gustatory neocortex lesions (Bermudez-Rattoni, Fernandez, Sanchez, Aguilar-Roblero, & Drucker-Colin, 1987; Escobar et al., 1989; Yirmiya, Zhou, Holder, Deems, & Garcia, 1988).

The mechanisms by which the brain transplants produce functional recovery are not well understood. In this regard, several authors explain the behavioral improvement after fetal brain transplants in previously lesioned animals as being due to the release of "trophic" factors (Labbe, Firl, Mufson, & Stein, 1983). Other groups have pointed out that new connections between the graft and the host are responsible for the behavioral recuperation (Dunnett, Low, Iversen, Stenevi, & Bjorklund, 1982). Kesslak and co-workers (1988) reported that hippocampal but not glial transplants to adult rats produced partial recovery of a forced-choice alternation task. These results suggest that morphological recovery is necessary for the functional recovery. In agreement with this hypothesis, we recently demonstrated, using the horseradish peroxidase histochemistry technique (HRP), that cortical but not tectal brain transplants were able to produce behavioral recovery and reestablish connections with the amygdala and the ventromedial nucleus of the thalamus (Escobar et al., 1989).

Several authors have found connections between the cortical grafts and the host brain. Floeter and Jones (1985) reported that the cortical transplant projected fibers to the thalamus, the contralateral cortex, the striatum, and the hippocampus. Castro and co-workers observed that 10 months after cortical grafting, the transplanted tissue had received fibers

from the basal forebrain, locus coeruleus, and raphe (Castro, Tonder, Sunde, & Zimmer, 1988). These results clearly show that the cortical grafts are able to survive in the brain parenchyma and to establish afferent and efferent connections with the host tissue (Ebner, 1988; Escobar et al., 1989).

In the present study we report the time course of the behavioral recovery induced by gustatory neocortex transplants after GN lesions, as well as the time course of the appearance of connections with the amygdala and thalamus. In addition, we report the time course of the appearance of acetylcholinesterase reactivity and the development of grafted tissue using the Golgi staining technique.

METHODS

Subjects

Fifty-four male Wistar rats weighing 250–280 g were individually housed in Plexiglas boxes and had *ad lib* access to food and water, except during the CTA procedures (see below). The animals were kept on a strict 12:12-h light–dark cycle (08:00 h on; 20:00 h off).

Surgery

Large bilateral electrolytic lesions were made under pentobarbital anesthesia (50 mg/kg) to encompass the gustatory neocortex (AP = +1.2 mm, L +5.3 mm, DV −5 mm) in 30 experimental animals. Lesions were made by passing a direct anodal current (1 mA/60 s) through a stainless steel electrode coated with epoxyle except for the cross section of the tip. Twenty-four animals were used as unoperated controls.

Behavioral Procedure

Following the 7-day recovery period, the experimental and control animals were habituated to drinking their entire daily water ration in the home cages during a 10-min period in the morning and an equal period in the afternoon. This water deprivation schedule was maintained from Day 8 throughout the remainder of the experiment unless otherwise stated. The volume of water consumption was measured every day with 50-ml calibrated test tubes equipped with a rubber stopper and a glass drinking spout. Water consumption was recorded to the nearest 0.5 ml.

On Day 13 (the acquisition trial), 0.1 *M* LiCl was presented instead of water in the afternoon period. It has been demonstrated that the taste of LiCl can readily be aversively conditioned to its gastric aftereffects, since it is also the agent inducing illness (Nachman, 1963). The first extinction trial was given after five water intake baseline measures on Day 16 in the afternoon. Extinction trials consisted of the presentation of 0.1 *M* of NaCl instead of the LiCl. Two extra extinction trials (Days

tographed under bright- and dark-field microscopy for the presence and location of retrogradely labeled neurons.

Golgi stain. Six animals from groups G60, G30, and G15 (two each) were anesthetized with pentobarbital and perfused through the heart with 10% neutral buffered formalin, and the brains were removed the following day. In each rat a 4-mm-thick coronal block of tissue including the gustatory cortex was prepared for the rapid-Golgi technique. The immersion–fixation solution consisted of 4.5% potassium dichromate (K2Cr207) and 1% osmium tetroxide in distilled water (3:1). After 10 days of fixation the solution was poured off and the tissue was drained briefly on absorbent paper, transferred to a 0.75% silver nitrate (AgNO3) solution, and stored in brown glass bottles. Twenty-four hours later the tissue was removed from the silver solution, drained briefly on absorbent paper, and then washed with an ethanol impregnated brush. The tissue was then gradually dehydrated with ethanol and stored for 24 h in absolute ethanol and ether. Following that, the tissue was gradually exposed to gradually more concentrated solutions of nitrocellulose (from 2 to 30%) over the course of not more than 5 days at the maximum. The blocks were embedded in low viscosity 30% nitrocellulose and hardened overnight in a container with chloroform vapors.

Serial sections were cut at 120-μm thickness on the sliding microtome, dehydrated in ethanol (70, 80 and 95%, 10 min each), and passed through 98% isopropanol and 98% terpineol (10 min each). The sections were transferred to reagent grade xylene and mounted with synthetic resin.

Acetylcholinesterase histochemistry. The remainder of the animals (two from each experimental group) were anesthetized with pentobarbital and perfused transcardially with the same formula described above for HRP injections. The brains were cut coronally (40 μm thickness), mounted, and then immersed in the incubating solution described by Paxinos and Watson (1982). The following day the slices were developed in sodium sulfide (pH 5) and mounted with synthetic resin.

RESULTS

Behavior

One-way ANOVA was done on the test day consumption volume for all groups, with post hoc group comparisons, when appropriate, using the Student–Newmann–Keuls'test (Fig. 1). During the pregraft test trial, there were significant differences among groups ($F(7, 51) = 11.4$; $p < .001$). As expected, the four control groups showed strong taste aversions in the first test trial. The experimental (GN lesions) groups showed significant disrupted taste aversions when compared with their own controls (p's $< .05$). Postgraft ANOVA comparisons (Fig. 1, right) revealed that there were significant differences among the groups ($F(7, 51) = 6.88$; p

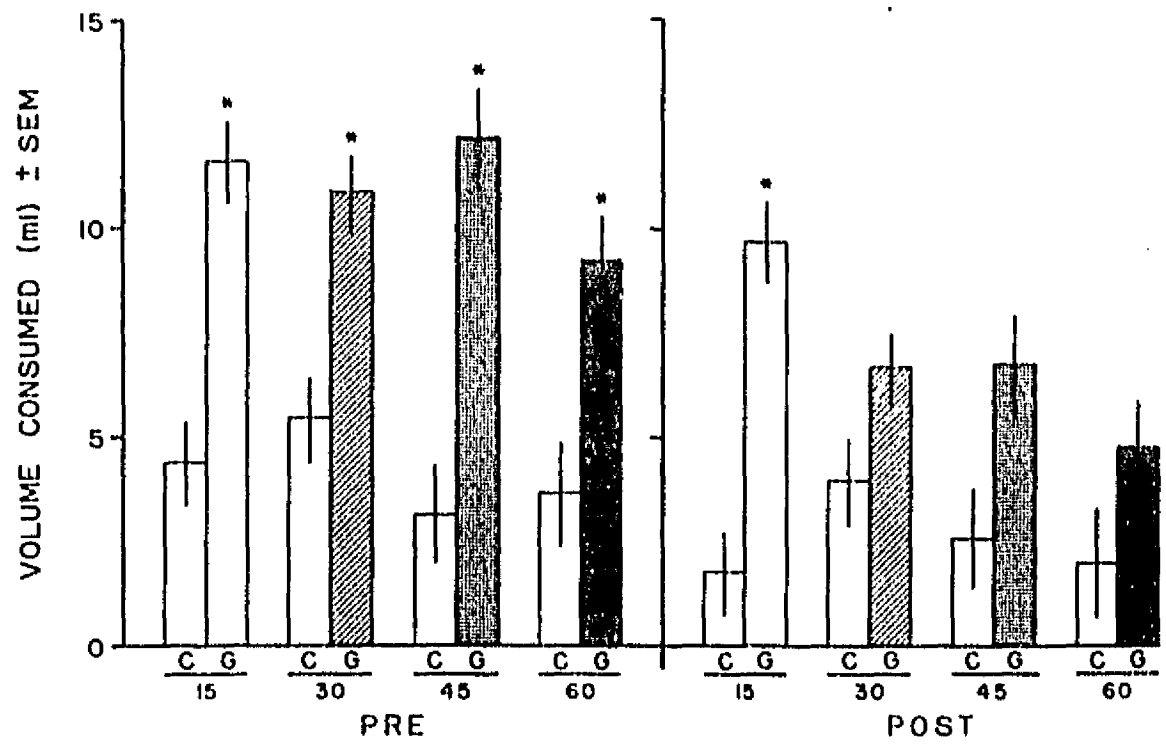

FIG. 1. The graph illustrates the amount of NaCl consumed by control (C) and grafted groups (G) pre- and postgraft. The left side shows the results from the test trial prior to transplant. The right side shows the results of one test trial after transplant for the 30-, 45-, and 60-day groups (see text). $^*p < .05$ (Newmann–Keuls test).

$< .001$). The control groups again showed excellent aversion. The G15 group showed a disrupted taste aversion, consuming significantly more NaCl solution when compared with its own control ($p < .05$). The G30 and G45 groups consumed more saline solution than their respective control, although there were no significant differences. The G60 group showed a strong water intake suppression in the presence of the CS, which was similar to its own control.

In addition, paired *t* tests between pre- and postgraft volume consumption were done. The postgraft scores revealed that groups G30, G45, and G60 showed a significant aversion as they reduced their water consumption in the presence of the CS when compared with their pregraft scores (p's $< .05$). In contrast, group G15 showed a disrupted taste aversion both pre- and postgraft, as they had similar NaCl water consumptions.

HRP Histochemistry

The analysis of the brain tissue injected into the VPM and VM nuclei of the thalamus or amygdala with HRP in the G15 experimental animals showed that there were no HRP-labeled cells in the grafted tissue. Nevertheless, we found labeled host cells in the ipsilateral thalamus and amygdala projections of the same animals, indicating that the labeling procedure was working. In the graft tissue of G30 animals the HRP neurons were

scarcely labeled. In contrast, in the 45- and 60-day graft tissue, a great number of labeled neurons were found, though not as many as in control tissue, as we have described previously (Table 1; Fig. 2; Escobar et al., 1989). The cell distribution inside the transplant did not follow any distinguishable pattern in all the grafts in which HRP-labeled cells were found. In addition, we did not find differences in the amount of labeled cells depending upon the place where the injection was made, i.e., amygdala or thalamus.

Golgi Stain

The Golgi stain results were obtained from six adult brains with fetal brain transplants. We observed differences at each age of the transplanted tissue. The difference in the tissues taken at 15, 30, and 60 days was that they were all in different stages of neuronal development and maturation. In all experimental groups the grafted tissue showed a neural reorganization in both tissue types (grafted and host) with a greater neuronal density in the transplanted tissue, particularly in those of 60 days. In general, the fetal transplants adhered to the host tissue with abundant vascularization and great proliferation of glial cells in the transplant border as well as fibers that crossed the interface. The following chronological changes were observed. At 15 days, transplanted tissue showed scarce development of neurons and blood vessels. Round-shaped neurons appeared with few dendritic processes; some of them had no spines at all in their dendrites (Fig. 3A). There were few glial cells in the border of the transplant. In an overall view the grafts appeared to be at an initial state of neuronal development, with an incipient vascularization process between transplant and host tissue. At 30 days, graft tissue appeared to be in a more advanced stage of development (Fig. 3B). That is, neurons showed a great number of dendritic processes growing in all directions from the cell body. The axons were apparent in the majority of the neurons, and blood vessels were found in the border and inside the transplanted tissue. Many pyramidal and multipolar neurons were found inside the transplant. Glial cells were found in many parts of the transplanted tissue, without any regular pattern. At 60 days, the transplanted tissue showed a further advance in the development of neurons and glial cells (Fig. 3C). Neurons in the graft presented multipolar, piriform, and triangular-shaped somas that appeared quite similar to the host cortex but not in the same stage of development. The cells in the transplanted tissue were surrounded by abundant vascularization and well-developed glial cells (Fig. 3C, g). These glial cells were identified as astrocytes (Fig. 3C, ga) and oligodendrocytes (Fig. 3C, go). Ramifications of the axons can be observed in the cell's own dendritic field (Fig. 3C, neuron 5) or in a straight direction (Fig. 3C, neurons 2 and 6). Fibers were more abundant in the border of the transplant.

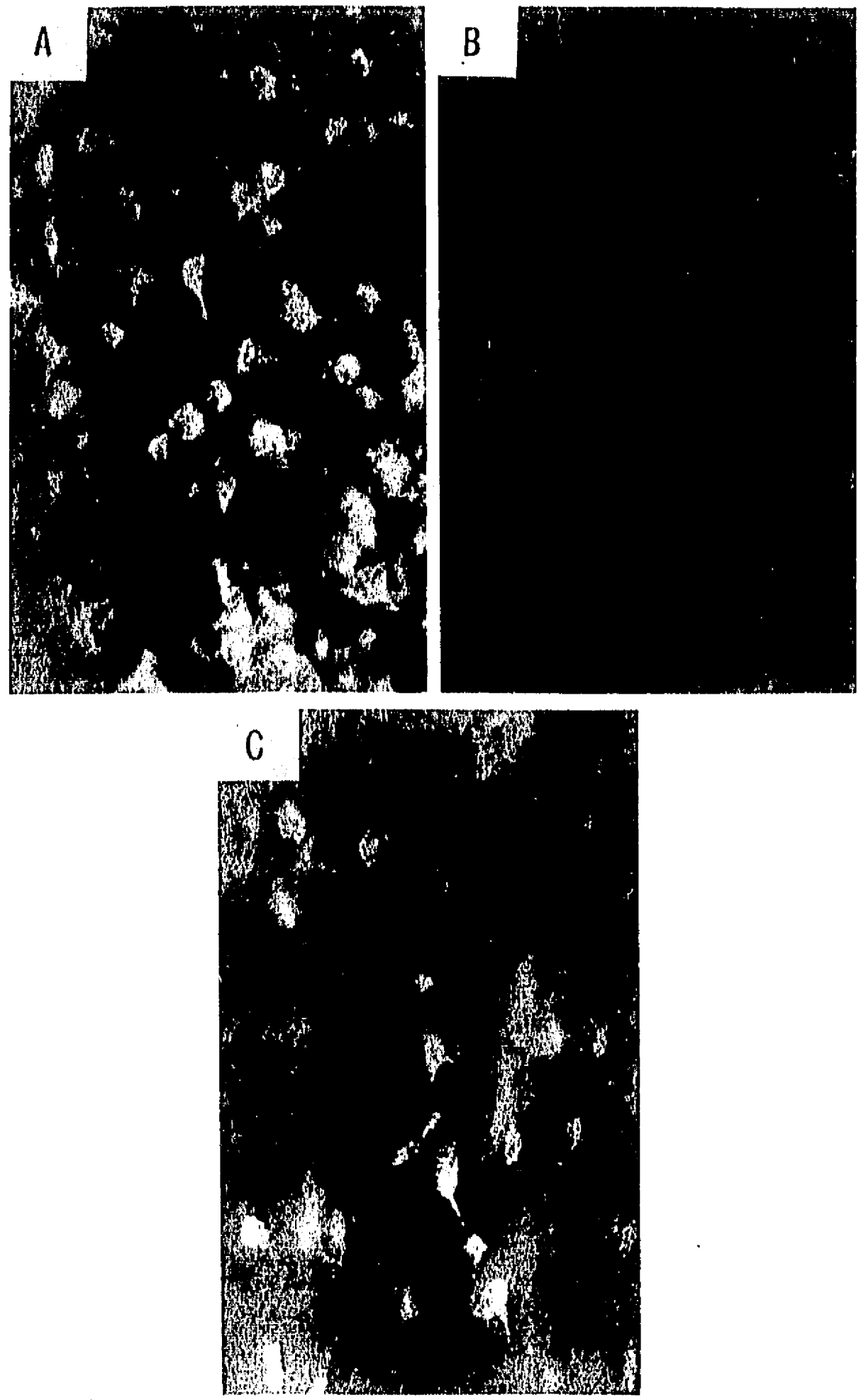

FIG. 2. Representative coronal sections in dark-field illumination of a control subject (A). (B and C) HRP-labeled neurons within homotopic grafts 30 and 60 days after transplant, respectively. A, B, and C × 160.

TABLE 1
Quantitative Analysis of the Presence of the HRP-Labeled Neurons inside the Cortical Grafts

	Days post-transplant			
Injection site	15	30	45	60
Thalamus				
Mean	0	13.5	37.5	38.5
SE	—	1.5	7.5	3.5
n	2	2	2	2
Amygdala				
Mean	0	9.5	33.5	31
SE	—	0.5	1.5	2.0
n	2	2	2	2

Note. See Fig. 2.

Acetylcholinesterase Reactivity

The 15-day animals showed some labeled cells and there were few processes in the transplant. In the 30-, 45-, and 60-day groups there was an increased number of AChE fibers inside the transplants (Fig. 4). These fibers formed patches along the grafts. The 15-day post-transplant groups did not show these AChE patches, since there were few AChE-stained fibers (Fig. 4A). We could not observe any difference in the number of cells among the different transplant groups, although there was an increased AChE reactivity within the G30, G45, and G60 (Fig. 4).

DISCUSSION

The behavioral data obtained in these experiments showed that it took the grafts at least 30 days to start producing functional recovery in the host animals. During the initial 15 days post-transplant, the subjects did not show any recuperation in the CTA paradigm (Fig. 1). The animals were able to learn the aversive response to the noxious stimulus after 30 and 45 days post-transplant. At 60 days postgraft the behavioral recovery was almost complete (G60; see Fig. 1), as the grafted group did not show any significant differences with its own control (Fig. 1). The time-dependent behavioral recovery was accompanied by time-dependent histological changes. At 15 days postgraft the cortical transplants did not establish any demonstrable connections with the thalamus or with the amygdala (Table 1). In the 30-, 45-, and 60-day postgraft groups, the brains showed increased connections with both the thalamus, (VPM–VM nuclei) and the amygdala (see Table 1). We could not find a correlation between the number of cells labeled in the transplants and the behavioral

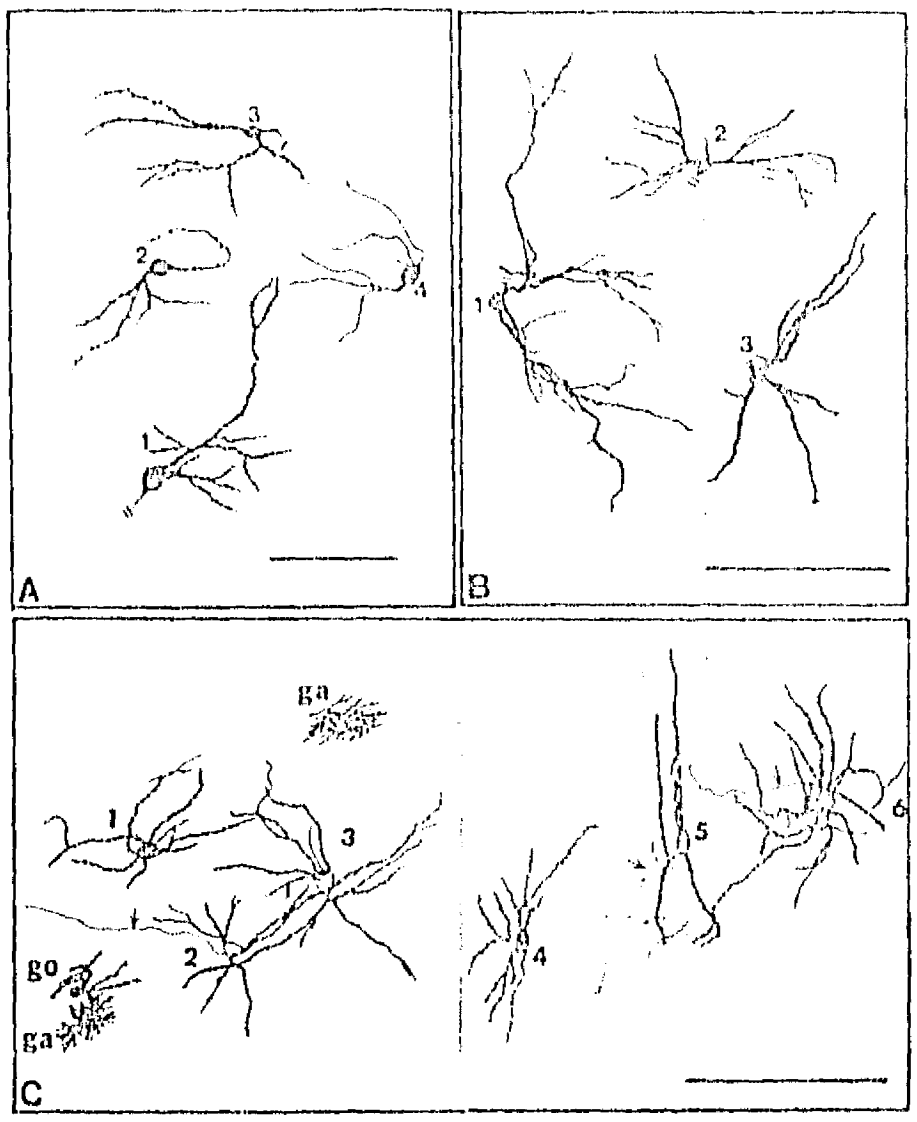

FIG. 3. Camera lucida drawings of Golgi-rapid impregnated neurons from 15-, 30-, and 60-day-old transplants. (A) Drawing of Golgi rapid-impregnated neurons from 15-day-old transplant. Some neurons show a round-shaped soma and dendrites supporting sparse spines (see, cells 2, 3). Cell 1 shows more spines and cell 4 is a nonspiny neuron. (B) Drawing from Golgi-impregnated neurons from 30-day-old transplant. Neuron 1 shows a multipolar shape with spines on its dendrites. (C) Golgi rapid-impregnated neurons from 60-day-old transplant. Neurons 1, 2, 3 show multipolar shape, their dendrites are covered with spines, and they keep close relation with glial cells (g), astrocites (ga), and oligodendrocytes (go). Neurons 4 and 5 (from the border of the transplant) have large and ramified axons (arrows), and neuron 2 is a typical multipolar cell. Bars represent 100 μm at 400X magnifications.

recovery. However, in the case of the group (15 day) that did not show behavioral recovery, there were also no labeled cells in the transplants (see Fig. 1; Table 1).

The neurons of the transplants from the 30- and 60-day postgraft groups showed a more mature cell morphology. In these groups, the Golgi stain revealed that cell bodies were more mature, since they had more dendritic processes with more spines. Nevertheless, the stages of maturation were different from that of the neighborhood host cortical tissue. These results are in agreement with those that have used similar (60-day postgraft)

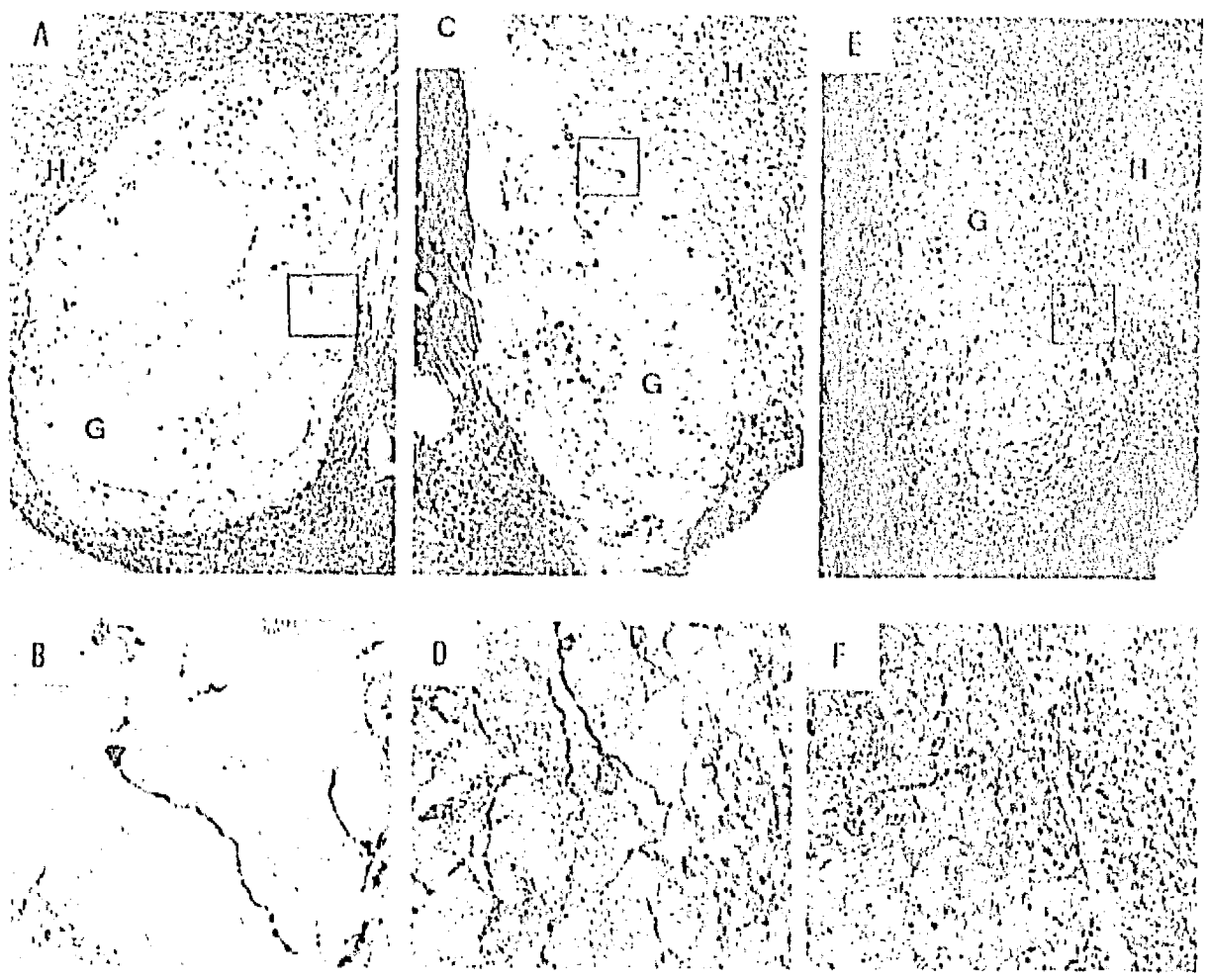

FIG. 4. Acetylcholinesterase reactivity of homotopic neocortical grafts, in 15 (A and B), 30 (C and D), and 60 (E and F) days postgraft. A, C, and E × 320. B, D and F × 160.

cortical fetal brain transplants (Yirmiya et al., 1988). In contrast, the 15-day post-transplant group showed an immature cell morphology with a small number of dendritic spines observed with the Golgi staining technique, compared to the adjacent host gustatory cortex (see Figs. 3A, 3B, 3C; Jaeger & Lund, 1981).

Our results suggest that some maturation of the transplanted tissue accompanies behavioral recovery. The maturity of the neurons can be determined by the number of their connections, which include, in part, those established between the transplant and the host. Recently, some authors have reported the establishment of connections between cortical grafts and the thalamus in neonatal rats 2 to 4 months after transplantation (Castro, Zimmer, Sunde, & Bold, 1985). Other studies have also shown that the thalamus of the adult brain could establish only a few connections with the cortical transplants, from 8 to 28 weeks after transplantation (Gonzalez, Sharp, & Loken, 1988). We had previously demonstrated that 60-day transplants of the gustatory neocortex could establish connections with the VPM–VM and the amygdala, although the number of labeled cells was not as great as in control animals (Table 1; Escobar et al., 1989). In the present paper, the results suggest that the transplanted neurons

required more than 15 days to start making connections with both the thalamus (VPM and VM) and the amygdala. In addition, with the use of the Golgi impregnation, in the 60-day grafts we were able to see some fibers crossing the boundaries of the transplant into the host tissue, indicating a dynamic process of interaction between the transplant and the host tissue. However, there was not a laminar arrangement in the grafts as in the normal host tissue, indicating that grafted neuronal tissue follows its own developmental and maturation neuronal patterns. These results are in agreement with those of others, who found partial functional recovery without a normal laminar arrangement of fetal cortical brain transplant. (Mufson, Labbe, & Stein, 1987; Yirmiya et al., 1988). However, fetal cortical transplants in the central hemisphere of newborn rats showed laminar patterns in a supra- and infragranular laminae, which resembled the normal cortical laminar distribution (Castro et al., 1987), since the factor for the lamination neocortical pattern appears in postnatal development.

One of the explanations for behavioral recovery, from the results presented here, is that some morphological recuperation is needed, since it is necessary to wait at least 30 days to start seeing any functional recovery in gustatory neocortex-lesioned rats. However, it has been suggested by other authors that structural and morphological integrity of fetal brain grafts may not be essential for behavioral recovery after brain injury (Dunnett, Ryan, Levin, Reynolds, & Bunch, 1987; Kesslak et al., 1986; Stein, Palatucci, Kahan, & Labbe, 1988). These authors have speculated that brain injury and/or brain grafts induced a release of neurotrophic substances that can reactivate neural function and/or prevent injury-induced degeneration in the damaged host brain (Dunnett, et al., 1987; Kesslak et al., 1986). In our results the possibility is low that neurotrophic factors alone may be involved in the functional recovery since it was necessary to wait at least 30 days to see any recuperation. The best recovery was reached at 60 days postgraft (see Fig. 1). This observation is supported by the finding that homotopic cortical but not heterotopic occipital, cortical, or tectal fetal brain transplants could restore the associations between taste cues and illness (Escobar et al., 1989; Lopez-Garcia, Fernandez-Ruiz, Bermudez-Rattoni, & Tapia, 1990). Therefore, if neurotrophic factors are involved they need to be associated with cortical homotopic and/or with some time-dependent factor to start producing functional recovery.

The demonstration of the AChE expression in the transplant in our results is congruent with previous observations (Hohmann & Ebner, 1988). Thus, other authors (Park, Clinton, & Ebner, 1984) found AChE expression after 7 days of cortical transplantation. However, it was only after 2 months that they found AChE reactivity similar to that of the cortical host tissue (Park et al., 1984). In this paper, we are showing the time

course of AChE graft expression. There was observed a great reactivity for AChE in the somas but few processes for the 15-day postgraft group. The number of all processes increased AChE reaction at 30, 45, and 60 days postgraft. Moreover, in a recent paper it was shown that acetylcholine can be released from homotopical but not from occipital cortical grafts and that this was correlated with recovery of CTA with 60-day fetal neocortical grafts (Lopez-Garcia et al., 1990).

Recently, several authors have demonstrated the presence of trophic factors delivered by specific systems. For example, Zhou and co-workers (Zhou, Averbach, & Azmitia, 1987) described the enhanced proliferation of processes from raphe-transplanted but not locus coeruleus-transplanted neurons when they were placed in serotonin-denervated hippocampus. Moreover, when a hippocampal transplant is placed near undamaged host hippocampus, the raphe neurons of the host are capable of innervating the new target sites, indicating that there is some kind of chemotaxis (Zhou, Averbach, & Azmitia, 1988). So, it seems that some trophic factors could guide the neuronal processes with some specificity and finally could lead to the formation of new connections.

In the case of the establishment of connections between GN and VPM, it may be possible that these connections are mediated by trophic factors (Zhou et al., 1987, 1988). In our model, there are at least two potential sources of factors: the transplant itself and the lesion-denervated host tissue (Cunningham, Haun, & Chantler, 1987). The interaction of these factors could promote the connection of the transplant with the VPM–VM and amygdala. One possible hypothesis to explain our behavioral results is based on the reconnectivity between VPM–VM and GN, as proposed by Sharp and Gonzalez (1986). These authors suggested that the reconnections between thalamus and cortex could stop the degenerative processes due to the lesion. In this manner the graft could help the restoration of the lost function.

In conclusion, we have demonstrated that after 30 days postgraft the animals with transplants were able to learn the aversive response in the CTA paradigm. The neurons in the transplant can express AChE in a time-dependent fashion. After 30 days the grafted neurons started to establish connections with the thalamus and amygdala of the host. Finally, the neurons in the older transplants showed a more mature morphology than those in the younger ones. All of these results suggest that cell maturation and reconnectivity are important for recuperation of the capacity for taste aversion learning in GN-lesioned rats.

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Effects of Excitotoxic Lesions of the Nucleus Basalis Magnocellularis on Conditioned Taste Aversion and Inhibitory Avoidance in the Rat

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LÓPEZ-GARCÍA, J. C., J. FERNÁNDEZ-RUIZ, M. L. ESCOBAR, F. BERMÚDEZ-RATTONI AND R. TAPIA. *Effects of excitotoxic lesions of the nucleus basalis magnocellularis on conditioned taste aversion and inhibitory avoidance in the rat.* PHARMACOL BIOCHEM BEHAV 45(1) 147–152, 1993.—The role of the nucleus basalis magnocellularis (NBM) in a variety of learning tasks is well known. Lesions of this nucleus result in a reduction of cholinergic transmission throughout a vast portion of the cortex. Because cholinergic transmission in the insular cortex seems to be important for the acquisition of conditioned taste aversion, the aim of the present work was to study the effects of bilateral chemically induced lesions of the NBM on this conditioning, as correlated with some cholinergic markers in the insular cortex. The effect on inhibitory avoidance was also studied. Lesions prevented the acquisition of the aversion and disrupted retention of the task in previously trained animals. Learning in the inhibitory avoidance paradigm was also notably affected. Postlesion reductions of choline acetyltransferase and acetylcholinesterase activities and of K^+-stimulated [^{3}H]acetylcholine release were found in the insular cortex. Further, in intact rats labeling of NBM neurons was observed by retrograde tracing after injection of Fluoro-Gold into the insular cortex. These findings indicate that the NBM is involved in the neural integration of feeding behavior and that its cholinergic projection to the insular cortex is one of the implicated neurotransmitter systems.

Nucleus basalis magnocellularis Insular cortex Conditioned taste aversion Choline acetyltransferase Acetylcholine release

THE nucleus basalis magnocellularis (NBM) is the major source of cholinergic projections to the cerebral cortex (25, 26,30) and it is known to be involved in a variety of learned behaviors (5,6,17,27,31,34,36).

Conditioned taste aversion (CTA) is a learning paradigm in which animals acquire aversion to a taste cue when it is followed by digestive malaise (14). The brain structures involved in CTA learning have been well established (19). The agranular insular cortex (IC), a region of the temporal cortex in the rat, corresponding to Krieg's areas 13 and 14 and referred to as gustatory neocortex (4), has been implicated as a neural substrate of CTA (4,20). Recently, it has been demonstrated that the IC is also involved in the acquisition and consolidation of spatial and inhibitory avoidance learning tasks (2,3).

We have previously shown that the adult IC is able to release significant amounts of radioactive acetylcholine (ACh) after K^+-depolarization in a Ca^{2+}-dependent manner and possesses choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities (21). However, it is not known whether this cholinergic input arises from the NBM and whether such projection is involved in CTA learning. Hence, the aim of the present work was to study the existence of a cholinergic pathway arising from the NBM to the IC and its possible role in the flow of taste information. For this purpose, we analyzed the effect of excitotoxin-induced lesions of the NBM on CTA and its relationship with neurochemical alterations in the cholinergic neurotransmission in the IC.

METHOD

Subjects and Surgical Procedure

Adult, male Wistar rats weighing 250 ± 10 g at the start of the experiment were used. They were housed individually,

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under an inverted 12 D : 12 L cycle, with food and water ad lib except when indicated.

Under pentobarbital anesthesia (50 mg/kg), bilateral lesions of the NBM were made in 40 animals by stereotaxic injection of 0.5 μl 0.12 M quisqualic acid (QA) dissolved in phosphate-buffered saline (PBS), using the following set of coordinates with respect to bregma: AP -1.8 mm, L ± 3.2 mm, V -6.8 mm (29). Bregma and lambda were set at the same horizontal level. The use of this neurotoxin differs from most articles, in which ibotenic acid has been employed to destroy the NBM. However, it has been reported that QA shows a higher degree of specificity for NBM neurons than ibotenate and that after QA-induced lesion of the NBM cortical ACh is decreased as after ibotenate acid but without the nonspecific behavioral deficits observed with the latter (9).

The duration of the stereotaxic injection was 5 min, with a further 5-min period allowed for diffusion. In preliminary experiments, it was observed that the bilateral injection of QA during the same period of anesthesia resulted in severe loss of weight and death of the majority of animals. Therefore, a 1-week interval between the QA injection into the right and left NBM was allowed. Control rats ($n = 13$) received injections of PBS only. Behavioral and neurochemical tests were started 2 and 4 weeks, respectively, after the second injection (see the Results section). Of the 40 rats injected, 14 were discarded because the histological examination showed that the injection site was misplaced.

Behavioral Procedures

Inhibitory avoidance. Training for inhibitory (passive) avoidance was carried out in a two-compartment box (30 × 40 × 15 cm) divided by a sliding door. One of the chambers was illuminated by a 40-W light bulb. The other chamber was not illuminated and its floor was a metal plate through which electric shocks were delivered. During the acquisition session, animals were placed in the illuminated chamber and after 30 s the sliding door was opened, allowing the rat to enter the dark compartment. The time elapsed since the door was opened until the rat moved into the dark chamber was recorded. Then, the door was closed and a 0.8-mA DC foot-shock was delivered for 3 s. The door was opened and the animal was allowed to return to the illuminated side. Twenty-four hours later, the same procedure was followed except foot-shock was not applied (retention trial). If the rat did not enter the dark chamber within 600 s, the test was stopped and the animal was returned to its home cage.

Conditioned taste aversion. A previously described experimental model of CTA was used (22). Briefly, animals were deprived of water for 24 h and trained to drink water twice a day during 10-min trials for 4 days. On the fifth day, a 0.1 M LiCl solution was given instead of water to induce taste aversion. After 4 more days of baseline consumption, water was substituted by a 0.1 M NaCl solution to test the aversion. LiCl and NaCl are indistinguishable by rats (38). A decrease of NaCl intake during the test trial was considered as aversion to the salty taste.

Neurochemical Procedures

Release of labeled neurotransmitters. The simultaneous release of [^{14}C]GABA and [^{3}H]ACh in IC slices was studied using a previously described superfusion method (21,28). Animals were decapitated and their brains quickly removed. The IC was dissected using the middle cerebral artery and the rhinal sulcus as references (4,20,37). IC slices (200 μm thickness) were obtained in a McIlwain tissue chopper and preincubated (about 15 mg tissue) for 10 min at 37°C in 1 ml of an oxygenated medium containing (in mM concentrations): NaCl 118, KCl 4.7, Na_2HPO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 5.5, Tris-HCl 25, pH 7.4, aminooxyacetic acid 0.1, and eserine 0.1. After this period, the slices were incubated for another 10-min period in the presence of both [^{14}C]GABA (2 μCi, 0.5 μM) and [^{3}H]choline chloride (2 μCi, 0.5 μM) and 0.8-ml aliquots were transferred to superfusion chambers holding 0.65-μm filters. Tissue was washed by superfusion during 5 min and then the collection of 1-min fractions of the superfusate was started. At the end of the fifth fraction, the medium was substituted by one containing 47 mM KCl and 10 more fractions were collected. The radioactivity in each fraction and that remaining in the filter was counted by scintillation spectrometry, with the double-channel procedure; correction for ^{14}C/^{3}H overlapping was made. Results are expressed as percent of total radioactivity released per minute. Total radioactivity is the sum of total released radioactivity plus that remaining in the filter at the end of the superfusion.

The identity of the radioactive compounds released by K^+-depolarization under these experimental conditions has been previously established. About 80% of the ^{14}C label released corresponds to GABA (28), whereas more than 95% of the tritium radioactivity released represents ACh (21).

Enzyme activities. Water homogenates of the IC were used for measuring enzymatic activities by previously described procedures. ChAT (13,21) and glutamate decarboxylase (GAD) (1,21) activities were measured by radioisotopic techniques and AChE activity according to a spectrophotometric method (10). Protein was determined using the Folin reagent method as described (23).

Histology

To assess the lesion induced by QA microinjection, some animals were anesthetized with an overdose of pentobarbital and perfused through the ascending aorta with 0.15 M NaCl followed by a 4% paraformaldehyde, 0.1% glutaraldehyde solution in PBS. The brains were subsequently immersed for 2 h in the fixation solution and then in 20% sucrose during 24 h, prior to sectioning. A series of coronal sections (40 μm thickness) were cut in a cryostat, collected in PBS, mounted on gelatin-coated slides, and stained with cresyl violet (Nissl staining) by standard procedures.

In another group of 10 nonlesioned rats, 0.5 μl of a 2% Fluoro-Gold solution (33) were unilaterally injected in the IC over a 5-min period. Four days later, animals were perfused, their brains sliced as described above, and the site of injection verified histologically by locating the needle track. The appearance of Fluoro-Gold in retrogradely labeled neurons in the NBM was studied with the aid of a fluorescence microscope.

Statistical Analysis

Overall differences among the data from the behavioral experiments were analyzed using one-way analyses of variance (ANOVAs). Posthoc comparisons were made using the Fisher's least significant difference test. Biochemical data were analyzed using Student's t-tests. In all cases, values of $p < 0.05$ were considered significant.

Chemicals

[U-^{14}C]Aminobutyric acid (sp. act. 192 mCi/mmol) and [acetyl-^{3}H]acetyl-coenzyme A (1.6 Ci/mmol) were purchased

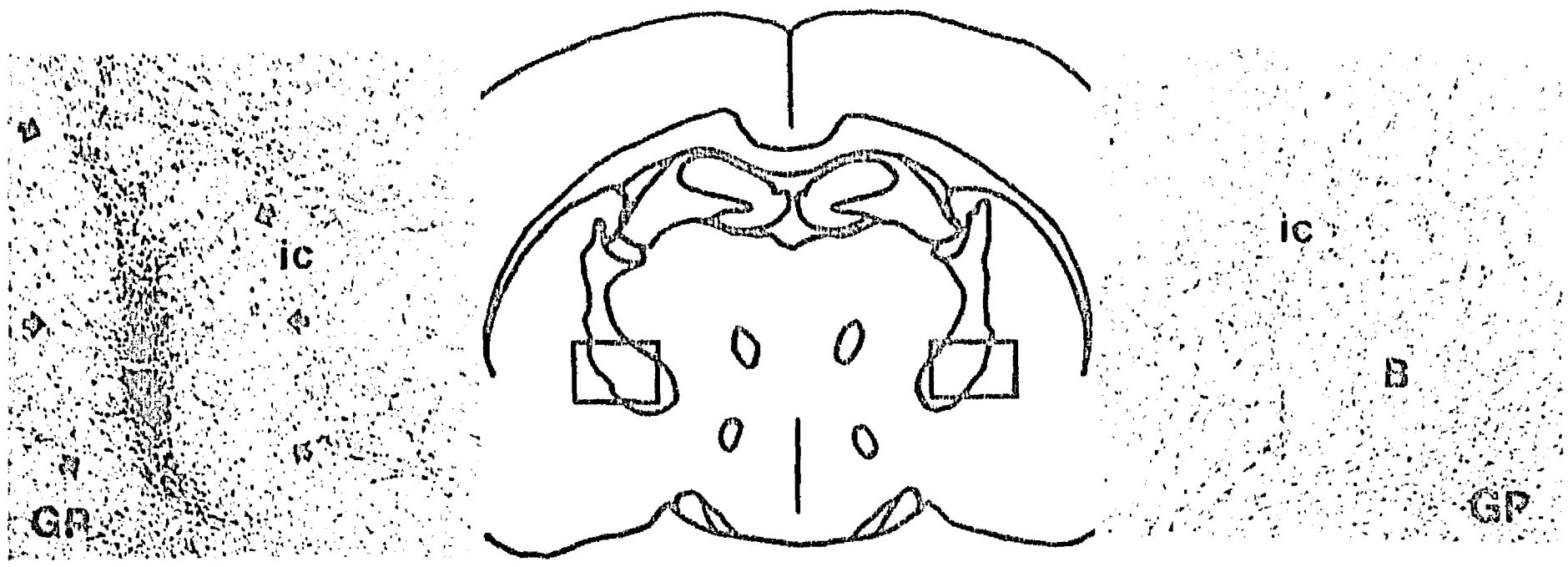

FIG. 1. Center: diagram of a section of rat brain at the NBM. Left: micrograph of a Nissl-stained section of the nucleus basalis magnocellularis (NBM) (B) 2 weeks after the lesion induced by quisqualic acid (QA) microinjection; the arrows indicate the limits of the lesion (×20). Right: micrograph of the same region in a control rat. GP, globus pallidus; ic, internal capsule. Note the important gliosis in the lesioned region.

from NEN Dupont (Boston, MA). [Methyl-^{3}H]choline chloride (80 Ci/mmol) and [1-^{14}C]L-glutamic acid (56 mCi/mmol) were obtained from Amersham (Buckinghamshire, UK). Quisqualic acid was purchased from Tocris Neuramin (Essex, UK). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Histological Observations

Retrograde tracing. Injection of Fluoro-Gold into the IC of intact animals resulted in labeling of a great number of neurons in the ventroposteromedial thalamic nucleus and in the basolateral amygdala ipsilateral to the injection site, as previously described (11). In agreement with other studies (32), in the ipsilateral NBM some Fluoro-Gold containing large neurons were observed, although they were less abundant than in the former structures (results not shown).

Assessment of the NBM lesion. In control as well as in lesioned animals, the needle track was readily identified by the presence of gliosis. In PBS-injected animals, gliosis was also observed in the NBM area but the appearance of the neurons within the injection site was normal. The injection of QA in the NBM, however, produced loss of magnocellular neurons in the area, as revealed by Nissl staining (Fig. 1).

Behavioral Observations

Inhibitory avoidance. The deleterious effect of NBM lesion on the inhibitory avoidance task has been previously reported (17,27). In the present study, no differences were observed in the latency to enter the dark chamber during the acquisition session between the control and the experimental group (<30 s in most cases) and, in agreement with the previously described deficits, 70% of control rats reached the maximum latency allowed during the retention trial, with a mean value of 485 s, whereas NBM-lesioned rats entered the dark chamber within the first 200 s of the test (Table 1). All animals vocalized as a response to the electric shock during the acquisition session, indicating that pain sensitivity was not affected by the lesion.

Conditioned taste aversion. A small, nonsignificant reduction in the intake of the LiCl solution vs. the baseline intake (neophobia) was found in the control group (Table 2). This trend was not observed in lesioned animals. During the test, however, a highly significant reduction in NaCl intake was observed in control animals, whereas NBM-lesioned rats drank as much of the solution as during their daily intake (Table 2). As mentioned above, a decreased intake during the test trial should be considered as aversion to the salty taste.

In another series of experiments, the effect of the NBM lesion in previously trained animals was studied. In this case, the LiCl solution was given 2 days before the first QA injection into the right NBM and animals were tested with the NaCl solution 2 weeks after the injection into the left side (see the Method section). As shown in Table 2, there was a marked decrease of NaCl consumption by the control group during

TABLE 1

EFFECT OF NBM LESION ON INHIBITORY AVOIDANCE

	Inhibitory Avoidance Latencies (seconds)	
	Acquisition	Test
Control (13)	20.1 ± 5.0	485 ± 62.5
Lesioned (26)	22.6 ± 5.6	167 ± 44.6*

Figures are mean values ± SEM for the number of animals shown in parentheses.

*$p < 0.001$ vs. control group.

TABLE 2

EFFECT OF NBM LESION ON CONDITIONED TASTE AVERSION

	Acquisition	Baseline	Test	% Intake
Lesion before acquisition				
Control (13)	9.1 ± 0.9	10.8 ± 0.5	5.2 ± 0.5^{+}	49 ± 5
Lesioned (26)	10.3 ± 0.7	10.5 ± 0.4	9.4 ± 0.8*	90 ± 7*
Lesion after acquisition				
Control (10)	9.6 ± 0.8	10.7 ± 0.6	6.3 ± 1.3^{+}	52 ± 14
Lesioned (9)	11.6 ± 0.9	10.4 ± 0.4	12.7 ± 1.1*	122 ± 10*

Results are expressed as absolute water intake (in ml) during each of the stages of the behavioral procedure and as percentage of water intake during the test as compared to baseline consumption (last column). Figures are mean values ± SEM for the number of animals shown in parentheses.

*$p < 0.001$ vs. control group.

$^{+}p < 0.001$ vs. baseline intake.

the test, whereas lesioned animals did not reduce the intake even though they had been previously trained.

Biochemical Determinations

Release of neurotransmitters. [^{14}C]GABA release from IC slices in response to high K^+ concentrations is shown in Fig. 2. As previously described (21), in IC slices from control rats K^+-depolarization produced a threefold peak increase of labeled GABA release as compared with the basal prestimulation value. The NBM lesion did not affect this release because identical results were obtained with IC slices prepared from lesioned animals.

[^{3}H]ACh release was studied in the same set of IC slices used for [^{14}C]GABA release experiments (see the Method section). As shown in Fig. 2, and in agreement with previous findings (21), K^+-depolarization resulted in a 100% peak stimulation of ACh release in control IC slices. In tissue obtained from lesioned animals, there was a 37% reduction of the K^+-stimulated [^{3}H]ACh release (stimulation peak related to the basal release) as compared to control tissue.

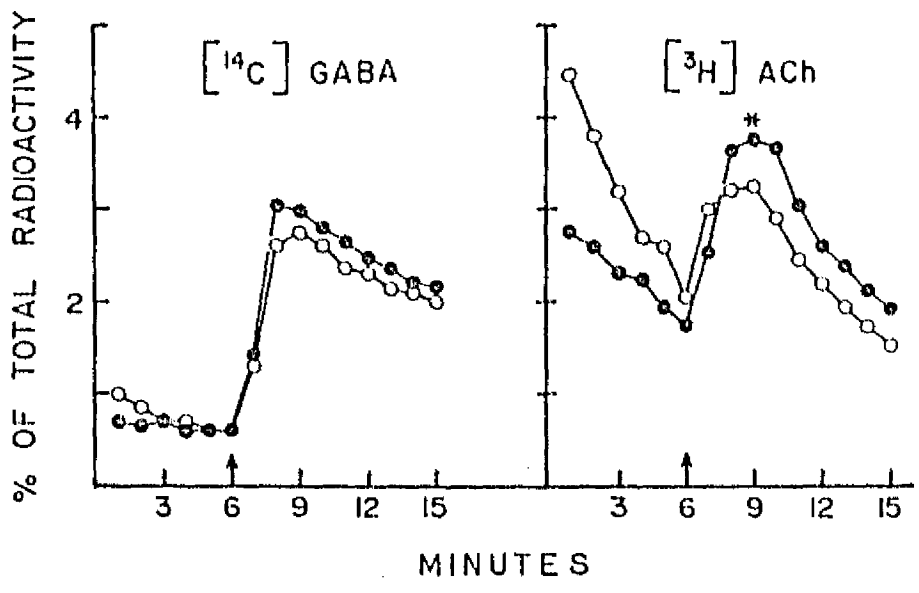

FIG. 2. K^+-stimulated release of labeled GABA (left) and acetylcholine (ACh) (right) from insular cortex (IC) slices obtained from nucleus basalis magnocellularis (NBM)-lesioned animals (○) and control rats (●) in the presence of Ca^{2+}. After loading the slices simultaneously with [^{14}C]GABA and [^{3}H]choline, they were superfused as described in the Method section. At 6 min (arrow), the superfusion medium was substituted by medium containing 47 mM KCl. Mean values of 26 experiments for lesioned rats and 13 for control animals. The maximum SEM was 20% of the corresponding mean but for most points it was smaller than 10%. *$p < 0.05$ vs. control group.

Enzyme activities. As shown in Table 3, IC homogenates from control and lesioned animals had similar GAD activity. When the incubation medium was supplemented with pyridoxal-5′-phosphate, GAD activity is enhanced by 100% and the NBM lesion had no effect on GAD activation by its coenzyme. In contrast, ChAT activity in IC homogenates was notably reduced (46% decrease as compared to controls) after the NBM lesion. AChE activity was also decreased (by 30%) in lesioned animals, although this reduction did not reach statistical levels of significance (Table 3).

DISCUSSION

The major cholinergic input to the cerebral cortex is the NBM, and this seems to include a specific projection to the IC (32). The histological, neurochemical, and behavioral results of the present work clearly support this possibility and further indicate that this pathway may be involved in CTA. Besides the appearance of labeled neurons in the NBM when the retrograde tracer Fluoro-Gold was injected into the IC of intact rats, our neurochemical observations after the QA-induced lesion indicate the putative cholinergic nature of this pathway.

TABLE 3

EFFECT OF NBM LESION ON ENZYME ACTIVITIES IN THE IC

	Control (13)	Lesioned (26)
GAD		
+PLP	45.3 ± 5.4	40.8 ± 6.3
−PLP	16.9 ± 2.5	21.0 ± 3.1
ChAT	62.0 ± 8.9	33.4 ± 4.2*
AChE	942 ± 196	672 ± 88

Results are expressed as nmol/h/mg protein. Figures are mean values ± SEM for the number of animals shown in parentheses. GAD activity was determined in the presence and absence of pyridoxal-5′-phosphate (PLP).

*$p < 0.01$ vs. control group.

The notable reduction of ChAT activity in the IC after QA-induced lesion of the NBM is in agreement with previously reported decreases in other cortical regions using QA as the neurotoxic agent (8). In addition, we observed a 30% decrease of AChE activity, although with considerable experimental variability, and a 37% reduction in [^{3}H]ACh release. A greater release reduction was expected because ChAT activity was considerably decreased and the tissue was loaded with labeled choline. This disagreement can be explained by the remaining unlesioned cholinergic fibers, which might take up choline and synthesize and release ACh in a normal fashion. In fact, at least 20% of the ACh present in the cortex does not arise from the NBM (9,16) and it may be presumed that this value applies also to the IC.

The fact that [^{14}C]GABA release and GAD activity in the IC were not altered by the NBM lesion rules out the possibility of nonspecific damage of the IC and indicates that GABAergic neurotransmission, if involved, is not sufficient for CTA learning. The possibility that other neuroactive substances known to be present in the IC, such as glutamate (21), cholecystokinin, somatostatin, or enkephalin (24), may be involved in gustatory neural processing remains open.

Several avoidance and spatial learning tasks are affected by NBM destruction. The impairment of inhibitory avoidance in NBM-lesioned rats seen in the present study agrees with previous findings on the role of NBM in this model (17,27). Because lesions of the IC also disrupt passive avoidance (2,3) and because, as discussed above, there is a NBM projection to the IC, the loss of this connection may be involved in this behavioral deficit.

The main and novel behavioral finding of the present study was the marked impairment of CTA learning after NBM damage. Our results do not establish the particular stage of CTA learning in which the NBM is involved. Because central cholinergic blockade prevents the acquisition of CTA (15), the NBM projection to the IC might be the anatomic substrate of such interference. On the other hand, the NBM may also be involved in CTA information retrieval. Indeed, our present results show that animals that were trained before the surgery did not retain the CTA after NBM destruction, suggesting a role for NBM in CTA memory.

Opposite the present results, in a previous study in which the NBM was lesioned with ibotenate no effect on CTA was observed (12). This discrepancy might be due to the fact that in the latter work animals were preexposed to the conditioned taste several times prior to the induction of the digestive malaise.

Finally, it has been proposed that the learning deficits observed after NBM lesions are due to an effect on its limbic targets, mainly the amygdala, and not to the interruption of its neocortical projection (18). However, it has been shown that CTA learning is not mediated by the amygdala (3,7), and a recent report showed no participation of the NBM cholinergic projections to the amygdala in CTA (35).

In conclusion, our data indicate the existence of a cholinergic NBM projection to the IC and suggest that it may be one of the neurotransmitter systems involved in the neural integration of feeding behavior.

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TRABAJO IV

EFFECTS OF NERVE GROWTH FACTOR ON THE RECOVERY OF CONDITIONED TASTE AVERSION IN THE INSULAR CORTEX LESIONED RATS

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INTRODUCTION

Recent research in our laboratory has focused on the influence of brain grafts on the recovery of learning ability in cortical-lesioned animals. The findings suggest that graft maturation and/or cholinergic activity may play a role in the graft-mediated behavioral recovery following brain lesions.

THE BEHAVIORAL MODEL: CONDITIONED TASTE AVERSION

Our studies have used conditioned taste aversion paradigms (CTA) to examine the effects of brain grafts on learning in cortically lesioned rats. Animals can acquire aversions to flavors if the taste stimulus (CS) is followed by gastrointestinal illness (Garcia et al., 1985) as an unconditioned stimulus (US). Taste is readily associated with illness and can be observed after a single taste-illness experience. Flavor-illness association has been demonstrated in several laboratories and in different animal species. A major advantage of this model in the study of the neurobiology of learning and memory is the knowledge of the neural pathways involved. The pathways for CTA have been established with the use of anatomical, electrophysiological and behavioral methods (for review see Garcia et al., 1985; Kiefer, 1985).

THE INSULAR CORTEX

The insular cortex (IC) has been referred to as gustatory or visceral cortex, since it receives taste and visceral information from the ventromedial nucleus of the thalamus that, in turn, receives afferences from the pontine parabrachial nucleus, which is a second-order relay for taste and visceral information (for review see Norgren, 1989; Kiefer, 1985). The anatomical connections of the IC clearly suggest that this brain region may play a role in integrating and possibly storing visceral information (see Garcia et al., 1985). Moreover, it has been postulated that the IC receives convergence of limbic afferences and primary sensory inputs that is not seen within any other sensory area in the cortex (Krushel and van der Kooy, 1988). Among the IC connections that may be important for memory processing are those with the limbic system; i.e., amygdala, dorsomedial nucleus of the thalamus and prefrontal cortex (Krushel and van der

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Kooy, 1988; Bermúdez-Rattoni and McGaugh, 1991; Escobar et al., 1989; van der Koy, 1984).

Although the IC connectivity is fairly well known, the neurotransmitters of the IC have not been extensively studied. In this regard, we have recently demonstrated that slices taken from the IC are able to release labeled GABA, ACh and glutamate but not dopamine. Additionally, there are significant glutamic acid decarboxylase, choline acetyl transferase and acetylcholinesterase activities in IC homogenates (Lopez-Garcia et al., 1990a).

Several studies have shown that the insular cortex area is involved in mediating the associative aspects of taste response, but is not involved in the hedonic responses to taste. Rats lacking the IC are impaired in acquiring and retaining taste aversion. That is, when the IC lesions are made either before or after acquisition of the CTA animals do not show taste aversion. However, the hedonic responses of lesioned IC rats appear to be normal: like normal rats, IC-lesioned animals prefer sucrose as well as low concentrations of sodium chloride over water and reject quinine and acid solutions. Also, it is known that taste responsiveness remains intact even in decerebrate rats (Kiefer, 1985; Braun, et al., 1982; Grill & Norgren, 1978).

RECOVERY OF FUNCTIONS BY FETAL BRAIN GRAFTS

Functional behavioral recovery from brain injury has recently been demonstrated using the fetal brain transplant technique in adult mammalian brains. It has been established that transplanted neurons differentiate and make connections with the host brain (Bjorklund and Stenevi, 1985). We recently showed that the fetal brain transplants produced a significant recovery in the ability of IC-lesioned rats to acquire a CTA. The possibility of spontaneous recovery was excluded, because the animals with IC lesions that did not receive transplants were unable to acquire the CTA 8 weeks after the transplantation, even with two series of acquisition trials (Bermúdez-Rattoni et al., 1987). In contrast, animals with lesions in the amygdala showed spontaneous recovery eight weeks after the lesion was induced. Similar spontaneous recovery of performance in an alternation task has been found in animals tested 6 weeks after having received large cortical ablations (Dunnett et al., 1987).

Elsewhere we have discussed in detail these functional differences between the amygdala and IC (Bermúdez-Rattoni et al., 1987). One possible explanation is that amygdala lesions may have resulted in reorganization of other elements in the neuronal network. Another plausible explanation is that for taste-aversion learning the IC may be a permanent memory store, whereas the amygdala may only serve to influence an initial step in the storage of CTA (Bermúdez-Rattoni et al., 1987; Bermúdez-Rattoni et al., 1989).

We have further shown that the degree of functional recovery induced by fetal brain tissue grafts depends on the place from which graft tissue was taken. Animals which received homotopic but not occipital cortical tissue recovered the CTA. Further, the animals that received either tectal heterotopic tissue or no transplant showed no behavioral recovery. Results based on horseradish peroxidase (HRP) histochemistry revealed that cortical, but not brain-stem grafts, established connections with amygdala and with the ventromedial nucleus of the thalamus (Escobar et al., 1989). Biochemical analyses revealed that IC fetal grafts released GABA, ACh and glutamate in response to K+ depolarization. In contrast, occipital grafts

released labeled GABA and glutamate, but not ACh (Lopez-Garcia et al., 1990b). These results suggest that cholinergic transmission is important for CTA and that ACh may play a role in graft-mediated behavioral recovery.

The results discussed above indicate that some morphological recovery is necessary for the acquisition of taste aversion. However, it has been suggested that structural and morphological integrity of fetal brain grafts may not be essential for behavioral recovery after cortical brain injury (Dunnett at al., 1987; Kesslak et al., 1986). These investigators have speculated that brain injury or brain grafts induce a release of neurotrophic factors that can reactivate neural function and/or prevent injury-induced degeneration in the damaged host brain (Dunnett et al., 1987: Kesslak et al., 1986). Labbe and coworkers (1983) reported that rats lesioned in the frontal cortex and given cortical transplants were able to learn a spatial alternation task in fewer trials than lesioned controls or rats with cerebellar implants. The recovery effects were seen just one week after transplantation. In this regard, Dunnett and coworkers (1987) found that neocortical grafts produced short-lasting improvement in the T-maze alternation performance. They concluded that the short-lasting effects were attributable to diffused influences of the embryonic tissue on the lesioned host brain instead of a reconnection of the damage circuits. In contrast, findings from our laboratory clearly suggest that, with CTA, recovery of function increases with time after transplant.

TIME-DEPENDENT RECOVERY

In a series of experiments in our laboratory, rats with lesions of IC showing disrupted taste aversion received neocortical transplants and were retrained at 15, 30, 45 and 60 days after transplantation. The behavioral results showed almost complete functional recovery at 60 days, slight recovery at 45 and 30 days and a poor recovery at 15 days post-graft. HRP histochemistry revealed that at 15 days there were no HRP labeled cells in the ventromedial nucleus or into the amygdala. At 30, 45 and 60 days post-graft, there were increasing connections, almost as many as those seen in the controls, with the thalamus and with the amygdala. The behavioral recovery was correlated with increased acetylcholinesterase activity, detected histochemically, and with morphological maturation, revealed by Golgi staining (Fernández-Ruiz et al., 1991). The possibility that neurotrophic factors alone may be involved in the functional recovery is unlikely, because it is necessary to wait at least 30 days to see any recuperation. Therefore, such findings suggest that if neurotrophic factors are involved, they need to be associated with cortical homotopic grafts and/or with some time dependent factor essential for producing functional recovery. The implication of these series of experiments is that, for the IC and CTA, functional recovery is related to the morphological maturation of the graft.

THE ROLE OF THE NERVE GROWTH FACTOR IN FUNCTIONAL RECOVERY

Several lines of evidence have demonstrated that the nerve growth factor (NGF) produces a significant regeneration, regrowth and penetration of cholinergic axons in the hippocampal formation (Gage 1990; Hagg et al., 1990). Chronic perfusion of NGF, in fimbria-fornix lesioned animals with severe learning impairments, produce functional and anatomical recovery (Varon et al., 1989). It has also been demonstrated that chronic intracerebral infusion of NGF improves memory performance in cognitively impaired aged rats (Gage and Bjorklund, 1986). Nevertheless, long-term impairments by application of NGF in combination with intrahippocampal septal grafts have been observed (Pallage et al., 1986).

In another series of experiments, we have assessed the role of NGF on the recovery of acquired conditioned taste aversions in cortical lesioned rats. For CTA, the animals were habituated for 10 days to drink tap water daily during 5 min sessions. After surgery, the animals were given one acquisition trial and two testing trials conducted every fourth day with baseline access to distilled water on the intervining days. The acquisition day involved the presentation of 0.1 M LiCl instead of water. It has been demonstrated that the taste of LiCl can readily be aversively conditioned to its gastric aftereffects, since it is also the agent inducing illness (Nachmann, 1963). The tests consisted of the presentation of 0.1 M of NaCl instead of the LiCl with three water intake baseline measures in between (see Bermúdez-Rattoni et al., 1987; Fernández-Ruiz et al., 1991). Rats cannot discriminate between the flavors of NaCl and LiCl (Nachmann, 1963). Three groups of rats showing disrupted taste aversions due to IC electrolytic lesions were grafted as follows: the first group received fetal (15E) cortical graft + NGF (ICNGF), the second group received gelfoam + NGF (NGF), and the third group received fetal cortical graft alone (IC). Unoperated animals were used as a control group (CON). The three grafted and control groups were subdivided in three subgroups (ICN, ICNGF, NGF and CON) that were retrained for CTA at 15-, 30- and 60-days post-graft respectively. As expected, the control groups showed strong taste aversions in all the post-graft times (see Fig. 1). The IC group showed a disrupted taste aversion at 15 days post-graft. During the 30 and 60 post-transplantation day, the IC groups showed recovered taste aversion when compared with the CON group. The ICNGF group showed a significantly recovered ability to acquire the taste aversions at the three post-graft times when compared with the NGF alone group. These results indicate that the application of NGF with the cortical graft accelerate the functional recovery up to 15 days after grafting.

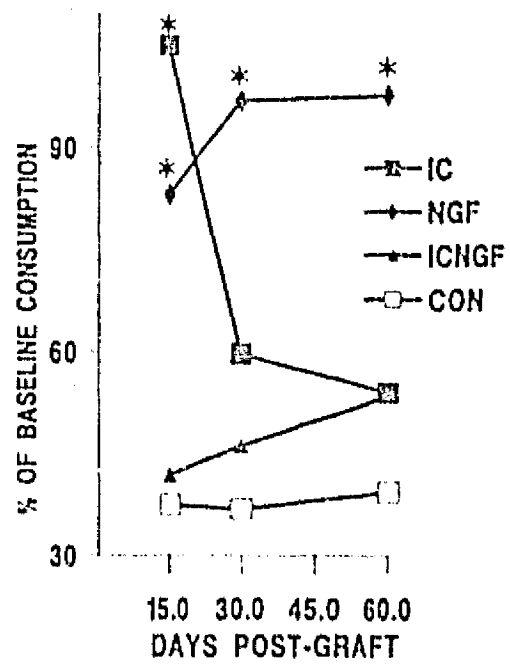

Fig. 1. The amount of NaCl consumed by control (CON), Insular Cortex (IC), Insular Cortex + NGF (ICNGF), and NGF with gelfoam grafted groups at 15-, 30-, and 60-days postgraft. * $p < 0.05$ (Newman-Keuls' test). For comparison the group CI was redrawn from Fernández-Ruiz et al., 1991.

The possibility that combinations of NGF with any other brain tissue could produce functional recovery in IC lesioned animals is currently being evaluated in our laboratory. Briefly, four groups of IC-lesioned rats showing disrupted taste aversions received either cortical graft + NGF, cortical graft + DMEM (Dulbecco's Modified Eagle Medium), mesencephalic tissue with NGF, or mesencephalic tissue alone. All the groups were retrained 15 days after transplantation. The results clearly indicate that the combination cortical grafts with NGF produce significant recovery in the lesioned animals to acquire taste aversions. Those animals that received mesencephalic grafted tissue in combination with NGF, mesencephalic tissue alone, or the vehicle with cortical grafts were unable to acquire the taste aversions after 15 days postgraft. The histochemical results show the presence of positive immunoreaction for NGF and many AChE positive labeled fibers and somas into the grafts of the cortical with NGF group. The grafts from the same group showed a noticeable growth and integration with the host tissue. In addition, preliminary biochemical experiments showed that choline-acetyltransferase (ChAT) activity in this group was very similar to that of the unoperated controls.

CONCLUSIONS

In our model, the application of NGF alone did not produce significant functional recovery in any of the post-graft times tested. In contrast, other authors using different learning models and different brain regions have found, after few days post-graft, recovery following acute application of NGF or other trophic factors (Kesslak, et al., 1986; Will and Hefti, 1985; Hefti et al., 1984; Hefti, 1986). Varon and coworkers have found functional recovery by chronic administration of NGF in fornix lesioned animals that had evidenced severe impairments in a Morris spatial task (Varon et al., 1989). In our study, the best functional recovery was seen when the NGF was associated with homotopical cortical grafts but not with heterotopical mesencephalic grafts or the application of NGF alone. These behavioral results appear to be related with the integration and maturity of the grafted tissue. Preliminary results using the Golgi staining technique indicate that the cortical grafts with NGF showed more neuronal maturation compared to the other groups. Therefore, as mentioned, if neurotrophic factors are involved, they need to be associated with cortical homotopic grafts and/or certain cortical factors essential for producing functional recovery.

ACKNOWLEDGMENTS

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TRABAJO V

Journal of Neural Transplantation and Plasticity (in press)

Nerve Growth Factor with Insular Cortical Grafts Induces Recovery of Learning and Reestablishes Graft Choline Acetyltransferase Activity

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SUMMARY

Rats showing disrupted taste aversion due to insular cortex (IC)-lesions received either IC-grafts with NGF, grafts without NGF, or NGF alone. An additional group served as lesioned controls. Only those animals that received IC-grafts with NGF recovered the ability to learn the conditioned taste aversion task, at 15 days post-graft. Choline acetyltransferase (ChAT) activity in the IC-grafts with, but not without NGF, was similar to the IC activity of unoperated controls. In contrast, glutamate decarboxylase activity was similar in all the groups. These findings suggest that IC-grafts associated with NGF induce recovery of learning abilities in IC-lesioned rats, which correlates with reestablishment of ChAT activity in the grafts at 15 days post-implantation.

KEY WORDS

nerve growth factor, insular cortex, choline acetyltransferase, grafts, learning, conditioned taste aversion

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It has been demonstrated that chronic perfusion of NGF in fimbria-fornix lesioned animals with severe learning impairments induces functional and anatomical recovery /23/. Moreover, chronic intracerebral infusion of NGF improves performance in cognitively impaired aged rats /24/. Recently Otto *et al.* /21/ showed that the presence of NGF-soaked gelfoam reduces the death rate of medial septal neurons following fimbria-fornix lesions.

Conditioned taste aversion (CTA) is a learning paradigm in which rats acquire aversion to a taste cue when it is followed by digestive malaise /13/. It has been shown that bilateral lesions of the insular cortex (IC) disrupt acquisition and retention of CTA /3,5,16/. Recent research in our laboratory has focused on the influence of brain grafts on the recovery of learning ability in IC-lesioned animals /2,9,10/. Analysis of the time course of the behavioral recovery induced by IC-grafts after IC-lesions, demonstrated that at the initial 15 days post-implant the subjects did not show any recuperation in the CTA paradigm, whereas a good recovery was observed at 45 days after graft. In addition, behavioral recovery was correlated with increased acetylcholinesterase activity, detected histochemically /10/ and biochemically /18/.

These findings suggest that graft maturation and/or cholinergic activity may play a role in the graft-mediated behavioral recovery following brain lesions. In the present study we evaluated the role of NGF in the recovery of CTA induced by cortical grafts, and attempted to correlate such behavioral effects with ChAT and glutamate decarboxylase (GAD) activities, as indicators of cholinergic and GABAergic neuronal systems, respectively, in the grafted tissue.

Adult male Wistar rats weighing 250-300 g at the start of the experiment were used. They were

housed individually, under an inverted 12 h light-dark cycle, with food and water *ad libitum* except where indicated. Bilateral stereotaxic electrolytic lesions (2 mA/45 s) of the IC were made in 41 animals under pentobarbital anesthesia (50 mg/kg) using the following coordinates with respect to bregma: AP = +1.2 mm; L = -5.5 mm; V = -5.5 mm. Eleven animals were used as unoperated controls.

A previously described experimental procedure for CTA was used /10/. Briefly, animals were deprived of water for 24 h and trained to drink water twice a day, during ten min trials for four days. On the fifth day, a 0.1 M LiCl solution, which induces digestive malaise, was given instead of water in order to induce taste aversion. After four more days of baseline consumption, the water was substituted by a 0.1 M NaCl solution to test the aversion. LiCl and NaCl are indistinguishable by rats /20/. The NaCl consumption volume was taken as the taste aversion score.

After the first behavioral CTA training and test, the experimental animals were divided randomly into five groups: Group one received IC-grafts in combination with NGF (IC-NGF, n=13); group two received IC-grafts with vehicle (IC-VEH, n=7); a third group received gelfoam soaked in the NGF solution, without graft (NGF, n=10); the fourth group was an IC-lesioned group (LX, n=11) and the fifth was an unoperated control group (CON, n=11). Sixteen-day-old fetuses were removed from the abdominal cavity of pregnant rats under barbiturate anesthesia (50 mg/kg). The fetal brains were removed, and the temporoparietal area (above the rhinal sulcus) was dissected under a field microscope. The tissue (about 2 mm^3) was soaked in a highly concentrated solution (20 µg/ml) of NGF 7S (Sigma, St. Louis, MO) in Dulbecco's Modified Eagle's Medium (GIBCO, Grand Island, NY) containing 0.25% bovine serum albumin (Sigma, St. Louis, MO), according to the procedure of Otto *et al.* /21/. After soaking, the tissue was aspirated into a 100 µl Hamilton microsyringe and then stereotaxically injected into the IC area, with the same stereotaxic coordinates used previously for producing the lesion. After implanting, gelfoam (about 1 mm^3) soaked in the same NGF solution was inserted into the cavity. For group two the soaking solution and gelfoam did not contain NGF. Fifteen days after the transplantation procedure all the animals were *retrained* for CTA.

At the end of the behavioral procedure (post-implant) IC water homogenates from the IC region (group CON) or from dissected IC-implants (groups IC-NGF and IC-VEH) were used for measuring enzymatic activities. Animals were killed by decapitation and their brains quickly removed. The IC region was localized using the middle cerebral artery and the rhinal sulcus as references /16,17/ and was dissected on ice and homogenized as required. The grafted tissue was carefully dissected from the host tissue and processed in the same manner. ChAT /11,17/ and GAD /1,17/ were measured by the radioisotopic techniques previously described in detail, using [^{3}H]acetyl-coenzyme A (1.6 Ci/mmol) (NEN Dupont, Boston, MA) and [1-^{14}C]L-glutamate (56 mCi/mmol) (Amersham, Buckinghamshire, U.K.) respectively. Protein was determined using the Folin reagent method, as described /19/.

Two animals from each grafted group were perfused through the ascending aorta with 0.15 M NaCl followed by a 4% paraformaldehyde, 0.1% glutaraldehyde solution in PBS. The brains were subsequently cut and processed for cresyl violet (Nissl staining) by standard procedures /2/ to determine the transplants' characteristics.

A simple ANOVA was done on the test day consumption volume for all groups, pre and post graft, with post-hoc group comparisons, when appropriate, using the Student-Newmann-Keuls' test, at a 0.05 significance level. The results of the CTA experiments are shown in Fig. 1. During the pregraft test trial, there were significant differences among groups ($F_{4,50}$=25.63, $p<0.001$). As expected, the control group showed strong taste aversion, whereas the IC-lesioned (IC-NGF, IC-VEH, NGF and LX) groups showed significantly disrupted taste aversion. Fifteen days after grafting, ANOVA comparisons revealed significant differences among the groups ($F_{4,50}$=11.61, $p<0.001$). The IC-NGF group showed markedly reduced NaCl consumption, which was similar to that of the control group, and significantly different from its pregraft score, indicating a recovery of taste

aversion, while the LX, NGF and IC-VEH groups did not show any improvement (Fig. 1).

The results of the determination of enzyme activities are shown in Fig. 2. ANOVA analysis of ChAT activity revealed significant differences among groups ($F_{2,27}$=9.01, p<0.001). ChAT activity in IC-NGF grafts was similar to that in intact controls IC, whereas that in IC-VEH grafts was only 41% and 47%, as compared with the controls and the IC-NGF tissues, respectively. In contrast, GAD activity was similar in IC homogenates in control, IC-VEH and IC-NGF groups ($F_{2,27}$=0.56) (Fig. 2). The IC-grafts with or without NGF showed a well integrated and healthy aspect (Fig. 3).

It has been demonstrated that fetal brain grafts can produce functional recovery in a variety of behavioral tasks /4,6,8/. We have found that in the CTA paradigm cortical brain grafts produce a significant recovery of the ability to learn after 60 days post-graft in IC-lesioned animals, and that such recovery requires at least 30 days post-graft /10/. Other studies have shown that ChAT activity in the IC is higher than in other cortical areas, and that IC-graft ACh release is correlated with the recovery of CTA /17,18/, suggesting that the cholinergic system is involved in the functional recovery mediated by IC-grafts. The purpose of the present experiments was to study the effect of supplementing grafts with NGF on the behavioral recovery induced by fetal IC-implants, and to relate such effects to biochemical changes in the cholinergic or GABAergic systems.

The behavioral data obtained indicates that at 15 days post-graft the only group that promotes recovery of the ability to acquire the CTA was the group with homotopic IC-grafts in combination with NGF. In view of the above-mentioned data, this indicates that the administration of NGF in the cortical grafts significantly affects the functional recovery observed at 15 days post-graft. Experiments assessing the effects of NGF alone or in combination with grafts, in a time-dependent fashion, are currently being evaluated in our laboratory.

Our biochemical analyses showed that ChAT activity in the homotopic IC-grafts plus NGF was similar to that in the IC of intact control animals, whereas in the IC-grafts plus vehicle it was considerably reduced. Furthermore, preliminary studies using *in vivo* assays of ChAT and ACh levels have demonstrated that IC but not heterotopic grafts with NGF reestablish ChAT activity (Russell R.W., Escobar M.L., Booth R.A. and Bermúdez-Rattoni F., in preparation). These data, together with the above-mentioned findings, suggest a participation of cholinergic neurotransmission in the graft-mediated functional recovery. In this regard, it has been demonstrated that NGF application produces a significant regrowth and reconnectivity of cholinergic fibers using the septo-hippocampal lesion model /14/. Other authors, using different learning models and different brain regions, have found recovery after acute application of NGF or other trophic factors. Moreover, that recovery has been correlated with the survival and improvement of the functional capabilities of cholinergic cells /7,12,15,22,24/. In this regard, Varon and coworkers /23/ using chronic NGF administration in animals with fornix lesions, have described functional recovery in a Morris spatial task, that also correlated with regrowth of cholinergic fibers.

In the present experiments, measurement of GAD activity at 15 days post-graft, in contrast to ChAT, did not show any significant differences between groups. These results suggest that GABA mediated neurotransmission does not play an important role in graft-promoted behavioral recovery. Obviously, the involvement of other neurotransmitter systems cannot be dismissed. Despite the apparent similarity in the morphological appearance of the IC-NGF and IC-VEH grafts, the results indicate that the biochemical differences in ChAT activity are more relevant to the behavioral recovery observed.

The present findings suggest that NGF, when associated simultaneously with homotopic IC-grafts, produces recovery of learning abilities in IC-lesioned rats and the reestablishment of ChAT activity in the grafts at 15 days post-graft.

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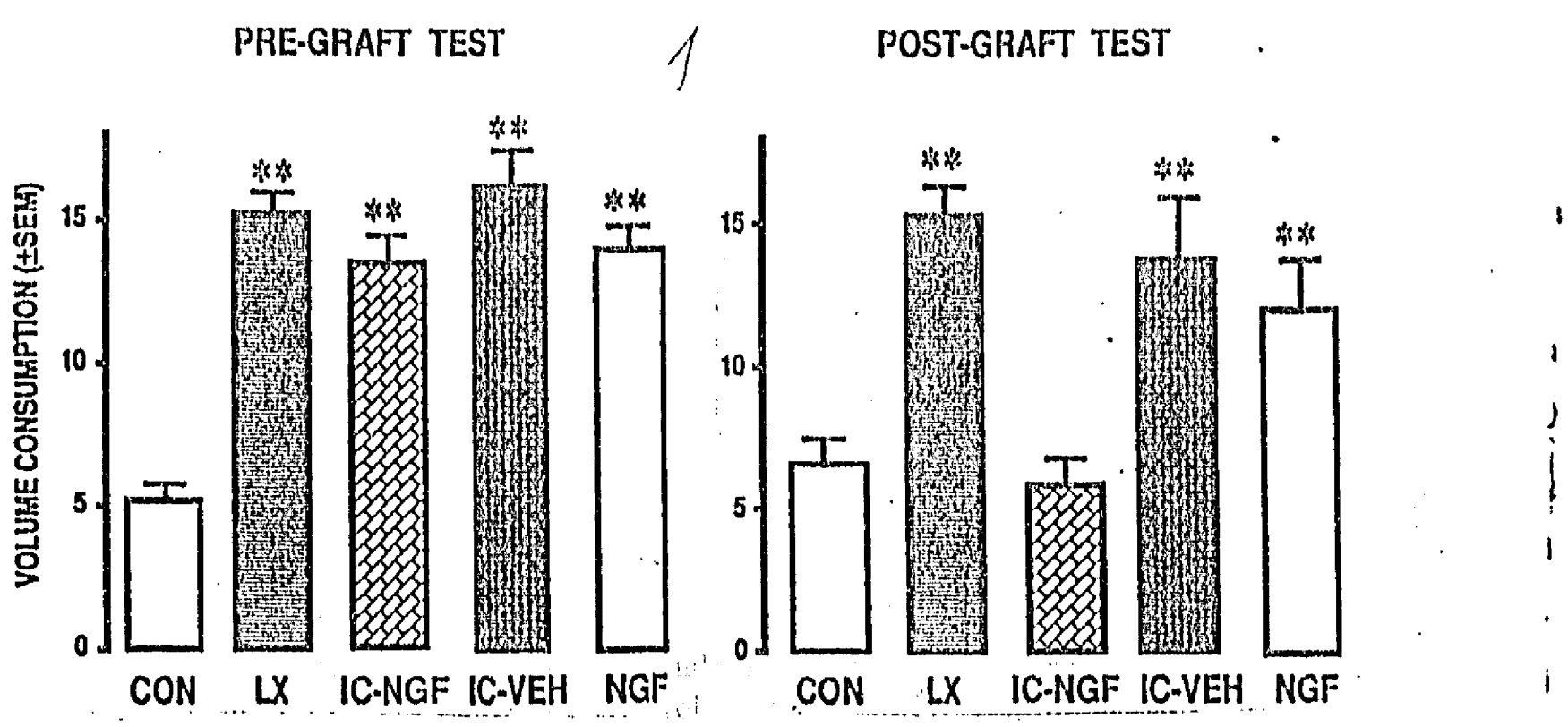

Fig. 1: Amount of NaCl consumed by control (CON), lesioned (LX), insular cortex + NGF graft (IC-NGF), NGF alone (NGF) and insular cortex + vehicle graft (IC-VEH) groups. Left panel shows the results from one taste test trial prior to implant. Right panel shows the results of one taste test trial 15 days after implant. Values are means ± SEM. **p<0.01 as compared with controls (Newmann-Keuls' test).

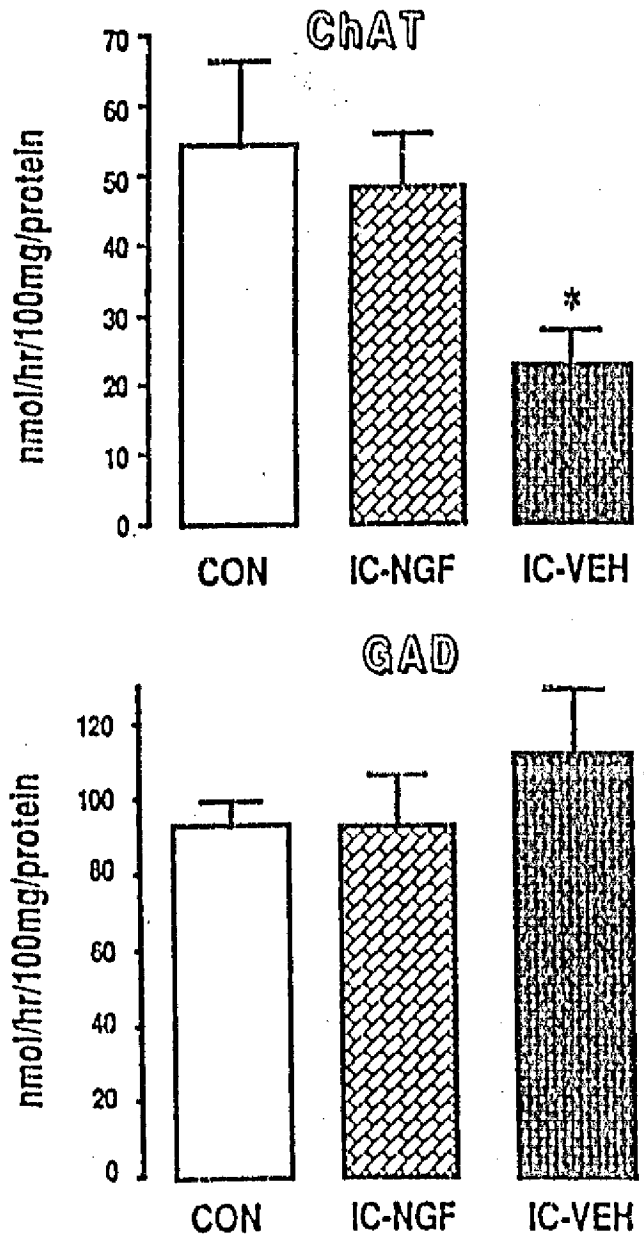

Fig. 2: ChAT (top panel) and GAD (bottom panel) activity in IC of intact control (CON), IC-NGF and IC-VEH

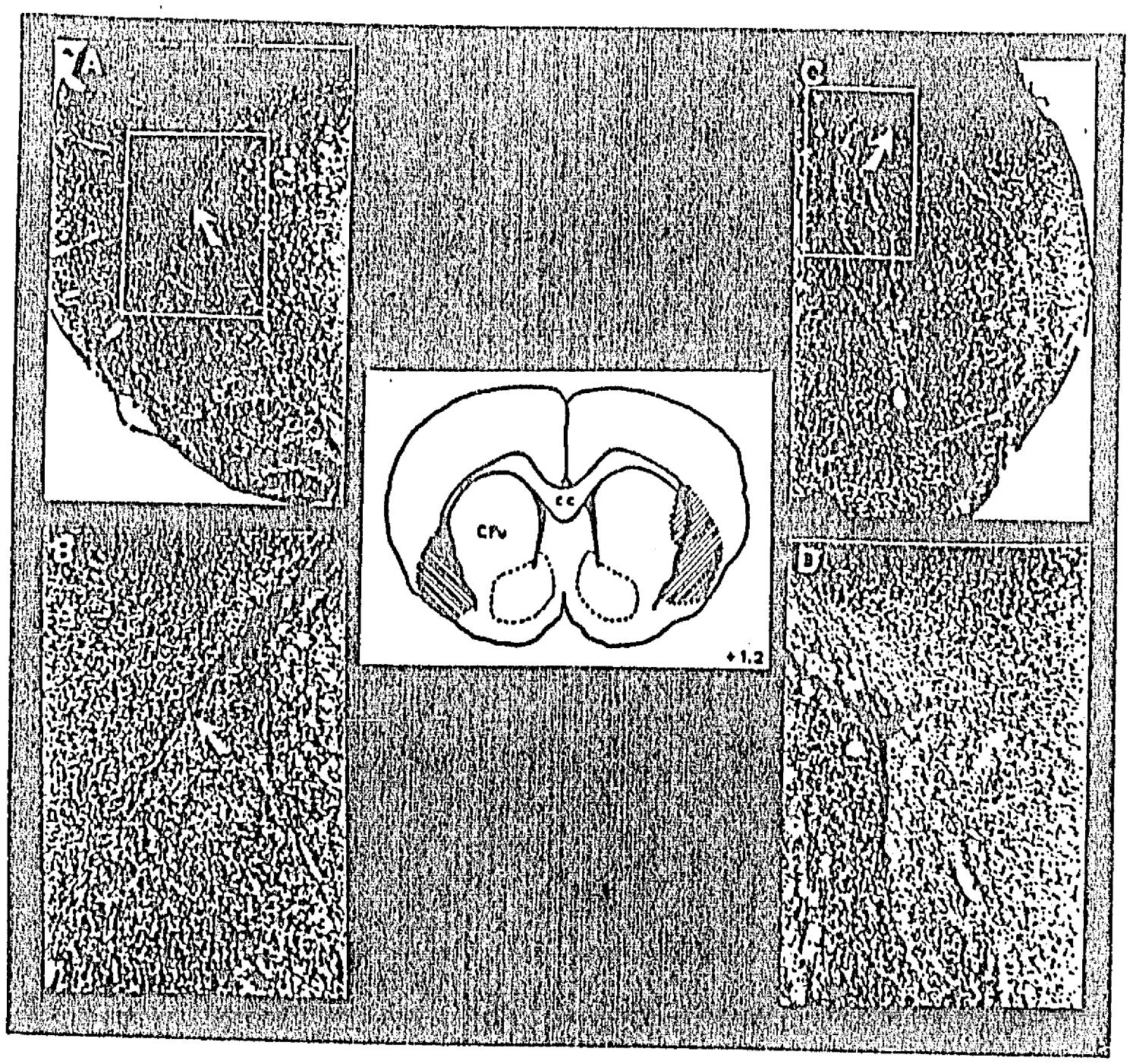

Fig. 3: Nissl staining of coronal sections from homotopic insular cortex grafts with (A and B) or without NGF (C and D). The position of the grafts in the host brain is schematized (center). B and D (40x) magnifications of the boxed regions in A and C (20x) respectively.

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TRABAJO VI

ACCELERATING BEHAVIORAL RECOVERY AFTER CORTICAL LESIONS: I. HOMOTOPIC IMPLANTS PLUS NGF.

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Key words: Insular cortex lesion; Fetal brain implant; Nerve growth factor; Behavioral recovery; Conditioned taste aversion; Inhibitory avoidance.

Running head: Homotopic implants +NGF

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ABSTRACT

We recently demonstrated that fetal brain implants produced a significant recovery in the ability of insular cortex (IC)-lesioned rats to learn a conditioned taste aversion (CTA). We now report effects on the recovery of CTA and of a second measure of learning, inhibitory avoidance (IA), of supplementing the implants with nerve growth factor (NGF). Four groups of male Sprague-Dawley animals showing disrupted taste aversion following IC lesions, plus two control groups, received different experimental treatments: Group 1, unlesioned control; Group 2, homotopic IC implants without NGF; Groups 3 and 4, IC implants + NGF; Group 5, heterotopic occipital cortical implants + NGF; and Group 6, without an implant as a lesioned control. All groups except Group 4 were trained pre- and post-implant in the CTA paradigm. Two days after CTA testing post-implant, all groups received IA training. Behavioral results showed that insular cortex implants with NGF promoted recovery to control levels of the ability to learn both tasks at 15 days post-implant. Those animals that received occipital implants with NGF, or insular cortex with vehicle or remained without implants did not show any significant behavioral recovery at 15-days post-implant. These findings suggest that NGF should be associated with homotopic implants facilitates recovery of learning abilities in insular cortex lesioned rats and suggest that similar treatments with NTFs may have analogous effects when lesions involve other brain areas.

INTRODUCTION

Some 30 years ago evidence for the control of growth in nerve cells by a protein factor, i.e., neurotrophic factor (specifically, NGF) (Levi-Montalcini, 1964) suggested "...the interesting possibility that interactions may occur between behavior and biochemical events involved in the synthesis of NGF...."(Russell, 1966). During the past decade a number of reports have shown the relations between NGF and the recovery of behavioral impairment with and without brain "implants" following lesions of cholinergic pathways in the brain. Thus, lesions of the cholinergic septohippocampal pathway have been shown to produce severe impairment of behaviors involving sensory-motor processes, learning and memory. It has also been reported that, continuous infusion of exogenous NGF icv reduces total neuronal degeneration measured at 2 weeks after fimbria-fornix transection in adult rats, sparing being 50% in the medium septum and 100% in the diagonal band of Broca (Williams, Varon, Peterson, Wictorin, Fischer, Bjorklund & Gage, 1986). Such relations between neurochemical events and changes in behavior have encouraged a spate of experimentation designed to manipulate NGF and neuronal regeneration as means of compensating for adverse behavioral effects of experimental lesions. Two general experimental approaches have been used: (a) repeated (chronic) injections, infusion of NGF or insertion of NGF-soaked gelfoam and (b) implantation of neuronal or target tissue. Both of these have been reported to have produced at least partial compensation for effects of brain damage, restoring the pattern of cholinergic innervation and producing concomitant improvements in some but not all behavioral patterns.

Conditioned taste aversion (CTA) is a learning paradigm in wich graft-mediated recovery has been observed (Bermúdez-Rattoni, Fernández,

Sánchez, Aguilar-Roblero & Drucker-Colin, 1987; Escobar, Fernández, Guevara-Aguilar & Bermúdez-Rattoni, 1989; Yirmiya, Zhou, Holder, Deems & García, 1988). In this behavioral model, rats acquire aversion to a taste cue when it is followed by digestive malaise (García, Lasiter, Bermúdez-Rattoni & Deems, 1985). The anatomical substrates responsible for CTA learning have been well established (Kiefer, 1985). It has been shown that bilateral lesions of the insular neocortex (IC), a region of the temporal cortex in the rat, disrupt acquisition and retention of the aversion in an experimental model of CTA (Lasiter & Glanzman, 1985). Recent research in our laboratory has focused on the influence of brain grafts on the recovery of learning ability in IC-lesioned animals (Bermúdez-Rattoni, et al., 1987; Bermúdez-Rattoni, Escobar, Piña, Tapia, López-García & Hiriart, 1992; Escobar et al., 1989; Fernández-Ruíz, Escobar, Piña, Díaz-Cintra, Cintra-McGlone & Bermúdez-Rattoni, 1991). Analysis of the time course of the behavioral recovery induced by IC-grafts after IC-lesions, demonstrated that at 15 days post-implant the subjects did not show any recuperation in the CTA paradigm, whereas a good recovery was observed at 45 days after implant. In addition, behavioral recovery was correlated with increased acetylcholinesterase activity, detected histochemically (Fernández-Ruíz et al., 1991) and biochemically (López-García, Fernández-Ruíz, Bermúdez-Rattoni & Tapia, 1990). In the paragraphs to follow we report experiments designed to investigate the role of NGF in the recovery of CTA and IA induced by cortical grafts.

Homotopic implant + NGF

MATERIALS AND METHODS

Research Design

The research design is summarized in Table 1. Each of six groups of rats received different treatments. Group 1 served as an unlesioned control group. Group 2 provided the data required to determine effects of IC implants without NGF. Groups 3 and 4 were both implanted with IC + NGF, differing only in that Group 4 was never exposed to the lithium involved in the conditioned taste aversion paradigm (CTA). Group 5 received heterotopic occipital cortex (OCC) for comparison with animals receiving homotopic implants. Group 6 was lesioned in the IC and did not receive an implant.

Animals

Sixty male Sprague-Dawley rats (Charles River Laboratories, Wilmingtom, MA), participated at the start of the study. They weighed between 200-225 g when they arrived in the vivarium. They were assigned one per cage and were fed on standard laboratory chow. A 12 hr light/dark cycle (lights on at 7:00 a.m.) was maintained throughout the experiment. During their stay the animals were checked regularly for signs of infection; additional "sentinel" animals showed negative serologies throughout. One animal failed to survive surgery following lesioning; data on amount of water consumed by another subject during a critical CTA trial were lost, eliminating that animal from further participation in the study.

Lesions

Stereotaxic surgery was used to create bilateral electrolytic lesions in the IC. Animals were kept under deep pentobarbital anesthesia (50 mg/kg) during the procedure. A monopolar stainless steel electrode, coated with

epoxyle except for the tip (0.5 mm), was inserted using the following coordinates with respect to bregma: AP, +1.2; L, ± 5.5; and V, -5.5. The lesions were produced by passing a 2 mA anodal current through the electrode for a duration of 45 s.

IC Implants

Sixteen-day-old fetuses were removed from the abdominal cavities of pregnant rats under pentobarbital anesthesia and their brains were extracted. Tissue blocks of approximately 3 mm^3 in volume were dissected from the region corresponding to the temporoparietal area (above the rhinal sulcus) and from the occipital cortex for homotopic and heterotopic implants, respectively. Using a 100 µl Hamilton syringe (1700 series gastight, needle I.D. 0.006 in.) tissue was placed stereotaxically into the cavity produced by the lesion, using the same set of coordinates given above. Prior to the implants the tissues were embedded in a high concentration solution (20 µg/ml) of NGF 7S (Sigma, St. Louis, MO) + DMEM (Dulbecco's Modified Eagle's Medium, GIBCO, Grand Island, NY/0.25 BSA Bovine Serum Albumin (Sigma, St. Louis, MO) according to Otto, Frotscher and Unsicker, 1989. Following implant, a cap of gelfoam embedded in the same solution was inserted into the cavity and the external (cranial) opening was sealed with nylon screws. Implant surgery started 12 days after the lesion procedure.

Conditioned Taste Aversion (CTA)

The CTA procedure used in the present experiment has been described elsewhere (Fernández-Ruíz et al., 1991; López-García, et al., 1990). In brief, animals were first deprived of water for 24 hr and then given 10 min drinking sessions twice a day (9:00 a.m. and 4:00 p.m.) for two days,

by which time the amount consumed had become asymptotic. On the morning of the third day a 0.1 M LiCl solution replaced the tap water in order to induce taste aversion. There followed four drinking sessions with tap water before a test for retention of the CTA using a 0.1 M NaCl solution, the latter being indistinguishable from LiCl by rats (Nachman, 1963). A second retention trial with NaCl was given after two further sessions with tap water. Fifteen days after the implant surgery the CTA procedure was repeated, this time with only one NaCl test.

Inhibitory (Passive) Avoidance

Inhibitory (passive) avoidance memory was measured in a "step through" apparatus. The apparatus consisted of (a) a small compartment made of white plastic, (b) a larger dark compartment of stainless steel, and (c) a shock delivery unit with a set duration (0.5 s) and adjustable intensity (mA) of the mild electric shock used as an aversive stimulus. The procedure involved two types of trials. During a single training trial the animal was placed in the white compartment; entry into the dark compartment led immediately to the closing of a guillotine door and the administration of a 0.40 mA footshock. A test trial without shock was given 2 days later, the measure taken being time to enter the dark compartment after release from the white. The times for animals not entering within 10 min were recorded as 600 s. The IA procedure was carried out at the end of the second CTA training and testing. Six days later, all animals were killed and their brains were dissected and processed for cholinergic assays (Jenden, 1977).

Data Analysis

Analyses of all data centered on ANOVA. Parametric or nonparametric procedures were applied depending upon the nature of the measure being treated. When F-values were significant ($p<5.0x10^{-2}$ level of confidence), parametric (Zar, 1974) for CTA or nonparametric (Siegel & Castellan, 1988) for IA techniques for multiple comparisons were used in determining the source(s) of the significance. F and p values are usually given only when significant, precise p values being recorded to $p<5.0x10^{-2}$.

RESULTS

The results are summarized in two major sections. The first examines effects of the lesions and implants on CTA and the second, effects of implants on IA.

Conditioned Taste Aversion

CTA Conditioning. Analyses of results during the postlesion period provided opportunities to examine several treatment effects independently from interactions with effects of the various implants. Potential effects of the IC lesions per se were reflected in water intakes during the two drinking sessions (A.M. and P.M.) which followed the surgery by five days and preceded the LiCl treatment the following day. However, there were no significant differences among the treatment groups during either of these sessions. The mean ± SEM for the combined groups were: A.M., 14.97 ± 0.318; and P.M., 13.53 ± 0.351. These analyses indicate that any

postoperative effects on drinking behavior that may have accompanied the surgery had disappeared by the time conditioning of the CTA began.

As has been reported previously, there was a clear disruption of the neophobic response by IC-lesions (Bermúdez-Rattoni et al., 1987; Escobar et al., 1989). ANOVA was calculated on percentages from previous day baseline volumes within each group. During the postlesion acquisition trial, there were significant differences in immediate responses to the LiCl experience per se between groups: $F(4,49)=3.16$, $p=2.2\times10^{-2}$. Analysis by Scheffé contrasts indicated that the control group drank significantly less (84.79±3.68) than the four lesioned groups exposed to LiCl (101.77±3.13) and the non conditioned group (103.68±6.45).

Retention of CTA. After four normal drinking sessions, the first of two tests for retention of the CTA was administered, NaCl solution being substituted for tap water. Figure 1A shows the effects of IC-lesions during the NaCl test session. Differences among groups were highly significant: $F(4,49)=11.27$, $p=1.0\times10^{-4}$. Examination of the means for the various groups showed a clear suppression of NaCl-solution intake in the control animals despite the reduced consumption of LiCl during the acquisition session, but not in the other groups, all of which had been lesioned (control, $\bar{x}=59.75\pm11.39$; lesioned, $\bar{x}=122.2\pm7.6$) and the non conditioned group (140±10.02). This trend was similar to that on the normal tap water session following the LiCl trial. Scheffé contrasts also showed that the control group drank significantly less than the other animals. In terms of established CTA interpretation, the controls remembered the LiCl experience, while the others did not (Figure 1A).

Extinction of CTA. Two normal tap water sessions intervened before the second NaCl test. There were no significant group differences in water

intake during that session. Apparently extinction of the CTA had occurred in the control animals.

Effects of implants. Retraining of the CTA began 15 days after implant surgery, with LiCl solution substituted for tap water during the A.M. session on the third day. A NaCl test for retention of the CTA took place during the P.M. trial on the fifth day of the series, differences between groups being highly significant: $F(5,53)=27.12$, $p=1.0\times10^{-4}$. Scheffé contrasts revealed that the control ($\bar{x}=32.78\pm4.78$) and IC-V-NGF ($\bar{x}=28\pm2.84$) animals differed from the other groups, but not from each other. In other words, the animals receiving IC implants supplemented with NGF had recovered the ability to learn the CTA task (Figure 1B). It is strikingly clear that, although a high level of retention as evidenced in low water intake continued to characterize the behavior of the control group, a major improvement occurred in animals with IC + NGF implants. Retention by subjects in the other treatment groups showed very little change postimplant, despite the earlier practice during the postlesion period.

Of particular significance to the interpretation of our present results are comparisons between the performances of the various groups on retention (NaCl) tests postlesion and postimplant. Such comparisons provide information about effects of the various experimental treatments. They were carried out using paired t-tests. Retention of the CTA by control and LX subjects were not significantly different when tested postlesion and postimplant. Both lesioned groups receiving exogenous NGF drank less after treatment than before, indicating an improved retention ($p<5.0\times10^{-2}$). The IC-V group (without NGF) also improved significantly ($p<5.0\times10^{-2}$). Animals receiving homotopic implants (IC) plus

NGF evidenced by far the greatest recovery, reaching control levels within 15 days postimplant ($p<1.0x10^{-3}$). Those with heterotopic implants (OCC) plus NGF showed a small but reliable recovery that could be attributed to the exogenous NGF included in their treatment ($p<5.0x10^{-2}$).

Inhibitory Avoidance

Performance in the inhibitory avoidance situation was introduced as a second measure of differences in learning among the various treatment groups. Training sessions were conducted on the day immediately following the CTA retention trials; tests for IA memory, on the second day thereafter. Because of the arbitrary limit of 600 sec imposed on retention trials, the distributions of measure of retention times were truncated, leading to the use of nonparametric statistics for analyzing the data. Training times were not so artificially limited and were normally distributed.

Means±SEM for retention times (sec) are given in Figure 2. There were no significant differences in training times among the groups: $F(5,53)=0.32$, $p=9.0x10^{-1}$. This indicated that the preceding treatments (lesion, implant, CTA) had no carryover effects on the responses of the animals to the negative phototropism involved when placed in the IA apparatus. By comparison, performances during the retention session were highly significant: Kruskal-Wallis (chi-square approximation) analysis yielded a $Chi^2(5)=44.517$, $p<1.0x10^{-4}$. This indicated both that acquisition of the avoidance response had occurred during the training trials and that the training had differential effects on the various treatment groups.

Examination of Figure 2 suggests that three treatment groups had significantly longer retention times than did the others, i.e., control, IC-V-NGF, and IC-V-NGF-N. Multiple comparisons using rank sums (Siegel &

Castellan, 1988) confirmed this observation. It is also reasonable to predict that the two IC-V-NGF groups, differing only in that one had been tested for CTA and the other had not, would not be significantly different. This prediction was corroborated by a rank sums comparison, indicating that there were no carryover effects of exposure to LiCl three days earlier.

A central hypothesis in the present studies was designed to test whether supplementation of the fetal IC implant with NGF would induce recovery from the IC lesions at 15 days post-graft. This hypothesis may be tested by comparing the rank sums of retention times for the two IC-V-NGF groups with the groups, IC-V, that did not receive the NGF, and the OCC-V-NGF that received heterotopical grafts plus NGF supplement. Clearly supplementing the homotopic fetal implant tissue with NGF produced a significant recovery of learning performance. These effects were not evidenced when heterotopic grafts were supplemented with NGF or when only vehicle was applied in the homotopic grafts. Differences among the other three groups were not significant and showed little evidence of learning.

DISCUSSION

Lithium: lack of extended carryover effects

The research was also designed to clarify a secondary matter, i.e., that the use of LiCl in the CTA paradigm might influence the conditioning process by affecting levels of ACh in brain. It has been reported that, after acute injection, lithium accumulates slowly in brain, reaching a maximum concentration in approximately 24 hr and being removed slowly (Kling, Manowitz & Pollack, 1987). It has also been shown that corresponding increases occur in rate of synthesis of ACh (Jope, 1979). Behavioral

effects of chronic LiCl treatment have been reported to include suppression of hyper-reactivity to changes in external environmental stimulation (Russell, Pechnick and Jope, 1981), effects reminiscent of the use of Li as a therapy in human manic and manic-depressive disorders. To ensure that interpretation of the results of the present experiments would not be complicated because of the exposure to Li, the research design included animals treated exactly as the experimental group, IC-V-NGF, but not tested for CTA conditioning, i.e., receiving no LiCl. It would be predicted that any carryover effects would appear as differences between these two groups. As the results showed, behavioral measures of the two were not significantly different when tested three days after exposure to LiCl.

Behavioral recovery

A case for the involvement of the cholinergic neurotransmitter system in behavioral recovery from brain lesions when the latter are followed by fetal implants has received extensive support from a variety of experimental studies (Bermúdez-Rattoni et al., 1992; Gage & Buzsaki, 1989; Russell, 1988). Lesions have been shown to produce highly significant morphological, electrophysiological and neurochemical changes that are reflected in behaviors known to be influenced by the functioning of that system. Given such effects, attention has turned to means by which they may be ameliorated, with particular emphasis on the use of exogenous NGF and/or its combined actions with tissue implants. The present experiments have defined the success of such approaches, i.e., "recovery" from lesions, in terms of behavioral criteria. Investigations using other criteria have demonstrated that treatments involving homotopic implants supplemented by exogenous NGF can enhance and accelerate regrowth and penetration of cholinergic axons into a lesioned area (Hagg, Vahlsing,

Manthorpe and Varon, 1990; Vahlsing, Hagg, Spencer, Connor, Manthorpe & Varon, 1991), restore rhythmic slow (theta) activity in the denervated hippocampus (Buzsaki, Gage, Czopf & Bjorklund, 1987) and stimulate the recovery of cholinergic functions (Lapchak, Jenden and Hefti; 1991). Results of the present research have extended knowledge of the latter and related it to changes in behavior.

The ultimate criteria for recovery in the present experiments were measures of learning in two quite different paradigms: conditioned taste aversion and inhibitory avoidance. Previous research had shown a form of specificity in reactions between the sites of lesions and their effects on these two behaviors: IC lesions resulted in disruption of both, amygdaloid lesions only IA (Bermúdez-Rattoni & McGaugh, 1991). The research had also established convincing evidence for the importance to behavioral recovery of homotopic implants, heterotopic being ineffective (Bermúdez-Rattoni et al., 1992). Our present results are consistent with both these conclusions. These results are also consistent with an earlier report that recovery of IA from the debilitating effects of IC lesions is greatly facilitated by the simultaneous implant of homotopic fetal tissue and NGF (Escobar, Hiriart, Piña & Bermúdez-Rattoni, 1991). NGF in gelfoam had been found to reduce death of medial septal neurons following fimbria-fornix lesions, the success rates being only partial and related to the level of NGF administered (Otto et al., 1989).

Roles of NGF in behavioral recovery

During the past decade interest in behavioral effects of experimentally administered exogenous NGF has grown rapidly. Almost without exception reports of such research have indicated improvement in behavioral deficits induced by brain lesions. In our laboratory we have

been especially interested in looking for neural and vascular changes which may coincide in these time frames with the behavioral modifications. We have shown (Fernández-Ruíz et al., 1991) that tissues taken at various times after fetal brain implants without NGF supplementation were in different stages of maturation. At 15 days there was very little development of neurons and blood vessels in the implanted tissue. Increasing maturation occured at 30 and 45 days. By 60 days a greater neuronal density had developed; fetal implants adhered to the host tissue with abundant vascularization; proliferation of glial cells was clearly apparent; and, fibers increasingly crossed the border between the implant and the host. We have also demonstrated that, using the same experimental designs, recovery of learning and memory followed a similar cause: starting at 45 days post-implant (Fernández-Ruíz et al., 1991) and reaching a significant recovery by 60 days (Bermúdez-Rattoni et al., 1987; Escobar et al., 1989).

The results we now report fit well within this general time frame. In the present experiments we have introduced exogenous NGF using two techniques employed by other investigators: implants of fetal brain tissue and infusion using NGF-saturated gel foam. The nature of our research design makes it possible to isolate the behavioral effects of each, as well as their combined effects. Neither fetal implants alone nor NGF supplement of heterotopic implants induced recovery of CTA or IA to normal control levels within 20 days post implant. The contrast with performances of animals receiving both homotopic implants and NGF supplements was striking, they having reached parity with the unoperated control subjects within this period These findings indicate that NGF associated with homotopic implants significantly facilitated behavioral recovery and

suggest that similar treatments with NTFs may have analogous effects when lesions involve other brain areas.

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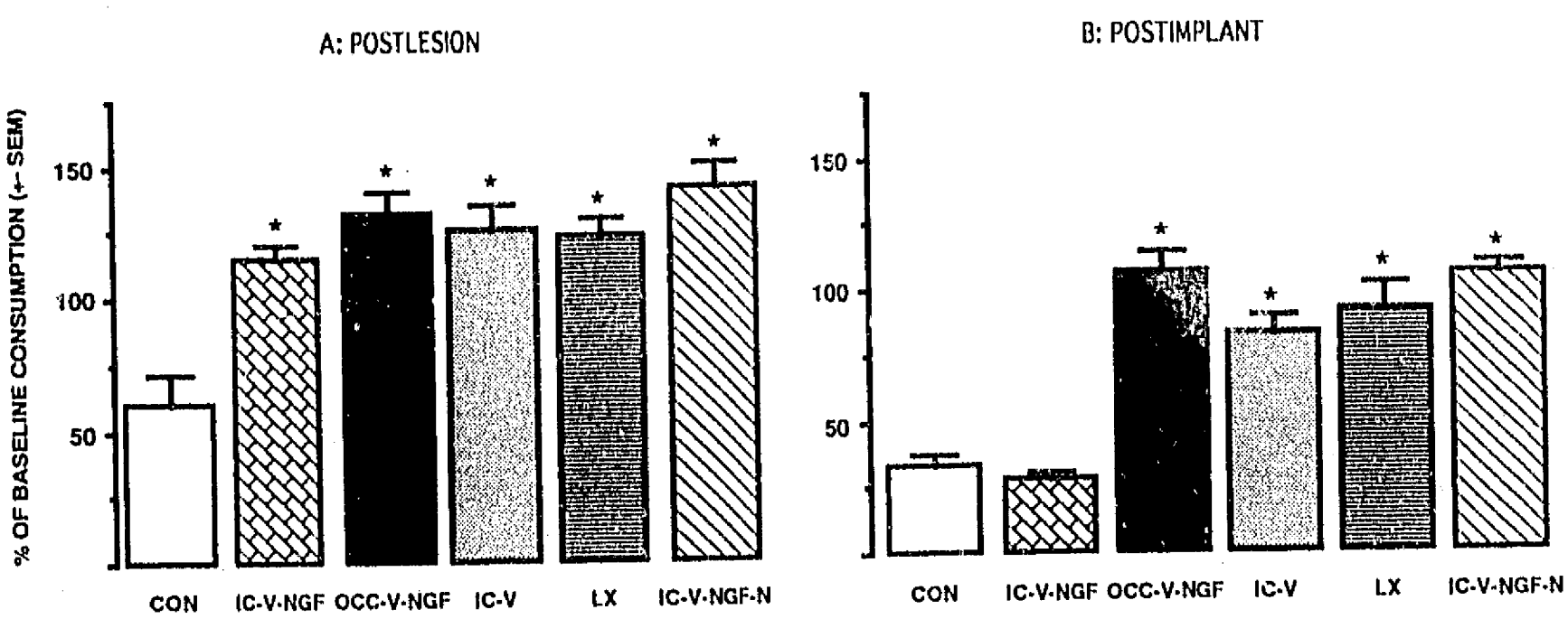
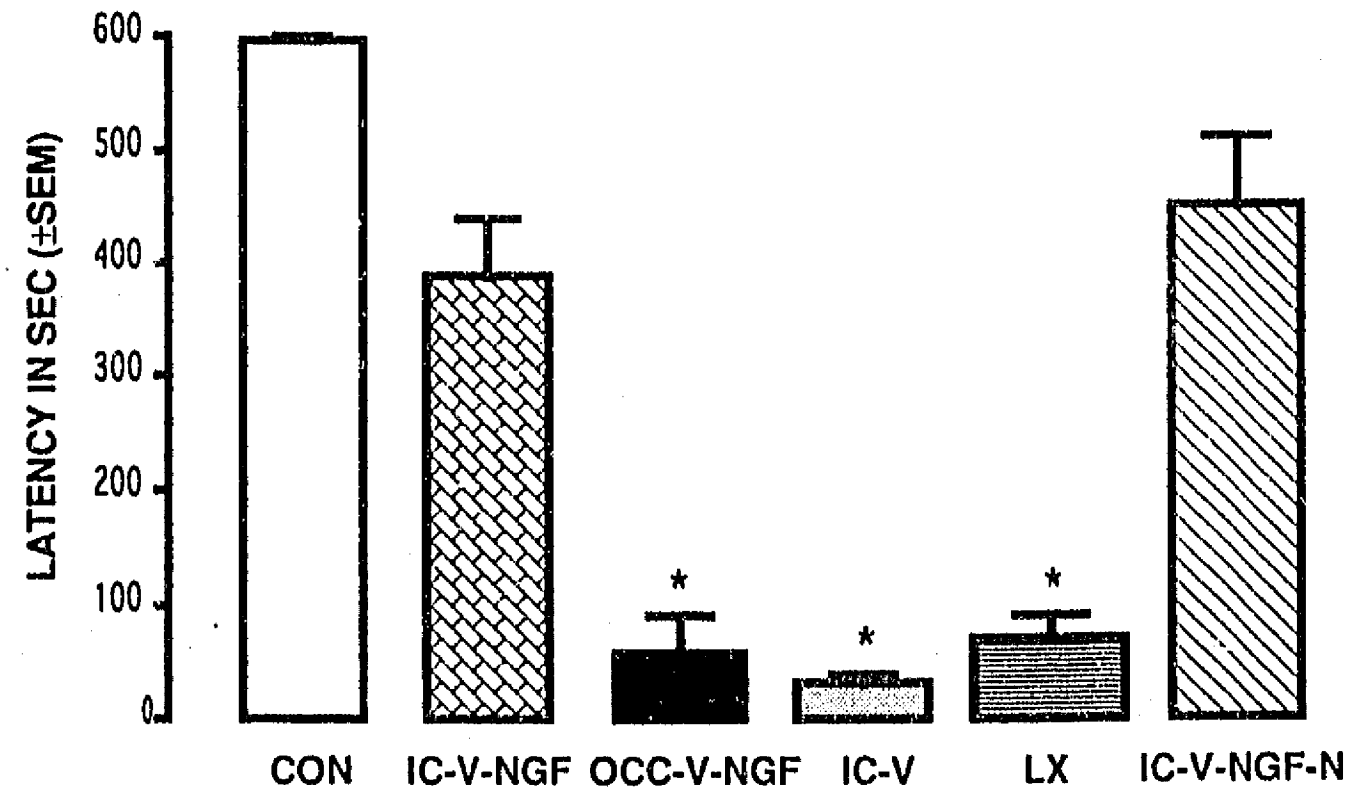
EFFECTS OF NGF ON TASTE AVERSION



* Significantly different from control: $p<5.0 \times 10^{-2}$

EFFECTS OF NGF ON INHIBITORY AVOIDANCE: POSTIMPLANT



* Significantly different from control: $p<5.0 \times 10^{-2}$

TABLE I
SUMMARY OF RESEARCH DESIGN*

Group	n	Treatment 1 Lesion	2 CTA	3 Implant	4 CTA	5 IA	6 Neurochem
Days of experiment		0	8	16	31	37	Assays 43
Control	10	-	+	-	+	+	+
IC-V	9	+	+	+	+	+	+
IC-V-NGF	10	+	+	+	+	+	+
IC-V-NGF-N	9	+	-	+	-	+	+
OCC-V-NGF	10	+	+	+	+	+	+
LX	10	+	+	-	+	+	+

* "+", treatment given, "-", treatment not given

Fig. 1 Amount of NaCl consumed by CON control; IC-V insular cortex implants; IC-V-NGF insular cortex implants with nerve growth factor (-N, not exposed to lithium); OCC-V-NGF, occipital cortex implants with nerve growth factor and LX, lesions only. Consumption is expressed as the percentage of each group's previous day water baseline. Left panel (A) shows the results of one taste test trial prior to implant. Right panel (B) shows the results of one taste test trial 15 days after implant. Values are means ± SEM * $p<5.0x10^{-2}$ as compared with controls (Sheffé test).

Fig. 2 The bars illustrate the latency in seconds during the inhibitory avoidance test, of control (CON); insular cortex implants(IC-V); (IC-V-NGF) insular cortex implants with nerve growth factor (-N, not exposed to lithium); (OCC-V-NGF) occipital cortex implants with nerve growth factor and (LX) lesioned groups at 15 days post-graft. * $p<5.0x10^{-2}$ (Kruskal-Wallis) as compared to control group.

TRABAJO VII

ACCELERATING BEHAVIORAL RECOVERY AFTER CORTICAL LESIONS, II: *IN VIVO* EVIDENCE FOR CHOLINERGIC INVOLVEMENT.

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Key words: Neocortical lesions; fetal brain implants; conditioned taste aversion; inhibited avoidance; endogenous ACh and Ch; uptake of exogenous [^{2}H4]Ch; synthesis of [^{2}H4]ACh; mole ratios of ACh and Ch; rates of synthesis of ACh.

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ABSTRACT

We recently demonstrated that insular cortex (IC) fetal implants supplemented by nerve growth factor (NGF) can accelerate the recovery of behavioral deficits induced by IC brain lesions. In the present report we describe results of *in vivo* assays of acetylcholine (ACh) turnover in the IC of rats subjected to the same brain lesion and implant treatments used in that research and for which detailed behavioral data are available. The neurochemical assays were carried out immediately after completion of the behavioral measurements. The assays showed that implants or NGF with heterotopic tissue continued to be associated with elevated levels of ACh and with deficits in learning and memory at a time postlesion when both behavior and ACh turnover *in vivo*, after treatment with homotopic implants and NGF combined were at nonlesioned control levels. The results support the concept that, *in vivo*, the cholinergic neurotransmitter system is intimately involved in recovery from IC lesion-induced deficits in behavior and show that a combination of homotopic implant and NGF may be used as a means of manipulating that system to accelerate the repair of such deficits. Mechanisms by which this combination produces its effects are considered and the possibility suggested that other neurotrophic factors (NTF) may also be useful when other types of brain lesions are involved.

INTRODUCTION

Recently we have demonstrated that behavioral recovery after cortical lesions involving cholinergic pathways in the brain can be very significanlty accelerated by homotopic fetal implants supplemented by NGF (Bermúdez-Rattoni, Escobar, Piña, Tapia, López-García & Hiriart, 1992; Escobar, Russell, Booth & Bermúdez-Rattoni, submitted). Research using other criteria for "recovery" had concluded that similar combinations of implants plus NGF can enhance and accelerate regrowth and penetration of cholinergic axons into a lesioned area (Hagg, Vahlsing, Manthorpe, & Varon, 1990; Vahlsing, Hagg, Spencer, Connor, Manthorpe & Varon, 1991), restore rhythmic electrophysiological activity in the denervated hippocampus (Tuszynski, Buzsaki &Gage, 1990) and stimulate recovery of ACh synthesis and release *in vitro* and *in vivo* (Lapchak, Jenden & Hefti, 1991). Such observations are consistent with the wealth of evidence supporting the central roles played by the cholinergic system in a variety of neurodegenerative diseases (Candy, Perry, Court, Oakley and Edwardson, 1986; Perry, 1990) in brain aging (Wenk, Pierce, Struble, Price & Cork, 1989) and in learning and memory (Decker & McGaugh, 1991).

Research described in the present report focuses on interactions between two of these basic parameters: neurochemical events and behavior. Much attention has been given to changes in activity levels of enzymes involved in the synthesis and degradation of ACh (Gage & Buzsaki, 1989; López-García, Bermúdez-Rattoni & Tapia, 1990a). Changes in ACh itself have been seen to relate to behavioral recovery, neurotransmitter turnover rates of newly established neuronal connections operating at rates similar to those of undisturbed pathways (Bjorklund, Gage, Schmiddt, Stenevi & Dunnet, 1983). Recovery from very significant lesion-induced reductions in rates of [^{14}C]2-deoxyglucose utilization has been observed

following cholinergic reinnervation, suggesting a relation between the recovery and this essential component of ACh synthesis (Kelly, Linvall, Stenevi & Bjorklund, 1985). Chronic administration of exogenous NGF "...significantly increases the activity of hemicholinium sensitive Ch transport..." (Williams & Rylett, 1990), which has been shown to affect memory processes (Russell & Macri, 1978; Russell, 1988). It has been reported that muscarinic receptors, both M_1 and M_2, "...respond to cholinergic denervation and reinnervation similarly" (Joyce, Gibbs, Cotman & Marshall, 1980).

Such a constantly expanding body of knowledge about relations between NGF and parameters of the cholinergic neurotransmitter system provides a basis for understanding not only the modes of action of fetal implants and of NGF, but also of how these may participate in the recovery of various behavioral functions. However, when behavior has been the dependent variable measured, questions have been raised about (a) limitations to the extrapolation of data collected *in vitro* to the state of affairs *in vivo*, and (b) the specificity of the various markers used to measure cholinergic functions. A lack of complete parallelism between *in vitro* and *in vivo* has been observed in studies of behavioral effects of drug treatments (Bollinger, Palacios, Closse, Gmelin & Malanowsky, 1986). Recently it has been reported that: "In contrast to the significant reductions in cholinergic parameters measured *in vitro* after partial fimbrial lesions, such partial lesions did not significantly alter *in vivo* measures of hippocampal cholinergic function" (Lapchak, et al., 1991). Presumably, residual cholinergic neurons remaining after partial lesions may compensate for losses by upregulating their capacity to synthesize and store ACh. Such results indicate the importance of measuring parameters of the cholinergic system *in vivo* when testing hypotheses about their interactions with behavior.

In principle, each of its parameters may be used as a marker of the cholinergic system. However, the values of markers depends upon their specificity and sensitivity. Among markers reported in many studies, choline acetyltransferase (ChAT) is often considered a specific marker for cholinergic neurons, yet ChAT identical with brain ChAT has been found in tissues without such innervation (Haubrich, 1976; Rama-Sastry, Stathan, Axelrod & Hirata, 1981). Another enzyme often reported as a marker is acetylcholinesterase (AChE), which also is not associated specifically with cholinergic cells. Loss of cholinergic neurons may not result in a corresponding decrease in muscarinic receptors (mAChR); indeed, the result may be a regulatory increase. Problems in the selection of cholinergic markers have been summarized (Jenden, 1985).

For our present purposes, the preferred approach to obtaining information about interactions between behavioral and cholinergic functions was to assay ACh turnover *in vivo* at a critical time in the recovery of behavior under various experimental conditions, e.g., following fetal tissue implants with and without NGF supplements. The availability of animals participating in our experiment designed to study recovery of learning and memory after gustatory neocortex-lesions (Escobar, et al., submitted) provided particularly favorable circumstances within which to carry out such assays. Fernández-Ruíz, Escobar, Piña, Díaz-Cintra, Cintra-McGlone and Bermúdez-Rattoni (1991), had described the time course for recovery of taste aversion learning and memory following homotopic fetal brain implants: "Behavioral results showed a very good functional recuperation at 60 days, slight recovery at 45 and 30 days, and a poor recovery at 15 days postgraft." Our more recent research has shown that recovery of CTA to control levels could be induced by 16 days postimplant when the implants were supplemented with NGF. In the present report we describe results of *in vivo* assays

of ACh turnover in the gustatory neocortices of rats subjected to the various brain lesion and implant treatments described in our earlier study and for which detailed behavioral data were available. Because of the involvement in CTA of the nucleus basalis magnocellularis (NBM) through cholinergic projections to the IC (López-García, Fernández-Ruíz, Escobar, Bermúdez-Rattoni & Tapia, in press) cholinergic assays were also carried out on NMB tissue. The neurochemical assays took place immediately after completion of the behavioral measurements, i.e., with brain tissues taken at 26 days postimplant. The combined behavioral and neurochemical data make it possible to determine the status of the cholinergic system *in vivo* at a time when behaviors had returned to normal control levels and to compare this with treatments that were not associated with such recovery.

MATERIALS AND METHODS

Behavioral Measures and Research Design

With the exception of assays for ACh turnover, the research design, materials and methods have been described in full in our earlier report (Escobar, et al., submitted). They are summarized briefly here. The research design included six groups of male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). They were maintained on standard laboratory chow and lived under a 12 hr light/dark cycle throughout the experiment. Each of the six groups received different treatments. Group 1 (control) served as an unlesioned control. Group 2 (IC-V) provided data for determining effects of IC implants without NGF supplementation. Groups 3 and 4 (IC-V-NGF) were both implanted with IC+NGF, differing only in that the latter was not subjected to assays of conditioned taste aversion (CTA) involving ingestion of lithium. Because lithium was shown to have no carryover effects when the neurochemical assays were conducted, these

groups were combined for purposes of the present analyses. Group 5 (OCC-*in situ*) was a heterotopic control for comparison with animals receiving homotopic implants and involved tissue taken directly from the occipital cortex.

Learning and memory were measured in two well-standardized behavioral tasks (Bermúdez-Rattoni & McGaugh, 1991). Conditioned taste aversion involved substituting lithium chloride solution (0.1 M) for normal tap water in inducing taste aversion and measuring retention of the conditioned response at intervals thereafter. Inhibited avoidance (IA) took advantage of the fact that rats are negatively phototropic, punishing animals for making such a response (mild electric shock) and measuring memory in terms of the inhibition of responding on later test trials.

Lesions and Implants

Stereotopic surgery was used to create bilateral electrolytic lesions in the IC. Animals were kept under deep pentobarbital anesthesia (50 mg/kg) during the procedure. A monopolar stainless steel electrode, coated with epoxyle except for the tie (0.5 mm), was inserted using the following coordinates with respect to bregma: AP, +1.2; L, ±5.5; V, -5.5. The lesions were produced by passing a 2 mA anodal current through the electrode for a duration of 45 s.

Sixteen-day-old fetuses were removed from the uteri of pregnant rats under pentobarbital anesthesia and their brains were extracted. Tissue blocks of approximately 3 mm^3 in volume were dissected from the region corresponding to the temporoparietal area (above the rhinal sulcus) and from the occipital cortex for homotopic and heterotopic implants, respectively. Using a 100 µl Hamilton syringe, tissue was placed stereotaxically into the cavity produced by the lesion, using the same set of coordinates given above. Prior to the implants the tissues

were embedded in a high concentration solution (20 µg/ml) of NGF 7s (Sigma, St. Louis, MO) + DMEM (Dulbecco's Modified Eagle's Medium) GIBCO(Grand Island, NY)/0.25 BSA Bovine Serum Albumin, (Sigma, St. Louis, MO). Following implant, gelfoam embedded in the same solution was inserted into the cavity (Otto, Frotscher & Unsicker, 1989) and the external (cranial) opening was sealed with nylon screws. For purposes of comparison, tissue samples were taken from the occipital cortex *in situ*.

Measurement of ACh and Ch Turnover

For measurement of acetylcholine (ACh) and choline (Ch) turnover in the two brain regions, the rats were injected i.v. via the tail vein with [$^2H_4$]Ch (20 nmol/gm) and sacrified 1 min later by focused microwave irradiation of the head (4 kw for 2.9 sec; Gerling-Moore, Palo Alto, CA). In dissecting the tissues to be assayed, the IC region was localized using the middle cerebral artery and the rhinal sulcus as reference points (López-García, Fernández-Ruíz, Bermúdez-Rattoni & Tapia, 1990b; Lasiter, & Glanzman, 1985) and the implanted tissue was carefully teased from the host tissue. Because of our earlier observations showing only very nedomentary recovery within the time frame of the present experiment (Fernández-Ruíz, et al., 1991), it was not possible to include the lesion--only group in our assays. After dissection, brain regions were weighed and immediately homogenized in 5 volumes of a mixture of cold 15% 1 N aqueous formic acid and acetone (v/v) containing [$^2H_9$]Ch and [$^2H_9$]ACh as internal standards. The extraction procedure utilized ion pair extraction with dipicrylamine as described previously (Freeman, Wictorin, Bjorklund, Williams, Varon & Gage, 1987). The concentrations of endogenous ACh and Ch and of [$^2H_4$]-labelled ACh and Ch were determined by GCMS (Jenden, Roch & Booth, 1973). Total concentrations

were calculated as the sum of endogenous + [^{2}H4]ACh or [^{2}H4]Ch and mole ratio, as the ratio of [^{2}H4]ACh or [^{2}H4]Ch to their respective total amounts. Rates of ACh synthesis were calculated according to the procedure described by Schuberth, Spart and Sundwall, 1970 and by Jenden, Choi, Silverman, Steinborn, Roch & Booth, 1974) which corrects for changes in [^{2}H4]Ch availability during the interval between injection and microwave irradiation.

RESULTS

Behavioral Effects

Behavioral effects of the various treatments have been described in detail elsewhere (Escobar, et al., submitted) and need only to be summarized here. Two paradigms for measuring learning and memory were involved. (1) Retention of the CTA by normal control subjects did not differ significantly when tested at pre- and postimplant intervals. Animals receiving homotopic implants plus NGF evidenced by far the greatest recovery, reaching normal control levels within 16 days postimplant. The group treated with homotopic implants without NGF and the group with heterotopic implants plus NGF showed significant, but much less pronounced, improvements. (2) In tests of memory using the IA paradigm the two groups treated with homotopic implants plus NGF did not differ significantly from the performance of the normal control animals. The other treatments were not different from each other, showing no significant evidence of recovery from memory deficits. Given these behavioral effects, the objective of the present study was to determine the extent to which the differences among the treatment groups might be related to concomitant differences in the various parameters of the cholinergic system.

ACh and Ch Levels and Synthesis

In vivo assays for neurochemical parameters in two brain regions, insular cortex (IC) and nucleus basalis magnocellularis (NB), were carried out after completion of the tests for retention of the inhibited avoidance task. Means ± SEM for each of the parameters assayed are presented in Tables 1 and 2. Table 3 reports the rates of synthesis of ACh in samples of tissues from implants in the two brain regions under the three major treatment conditions and in the control animals.

Two-way ANOVAs established the existence of highly significant differences between the two brain regions among the various treatments (experimental groups) and for interactions between them. The data for each of the neurochemical parameters were next subjected to one-way ANOVAs across treatment groups. These analyses were carried out independently for tissues from the IC and the NB. The results are included in Tables 1 and 2. When significant F-values were found, further post-hoc analyses (Scheffé, 1953) were used to identify the responsible treatment(s). Because there were no significant differences between the IC-V-NGF and IC-V-NGF-N groups, the two were combined in the analyses. The failure to find differences between these two groups indicated that the exposure to LiCl during the CTA conditioning 10 days earlier had no carryover effects at the time the GCMS assays were conducted.

Endogenous ACh and Ch. ANOVA showed that levels of endogenous cortical [$^2H_0$]ACh and [$^2H_0$]Ch differed very significantly among the treatment groups. Scheffé contrasts failed to detect significant differences in ACh levels when the IC-V-NGF animals were contrasted with controls. Similarly, endogenous levels did not differ between the IC-V and OCC-V-NGF groups. Quantitatively the latter were significantly higher than the IC-V-NGF and control levels. ACh levels in the NB did not differ among the various treatments.

ANOVA provided evidence that endogenous Ch levels were different in both the cortex and in the NBM, the F-ratio for the former having a more significant p-value than that for the latter. Once again, Scheffé contrasts failed to detect significant differences between cortical levels in animals receiving IC-V-NGF implants and controls, these two treatments having significantly lower levels than the group receiving the IC-V implant without NGF. Multiple contrasts showed that, as was observed in analyses of cortical tissue, the IC-V and OCC-V-NGF groups had higher levels of endogenous Ch in the NBM than did the control and IC-V-NGF animals.

Uptake of labelled Ch. Despite the significant differences in endogenous levels of ACh and Ch described above, there was no evidence from the GCMS assays that the uptake of [^{2}H4]Ch into the cortex varied among the treatment groups. However, there were differences in the NBM. Inspection of the mean levels presented in Table 2 suggests that uptake in the IC-V group was significantly greater when compared to the IC-V-NGF animals. Indeed, a multiple contrast showed that the IC-V animals had a greater uptake than all other groups combined.

Levels of [^{2}H4]ACh. During the one-minute interval between the injection of [^{2}H4]Ch and the inhibition of cholinergic activity by microwave fixation, some of the [^{2}H4]Ch was converted to ACh. As with Ch uptake, there were no significant differences among tissues taken from the cortex. Once again levels in the NBM did differ, those in IC-V animals being highest, closely followed by levels in OCC-V-NGF samples. Scheffé contrasts showed that the source of the significance reflected in the one-way ANOVA was due to differences between these two treatments, which did not differ between themselves, and the combined control plus IC-V-NGF groups.

Mole Ratios. The final columns in Table 1 summarize the mole ratios of [^{2}H4]ACh to the total ACh in cortex and NB for each of the various treatment groups. This is one measure of the synthesis of ACh during the interval (1 min) between injection of the [^{2}H4]Ch and the inactivation of the cholinergic system by microwave fixation. It is, of course, a measure which involves interactions between synthetic and metabolic processes within the cholinergic pathway, influenced by the activity levels of the enzymes ChAT and AChE. As Table 1 shows, 1-way ANOVAs by treatments provided significant p-values for both cortex and NB. Further analyses by Scheffé comparisons showed that the overall significance among the cortical tissues was due entirely to the elevated specific activity of the occ *in situ* groups relative to other treatments. It will be recalled that tissues for this group had been taken from the occipital cortex *in situ* and not from an implant. The Scheffé contrasts detected signficant differences between ACh mole ratios in such tissues and those for the IC-V and OCC-V-NGF groups, but failed to detect differences in tissues taken from the control and IC-V-NGF animals.

The control and occ *in situ* groups provided information about mole ratios in tissues not involved as implants, i.e., tissues sampled directly from the insular and occipital cortex, respectively. The preceding sections have already indicated that differences were not detected between control tissues and those of the IC-V-NGF treatment group, i.e., that in which behavioral recovery had occurred. Analyses of tissues taken directly from the occipital cortex deviated from this result. None of the choline parameters showed significant differences from those of the OCC-V-NGF implant tissues. However, differences in acetylcholine parameters did appear in both the cortex and NB. In the former, endogenous ACh and AChTot were significantly lower for the *occ in situ* tissues and their mole ratios were

significantly higher. In NB, [^{2}H4]ACh and ACh mole ratios were lower in the *occ in situ* tissues in relation to those from the OCC-V-NGF implants.

Rates of ACh synthesis. Schuberth, et al., 1970, and Jenden, et al, 1974, have provided a means for calculating a rate of synthesis for ACh *in vivo*. This rate represents the product of [^{2}H4]ACh and the ratio of ChTotal to [^{2}H4]Ch, thus allowing for changes in the availability of [^{2}H4]Ch during the time between its injection i.v. and microwave fixation of the cholinergic system one minute later. Rates for the synthesis of ACh in cortex and NB as provided by our present analyses are summarized in Table 3. One-way ANOVAs showed that rates in both regions differed significantly among treatment groups. Scheffé contrasts failed to detect differences between rates for the pair, control and IC-V-NGF groups, nor between the pair, IC-V and OCC-V-NGF treatments. In both cortical and NBM tissues, multiple comparisons detected significantly higher rates of ACh synthesis in the second of these pairs.

DISCUSSION

The present experiment was designed to investigate the involvement of the cholinergic neurotransmitter system in behavioral recovery following lesions to the brain's insular cortex. Earlier research had shown that a combination of homotopic fetal brain implants supplemented with NGF accelerated recovery at a time when these two treatments by themselves were not effective. Because cholinergic innervation is related to levels of NGF in the CNS (Korshing, Auburger, Heumann, Scott & Thoenen, 1985), it could be predicted that variations in those levels would be measurable as concomitant changes in cholinergic function and these, in turn, as changes in behaviors for which they are substrates. Our research and reports from other laboratories generated two broad hypotheses: (a) that the lesions would produce elevated ACh turnover *in vivo*, and (b) that the turnover would return to normal control levels when behavioral deficits induced by the lesions had disappeared. The results of the present experiments are consistent with both these hypotheses. They raise questions about mechanisms that may underlie the observed effects.

NGF-Cholinergic System -- Behavior Interactions

In evaluating various possibilities the time frame required for a proposed mechanisms to function is of paramount important, i.e., changes in morphological and/or neurochemical parameters could be expected to occur within a time frame consistent with behavioral recovery. That such recovery after IC lesions does, in fact, follow a time-dependent course has been reported in studies where all experimental animals received the same NGF treatment, but CTA behavior was assayed at different times post-implant (Fernández-Ruíz, et al., 1991). During the first two weeks post-implant, the experimental animals gave no evidence of the

recovery which later characterized behavior progressively and which was paralleled by increases in cell maturity and reconnectivity between implanted neurons and host tissues. Assays of neurochemical events occuring within the same time frame have also shown progressive changes.

Upon completion of the two assays for memory (CTA and IA), at 23 days postlesion, the behaviors of animals in the present experiment receiving the four treatments could be classified into two groups, there being no significant differences between treatments within each group. Behaviors of animals with combined implants plus NGF treatment performed at the same levels as control animals. By comparison, the performances of those with implants only or with heterotopic implants plus NGF had retention significantly poorer than controls. Behavioral assays conducted immediately prior to the respective experimental treatments had shown comparable performance levels for animals from all groups. Presumably, effects that developed thereafter were related to differences in the treatments they received.

Differences among treatment groups in cholinergic functions in the insular cortex were highly significant and paralleled the differences in behavior. Both levels of ACh (nmol gm^{-1} min^{-1}) and rates of [$^{2}H4$]ACh synthesis (nmol gm^{-1} min^{-1}) in animals receiving the combined treatment were comparable to control levels, while those of animals with the other two treatments were significantly elevated. Comparisons between cholinergic function in tissues from animals with heterotopic implants supplemented with NGF (OCC-V-NGF) and tissues taken from the occipital lobes *in situ* showed the former to be elevated and the latter to be at control levels, thus the difference can be attributed to the presence of NGF *per se*.

Effects of the various treatments on cholinergic function in the NBM differed in an important regard. The NBM had been chosen for study because of its cholinergic projections to the IC and to other areas of the cortex (Rye, Wainer, Mesulam, Mufson & Saper 1984; Decker & McGaugh, 1991). It has been shown to be involved in a variety of learned behaviors (Hepler, Wenke, Gibbs, Olton & Coyle, 1985; Smith, 1988). Recent studies of effects of excitotoxic lesions of the NBM have reported significant reductions in cholinergic markers in the IC, indicating interaction between the two brain regions (López-García, et al., in press). Accompanying the neurochemical effects of the lesions were suppression of CTA learning and disruption of its retention in previously trained animals. In the present experiment, the NBM was not lesioned, yet certain significant neurochemical differences in treatment effects were observed: ACh levels were not affected, while rates of ACh synthesis were elevated in the IC implant and OCC-V-NGF animals when compared to controls.

Potential Mechanisms of Action

Taken together, results of research reported here and elsewhere indicate that the upregulation of cholinergic function which accompanies IC lesions is involved in the recovery of behavioral deficits induced by the lesion. Recovery can be facilitated by treatment with homotopic implants or NGF supplements and accelerated still further when the two treatments are combined. Cholinergic neurons in the basal forebrain both express NGF and respond to NGF (Koliatsos, Clatterbuck, Gouras & Price, 1991). What hypotheses about mechanisms of action may be generated to encompass these processes? The possibilities have been reviewed on several occasions as the body of relevant information has grown

(Clark, Gage, Dunnett, Nilsson & Björklund, 1987; Gage & Buzsaki, 1989; Thoenen, Aubruger, Hellweg, Heumann & Korsching, 1987).

Survival after lesion: "silent" neurons. It has recently been reported that, in the axotomized septohippocampal system "... about one-third of the axotomized septal cholinergic neurons may survive for a long time in a down-regulated atrophic state" (Fischer & Bjorklund, 1991). These cells remained undetected by conventional markers, AChE histochemistry and NGF receptor immunocytochemistry, but were detected by the fluorescent retrograde tracer, FluoroGold (FG). Reduced numbers of AChE and NGF-positive cells were observed within four to seven days postlesion, while loss of FG-prelabeled cells was confirmed only after four weeks. Such results suggest that erroneous interpretations of neuronal loss are possible. NGF produced endogenously or imported exogenously either directly or through implants may attenuate both the atrophic and degenerative processes through its action on such "silent" neurons (Gasser, Weskamp, Otten & David, 1986; Fischer & Bjorklund, 1991). Indeed, it has been reported that delayed treatment with NGF "reverses the apparent loss of cholinergic neurons after acute brain damage" (Hagg, Vahlsing, Manthorpe & Varon, in press).

Extent of the lesion. In interpreting the present results it is also important to consider the possibility that not all relevant IC cholinergic neurons were lesioned in the present experiments. Significant differences in effects on transmitter functions of "full" and "partial" fimbrial lesions have been reported (Lapchak, et al., 1991) and are indicative of what may have happened in our study. Results following partial destruction of cholinergic fibers suggest that "...residual cholinergic neurons are able to upregulate their capacity to synthesize and store ACh *in vivo*..." thus compensating "...for the loss of neighboring neurons of their

population." In the present experiments cholinergic functions have been measured *in vivo* using the same methods and parameters. The tissue studied was from another brain area, IC vs septohippocampal.

Assuming that in our studies destruction was partial rather than full, it could be predicted that residual neurons would "compensate" for lesion-induced losses. Furthermore, it could be hypothesized that this process would continue as recovery to normal population densities of cholinergic neurons progressed. That such progressive morphological recovery occurs is reported in recent results based upon horseradish peroxidase assays following gustatory neocortical lesions and fetal brain implants using the same techniques as in our present experiment (Escobar, Fernández, Guevara, & Bermúdez-Rattoni, 1989; Fernández-Ruíz, et al., 1991). It has also been suggested that certain neurochemical effects of lesions in a related brain region (the NBM) "...can be explained by the remaining unlesioned cholinergic fibers, which might take up choline and synthesize and release ACh in a normal fashion (López-García, et al., in press).

"Reactive synaptogenesis." There is evidence that, following brain lesions, reinnervation may occur through both intrinsic and extrinsic processes. That central neurons exhibit an intrinsic capacity for axonal sprouting and generation of new connections in response to injury is well established (Cotman & Nadler, 1978). When intrinsic reinnervation occurs without supplementation, progress has been reported to occur at a diminishing rate (Matthews, Cotman & Lynch, 1976). Plots of the reappearance of morphologically-intact synapses show rapid increases through 30 days postlesion, when the number of synapses reached approximately 50% of control values. Complete recovery was approached at 240 days. Lesions of the septohippocampal pathway are followed by an increase in endogenous NGF content in the hippocampus and septum, injury to the cholinergic system appearing

to be intimately involved (Korsching, Heumann, Theonen & Hefti, 1986; Gasser, et al., 1986).

The possibility that "spontaneous recovery" dependent solely on the intrinsic capability of neurons for axonal sprouting and formation of new connections could account for effects observed within the time frame of the present experiment appears to be unlikely. Animals with IC lesions that have not received implants or NGF supplementation have been found to be unable to learn the CTA even when given extra acquisition trials over a period of eight weeks (Bermúdez-Rattoni, Fernández, Sanchez, Aguilar-Roblero & Drucker-Colin, 1987). Functional recovery within the time frame of the present experiments depended upon supplementation of the lesioned area with homotopic fetal implants plus NGF.

It has been shown (Fernández-Ruíz, et al., 1991) that tissues taken at various times after fetal brain implants without NGF supplementation were in different stages of maturation. At 15 days there was very little development of neurons and blood vessels in the implanted tissue. Increasing maturation occurred at 30 and 45 days. By 60 days a greater neuronal density had developed; fetal implants adhered to the host tissue with abundant vascularization; proliferation of glial cells was clearly apparent; and, fibers increasingly crossed the border between the implant and the host. It has also been demonstrated that under the same general conditions, recovery of learning and memory followed a similar course starting at 45 days post-implant. In another study of adult rats, exogenous NGF administered immediately after unilateral decortication induced changes in terminal fields and synaptic connections which compensated for the deficits produced by the lesions: "...increased to supernormal levels both the size of cholinergic boutons and the number of synaptic connections..." parameters unaltered in unlesioned rats treated with NGF (Garofalo, Ribeiro-da-Silva & Cuello, 1992).

Interaction between neurotransmitter systems. The finding in our present experiment that ACh synthesis in the NBM showed effects of the various treatments that were analogous to these found in the IC suggests the possibility that interactions between neurotransmitter systems may have been involved. A likely candidate is γ-aminobutyric acid (GABA). Contacts between GABAergic and cholinergic neurons have been identified as a general feature in the basal forebrain (Zaborszky, 1992). GABAergic manipulations of the NBM affect performance on tasks sensitive to cholinergic manipulation (Decker & McGaugh, 1991). GABA has been shown to play an important role in memory processing (Sarter, Dudchenko, Moore, Halley & Bruno, 1992). More specifically, out of such research has come the conclusion that the NBM is involved in taste aversion learning (Tardiff, Kesner & Berman, 1988).

The design of the present experiment included assays for the same parameters of cholinergic function in both the IC and NBM. At the time of neurochemical assays, tissues were taken from the same animals and the same treatment groups. Comparisons among the groups revealed a major similarity and a major difference between IC and NBM. In both tissues, rates of [$^2H_4$]ACh synthesis in animals that had received the combined IC plus NGF treatment were at control levels, significantly less than rates in tissues from the other two treatment groups. Comparisons of ACh levels, however, showed similar differences in treatment effects for cortical tissues, but not for tissues from the NBM.

The possibility that interactions between cholinergic and GABAergic neurotransmitter systems might be involved in the accelerated behavioral recovery observed in the present study has been examined by two experimental approaches. One studied effects of excitotoxin-induced lesions of the NBM on CTA, IA and on cholinergic markers in the IC (López-García, et al., in press). NBM lesions

markedly impaired both learning paradigms. Postlesion reductions in cholinergic markers were found in the IC, indicating involvement of NBM cholinergic projections to that area. This was in contrast to the observation that NBM lesions failed to affect GABAergic markers, [^{14}C]GABA release and glutamate decarboxylase (GAD) activity, in the IC. The latter fact led to the conclusion "...that GABAergic neurotransmission, if involved, is not sufficient for CTA learning."

A second series of experiments using a design similar to that of the present study, has corroborated our observation that recovery of CTA learning to control levels occurs at 15 days postimplant only when treatment of IC lesions includes both IC-implants and NGF, other treatments remaining significantly different from controls. By comparison, GAD activity in the IC at the 15-day point did not show any significant differences among the experimental treatments. These results have been interpreted as suggesting that "...GABA-mediated neurotransmission does not play an important role in graft-promoted mediated behavioral recovery" (Escobar, Jiménez, López-Garcia, Tapia & Bermúdez-Rattoni, in press).

Cholinergic functions. Considerable attention has been given to the role of NGF in the regulation of cholinergic functions in the brain and subsequent effects at the behavioral level (Araujo, Lapchak, Chabot, Nair & Quirion, 1989; Calamandrei, Valanzano & Alleva, 1991; Vahlsing, et al., 1991). Evidence points to cholinergic neurons in the basal forebrain nuclei as predominantly responsible for cognitive defects in progressive degenerative dementias. The same neural population is responsive to NGF and depends upon it for normal development and for the maintenance of normal function (Thoenen, Bandtlow & Heumann, 1987). NGF is involved in cholinergic regulatory processes of the rodent CNS, the animal model used in the present experiments. It is among these processes that bases for

the interactions between NGF, the cholinergic neurotransmitter system and behavior reported in our results may well be found. Consideration of some of the major possibilities follows. Cholinergic neurons in the basal forebrain both express NGF and respond to NGF. Involved in these functions are two species of receptors. The distribution of one of these, NGF receptor molecules (NGF-R), in the CNS resembles the distribution of cholinergic neurons of the basal forebrain (Calamandrei, Valanzanox & Alleva, 1991). They and their mRNA are particularly evident in areas where cholinergic innervation is dense. Cholinergic neurons in the basal forebrain are supplied with NGF by retrograde axonal transport from their target regions. NGF also modulates the expression of its own receptor within the CNS (Fusco, Polato, Vantini, Cavicchioli, Bentivoglio & Leon, 1991). NGF-Rs have been shown to be downregulated in aging and in such disorders as Alzheimer's disease (Hefti & Mash, 1989). Studies using the same basic research design as in our present experiment have reported that upregulation of NGF receptors to control levels accompanied post IC lesions with homotopic implants plus NGF, at a time (15 days) when neither of these treatments alone showed similar effects (Bermúdez-Rattoni et al., 1992). Such evidence suggests a kind of compensatory mechanism that might account for the accelerated recovery from IC lesions seen in our present experiment.

Similar observations have suggested that increases in cholinergic binding sites in rats basal forebrain lesions may reflect compensatory regulation of spared neurons at the level of the presynaptic vesicle (Ruberg, Mayo, Brice, Duyckaerts, Hauw, Simon, LeMoal & Agid, 1990). More detailed examination of cholinergic receptor binding has revealed differences between high- and low-affinity binding sites (Deckel & Robinson, 1987). Kainic acid lesions followed by fetal implants in the striatum induced significant upregulation in high-affinity sites and significant

downregulation in low-affinity sites. The former are involved in the synthesis of ACh and the latter with processes related to phospholipids. Such effects following IC lesions with heterotopic implants supplemented by NGF would be expected to induce elevated synthesis and levels of ACh in the brain, as seen in the present experiment. After IC lesions were treated with homotopic implants plus NGF effects were consistent with the view that recovery from the lesions included the return of neurochemical, neurohistological, and neurobehavioral functions to normal limits.

To put to work results of the kind we have reported here requires fuller understanding of the mechanisms underlying interactions among brain implants, neurochemical processes, and behavior. Some likely models have been considered above. Each needs further study. The potential value of such knowledge about NGF and other neurotrophic factors can already be seen as the uses of brain implants in human therapies gain increasing attention.

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TABLE 1
ACETYLCHOLINE: MEANS ($\bar{x}$) ± SEM FOR LEVELS AND MOLE RATIOS

Treatment	n	$[^2H_0]$ ACh* $\bar{x}$	$[^2H_0]$ ACh* SEM	$[^2H_4]$ ACh* $\bar{x}$	$[^2H_4]$ ACh* SEM	ACh TOT* $\bar{x}$	ACh TOT* SEM	ACh Mole Ratios $\bar{x}$	ACh Mole Ratios SEM
A. CORTEX									
Control	10	35.371	± 2.8477	0.957	± 0.1490	36.328	± 2.7588	0.029	± 0.0060
IC-V	9	47.336	± 3.6642	0.739	± 0.0607	48.075	± 3.6562	0.016	± 0.0019
IC-V-NGF	19	30.643	± 3.1156	0.693	± 0.0332	31.337	± 3.1257	0.024	± 0.0024
OCC-V-NGF	10	57.848	± 9.3424	0.923	± 0.0718	58.772	± 9.3871	0.019	± 0.0024
OCC-*in situ*	10	22.218	± 1.3064	0.829	± 0.0688	23.047	± 1.3204	0.037	± 0.0033
F (5,52)	10	7.78, $p=1.6X10^{-5}$		1.94, $p=1.0x10^{-1}$		7.80, $p=1.5x10^{-5}$		4.99, $p=8.3x10^{-4}$	
B. NUCLEUS BASALIS MAGNOCELLULARIS									
Control	10	46.256	± 3.2300	0.761	± 0.1797	47.017	± 3.3117	0.016	± 0.0029
IC-V	9	55.424	± 9.5286	1.025	± 0.0804	56.449	± 9.5447	0.022	± 0.0046
IC-V-NGF	19	51.906	± 3.6035	0.617	± 0.0363	52.464	± 3.6250	0.012	± 0.0009
OCC-V-NGF	10	68.869	± 9.4234	0.993	± 0.0941	69.863	± 9.4789	0.017	± 0.0034
OCC-*in situ*	10	55.368	± 4.5322	0.620	± 0.0904	55.989	± 4.6202	0.011	± 0.0008
F (5,52)		1.48, $p=2.1x10^{-1}$		3.51, $p=8.3x10^{-3}$		1.50, $p=2.0x10^{-1}$		2.50, $p=4.2x10^{-2}$	

* nmol gm^{-1} wet weight
** Mole ratio: $[^2H_4]$ACh/Total ACh

TABLE 2
CHOLINE: MEANS ($\bar{x}$) ± SEM FOR LEVELS AND MOLE RATIOS

A. CORTEX

Treatment	n	$[^2H_0]$ Ch* $\bar{x}$	$[^2H_0]$ Ch* SEM	$[^2H_4]$ Ch* $\bar{x}$	$[^2H_4]$ Ch* SEM	Ch TOT* $\bar{x}$	Ch TOT* SEM	Ch Mole Ratios** $\bar{x}$	Ch Mole Ratios** SEM
Control	10	26.517	3.0994	6.745	0.9036	33.263	3.5085	0.207	0.0202
IC-V	9	58.203	11.2135	6.629	0.9164	64.832	10.8385	0.127	0.0228
IC-V-NGF	19	26.890	1.7024	7.169	0.4100	34.059	1.8446	0.221	0.0126
OCC-V-NGF	10	34.757	2.8830	6.797	0.4872	41.554	3.0524	0.169	0.0146
OCC-*in situ*	10	35.930	4.7055	6.304	0.7785	42.234	4.9709	0.160	0.0211
F (5,52)	10	5.49, $p=3.9X10^{-4}$		0.25, $p=9.4x10^{-1}$		5.25, $p=5.6x10^{-4}$		4.66, $p=1.4x10^{-3}$	

B. NUCLEUS BASALIS MAGNOCELLULARIS

Control	10	21.962	1.1669	4.548	0.7705	26.510	1.6690	0.164	0.0202
IC-V	9	31.134	3.1600	6.236	0.8182	37.370	3.5736	0.167	0.0164
IC-V-NGF	19	26.814	1.7409	3.688	0.2145	30.501	1.8511	0.124	0.0067
OCC-V-NGF	10	32.555	2.6632	3.884	0.3842	36.439	2.5585	0.113	0.0147
OCC-*in situ*	10	26.131	1.9347	4.072	0.4269	30.203	2.1506	0.135	0.0166
F (5,52)		2.66, $p=3.3x10^{-2}$		3.11, $p=1.6x10^{-2}$		2.63, $p=3.4x10^{-2}$		2.69, $p=3.1x10^{-2}$	

* nmol gm^{-1} wet weight
** Mole ratio: $[^2H_4]$Ch/Total Ch

TABLE 3
RATES OF [$^2H_4$]ACh SYNTHESIS* FROM SYSTEMICALLY INJECTED [2H4]CHOLINE IN THE CORTEX AND NUCLEUS BASALIS MAGNOCELLULARIS (NBM)

Treatment	n	Cortex $\bar{x}$ SEM	NBM $\bar{x}$ SEM
Control	10	4.61 ± 0.466	4.48 ± 0.520
IC-V	9	5.31 ± 0.522	7.16 ± 1.026
IC-V-NGF	19	3.36 ± 0.220	5.34 ± 0.535
OCC-V-NGF	10	5.81 ± 0.655	10.52 ± 2.136
		$F(3,44)=7.43$, $P=3.9X10^{-4}$	$F(3,44)=5.59$, $P=2.4X10^{-3}$

*nmol gm^{-1} min^{-1}

LEGENDS FOR TABLES

Table 1. Animals were injected intravenously with [$^2H_4$]choline 1 min before focused microwave irradiation. Tissues were analyzed by GCMS. "Control," unlesioned control; "IC-V", IC implants without NGF; "IC-V-NGF," IC implants with NGF; "OCC-V-NGF," heterotopic implants with tissues from the occipital cortex; "OCC-*in situ*," tissues taken directly from the occipital cortex.

Table 2. The procedure used was identical to that stated in Table 1.

Table 3. Data were obtained from the same animals and tissues as those shown in Table 1. The rates of synthesis represent the product of [$^2H_4$]ACh and total Ch/[$^2H_4$]Ch availability.

DISCUSION GENERAL

Se ha probado que los transplantes de tejido cerebral fetal tienen la capacidad de sobrevivir y establecer conexiones normales con el tejido huésped denervado de animales previamente lesionados (Kromer y col, 1981a; b; Bjorklund y col, 1983; Lewis y Cotman, 1983), y que la integración anatómica y funcional de los transplantes, se traduce en la recuperación conductual de los sujetos implantados (Bjorklund y col, 1980; Low y col, 1982; Dunnett y col, 1982; Labbe y col, 1983).

Existen numerosas evidencias de que durante el desarrollo o después de una lesión, las neuronas del SNC y periférico requieren de agentes tróficos para sobrevivir, crecer y emitir proyecciones hacia sus tejidos blanco (Bjorklund y Stenevi, 1981; Kromer y col, 1981a; Gage y col, 1984). Se ha reportado también, que las lesiones cerebrales originan la producción de factores neurotróficos en los sitios lesionados, tanto en ratas neonatas como en adultas (Nieto-Sampedro y col, 1982; 1983; Manhorpe y col, 1983), y que la sobrevivencia del tejido neuronal transplantado se incrementa por la administración de extractos de tejidos lesionados (Nieto-Sampedro y col, 1984) o directamente de factores neurotróficos (Toniolo y col, 1985; Pallage y col, 1986; Frim y col, 1993).

Experimentos previos efectuados en nuestro laboratorio, demostraron que los transplantes homotópicos fetales de corteza insular produjeron recuperación en la capacidad de adquirir el CAS, en ratas que previamente la habían perdido debido a la lesión de la CI (Bermúdez-Rattoni y col, 1987). Se demostró asimismo, que los

transplantes homotópicos de CI, pero no los heterotópicos de tejido tectal, podían no solo restablecer las funciones cognoscitivas sino también la conectividad con el tálamo y la amígdala del tejido huésped, con quienes la CI mantiene conexiones normalmente. Estas observaciones subrayan la importancia de la especificidad de los tejidos transplantados en los procesos de integración (Escobar y col, 1989). Estudios realizados por Bermúdez-Rattoni y McGaugh (1991), demostraron que la lesión de la CI produce deficiencias en la adquisición, no solo de la tarea de CAS sino también de prevención pasiva (PP) en ratas.

En la primera etapa del presente trabajo, efectuada con la finalidad de comprender los procesos temporales que subyacen a la recuperación funcional y anatómica observadas, llevamos a cabo un análisis conductual y citoarquitectónico siguiendo el curso temporal (15, 30, 45 y 60 días) de desarrollo de los transplantes de CI. Los resultados de estas investigaciones muestran que la recuperación conductual comienza a manifestarse a partir de los treinta días de desarrollo post-transplante al tiempo que aparecen los primeros indicios de reconectividad, vascularización y maduréz estructural, alcanzando su mejor expresión hacia los 60 días. Durante los primeros 15 días de desarrollo post-transplante, los sujetos no mostraron ningún indicio de recuperación en el paradigma del CAS, en tanto que las observaciones histológicas mostraron la presencia de neuronas poco desarrolladas, con escasas proyecciones y revascularización incipiente.

El análisis de los resultados obtenidos con las técnicas de Golgi e impregnación argéntica, reveló la presencia de un proceso gradual de

desarrollo, caracterizado por una reorganización neuronal, tanto en el tejido huésped como en el transplante, con una mayor densidad neuronal en el tejido transplantado.

Por su parte, la técnica de histoquímica para AChE reveló que las neuronas de los transplantes homotópicos expresan una reacción positiva para esta enzima de manera tiempo-dependiente. Aunados a estas observaciones, los trabajos efectuados por Woolf y Butcher (1982) y Bermúdez-Rattoni y col. (1983), señalan que la acetilcolina juega un papel importante en el CAS. Existen evidencias de que la corteza insular presenta considerable actividad colinérgica, así como de que el bloqueo farmacológico de la transmisión colinérgica provoca deficiencias en la adquisición del CAS (López-García y col, 1990a). Estudios previos de nuestro laboratorio mostraron que los transplantes homotópicos neocorticales, que promueven la recuperación del aprendizaje en el CAS, liberan ACh, en tanto que en los transplantes heterotópicos (corteza occipital), que no inducen recuperación funcional, no se registra liberación del citado neurotransmisor. Esto sugiere una participación colinérgica en la recuperación conductual mediada por los transplantes dentro del CAS (López-García y col, 1990b).

Numerosos estudios farmacológicos han sugerido reiteradamente el papel de la acetilcolina, en un gran número de funciones cerebrales, incluyendo el aprendizaje y la memoria (Dunnett y col, 1985; Candy y col, 1986; Perry, 1990; Decker y McGaugh, 1991). Recientes investigaciones han señalado que el FCN promueve la sobrevivencia, crecimiento y capacidades funcionales de las neuronas colinérgicas centrales del cerebro basal anterior (Gage y

col, 1986; Dekker y Thal, 1993; Cuello y col, 1992; Frim y col, 1993). Administraciones intraventriculares de este factor, incrementan la sobrevivencia de las neuronas colinérgicas axotomizadas, tras la lesión del fórnix (Hefti, 1990; Kromer, 1987; Varon y col, 1989). Se ha reportado también que la administración de FCN embebido en gelfoam reduce la tasa de mortalidad de las neuronas del septum medial, originada por lesiones de la fimbria-fórnix (Otto y col, 1989). Claramente en nuestro modelo la aplicación del FCN sólo, no produjo recuperación funcional significativa a ninguno de los tiempos post-transplante probados. En contraste, otros autores, usando diferentes modelos de aprendizaje y diferentes regiones cerebrales han encontrado recuperación tras la aplicación aguda del FCN u otros factores tróficos, después de pocos días de desarrollo post-transplante (Kesslak y col, 1986; Will y Hefti, 1985; Hefti y col, 1984; Lapchak y Hefti; 1992; Hagg y col, 1990; Lapchak y Hefti, 1991). Varon y colaboradores, por ejemplo, han encontrado recuperación funcional debida a administraciones crónicas de FCN en animales lesionados en el fórnix, los cuales presentaban severas deficiencias en la ejecución de la tarea espacial de Morris (water maze) (Varon y col, 1989).

En nuestros estudios, la mejor recuperación de la habilidad para aprender las tareas de CAS y PP fue observada cuando el FCN se asoció con transplantes corticales homotópicos, no así cuando se administró en combinación con transplantes heterotópicos provenientes ya sea de mesencéfalo o de corteza occipital. A este respecto, Heusching y col. (1988), efectuaron un estudio en el que fue probada la actividad trófica de diferentes regiones del SNC (cortical,

hipocampal, septal y estriatal) sobre neuronas embrionarias transplantadas, provenientes de diferentes áreas cerebrales, encontrando que mientras los transplantes de corteza e hipocampo se integraron y crecieron adecuadamente en las 4 regiones, los transplantes de septum, sufrieron atrofia al ser colocados en el estriado. Por su parte, los transplantes de estriado, se integraron y crecieron adecuadamente tan solo cuando fueron colocados en la región estriatal. Los autores destacan el hecho de que en todos los casos los transplantes homotópicos, mostraron una mejor integración, caracterizada por un alto y constante número de neuronas sobrevivientes. Así Heusching y colaboradores, sostienen que cualquier área del cerebro adulto lesionada, presenta actividad trófica específica, preponderantemente dirigida hacia las células homotópicas embrionarias correspondientes. La habilidad para responder a la mencionada actividad, parece cambiar con el grado de afinidad entre el transplante y la zona receptora del huésped.

Nuestros resultados conductuales parecen estar relacionados con la integración y maduréz del tejido transplantado. Resultados preliminares empleando la tinción de Golgi indican que los transplantes corticales con FCN, mostraron un mayor desarrollo neuronal, una hipertrofia de las poblaciones celulares en torno al transplante, una menor cantidad de glía reactiva en su interior así como una extendida vascularización, cuando fueron comparados con el resto de los grupos. Por lo tanto, tal como mencionamos anteriormente, si bien el FCN está involucrado en los procesos de recuperación requiere, en nuestro modelo, de la presencia de los transplantes homotópicos neocorticales y/o de algún factor cortical

esencial para la producción de la recuperación funcional. Asimismo, nuestros resultados señalan reiteradamente que la acelerada recuperación mostrada en presencia del factor de crecimiento neuronal, se halla estrechamente asociada a la participación del sistema colinérgico, medido a través de los niveles endógenos del neurotransmisor y de la actividad de la ChAT (*in vivo* e *in vitro*), en los procesos de integración neuroquímica de los transplantes.

Diferencias en la actividad colinérgica similares a las detectadas en nuestros experimentos empleando marcadores *in vivo* e *in vitro*, han sido ampliamente documentadas en la literatura (Jenden, 1985; Lapchak y col, 1991; Dekker y col, 1991). Sin embargo a pesar de las discrepancias inherentes a las metodologías utilizadas, nuestros resultados demuestran que la actividad colinérgica en los implantes de los grupos controles intactos y los que recibieron transplantes de CI combinados con FCN, es similar en todos los casos, exhibiendo diferencias significativas con respecto a los grupos que recibieron implantes heterotópicos con FCN u homotópicos con vehículo sin factor.

Existen neuronas receptoras a la ACh en prácticamente toda la neocorteza, investigaciones a este respecto han mostrado que entre el 60 y el 80% de la ACh cortical, proviene de fuentes extrínsecas a la corteza (Hebb, 1963; Emson y col, 1985). Entre tales estructuras extracorticales destaca el núcleo basalis magnocellularis (NBM) del cerebro ventral anterior (Shute y Lewis, 1967; Hartgraves y col, 1982; Rye y col, 1984). Se ha probado asimismo, que la presencia de transplantes de tejido embrionario procedente de cerebro anterior ventral, en la neocorteza de ratas con lesiones de NBM, restablecen

algunas de las deficiencias sensoriomotoras y de aprendizaje, al tiempo que restablecen parte de la inervación colinérgica original (Fine y col, 1985; Dunnett y col, 1985).

Los estudios de nuestro laboratorio en los que se muestra la existencia de una proyección colinérgica específica entre el NBM y la CI, y que a través de métodos histológicos, neuroquímicos y conductuales apoyan la posibilidad de que tales proyecciones se hallen íntimamente relacionadas con el CAS, aunados a nuestros hallazgos con las técnicas de detección para acetilcolina *in vivo*, sugieren la participación del NBM en el restablecimiento de la comunicación neuroquímica que a su vez interviene en la recuperación promovida por los transplantes de CI en presencia de FCN. En este sentido, los estudios de Dekker y Thal (1993) demuestran que la administración intracerebroventricular de FCN en animales con lesiones del NBM, incrementa la inervación colinérgica de la neocorteza frontal procedente del NBM, estimulando además la sobrevivencia y producción de acetilcolina de las neuronas de este núcleo. Resultados similares empleando marcadores colinérgicos *in vivo*, fueron encontrados por Cuello y col. (1992). Nuestros análisis bioquímicos mostraron también, que la actividad de la GAD fue similar en todos los grupos probados, de manera que el sistema GABAérgico a diferencia del colinérgico parece no tener una participación determinante en la recuperación funcional de los transplantes de CI con FCN, obviamente no se descarta la participación de otros neurotransmisores, neuromoduladores y moléculas tróficas en la integración de los tejidos implantados.

Hace más de un cuarto de siglo que se sabe que las acciones derivadas de la actividad biológica del FCN se inhiben por la administración de los anticuerpos contra este factor. Cohen, Levi-Montalcini y colaboradores reportaron por primera vez en 1960, la destrucción del SNP simpático, debida a la administración exógena de anticuerpos dirigidos contra el FCN (anti-FCN) (Cohen, 1960; Levi-Montalcini y Booker, 1960). Dos décadas después (Gorin y Johnson, 1979) mostraron que la exposición prenatal al anti-FCN promueve la muerte de las células sensoriales. Efectos similares se han registrado en las células colinérgicas del SNC (Vroegog y col, 1992). A este respecto, llevamos a cabo estudios preliminares con la finalidad de probar la especificidad de las acciones derivadas de la presencia del FCN en nuestro modelo, los resultados de estas investigaciones muestran que la combinación del factor con un anticuerpo anti-FCN, bloquea el efecto de aceleración de la recuperación de la habilidad para aprender, mediado por los transplantes de CI con FCN.

Finalmente, en este contexto en el que los transplantes de tejido cerebral fetal son capaces de integrarse al tejido huésped, de restablecer funciones e incluso incrementar su sobrevivencia y aún acelerar la recuperación si son combinados con algunos factores tróficos, es necesario señalar que algunas investigaciones en las que se han analizado los efectos a largo plazo tanto de la administración de factores tróficos como de los transplantes combinados con éstos, han mostrado efectos negativos sobre las manifestaciones conductuales (Will y Hefti, 1985; Pallage y col, 1986; Hefti, 1990).

En nuestro laboratorio, recientemente llevamos a acabo estudios en los que analizamos la respuesta conductual de ratas con

transplantes de CI con o sin FCN después de 130 días de desarrollo post-transplante. Los resultados de estas investigaciones revelaron que todos los grupos de animales transplantados muestran severas deficiencias en el aprendizaje de las tareas de PP y laberinto de agua de Morris, comparados con los grupos controles intactos y aún con los grupos que permanecen lesionados en la CI. La hipótesis más ampliamente documentada que intenta explicar este fenómeno, afirma que después de largos períodos de desarrollo post-transplante se establecen conexiones inadecuadas entre el transplante y el huésped al tiempo que se origina una invasión de los tejidos adyacentes al implante, con el consecuente trastorno de la comunicación neuronal (Milner y Loy, 1980; Bjorklund y Stenevi, 1981; Madison y Davis, 1983; Gage y col, 1984; Will y Hefti, 1985). De manera que la investigación en torno a los efectos a largo plazo de la administración de factores tróficos y su combinación con transplantes de tejido cerebral fetal, requiere de un análisis más profundo acerca de sus efectos sobre la evolución neuroquímica y los procesos de aprendizaje de los sujetos implantados.

En conclusión, nuestros estudios han demostrado que:

1) La recuperación funcional de los transplantes homotópicos de CI en ausencia de FCN comienza a manifestarse a partir de los 30 días de desarrollo post-transplante, alcanzando su mejor expresión hacia los 60 días post-transplante.

2) Los transplantes homotópicos de CI combinados con FCN aceleran la recuperación de la habilidad para aprender, hacia los 15 días de desarrollo post-transplante en ratas previamente lesionadas en la CI.

3) La proyección colinérgica existente entre el NBM y la CI participa en la integración neural de la información gustativa y en el restablecimiento de la comunicación neuroquímica inducida por los transplantes de CI combinados con FCN.

4) La actividad colinérgica medida *in vivo* e *in vitro* a diferencia de la GABAérgica, es similar en los implantes de las ratas controles intactas y las que reciben transplantes de CI en combinación con FCN, y significativamente diferente a la de los grupos restantes.

5) Ninguno de los grupos con implante heterotópico, vehículo o FCN sólo, son capaces de promover la citada recuperación, ni de restaurar los niveles colinérgicos hacia los 15 días de desarrollo post-transplante.

Estos resultados sugieren que el FCN cuando se asocia simultáneamente con transplantes homotópicos de CI, produce recuperación de la habilidad para aprender en ratas previamente lesionadas en la CI, y promueve el restablecimiento de la actividad colinérgica de los implantes a partir de los 15 días de desarrollo post-transplante.

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