"RETRASO DE ALGUNAS MANIFESTACIONES DEL ENVEJECIMIENTO POR TRANSPLANTES INTRACEREBRALES DE CELULAS CROMAFINES CULTIVADAS".

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GLOSARIO:

6-OHDA	6-hidroxidopamina
MPTP	1metil-4-fenil-1,2,3,6-tetrahidropiridina
FGF2	Factor básico de crecimiento de fibroblastos
NGF	Factor de crecimiento nervioso
CNTF	Factor neurotrófico ciliar
HVA	Acido homovanílico
DOPAC	Acido 3,4-dihidroxi-fenil-acético
IGF	Factor de crecimiento semejante a la insulina
EGF	Factor de crecimiento epidermal
BDNF	Factor neurotrófico derivado del cerebro
NT-3	Neurotrofina 3
NT-4/5	Neurotrofina 4 y 5
GDNF	Factor neurotrófico derivado de una línea celular de glia
trkA	Receptor del NGF
trkB	Receptor del BDNF, NT-4/5 y de NT-3
trkC	Receptor del NT-3
TGF	Factor de crecimiento transformante
PDGF	Factor de crecimiento derivado de plaquetas
$P75^{NGFR}$	Receptor de baja afinidad del NGF
$gp145^{trkB}$	Receptor trkB
$p145^{trkC}$	Receptor trkC
TrkC14	Isoforma del receptor trkC
TrkC25	Isoforma del receptor trkC

Summary:

Over the last 20 years it has become evident that motor detriments resulting by dopamine nigrostriatal pathway lesions, can be compensates with intracerebral transplants of catecholaminergic tissue. The motor compensation observed with fetal substantia nigra grafts will be produces by dopamine striatal reinnervation and by a constant supply of dopamine released by the graft. On the other hand, after being grafted chromaffin cells, motor compensation will results by dopamine released from grafted cells. However, chromaffin cells are able to release neurotrophic factors producing fiber sprouting in the remaining dopaminergic cells.

There was no evidence that chromaffin cells survive after being grafted into the brain throughout long periods and it is not know whether they are able to prevent neurodegeneration. Therefore, the first part of this thesis shows that chromaffin cells cultured with nerve growth factor (NGF), are able to survive grafted into the rat brain at least by 14 months. These cells grafted in adulthood reduce motor detriments when rats became older. The effect correlates with the number of dopamine neurons at the ventral mesencephalic region, that was between normal aged or grafted rats with no success transplant with younger rats.

Since these results suggest a neurotrophic effect, the second part of this thesis shows that brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) receptors are expressed along development of neural and no-neural tissue. Therefore these trophic factors will have a functional role on the

development. BDNF, NT-3 and NT-4/5 receptor are present along development of dopamine nigrostriatal system, suggesting a functional role on this system. However, constant infusion of BDNF into either the striatum or substantia nigra produces akinetic state of infused animals. This behavior correlates with a decrease on tyrosine-hydroxylase immunoreactivity. In contrast, intracerebral infusion of α-Glial derived neurotrophic factor produces a transient sprouting of dopaminergic fibers into infused side.

The results of the first part suggest that chromaffin cells will produce best results in Parkinson's disease if grafted on initial phases of illness. Results of the second part suggest that neurotrophins will drive peripheral innervation, but they will have other unknown functions. Finally, is suggested that Parkinson's disease etiology has relation with nigral GDNF supply reduction.

RESUMEN.

Durante los últimos veinte años se ha mostrado que las deficiencias motoras producidas por la denervación dopaminérgica se pueden compensar a través de transplantes intracerebrales de tejido catecolaminérgico. Con los transplantes de substancia nigra fetal, la compensación se ha explicado básicamente por la reinervación del cuerpo estriado y la restauración del aporte de dopamina. Sin embargo, con los trasplantes de células cromafines, se ha argumentado que la compensación se debe a la combinación del aporte de dopamina secretada por el transplante y a los efectos tróficos generados sobre las neuronas dopaminérgicas remanentes en el sistema dopaminérgico nigro-estriatal.

A la fecha no existe evidencia de que los transplantes intracerebrales de células cromafines sobrevivan por largos periódos y tampoco se sabe sí este tipo de transplante puede prevenir procesos neurodegenerativos, por lo que en la primera parte de la presente tesis, se muestra que cuando las células cromafines se mantienen en cultivo y en presencia del factor de crecimiento nervioso (NGF), sobreviven transplantadas un plazo mayor a los doce meses. Estos transplantes ejercen además un efecto preventivo en el deterioro motor debido al envejecimiento, el cual se correlaciona con la sobrevivencia de un número mayor de neuronas dopaminérgicas que el observado en animales viejos intactos y una frecuencia de disparo neuronal más cercana a la detectada en animales jóvenes.

Debido a que los resultados sugieren un efecto neurotrófico, en la segunda parte de este estudio se analizó la expresión de los receptores de los factores neurotróficos, derivado del cerebro (BDNF) y neurotrofina 3 (NT-3) a lo largo del desarrollo de distintos órganos, encontrandose que estos receptores se expresan en el sistema nervioso y en órganos periféricos, lo que sugiere que dichos factores podrían desempeñar un papel funcional durante el desarrollo del organismo. Subsecuentemente se muestra la expresión de los receptores del BDNF a lo largo del desarrollo del sistema nigro-estriatal, lo que planteó la posibilidad de que el BDNF participa en la funcionalidad del sistema nigro-estriatal. Se encontró que la perfusión crónica intraestriatal o intranigral de BDNF en animales adultos, parece activar un proceso de desdiferenciación neuronal e inhibe la expresión de la enzima tirosina-hidroxilasa (TH), pero no parece producir la muerte neuronal. De acuaerdo con lo anterior, la perfusión del anticuerpo contra el factor neurotrofico derivado de una línea celular de glia (α-GDNF), produjo un incremento en la expresión de TH durante los primeros días de la perfusión, para revertirse después de 10 días. La discusión de estos resultados plantea la posibilidad de que los transplantes intracerebrales podrían retardar la manifestación de la enfermedad de Parkinson si se utilizan en la fase inicial de la enfermedad. Asimismo, se suguiere que las neurotrofinas podrían participar en los procesos de inervación periférica, aunque no se descarta la posibilidad de otras funciones en tales órganos. Finalmente se plantea que la enfermedad de Parkinson podría deberse en parte a una disminución en el aporte de GDNF, ya que cabe la posibilidad de que a través del uso de GDNF ésta puede revertirse.

PRIMERA PARTE.

Capítulo 1.

ENVEJECIMIENTO.

Definición e hipótesis del envejecimiento.

El envejecimento es un proceso que se inicia poco después de la maduración sexual y se caracteriza por la acumulación de deficiencias en los mecanismos del equilibrio homeostático (Cutler, 1992). El estudio del envejecimiento se remonta a principios de este siglo cuando Rubner (1908) asumiendo que cada especie tiene una capacidad limitada para el procesamiento energético (PME), postuló que la esperanza máxima de vida de los organismos (EMV) es inversamente proporcional a su tasa metabólica (TME). Esta hipótesís supone que en los arganismos de vida corta la TME es alta, en tanto que los de vida larga presentan una TME baja (Símic, 1992; Ver tabla 1).

En 1935 McCay y colaboradores reportaron que en los roedores un régimen dietético bajo en calorias puede alargar hasta en un tercio la vida de éstos, por lo que propusíeron que la reducción en la TME produce la disminución de elementos dañinos generados durante el metabolismo (Símic, 1992).

Harman en 1954, propuso que durante el metabolismo se producen radicales libres, explicando que el envejecimento es el resultado del daño celular producido por estas substancias reactivas (Harman, 1991). Esto motivó la busqueda de una correlación entre la EMV con el aporte de enzimas anti-oxidantes como la catalasa,

superóxido dismutasa y glutatión peroxidasa, encontrándose una correlación entre la concentración de estas enzimas y substancias anti-óxidantes con la TME (Cutler, 1985).

TABLA 1. Valores promedio de la tasa metabólica especifica (TME), esperanza máxima de vida (EMV) y procesamiento metabólico de la energía (PME).

ESPECIE	TME cal/gr/día	EMV en años	PME kcal/gr
Ratón	180	3.5	232
Vaca	15	30	253
Caballo	14	49	152
Chimpancé	27	50	450
Humano	25	100	815
Elefante	13	70	220

En 1972 Harman replanteó la propuesta de que los radicales libres producen el envejecimento a través del daño mitocondrial. No obstante, se argumentó que ésto parecia poco probable ya que estos organelos podrían evitar tales efectos al replicarse. Sín embargo, se estableció que durante el envejecimiento las mitocondrias muestran deterioros bioquímicos además de que su número por célula se reduce significativamente (Miquel y cols, 1992).

Alternativamente Cutler (1982) propuso que el proceso de envejecimiento podría estar determinado por un programa genético establecido a lo largo de la evolución,

postulando que el envejecimiento "podría evitarse" sí se anulan los "genes de este programa". No obstante, ya se mostró que el deterioro del DNA es causado principalmente por el daño oxidativo y no por programas genéticos (Símic, 1992).

Radicales libres.

El oxígeno puede reaccionar con semiquinonas o ubiquinonas generando superóxido (reacción 1; Chance y cols, 1979; VER LA SECCION DE LA ENFERMEDAD DE PARKINSON), éste es transformado a peróxido de hidrógeno por acción de las enzimas superóxido dismutasa (SOD), en el citoplasma por la Cu/Zn-SOD y en las mitocondrias por la Mn^{++}-SOD (reacción 2; McCord Fridovich, 1969), el peróxido de hidrógeno es eliminado por acción de las enzimas catalasa y peróxidasa (reacción 3).

$$(O_2) + (\cdot UQ^-) \longrightarrow \cdot O_2^- + UQ \quad (1)$$

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (2)$$

$$H_2O_2 + H_2O_2 \xrightarrow{CATALASA} H_2O + O_2 \quad (3)$$

Sín embargo, sí el peróxido de hidrógeno no se elimina reaccionará con el radical superóxido generando hidroxilo (reacción 4) , el cual provoca una reacción en cadena de peroxidación de proteínas y lípidos de membrana, además de dañar al DNA (Fridovich, 1988; Miquel y cols, 1989, 1992).

La transformación del peróxido de hidrógeno a hidroxilo se puede acelerar sí el peróxido interactúa con el ión ferroso (Fe^{++}, reacción de Fenton; 5), el cual proviene generalmente de la reacción del ión ferrico (Fe^{+++}) con el superóxido (reacción 6; Chevion, 1988; Imlay y cols, 1988). Se ha estimado que en la cadena de transporte de

electrónes se generan alrededor de 10^7 moléculas de superóxido/mitocondria/día (Símic y cols, 1988, 1991; Emerit y cols, 1990).

$$H_2O_2 + \cdot O2^- \longrightarrow O_2 + OH^- + OH^{\cdot} \quad (4)$$

$$H_2O_2 + Fe^{++} \longrightarrow Fe^{+++} + OH^- + OH^{\cdot} \quad (5)$$

$$(\cdot O_2^-) + Fe^{+++} \longrightarrow O_2 + Fe^{++} \quad (6)$$

Estas evidencias sígieren que las deficiencias observadas en durante el envejecimiento pueden ser inducidas por acción de radicales libres generados durante el metabolismo aeróbico. Acorde con ésto esta el hecho de que conforme se envejece, la expresión de las enzimas anti-oxidantes disminuye progresivamente (Ver tabla 2; Cutler, 1992; Harman, 1992).

La secuencia de reacciones antes descrita podría ser muy importante en los procesos de neurodegeneración, ya que como se podrá observar en la sección dedicada a la enfermedad de Parkinson, durante el metabolismo de la dopamina y en los mecanismos neurotóxicos de las neurotoxinas 6-hidroxidopamina (6-OHDA) y MPTP también se generan aportes consíderables de radicales libres.

<u>Daño al DNA por radicales libres.</u>

Cuando el hidroxilo reacciona con la timina y guanina del DNA forma timina-glicol (Tg) y 8-hidroxiguanina (8-OHG), estos compuestos se eliminan del DNA y se excretan por la orina. Esto ha dado lugar a considerar que el aporte urinario de estos

compuestos por día representa la tasa de daño oxidativo al DNA total por día. La estimación del aporte urinario de TG y 8-OHG en 20 especies de animales mostró que el ser humano es el que tiene la menor tasa de daño al DNA. Asímismo, se detectó que la tasa de daño oxidativo al DNA es proporcional a la TME (Ames, 1989; Bergtold y cols, 1988). No obstante, en el ser humano y en la rata una dosís símilar de radíación ionizante (rayos γ ó electrones de alta energía), induce un incremento símilar en la tasa de daño genético (Bergtold y Símic, 1991).

TABLA 2. Mecanismos de anti-oxidación participantes en el metabolismo celular que pueden ayudar a reducir los efectos de los radicales libres por incrementar o disminuir su concentración.

LOS QUE DEBEN INCREMENTARSE	NO ES CLARO SI DEBEN O NO INCREMENTARSE	LOS QUE DEBEN REDUCIRSE
1.- SOD Cu/Zn	1.- Acido ascórbico	1.- Catalasa
2.- SOD Mn	2.- Retinol	2.- Glutatión
3.- Carotenos		3.- Glutatión peroxidasa
4.- α-Tocoferol		
5.- Urato		
6.- Ceruloplasmina		

Capítuo 2.

ENFERMEDAD DE PARKINSON.

Características clinicas.

La enfermedad de Parkinson se caracteriza por la aparición lenta y progresiva de temblor, rigidez muscular, hipocinesia y pérdida de los reflejos posturales. Estos síntomas ocurren después de que se han perdido más del 80% de las neuronas dopaminérgicas del mesencéfalo ventral, lo que ocurre de manera más frecuente entre los 50 y 60 años de edad (Riederer y Wuketich, 1976;Koller y cols, 1987; Kurland y cols, 1988).

El temblor es de reposo con un ritmo de 3 a 8 ciclos por segundo predominando en los segmentos distales de las extremidades y, sin importar si el sujeto es diestro o siniestro, comienza en una de las extremidades superiores. No obstante, en raras ocasiones se inicia en alguna de las extremidades inferiores, en las de un lado del cuerpo ó de manera simultánea en las cuatro (Oliver, 1953). Este síntoma disminuye durante el movimiento voluntario (característica que permite diferenciarlo del temblor senil), desaparece durante el sueño y en los primeros momentos después de despertar y se torna más violento cuando el paciente está bajo tensión emocional (Oliver, 1953).

La rigidez comienza en los dedos de una mano y se propaga hasta abarcar a todos los músculos del cuerpo, este síntoma se caracteriza por el fenómeno de la"navaja de muelle" (Oliver, 1953). La marcha es lenta y estereotipada con una reducción del balanceo de los brazos, tendiendo a la inclinación hacia el frente con la pérdida de los

reflejos posturales (Oliver, 1953). Consecuentemente se desarrolla una incapacidad motriz al punto que el paciente se vuelve dependiente de la gente que le rodea. Otros síntomas de esta enfermedad son la salivación exagerada, el rostro inexpresivo, la palabra monótona con voz apagada y la escritura micrográfica.

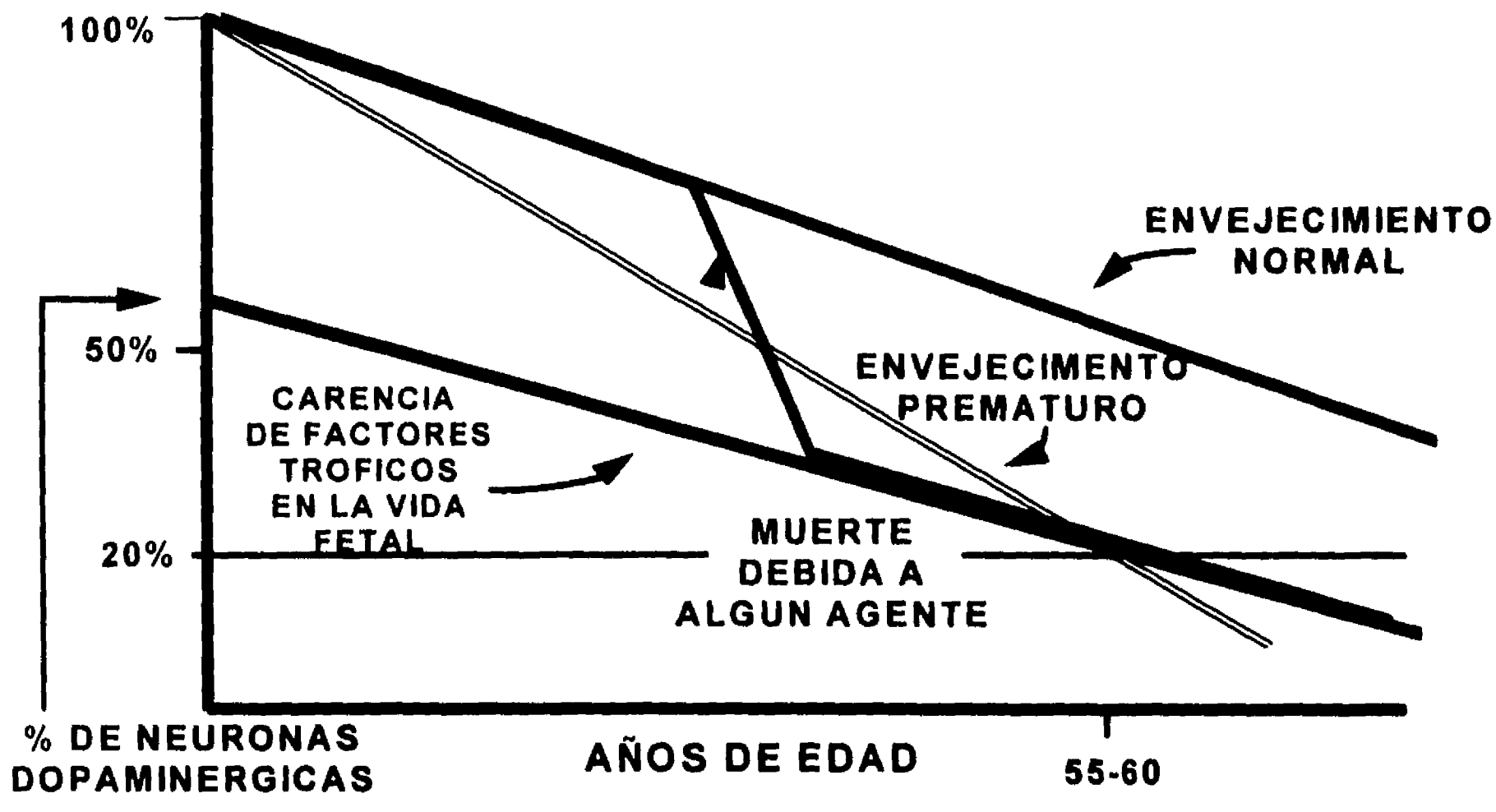
Caracteristicas bioquímicas como posibles causas de la enfermedad de Parkinson.

Se sabe que por cada década de vida mueren de manera natural del 5 al 8% de las neuronas dopaminérgicas del mesencéfalo, ésto significa que al final de la sexta década, la reducción del número de estas neuronas será del 40-50% (Carlsson y Winbland, 1976; McGeer y cols, 1977) y aunque las causas directas de la enfermedad se desconocen, se han propuesto varios factores. 1.- Como resultado de infecciones virales (Poskanzer y Schwab, 1963; Mattock y cols, 1988). 2.- Por una predisposición hereditaria (Ward y cols, 1983; Johnson y cols, 1990). 3.- Por la falta de algún factor de crecimiento esencial durante el desarrollo embrionario (Eldridge e Ince, 1984). 4.- Por acción de pro-tóxinas ambientales (Calne y Langston, 1983; Rajput y cols, 1984; Tanner y cols, 1987; Langston, 1989; Stern y cols, 1991). 5.- Como resultado del estrés oxidativo (Figura 1; Cohen, 1983; Mann e Yates, 1983; Graham, 1978; Fahn 1989; Riederer y cols, 1989; Kurth y cols, 1993).

De las posibilidades citadas, el estrés oxidativo podría ser uno de los principales inductores de la enfermedad de Parkinson, ya que en las neuronas dopaminérgicas existen diversos elementos que combinados favorecen la formación de radicales libres.

Estos elementos son: a) La actividad de la enzima monoamino oxidasa (MAO). b) Los productos de la auto-oxidación dopaminérgica. c) La acumulación de la neuromelanina y d) La acumulación de hierro oxidante en el soma.

FIGURA 1. POSIBLES CAUSAS DE MUERTE NEURONAL EN LA ENFERMEDAD DE PARKINSON.



a) Actividad de la MAO.

Knoll (1981) propuso que la actividad de la enzima MAO-B podría ser un promotor de la enfermedad de Parkinson ya que produce peróxido de hidrógeno al catalizar la deaminación de la dopamina, la propuesta se fundamentó en el hecho de

que en las neuronas dopaminérgicas la expresión de MAO-B se incrementa con la edad, lo cual favorece la producción de hidroxilo (reacción 1; Oreland, 1991; Cohen, 1983; Langston, 1986).

$$\text{Dopamina} + H_2O + O_2 \xrightarrow{\text{MAO}} \text{Dihidroxi-fenil-acetaldehido} + NH_3 + H_2O_2 \quad (1)$$

b) Auto-oxidación de la dopamina.

Tal vez, la característica que hace a las neuronas dopaminérgicas más suceptibles a la neurodegeneración es el hecho de que la dopamina puede reaccionar con el oxígeno de manera no enzimática produciendo quinonas y semiquinonas que pueden reaccionar con el peróxido de hidrógeno favoreciendo la síntesis de radicales libres (Graham, 1978; VER SECCION DE ENVEJECIMIENTO).

c) Papel de la neuromelanina.

Se ha sugerido que la neuromelanina generada durante el proceso de auto-oxidación dopaminérgica favorece también la producción de radicales hidroxilo al reaccionan con el hierro presente en las neuronas dopaminérgicas (Graham , 1978, 1979; Youdim, 1988).

d) Papel del hierro.

Estudios *post-mortem* muestran que en la substancia nigra de sujetos normales, el hierro ferrico (Fe^{+++}) se encuentra en una proporción 2.45 veces mayor a la forma ferrosa (Fe^{++}; Dexter y cols, 1989a, 1999b). Esta característica podría no ser considerada dañina por sí sola, ya que tanto en el globo pálido como en el cuerpo estriado existen concentraciones elevadas del metal. Sin embargo, lo que hace peligroso al hierro para las neuronas dopaminérgicas es que este metal en estado oxidado, acelera la conversión del peróxido de hidrógeno a hidroxilo (VER SECCION DE ENVEJECIMENTO). Un dato relevante es que en la pars compacta de la substancia nigra de sujetos que padecieron la enfermedad de Parkinson, la concentración total del metal se incrementa casi en un 100%, a expensas del hierro oxidado dando como resultado una proporción de 1.06 respecto del reducido (Riederer y cols, 1989; Dexter y cols, 1991; Sofic y cols, 1991).

Enzimas anti-oxidantes en la enfermedad de Parkinson:

En la substancia nigra de pacientes con mal de Parkinson la concentración de las enzimas glutatión peróxidasa y catalasa disminuyen proporcionalmente al grado de avance de la enfermedad (Ambani y cols, 1975; Perry y cols, 1982; Riederer y cols, 1989). Esto se correlaciona con la mayor proporción de lípidos peróxidados en la membrana celular (Dexter y cols, 1989a,1989b). Estas caracteristicas son muy desventajosas ya que en el cerebro la eliminación del peróxido de hidrógeno la realizan

presisamente la glutatión peróxidasa y la catalasa (Dexter y cols, 1989a,1989b). Sin embargo, en estos pacientes también se detectó un incremento de la enzima Mn^{++}-superóxido dismutasa, que se localiza en las mitocondrias, aunque no de la Cu/Zn-SOD, la cual se encuentra en el citoplasma (Saggu y cols, 1989).

Alteraciones de las mitocondrias en la enfermedad de Parkinson.

Las neuronas dopaminérgicas en pacientes con esta enfermedad, el complejo I de la cadena de transporte de electrones mitocondrial está alterado, ya que se detectó una reducción en la actividad de la enzima nicotinamido adenín dinucleotido-CoQ reductasa (NADH-CoQ). Esta deficiencia se produce también en primates cuando son tratados con 1-metil-4-fenil-1,2,3,6,-tetrahidropiridina (MPTP), por lo que es factible considerar la hipótesis de las protoxinas ambientales como una posible causa. Empero, en ambas condiciones el DNA mitocondrial no muestra daño aparente (Mizuno y cols, 1989; Schapira y cols, 1990a, 1990b, 1990c; Hittori y cols 1991; Lestienne y cols, 1990).

Efectos de la 6-OHDA, MPTP y la dopamina.

Los efectos neurotóxicos de la 6-hidroxidopamina (6-OHDA) y el MPTP son mediados por radicales libres a través de reacciones semejantes a las antes descritas en la sección de envejecimeinto. Es decir, después de que la 6-OHDA se incorpora a las neuronas dopaminérgicas, el oxígeno se consume en forma acelerada provocando

que se genere la hipoxia de la neurona y la síntesis de radicales libres, lo que favorce la reactividad de p-quinonas y aminocromos que se producen durante la autoxidación de la neurotoxina (Heikkila y Cohen, 1973; Cohen y Heikkila, 1974; Ballard y cols, 1985; Langston, 1987).

De manera similar se ha mostrado que en condiciones de cultivo, el estrés oxidativo se puede provocar en las células catecolaminérgicas cuando se incorporan al medio dopamina o l-dopa (Michel y Hefti, 1990; Carvey y cols, 1991; Tanaka y cols, 1991; Mena y cols, 1992). En concordancia con ésto, se observó que la l-dopa produce efectos deletereos en la sobrevivencia de transplantes intracerebrales de tejido catecolaminérgico (Steece-Collier y cols, 1990; Yurek y cols, 1991).

En animales intactos el uso de l-dopa no produce degeneración neuronal a menos de que ocurra una lesión parcial del sistema dopaminérgico nigro-estriatal. Este fenómeno puede ser significativo en relación con la terapia utilizada para la enfermedad de Parkinson (Blunt y cols, 1991).

Por otro lado, el uso de MPTP en primates seniles indujo la formación de inclusiones eosinófilas similares a los cuerpos de Lewy en la substancia nigra, locus coeruleus y los núcleos dorsal del raphe, dorsal del vago y basal de Meynert (Forno y cols, 1986). Si bien este resultado muestra homologías significativas respecto a la enfermedad de Parkinson, difiere de ésta en que en el putamen de los pacientes la dopamina disminuye hasta 98%, mientras que en el núcleo caudado la disminución es del 81% (Bernheimer y cols,1973; Kish y cols, 1988). Este patrón de disminución

dopaminérgica diferencial es la característica por la que se distingue a la enfermedad ideopática de otros estados de parkinsonismo, como el post-encefálico, el temblor supranuclear progresivo, la artereoesclerosis senil, o en donde ocurre la combinación de demencia, parkinsonismo y esclerosis lateral amiotrófica (Hornykiewicz y cols, 1989).

Comentario final.

El estrés oxidativo parece ser uno de los principales eventos involucrados durante el desarrollo de la enfermedad de Parkinson. Sin embargo, la evidencia no permite explicar las causas de la disminución diferencial del aporte de dopamina característico de la enfermedad. No obstante, es interesante buscar evidencias en torno a la participación del estrés oxidativo en las etapas iniciales de la enfermedad.

Capítulo 3.

TRANSPLANTES DE TEJIDO CATECOLAMINERGICO Y MODELOS DE LA ENFERMEDAD DE PARKINSON.

Introducción

Durante los últimos viente años se han mostrado evidencias experimentales que sugieren que los transplantes intracerebrales podrían promover la rehabilitación parcial de deficiencias debidas a la pérdida de poblaciones neuronales discretas. Esta compensación se ha asociado con el restablecimiento del aporte de algún neurotransmisor, asi como con fenómenos de plasticidad cerebral. Estas evidencias se han desarrollado principalmente en los modelos de la enfermedad de Parkinson.

En esta sección se describen brevemente los modelos de la enfermedad de Parkinson y se enumeran las evidencias acumuladas durante 20 años sobre los efectos de los transplantes de tejido catecolaminérgico y la aplicación de factores tróficos. Para finalizar se describen las perspectivas en el futuro cercano. Sin embargo, si el lector tiene interés por los aspectos históricos de los transplantes, se sugiere consultar las siguientes referencias (Björklund y Stenevi, 1984; Stenevi y cols, 1976).

Modelos utilizados para estudiar la enfermedad de Parkinson.

1.- Modelo de la conducta de giro.

Raab y Gigee (1951) establecieron que la dopamina se encuentra en el cerebro al inducir un incremento en su concentración con la administración de l-dopa. A fines de la década de los 50, Carlsson y cols (1957; 1958) mostraron que la reserpina (droga que disminuye el aporte de dopamina cerebral) produce un sindrome de acinesia similar al observado en pacientes con mal de Parkinson, revirtiéndolo con la administración de l-dopa. Posteriormente, se estableció que en sujetos con la enfermedad de Parkinson existe una considerable disminución del aporte de dopamina en los núcleos caudado y putamen (Ehringer y Hornykiewicz, 1960). El mismo año, Birkmayer y Hornykiewicz utilizando l-dopa (poco más de 150 mg i.v.) indujeron la disminución de la rigidez en pacientes con esta enfermedad. Sin embargo, fué hasta después del reporte de Cotzias y cols (1967) que se aceptó como terapia al uso de l-dopa (Fahn, 1989).

Con los antecedentes de que la enfermedad de Parkinson resulta de la carencia de dopamina, asi como del sindrome producido con reserpina, se propuso que la destrucción del sistema dopaminérgico nigro-estriatal en animales de laboratorio sería un modelo de parkinsonismo permanente. Durante la década de 1950 se demostró que la droga 2,4,5-trihidroxifeniletilamina (6-hidroxidopamina; 6-OHDA), administrada por vía sistémica, produce denervación simpática (noradrenérgica; Carlsson y cols, 1957; Thoenen y Tranzer, 1973; Jonsson, 1980), por lo que se comenzó a administrar en el sistema ventricular, en el mesencéfalo ventral, o en el haz del cerebro medio anterior, por donde

cursan los axones dopaminérgicos hacia el telencéfalo (Andén y cols, 1966; Ungerstedt, 1968; 1971a,b,c; Langston y cols, 1987; Zigmond y Stricker, 1989).

En principio el modelo consistió en producir una lesión bilateral del sistema nigro-estriatal y a pesar de que se produce un sindrome de acinesia semejante al que se observa en los estadios más avanzados de la enfermedad de Parkinson, no se utiliza frecuentemente porque los animales presentan estados de adipsia y afagia que al cabo de pocos días causan su muerte (Langston y cols, 1987; Zigmond y Stricker, 1989).

El modelo de hemiparkinsonismo en el que se lesiona sólo uno de los sistemas nigro-estriatales, se estableció como una alternativa más viable ya que además de que los animales desarrollan un desequilibrio motor fácil de evaluar, cursan con adipsia y afagia sólo por cinco o diez días (Ungerstedt, 1971c; Creese y cols, 1977; Creese y Snyder 1979; Joyce y Marshall, 1985, 1987; Nave y col, 1984, Marshall y cols, 1989).

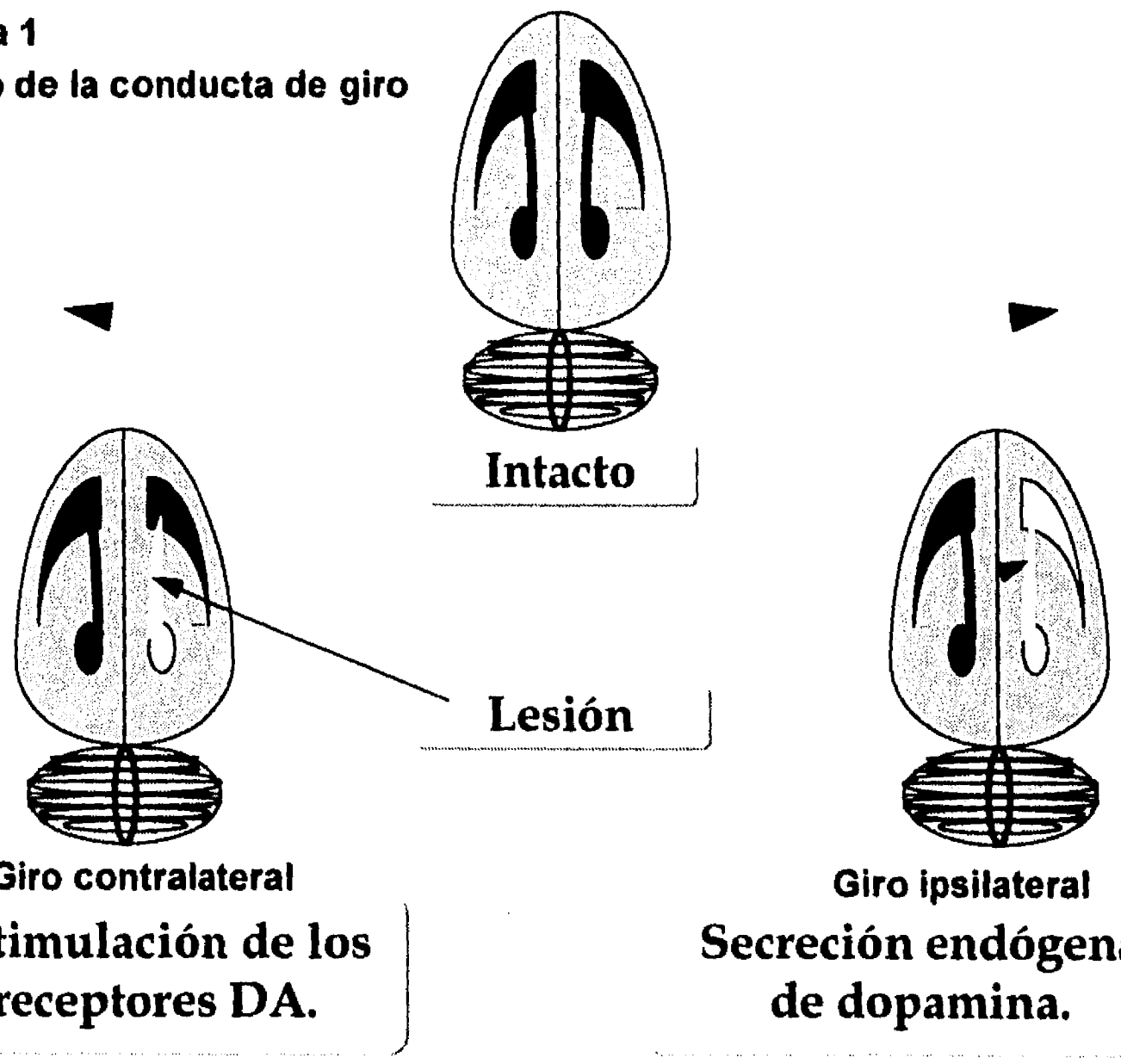
El sindrome se caracteriza por asimetría postural orientada haciael lado lesionado, con disminución sensorial en el lado contralateral (Ungerstedt, 1971b;Ungerstedt, 1971c; Creese y cols, 1977; Creese y Snyder, 1979; Meador-Woodruff y cols, 1989; Nave y cols, 1984, 1991). Asimismo, la carencia de dopamina provoca que, en las neuronas estriatales sensibles a dopamina, se produzca un incremento significativo en el número de receptores dopaminérgicos del tipo D2 y un ligero decremento de los D1 (Ungerstedt, 1971c; Creese y cols, 1977; 1979; Meador-Woodruff y cols, 1989; Nave y cols, 1991).

Este desequilibrio dopaminérgico se puede evaluar con agonistas del receptor dopaminérgico. Por ejemplo, si se estimulan los receptores post-sinápticos, la rata se

desplazará de manera estereotipada en círculos, hacia el lado contrario de la lesión (giro contralateral); en tanto que si se estimula la secreción endógena de dopamina, los animales se desplazarán hacia el lado de la lesión (giro ipsilateral) y dado que en ambas manipulaciones el animal se desplaza en círculos, se conoce a la preparación como "modelo de la conducta giratoria" (Ungerstedt 1971a,b,c; figura 1).

Cabe mencionar que el desequilibrio sólo es definitivo si se destruyen más del 95% de las neuronas dopaminérgicas, de lo contrario en un lapso de 30 a 60 días ocurrirá una rehabilitación espontánea del desequilibrio motor (Iversen, 1973; Creese y cols, 1977; 1979; Joyce y cols, 1985a; 1985b; 1987; Nave y cols, 1984; Marshall y cols, 1989).

Figura 1
Modelo de la conducta de giro



2.- Modelo del MPTP:

En 1982 en el norte de California algunos jóvenes adictos ala heroína comenzaron a presentar un estado de rigidez que se clasificó como parkinsonismo avanzado. Poco tiempo después se determinó que la droga que consumieron estaba contaminada con 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP; Langston y cols, 1983; 1984). La administración de esta droga en primates y en cepas de ratón, produce alteraciones motoras muy semejantes a las de la enfermedad de Parkinson y como en el caso del modelo de la conducta giratoria, se produce la "pérdida" de las neuronas dopaminérgicas del mesencéfalo ventral (Langston y cols, 1984, 1987; Langston e Irwin, 1986; Trevor y cols, 1987; Schultz y cols, 1989a,b; Zigmond y Stricker, 1989).

Efectos de los transplantes de tejido catecolaminérgico sobre los modelos de la enfermedad de Parkinson.

Con la evidencia de que las deficiencias motoras tanto en los modelos como en la enfermedad de Parkinson, son el resultado de la deficiencia del aporte dopaminérgico estriatal, las estrategias de transplante que se han utilizado son:

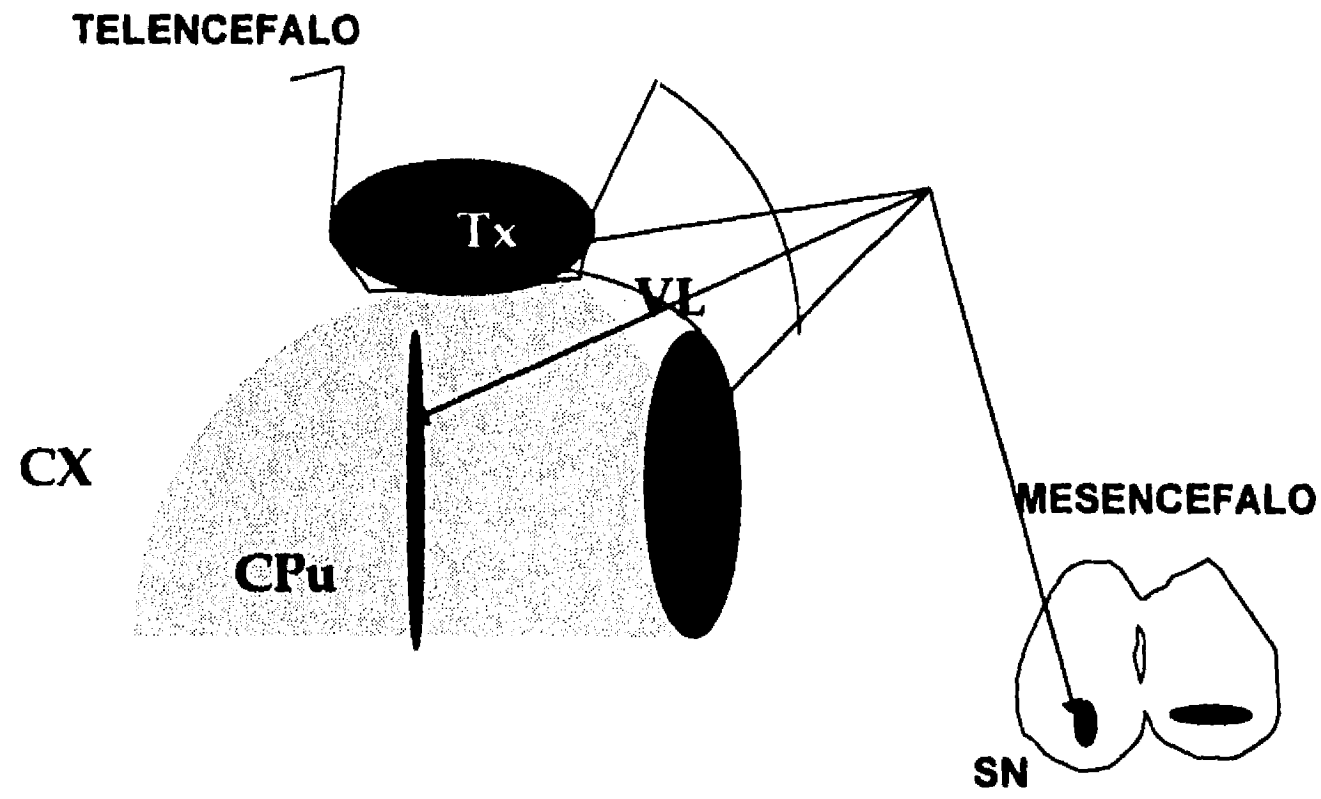
1.- Implantar junto al cuerpo estriado denervado un bloque de tejido catecolaminérgico, ya sea en una cavidad subcortical. 2.- Transplantar el tejido en la luz del ventrículo lateral. 3.- Inyectar una suspensión celular en el parénquima del cuerpo estriado o 4.- Inyectarla en el parénquima de la substancia nigra. Las células utilizadas han sido; neuronas dopaminérgicas del mesencéfalo ventral fetal, células cromafines de la

glándula suprarrenal o líneas celulares modificadas para sintetizar y secretar l-dopa o dopamina (Perlow y cols, 1979; Björklund y Stenevi, 1979; Freed y cols, 1980; Dunnett y cols, 1981a; Björklund y cols, 1980; 1983 Gage y cols, 1985). Aunque los efectos compensatorios son conductualmente equiparables, existen diferencias funcionales con cada tipo de tejido.

1.- Transplantes de Substancia Nigra fetal.

Los primeros reportes del efecto compensatorio de los transplantes en el modelo de la conducta de giro fueron publicados en 1979, Perlow y cols, colocaron el bloque de mesencéfalo fetal en la luz del ventrículo lateral ipsilateral al cuerpo estriado denervado, mientras que Björklund y Stenevi lo colocaron en una cavidad artificial subcortical.

Figura 2. Los sitios donde se ha colocado al transplante dopaminérgico (Tx) son en una cavidad cortical (CX), el ventrículo lateral (VL), en el parénquima estriatal denervado (CPu) o en la substancia nigra (SN)



La disminución en el número de giros ponderados con agonistas dopaminérgicos fue tan espectacular, que otros grupos de investigación utilizando el mismo paradigma, además de confirmar los resultados originales, han establecido mayores evidencias.

Se mostró que en la rata, la edad más adecuada para transplantar al mesencéfalo ventral fetal es de 14 a 16 días, ya que de 500,000 células dopaminérgicas implantadas sobrevive ± 1%, lo cual representa la mayor tasa de sobrevivencia (Freed y cols, 1980, 1983, 1985; Björklund y Stenevi, 1984; Brundin y cols, 1985; Simonds y Freed, 1990). Es interesante hacer notar que la nimodipina (antagonista de canales de calcio tipo L), puede favorecer la sobrevivencia de las neuronas dopaminérgicas de estadios de desarrollo mayor a los 17 días, incluyendo tejido de recién nacidos (Finger y Dunnett, 1989).

Los efectos compensatorios son específicos y directamente proporcionales al grado de reinervación dopaminérgica estriatal, ya que la remoción del transplante restablece la asimetría motora. En concordancia con ésto, se mostró que el transplante de tejido nervioso no-dopaminérgico, carece de efectos compensatorios (Björklund y Stenevi, 1984; Freed y cols, 1983; Arbuthnott y cols, 1985; Gage y cols, 1985; 1991; Brundin y cols, 1985, 1988, 1988a; Freund y cols, 1985; Dunnett y cols, 1981b; 1988; Zetterstrom y cols, 1986; Björklund y cols, 1987; Herman y cols, 1986, 1988; Doucet y cols, 1990; Zuddas y cols, 1990; Blunt y cols, 1991; Sirinathsinghji y cols, 1989, 1991; Strömberg y cols., 1989).

Acorde con lo anterior, Brundin y colaboradores (1988) establecieron que se requiere de al menos 100 neuronas dopaminérgicas viables post-transplante, para que ocurra una disminución significativa en la tasa de giro evaluada con anfetamina.

Este tipo de transplante parece restablecer parte del aporte dopaminérgico, pues se ha detectado una tendencia a la normalización del número de receptores D2 en las neuronas del cuerpo estriado denervado y transplantado (Freed y cols 1983; 1985; Dawson y cols, 1991).

Cuando el transplante se coloca en el ventrículo contralateral, el efecto conductual que se produce en términos cuantitativos es equiparable al reportado en los experimentos anteriores, aunque con una latencia mayor a la usual. Sin embargo, en tales condiciones no ocurrió el decremento del número de receptores D2 estriatales (Mendoza-Ramírez y cols, 1991). En concordancia con ésto, se mostró que en los animales en los que el transplante ipsilateral produce la disminución en la conducta de giro, la incorporación de glucosa radioactiva durante la estimulación con apomorfina se incrementa más en las estructuras del hemisferio contralateral, lo que sugiere que los efectos compensatorios son el resultado de cambios en diversas estructuras, más que un efecto local (Zainos-Rosales y cols, 1993).

Por otro lado y en relación a la regionalización funcional estriatal, se mostró que cuando el transplante se coloca adyacente a la región lateral cercana al cuerpo calloso, se inducen efectos compensatorios en la sensibilidad y orientación corporal, pero no en la conducta de giro (Dunnett y cols, 1981; Sladek y cols, 1986; Bankiewicz y cols, 1990).

Sin embargo, dada la evidencia producida por el transplante contralateral, es posible que a un período de tiempo mayor también pueda ocurrir una compensación motora.

En condiciones de reposo la frecuencia de disparo, el recambio de dopamina y la cinética de los autoreceptores de las neuronas dopaminérgicas transplantadas, es similar a las de aquellas estudiadas en el mesencéfalo de animales intactos (Wherthele y cols, 1981; Rose y cols, 1985; Strömberg y cols, 1985; Strecker y cols, 1987). Sin embargo, cuando se estimula con anfetamina, las células transplantadas secretan un aporte de dopamina mayor que el normal (Herman y cols., 1986; Forno y cols, 1989; Snyder-Kellery y Lund, 1990). Este resultado podría explicar lo sorprendente de la compensación motora evaluada con anfetamina y también el porque se requiere de muy pocas células en dichas condiciones.

El uso frecuente de l-dopa tiene efectos deletereos en la sobrevivencia del transplante (Steece-Collier y cols, 1990; Blunt y cols, 1991; Yurek y cols, 1991). No obstante, Gaudin y colaboradores (1990) reportaron que el tratamiento a corto plazo no parece producir efectos deletereos en la compensación conductual.

Por otro lado, algunos estudios muestran que conforme pasa el tiempo las células transplantadas mueren, se muestra que después de nueve meses las pocas células que aún están se encuentran deterioradas al grado que dejan de existir antes de un año (Gopinath y cols, 1991; Shetty y cols, 1991).

Por otro lado, el transplante de neuronas dopaminérgicas en neonatos reinerva al cuerpo estriado casi en su totalidad cuando el implante se realiza pocos días después de

la lesión del sistema nigro-estriatal (Schwarz y Freed, 1987; Carder y cols, 1988; Snyder-Keller y cols, 1989). Es posible que después del nacimiento el aporte de factores de crecimiento sea aun suficiente para restablecer un sistema de novo (VER MECANISMOS DE ACCION DE LOS FACTORES NEUROTROFICOS).

En concordancia con el concepto de que los órganos blanco tienen influencia trófica sobre las neuronas que proyectan a éllos, se ha mostrado que en condiciones de cultivo, el número de neuronas dopaminérgicas fetales que sobreviven no disminuye tanto cuando se les mantienen co-cultivadas con tejido estriatal fetal, estableciéndo incluso contactos sinápticos. Algo similar ocurre cuando ambos tejidos se co-transplantan en el estriado de animales adultos (Hoffman y cols, 1983; Tomozawa y Appel, 1986). Asimismo, las neuronas dopaminérgicas fetales implantadas en la región de la substancia nigra, pueden reinervar al cuerpo estriado cuando se interpone entre ambos núcleos un "puente" de tejido estriatal fetal, favoreciendose además la sobrevivencia de las neuronas dopaminérgicas transplantadas (Dunnett y cols, 1989; Yurek y cols, 1990).

2.- Transplantes de médula suprarrenal.

Con la idea de que los efectos compensatorios son mediados principalmente por el restablecimiento del aporte de dopamina estriatal, surgió la necesidad de buscar tejidos alternativos al uso del mesencéfalo fetal.

Aun cuando desde 1955 se sabía que en las células cromafines tiene lugar la biosintesis y secreción de las catecolaminas (Demis y cols, 1955), su utización como transplante en este paradigma ocurrió después de que se utilizara el mesencéfalo ventral.

Las células cromafines cuyo origen embrionario es la cresta neural, además de secretar catecolaminas sintetizan y secretan encefalinas, neuropéptidos y substancias tales como el factor básico de crecimiento de fibroblastos (FGF2) asi como pequeñas cantidades del factor de crecimiento nervioso (NGF) (Lillien y Claude, 1985; Freed y cols, 1990; Kordower y cols, 1990; Otto y Unsicker, 1990; Tischler y cols., 1992; Uschler y cols., 1980).

Estudios subsecuentes han mostrado que las propiedades endocrinas, morfológicas y funcionales de las células cromafines están reguladas en parte por los glucorticoides secretados por las células de la corteza suprarrenal (Notter y cols, 1989; Seidl y cols, 1987; Bohn y cols, 1981). Esto se estableció cuando en cultivo o como transplante a la cámara anterior del ojo, las células cromafines cambiaron morfológicamente semejando neuronas. Este cambio se acelera y es más evidente si se agrega en el medio o se inyecta en la cámara anterior del ojo NGF, factor neurotrófico ciliar (CNTF) o extractos del glioma C6. La adición de estos factores además de producir una disminución en el número de células que mueren, favorece la sintesis de dopamina a expensas de adrenalina y noradrenalina (Unsicker y cols, 1978, 1980, 1987; Aloe y cols., 1979; Livett, 1984). Es importante señalar que los cambios fenotípicos son dependientes de la edad, es decir, las células cromafines de animales fetales o neonatos, son más

susceptibles al cambio fenotípico que las de organismos adultos y éstas más que las de animales seniles (Aloe y cols., 1979;Livett, 1984; Unsicker y cols, 1978, 1980, 1987; Lieberman y cols., 1988; Bohn y cols, 1981; Tischler y cols, 1982; Doupe y cols, 1985; Müller y Unsicker, 1986; Shaw y Letoorneau, 1986; Ogawa y cols, 1986; Notter y cols, 1986; Herman y cols, 1992).

En 1981 Freed y colaboradores publicaron el primer estudio demostrando que el transplante intraventricular de médula suprarrenal, produce un decremento en el número de giros ponderados por apomorfina. Desde entonces varios grupos han confirmado dichos efectos además de establecer su caracterización funcional.

Se demostró que con este tipo de transplante los efectos compensatorios no dependen de la reinervación del cuerpo estriado, hecho en el que contrastan con los del transplante de neuronas dopaminérgicas, ésto motivó a que se propusiera que los efectos de los transplantes de médula suprarrenal son mediados por la difusión pasiva de substancias neuroactivas secretadas por el transplante (Freed et al 1990).

De igual manera que con los transplantes de neuronas dopaminérgicas, el número de células cromafines que sobreviven transplantadas es un factor que se correlaciona con el grado de compensación motora. Sin embargo, en este aspecto las células cromafines, a diferencia de las del mesencéfalo ventral, son más delicadas ya que requieren de un aporte de factores de crecimiento (ver más adelante; Freed y cols, 1981, 1990; Kamo y cols, 1987; Rosentein, 1987; Yong y cols, 1989; Hansen y cols, 1989; Becker y cols, 1990; Date y cols, 1990; Fine, 1990; Corran y Becker, 1991).

Se observó que en animales trasplantados con médula suprarrenal en los que se establecieron efectos compensatorios, el análisis del líquido cefalorraquídeo mostró consistentemente un incremento en la concentración de metabolitos dopaminérgicos: ácido homovanílico (HVA) y ácido 3,4-dihidroxi-fenil-acético (DOPAC), lo que sugirió que el efecto compensatorio también es mediado por el restablecimiento de la dopamina (Becker y Freed, 1988).

En este sentido y aunque la dopamina normalmente no atraviesa la barrera hemato-encefálica, se encontró que en las áreas parenquimales adyacentes al transplante, las catecolaminas que circulan por el torrente sanguíneo tienen acceso libre al cerebro (Becker y Freed, 1988). Esto puede explicarse por el hecho de que los vasos sanguíneos de los transplantes normalmente se anastomosan con los del tejido hospedero y dado que en los órganos periféricos no existe una barrera como la cerebral, el acceso al cerebro permanece libre (Rosentein, 1987; Ahlskog y cols, 1990; Broadwell y cols, 1990; Takei y cols, 1990).

Otro mecanismo que está presente en los transplantes de células cromafines y que no parece ocurrir con los transplantes de mesencéfalo ventral, es el hecho de que las células cromafines secretan factores de crecimiento, que promueven la proliferación de los procesos neuríticos en las neuronas dopaminérgicas remanentes del sistema nigro-estriatal. Este efecto fue detectado por primera vez en ratones que fueron lesionados con MPTP, dando lugar a lo que podriamos denominar como un "fenómeno de reactivación funcional del sistema nigro-estriatal remanente" (Bohn y cols, 1987). Esta reactivación

también se ha observado en rata, mono y en pacientes con enfermedad de Parkinson (VER EFECTOS TERAPEUTICOS DE LOS TRANSPLANTES).

Por otro lado, los transplantes de médula suprarrenal tienen efectos en el cuerpo estriado contralateral, produciendo un decremento en los niveles de dopamina (Becker y cols, 1990). Esto posiblemente se deba al hecho de que ambos sistemas nigro-estriatales interactuan de manera opuesta (Nieoullon y cols, 1977; Cheramy y cols, 1981); por lo que se podría esperar el mismo efecto con el uso de transplantes de neuronas dopaminérgicas fetales. Sin embargo, a la fecha no existe en la literatura evidencia al respecto.

Nishino y cols, (1988) asi como Mahalik y cols, (1989) han reportado que las células cromafines implantadas en el parénquima estriatal, son capaces de cambiar su fenotipo a algo que asemeja neuronas, presentando potenciales de acción y estableciendo lo que parecen ser contactos sinápticos con las neuronas de cuerpo estriado. Con base en esta evidencia se propuso que los transplantes de células cromafines podrían también ejercer sus efectos a través de contactos sinápticos.

En otros estudios se demostró que la infusión intra-estriatal de NGF, favorece la sobrevivencia de las células cromafines, además de que promueve el crecimiento de procesos hacia el sitio de infusión (Strömberg y cols, 1985).

De manera análoga a los efectos ponderados con transplantes de neuronas dopaminérgicas, se ha documentado que en el caso de los transplantes de médula suprarrenal, se produce un decremento en la hipersensibilidad de los receptores

dopaminérgicos D2 (Freed y cols 1985; Dawson y cols, 1991). Sin embargo, con el transplante de médula adrenal colocado en el ventrículo del lado contralateral, de manera semejante a lo que ocurrió con el transplante de mesencéfalo ventral, se produce un decremento en la tasa de giro pero no hay cambios en la densidad de receptores D2, lo que sugiere nuevamente que los mecanismos por los que los transplantes ejercen sus efectos, dependen de cambios en todo el cerebro y no solamente en las áreas aledañas al transplante (Mendoza-Ramírez y cols, 1991).

Con la idea de que parte de los efectos compensatorios se deben al halo de difusión dopaminérgica, se ha mostrado, in vitro que el uso de campos electromagnéticos de baja frecuencia, favorece la neurogenización de las células cromafines. Si este método no invasivo favoreciera la neurogenización después de que se transplanten las células cromafines, podría usarse para incrementar la eficiencia de los efectos compensatorios (Drucker Colín y cols, 1994).

3.- Transplante de líneas celulares que secretan L-DOPA o Dopamina.

La búsqueda de tejidos alternativos para transplante es importante ya que el mesencéfalo ventral fetal y la médula suprarrenal han presentado limitaciones, algunas de tipo técnico y otras de tipo ético. Asi, la primera línea celular utilizada en este paradigma fue la del feocromocitoma de médula suprarrenal de rata, las células PC12 que aunque en esencia son células cromafines, muestran diferencias con respecto a las normales.

Las células PC12 pueden inducir decrementos en la tasa de giro pero no son capaces de sobrevivir transplantadas más allá de unas cuantas semanas (Hefti y cols, 1985). No obstante, Freed y colaboradores (1986) establecieron que el tiempo de sobrevivencia puede ser hasta de cinco meses. Se ha argumentado que la disminución de la sobrevivencia se debe a la ausencia de NGF (Tresco y cols., 1992; Winn y cols., 1991).

Otra alternativa ha sido el uso de tejido adiposo combinado con NGF, pero a pesar de lo interesante del resultado por la participación de los factores tróficos como parte de los mecanismos compensatorios, no se ha publicado otro estudio en este sentido (Pezzoli y cols, 1988).

Itákura y colaboradores (1988) mostraron que el transplante de ganglio cervical superior fetal, el cual contiene neuronas dopaminérgicas, también produce disminución en la tasa de giro. Sin embargo, las limitantes de uso son similares a las del mesencéfalo ventral.

Con el desarrollo de las técnicas de la biología molecular se establecieron algunas líneas celulares en fibroblastos transfectadas con el fragmento que codifica la sintesis de l-dopa o dopamina. Los transplantes de estos tipos celulares han producido de manera cási inmediata una disminución del 100% en la tasa de giro. Sin embargo, las células continuan dividiéndose y ocupando el espacio del cuerpo estriado en un lapso de dos semanas, causando la muerte de los animales (Horellou y cols, 1990; Fisher y cols, 1991;

Crawford y cols., 1992; Cunningham y cols., 1991). Resultados similares se han reportado con otras líneas celulares como las células del glioma C6 (Uchida y cols, 1989).

4.- Efectos terapéuticos de los transplantes.

El uso clínico de los transplantes al cerebro se inició en 1985, cuando Backlund y colaboradores en Suecia, después de implantar en una cápsula fragmentos de la médula suprarrenal en el parénquima estriatal de dos pacientes con enfermedad de Parkinson, describieron que sólo durante algunos días ocurren ligeros efectos compensatorios.

Dos años después en México, se dieron a conocer los primeros efectos compensatorios en pacientes con dicha enfermedad. La técnica de transplante consistió en colocar bloques de médula suprarrenal del propio paciente, en una cavidad artificial en la cara interna de la cabeza del núcleo caudado (Madrazo y cols, 1987; Drucker-Colín y cols, 1988; Jiao y cols., 1988). Con este procedimiento se mostró que la compensación motora tarda más que la observada en los animales. De manera escéptica, algunos investigadores en el mundo calificaron los resultados del grupo mexicano como inespecíficos. No obstante, algunos años más tarde los mismos investigadores reportaron como un éxito que sólo en sus manos podría calificarse de espectacular, los mismos resultados que habían reprobado (Lindvall y cols, 1987, 1988a, 1988b; Frank y cols., 1988; Apuzzo y cols., 1990; Björklund, 1991; Hirsch y cols, 1990; Goetz y cols., 1988, 1989; Hansen y cols., 1988, 1989, 1990; Kelly y cols., 1989; Kordower y cols., 1990, 1991, 1992; Olson, 1990; Olanow y cols., 1988, 1990; López-Lozano y cols., 1990).

Allen y colaboradores (1989) han reportado como una sugerencia original, lo que años atrás plantearan los investigadores mexicanos,"...la edad del paciente es un factor determinante en la sobrevivencia del transplante, ya que si éste se practica en sujetos relativamente jóvenes, se incrementan las posibilidades de producir efectos terapéuticos significativos..."

En 1989 y 1990, Lindvall y colaboradores reportaron también como una aportación original, que el transplante unilateral produce mejorias a nivel bilateral, en este caso el transplante fue de mesencéfalo ventral fetal humano de 8 a 10 semanas de gestación. Esta compensación se correlacionó con un incremento del aporte dopaminérgico en ambos sistemas nigro-estriatales. Freed y cols en 1990, ratifican que el implante de substancia nigra fetal de siete semanas, ejerce efectos terapéuticos en ambos lados del cuerpo.

Sin embargo y aunque los efectos terapéuticos reportados con el transplante de mesencéfalo ventral humano son promisorios, cabe señalar que éstos no han sido más eficientes que los reportados con el auto-transplante de médula adrenal. En este sentido, es necesario puntualizar que los resultados obtenidos por el grupo sueco, han mostrado que la sobrevivencia de las neuronas dopaminérgicas fetales transplantadas es muy baja. Esto posiblemente se deba al hecho de que el transplante estaba constituido por tejido de varios fetos. Esta aseveración se apoya en los resultados obtenidos en primates por el grupo de Bankiewicz, quienes buscaron establecer directamente el efecto del uso de varios donadores simultáneos, encontrando que dicha práctica reduce significativamente

la sobrevivencia de las células implantadas y en consecuencia la compensación motora es baja (Bankiewicz y cols, 1990).

En contraste se demostró que con los auto-transplantes de médula suprarrenal se producen además efectos tróficos sobre las neuronas dopaminérgicas remanentes en el sistema nigro-estriatal (Hurtig y cols, 1989). Kordower y colaboradores (1991), encontraron que 30 meses después de ser transplantadas se detectan células cromafines viables en la zona del transplante y que éste induce la proliferación de fibras dopaminérgicas del hospedero hacia el sitio del implante.

Factores neurotróficos:

Con la evidencia de que las células cromafines y las neuronas estriatales fetales secretan factores tróficos que promueven el crecimiento de procesos neuríticos en las neuronas dopaminérgicas remanentes del sistema nigro-estriatal, se propuso que la caracterización de estos factores podría ser útil para inducir dicha reactivación artificialmente.

La búsqueda de substancias con actividad neurotrófica se habia iniciado con el descubrimiento de los efectos del NGF (Levi-Moltancini y Hamburger, 1953), lo que planteó la busqueda de substancias que definieran el fenotipo celular, el establecimiento de las conecciones neuronales, asi como aquellas que previnieran el fenómeno de "apoptosis" (muerte celular programada) o que pudieran revertirlo una vez iniciado.

Varios factores favorecen la sobrevivencia, maduración y crecimiento de procesos neuríticos en las neuronas dopaminérgicas. En la lista se incluye a: 1) Las neurotrofinas, el factor neurotrófico derivado del cerebro (BDNF); la neurotrofina 3 (NT-3) y el factor de crecimeinto nervioso (NGF). 2) El factor básico de crecimiento de fibroblastos (FGF-2). 3) El factor de crecimiento semejante a la insulina IGF. 4) El factor de crecimiento epidermal (EGF). 5) El factor neurotrófico derivado de una linea celular de glia (GDNF); y 6) El factor beta de crecimiento transformante (TGF-β), algunos además, ejercen un efecto protector a los efectos de las neurotoxinas 6-OHDA, MPTP y MPP+.

1). Neurotrofinas.

La secuencia de aminoácidos del NGF, BDNF, NT-3 y NT-4/5 muestra una homologia mayor al 50%, por lo que se les agrupó como la familia de las neurotrofinas. La expresión celular de éstas se ha detectado en el sistema nigro-estriatal con la excepción de NT4/5. Los receptores de las neurotrofinas son los miembros de la familia trk (tyrosine kinase receptor), trkA es el receptor del NGF, trkB es el del BDNF y NT-4/5, y en menor grado de NT-3; en tanto que trkC es el receptor de NT-3 (VER LA SECCION DE LOS MECANISMOS DE ACCION DE LOS FACTORES NEUROTROFICOS).

La expresión del RNA mensajero (RNAm) del NGF en el estriado de la rata es muy baja (Reivich y Kreutzberg, 1987; Shelton y Reichardt, 1986). No obstante, la combinación de estudios de fijación de ^{125}I-NGF con inmunohistoquímica, mostró que las neuronas

colinérgicas expresan los receptores sensibles al NGF (VER TrkA EN LA SECCION DE MECANISMOS DE ACCION DE LOS FACTORES NEUROTROFICOS).

Sin embargo, y aun cuando se ha demostrado que el receptor trkA no se expresa en las neuronas dopaminérgicas, se ha observado que la perfusión intra-ventricular de NGF en ratones tratados con MPTP, restaura el aporte de dopamina estriatal (Garcia y cols, 1992). Es posible entonces que el NGF ejerza su efecto de manera indirecta.

El BDNF promueve la sobrevivencia y desarrollo de procesos neuríticos en las neuronas dopaminérgicas en cultivo, protegiendolas de los efectos tóxicos de 6-OHDA o de MPTP (Knünsell y cols, 1991; Hyman y cols, 1991; Beck y cols, 1993; Spina y cols, 1992). Acorde con ésto, se mostró que el ^{125}I-BDNF inyectado en el cuerpo estriado se transporta y acumula en las neuronas dopaminérgicas de la substancia nigra (Wiegand y cols, 1991). Subsecuentemente se estableció la expresión de BDNF y trkB en las neuronas dopaminérgicas de la substancia nigra, tanto durante el desarrollo como en la vida adulta (Friedmann y cols, 1991; Maisonpierre y cols, 1990; Escandon y cols, 1994).

La inyección intranigral de BDNF en animales intactos produce conducta de giro cuando éstos son estimulados con anfetamina, en esta condición aumenta la tasa de recambio de dopamina por lo que se sugiere una posible participación en la fisiología del sistema nigro-estriatal (Altar y cols, 1992). Subsecuentemente se intentó rescatar a las neuronas dopaminérgicas de animales adultos después de la transección de la vía nigro-estriatal . Sin embargo, al inyectar BDNF en el ventrículo lateral no se producen los efectos que se habían detectado en condiciones de cultivo (Knüsell y cols, 1992).

La concentración de NT-3 no parece ser detectable en el cuerpo estriado, pero sí se detecta en las neuronas dopaminérgicas del área ventral tegmental (Gall y cols, 1992) y de manera semejante al BDNF, el NT-3 es capaz de promover la sobrevivencia de las neuronas dopaminérgicas en condiciones de cultivo, protegiéndolas también de los efectos de las neurotoxinas (Hyman, 1991).

LAS CARACTERÍSTICAS FUNCIONALES DE LAS NEUROTROFINAS SE PRESENTA EN EXTENSO EN LA SECCIÓN LOS MECANISMOS DE ACCIÓN DE LOS FACTORES NEUROTRÓFICOS EN LA SEGUNDA PARTE DE LA TESIS.

2). Factores de crecimiento de fibroblastos.

A la fecha la familia consiste de siete miembros y de éstos sólo tres están presentes en el cerebro, el FGF ácido (FGF1), el FGF básico (FGF2) y el FGF5. Se sabe que la concentración de FGF1 y FGF2 en el cerebro, es mayor a la de las neurotrofinas, sin que ésto signifique que las células sean más sensibles a estos factores.

Tanto en las neuronas del cuerpo estriado, como en las neuronas dopaminérgicas de la substancia nigra se co-localizan el FGF1 y el FGF2, mientras que en las células gliales sólo se expresa el FGF2 (Bean y cols, 1991; Cintra y cols, 1991). Esta expresión disminuye drásticamente después de la lesión con 6-OHDA y en pacientes con enfermedad de Parkinson avanzada (Cintra y cols, 1991; Tooyama y cols, 1993).

La infusión intracerebral del FGF2 en animales con lesión parcial del sistema nigro-estriatal con 6-OHDA, induce el fenómeno de proliferación de neuritas en las neuronas

dopaminérgicas remanentes (Cintra y cols, 1991; Tooyama y cols, 1993; Otto y Unsicker, 1990; 1993). Sin embargo, si la lesión se realiza con MPTP, se produce un incremento en los niveles del RNAm del FGF2 en la substancia nigra del lado lesionado durante los días previos a la reactivación del sistema nigro-estriatal (Leonard y cols, 1993).

Ahora bien, en cultivo la sobrevivencia de neuronas dopaminérgicas y GABAérgicas se promueve con FGF2, el cual puede protegerlas de los efectos tóxicos del MPTP. Sin embargo, esta protección no ocurre cuando se utiliza 6-OHDA. La protección observada con el uso de MPTP se correlacionó con la cantidad de células gliales presentes en el cultivo, por lo que se propuso que los efectos son mediados de manera indirecta (Ferrari y cols, 1989; Hartikka y cols, 1992; Mayer y cols, 1993; Matsuda y cols, 1990).

3). Factor semejante a la insulina (IGF).

Aunque la Insulina, el IGF-I e IGF-II se expresan durante el dasarrollo en varias estructuras cerebrales, el papel funcional que desempeñan se ha estudiado poco (Bondy, 1991; Rotwein y cols, 1988). No obstante, su uso en cultivos de neuronas dopaminérgicas muestra que estimula la sobrevivencia, la incorporación de dopamina y la actividad de la enzima tirosina hidroxilasa (Knüsel y cols, 1990; Spina y cols, 1992).

4). Factor de crecimiento epidermal (EGF).

La capacidad de estimular la proliferación y diferenciación de la glia, asi como la sobrevivencia de algunos tipos neuronales se ha establecido con el EGF (Leutz y Schachner, 1981; Honegger y Guentert-Lauber, 1983; Morrison ycols, 1987). Más recientemente se mostró que en animales lesionados con MPTP o 6-OHDA, la inyección intraventricular de este factor compensa las alteraciones motoras; pero sólo en el primer caso, induce un incremento en la reactivación del sistema dopaminérgico dañado, incrementando la actividad de la enzima tirosina hidroxilasa (Hadjiconstantinou y cols, 1991; Pezzoli y cols, 1991).

5). Factor neurotrófico derivado de una línea celular de glia (GDNF).

El GDNF es el factor que más recientemente ingresó a la lista. Es una proteína básica que en las neuronas dopaminérgicas tienen una EC50 de 1 pM. Su uso en cultivo estimula la sobrevivencia de dichas neuronas, evita que disminuya el número de éstas neuronas durante tres semanas (tiempo récord); estimula su maduración y crecimiento de procesos neuríticos y a diferencia de otros factores no parece tener efectos en las neuronas GABAérgicas, por lo que hasta el momento se ha sugerido como un factor específico (Lin, L.-F y cols, 1993; 1994). Este factor se expresa en el cuerpo estriado, la substancia nigra, tálamo, hipocampo, corteza crebral, cerebelo, médula espinal y en los astrocitos tipo 1 (Lin, L.-F y cols, 1994; Springer y cols, 1994; Strömberg y cols, 1993; Schaar y cols, 1993). Cabe señalar que la expresión estriatal de este factor se incrementa

casi en un 100%, en los primeros 10 días después de la lesión del sistema nigro-estriatal (Mendoza-Ramírez y cols, datos no publicados).

Conclusiones y perspectivas.

A partir de las evidencias generadas por el uso de los transplantes de neuronas dopaminérgicas fetales, células cromafines y más recientemente líneas celulares productoras de dopamina, se han planteado dos teorías complementarias.

La teoría dopaminérgica establece que es necesario restablecer el aporte de dopamina para que se compensen las alteraciones motoras. De aquí que es prioritario que se establezcan las condiciones en las que se asegure la sobrevivencia del total de células transplantadas, pues como ya se señaló menos del 1% sobrevive. Es prudente señalar que incrementar el número de células por transplante no ha sido una solución ya que con esta práctica el número de células que sobrevive por transplante es aún menor. En este sentido el uso de factores de cecimiento puede ser una buena alternativa.

La segunda teoría deriva de los efectos tróficos provocados por los transplantes de células cromafines y estriatales, por lo que se ha denominado como la teoría trófica de los transplantes. En este contexto, es importante determinar los eventos intracelulares que tienen lugar durante la reactivación de las neuronas dopaminérgicas.

Considerando los fundamentos de ambas teorías, parece fácil conceptualizar un modelo de la interacción de algunas estructuras cerebrales con los transplantes (ver fig 3).

Este modelo plantea que el transplante aporta una cuota constante de dopamina, pero además secretada factores neurotróficos que favorecen la secreción de factores neurotróficos endógenos en el tejido hospedero dando como resultado la reactivación del sistema nigro-estriatal remanente, y por ende la compensación motora.

Con las evidencias generadas con el uso de factores de crecimiento se puede proponer que una terapia factible para prevenir la muerte de las neuronas dopaminérgicas en los pacientes, sería el uso de un cóctel de factores tróficos aplicado durante los estadios iniciales de la enfermedad, para ésto se podría implementar el autotransplantes de células modificadas genéticamente para que los secreten evitando los problemas de rechazo. Sin embargo, ésto dependerá de la colaboración multidisciplinaria que se establezca, asi como de los recursos que para este propósito se destinen.

Acorde con lo anterior, el estudio multidisciplinario de los factores tróficos del tipo del NGF, BDNF, NT-3 GDNF, FGFs, EGF y otros que están por descubrirse, son fundamentales para establecer parte de los requerimientos anteriores, además de que serán útiles para el entendimiento de los mecanismos de la plasticidad cerebral.

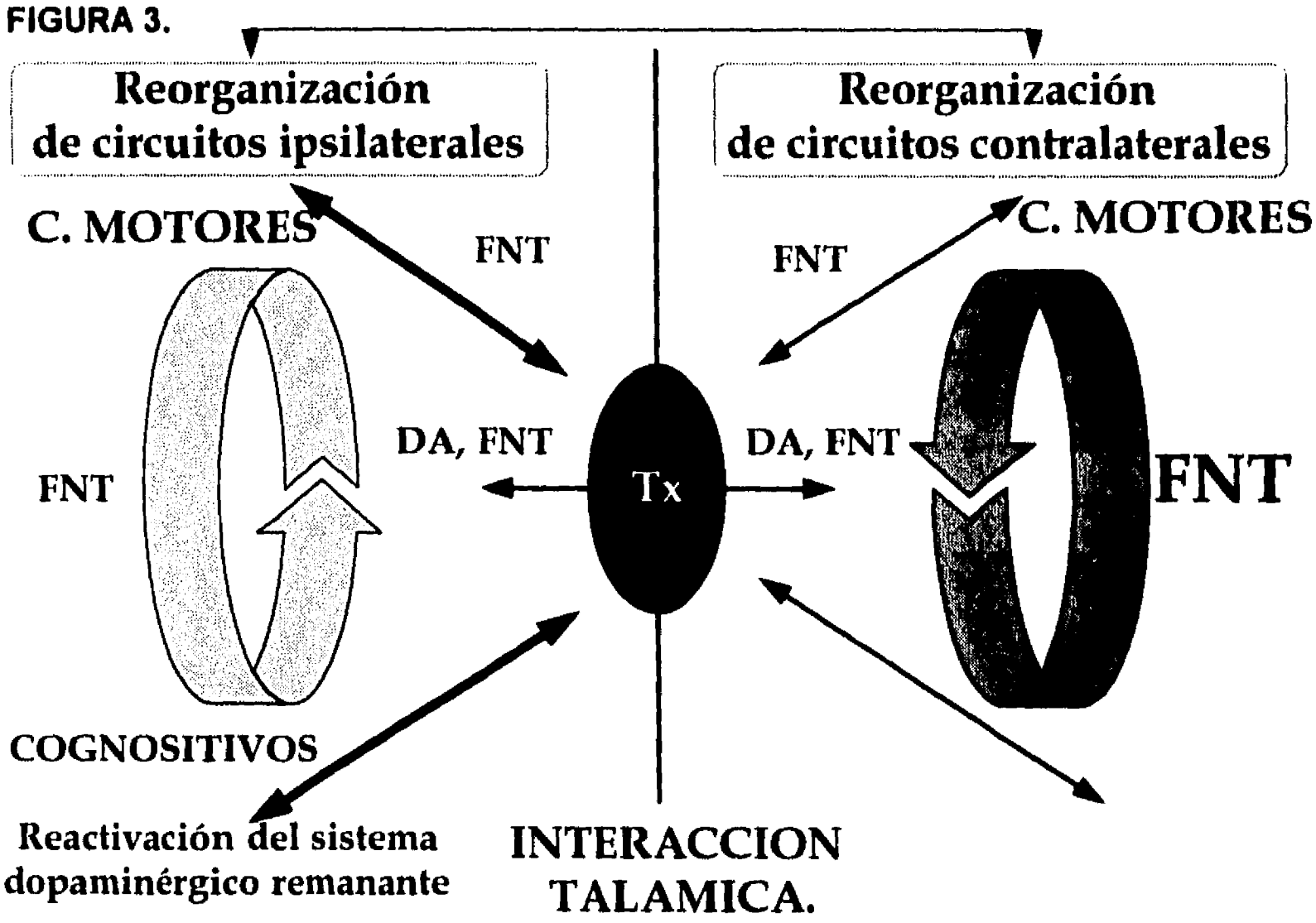
De lo anterior, es evidente que el uso de los transplantes intracerebrales en los próximos años se combinará con factores neurotróficos, lo que seguramente pondrá en claro la dinámica de la interacción transplante-hospedero, pero además ayudará al mayor entendimiento de los procesos de plasticidad cerebral que tienen lugar a lo largo de la vida de los organismos; es decir, los efectos que se han reportado en organismos juveniles, parecen ser diferentes de los observados en organismos seniles y dado que los

problemas de neurodegeneración son más frecuentes en la vejez, es necesario estudiar los efectos de los diferentes factores, asi como sus mecanismos en las diferentes etapas de la vida de los organismos.

En concordancia con la propuesta del párrafo anterior se planteó el estudio" Delay of electrophysiological and motor detriments in aged rats previously transplanted with NGF cultured Chromaffin cells in adulthood'.

REPRESENTACION DE LOS MECANISMOS QUE PARTICIPAN EN LA REACTIVACION DEL SISTEMA NIGRO-ESTRIATAL INDUCIDO POR LOS TRANSPLANTES DE TEJIDO CATECOLAMINERGICO.

FIGURA 3.



FNT= Factores neurotróficos, neurotransmisores o algún otro tipo de mensajero

DELAY IN MANIFESTATIONS OF AGING BY GRAFTING NGF CULTURED CHROMAFFIN CELLS IN ADULTHOOD.

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MENDOZA-RAMIREZ, J.L., L. BELTRAN, L. VERDUGO-DIAZ, C. MORGADO-VALLE AND R. DRUCKER-COLIN. *Delay in manifestations of aging by grafting NGF cultured chromaffin cells in adulthood.* NEUROBIOL AGING. Dopamine agonists or grafts compensate impaired motor functions in aged rats. However, there is no evidence showing whether grafting in adulthood retard aging manifestations. Motor performance of 13 mo old rats was tested on 2 meter long wooden beams which had a 15° inclination and whose thickness varied from 3,6,12,18 to 24mm. Rats at 14 mo were randomly assigned to 3 groups: sham graft (group 1); intrastriatal graft of chromaffin cells cultured with NGF (group 2); intrastriatal graft of chromaffin cells (group 3). Motor performance was tested at monthly intervals up until rats were 26 mo old. Two more groups were included: 26 mo old naive rats (group 4); and 3-5 mo old naive rats (group 5) both evaluated only once. At 26 mo, the basal activity of ventral mesencephalic dopaminergic neurons was recorded. Results showed in group 2 delay of motor detriments seen in aged rats, maintenance of basal firing rates of nigral cells comparable to those of younger rats and greater survival of substantia nigra cells. It is suggested that NGF cultured chromaffin cells produce a delay of motor detriments in aged rats, as a result of inducing survival and firing rates of nigral cells comparable to those seen in young rats.

Key words: Aging, Transplants, NGF, Motor activity, Substantia Nigra.

Detriment of many behavioral functions, including motor activity (28,29,42,55,57,70,80), learning (3,6,77), memory (3,6,67,78), sexual activity (43,54,70), sleep (21,44,66) and others are usually associated to the aging process. The deterioration of memory and/or motor performance in aging rats has been of particular interest, due to its relationship to neurodegenerative disorders such as Parkinson's Disease, Alzheimer, etc (6,25,30,38,41,48). Amongst the most studied alterations, stands out the motor deficits associated with the decrease of dopamine supply to striatal nuclei, which is accompanied by a decrease of striatal dopamine receptor density (1,11,50,57,58,59,60,62,71). The fact that motor deficits can be compensated by systemic injections of dopamine agonists (55,56) and ameliorated by transplants of either adrenal medullary tissue (chromaffin cells) or fetal substantia nigra (FSN) in rodents (4,5,6,12,13,14,19,24,26,27,69), primates (2,20,36,75,81) and humans (17,22,23,39,40,46,52,53), has further increased interest in the study of the aging process.

One of the pervading questions about the aging process, is whether manipulations which could retard the appearance of some of the deficits accompanying aging can be devised. There is evidence, for example, that transplantation of catecholaminergic tissue in neonate rats can reduce the effects produced by nigro-striatal dopamine pathway lesions, when rats are 3-4 months old (8,68,72,74,76). However, there are no studies determining whether

it is possible to delay symptoms of aging, by manipulating the brain much before aging sets in, since all the studies reported to this date manipulate the brain either pharmacologically or through transplants, once symptoms have been well established. There could be two ways to tackle this problem. The first could be to provide continuous infusion of substances, several months before the aging process sets in. In this case, subjects need to have a permanent cannula implanted with a continuous infusion procedure, which can become plagued by technical difficulties. The second, could be the use of chromaffin cells cultured with NGF, particularly since the latter, not only seems essential for survival, survival time and differentiation of many cell populations (37,47,87), but is also well known to induce neuronal traits in cultured chromaffin cells(15,63,84), and to enhance tyrosine-hydroxilase (TH) activity, the rate limiting enzyme of catecholamine biosynthesis (64,82,83).

The present study, therefore, attempts to determine whether NGF differentiated chromaffin cells transplanted into the striatal parenchyma of adult rats, survive long enough to induce a delay of motor detriments and of electrophysiological and morphological changes normally seen in aged rats.

METHODS

Seventy-two Wistar rats of various ages were kept up on a 12/12 hrs light/dark cycle with free access to water and food. Temperature was kept at 22°C.

Behavioral Tests

At 13 months of age 22 rats were subjected to a motor test which has been reported previously (16,28). Briefly, rats must traverse 2 meter long beams of wood having a 15° inclination and whose thickness varies from 3, 6, 12, 18 to 24 mm. The total time (up to a maximum of 120 sec) it takes animals to reach the upper part of the beam when placed on the lower extreme, is recorded for each width. The width sequence presentation of the beams is determined by using a table of random numbers. This test was chosen on accound of the fact that it was found to be very sensitive to dopaminergic function, since young animals administrated with haloperidol a D1 and D2 receptor blocker behaved very much like old rats on this test (16).

Once the traversing time was determined for each rat, they were randomly assigned to 3 groups at the age of 14 months.

Group 1. Control. Sham transplant (n=7).

Group 2. Transplant of Chromaffin cells cultured with NGF (n=8).

Group 3. Transplant of dissociated chromaffin cells (n=7).

After the transplants each animal was tested on the motor task described above every month up until the age of 26 months. Two additional groups were included as age controls:

Group 4. Non trained (naive) 26 mo old rats evaluated only once (n=20).

Group 5. Non trained (naive) 3-5 mo old rats evaluated only once (n=30).

No sham rats without training (naive) group was included, because it was previously reported that there are no differences between such a group and a group 4 type group (28).

Cell dissociation and tissue culture.

Rat chromaffin cells were prepared essentially as described by Unsicker et al (84) with slight modifications (18). Briefly, adrenal medulla from 1-3 day postnatal Wistar rats were released from cortical tissue and collected in calcium-free Spinner Salt Solution (Sigma) supplemented with 1 mg/ml bovine serum albumin (SSS-BSA). Tissue digestion was carried out in the same solution supplemented with 2 mg/ml collagenase (Worthington) and 15 μg/ml deoxyribonuclease I (DNAse, Sigma) for 45 min at 37°C by gentle agitation. Tissue was mechanically dispersed with a Pasteur pipette in SSS-BSA supplemented with 15 μg/ml DNAse, and the cell suspension was centrifuged at 100 g for 10 min at 20°C. Finally, cells were resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 4.5 μg/ml insuline (Sigma), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and 2.5 μg/ml fungizone (Gibco).

Chromaffin cells were plated at a density of 300,000 cells/well in 12-well Costar plates. Four hours later cell plating, 100 ng/ml Nerve Growth Factor (NGF, Sigma) was added to half of the culture plates. Cells were kept in tissue culture

condition for 7 days at 37°C and the culture media was replaced after 4 days in culture.

After 7 days in culture, chromaffin cell were exposed to 0.01% trypsin (Sigma) in 0.1M calcium-free phosphate buffer saline (PBS, Sigma) plus 1 mM EDTA (pH=7.4) for 10 min at 37°C (10). The enzymatic reaction was stopped with DMEM-FBS and then the cells were collected and centrifuged at 100 g for 5 min at 25°C. Cell viability and density was determined by the trypan blue dye exclusion method.

Transplant Procedures

All transplants were made intrastriatally at the following stereotaxic coordinates: AP=+1.0 mm, with respect to Bregma; L=-2.5 mm and V=from-6.0 mm to -4.5 mm (65) as follows: 7 rats in Group 1 received an injection of 5 μl of DMEM. 8 rats in Group 2 received approximately 500,000 viable NGF cultured chromaffin cells carefully alliquoted in DMEM and maintained in an incubator until required for grafting; 7 rats in Group 3 received approximately 500,000 viable dissociated chromaffin cells alliquoted in DMEM and maintained in an incubator until required.

Electrical Recording Activity

At the end of the behavioral trials, young (3-5 mo) age and grafted rats (26 mo) were anesthetized with halothane and the head positioned in a stereotaxic frame. Body temperature was maintained

around 37°C.

Single-unit extracellular spikes from spontaneously active neurons of ventral mesencephalic region at the following stereotaxic coordinates: 4.5 mm posterior to Bregma, 1.3-1.6 mm lateral to midline and 8 to 9 mm in the ventral axis (65) was recorded through glass micropipettes which were filled with 0.5 M sodium acetate. Electrode impedance typically measured 4-8 megohms at 135 Hz in physiological saline. The velocities and rate of deep penetration of the micropipettes was controlled by a hydraulic Micromanipulator (David Kopf, mod. 760). The action potentials were analyzed through a Dataway program installed in a PC, which allows a breakdown of images showing an oscilloscope window which monitors the action potentials, gives out a firing frequency histogram and graph the interspike intervals. At the same time signals were stored on tape (Hewlett Packard Model 3960), in order to be analyzed later on. The presence of two neuronal types in the ventral mesencephalic region have been shown (7,9,32,88). Those lying in the pars compacta of substantia nigra and ventral tegmental area have been shown to be dopaminergic and their electrophysiological characteristics have been described previously (32,33,34,35). These characteristics are: 1) the display of a biphasic or triphasic action potential, with a positive first phase 2) an action potential duration of between 2 to 5 msec and a peak-to-peak amplitude of 0.5-1.5 mV, 3) an irregular single spike firing pattern (0.5-9.0 spikes/sec) or a bursting frequency of 2-8

spikes of decreasing amplitude with increasing duration, 4) the display of a notch on the initial rising phase of the action potential and large late positive component. The second type has been proposed to be Gabaergic and is found in a zone extending from crus cerebri to the layer where dopaminergic neurons are predominant, their electrophysiological characteristics are 1) an action potential of 2-3 msec, 2) the notch on the initial rising phase is absent, 3) they do not display the large late positive component and 4) their firing rate varies over a wider range from 0.1-60 spikes/sec (35, 88).

Histology

At the end of the experiments, every rat was injected with Pentobarbital (i.p. 50 mg/kg of body weight), then transcardially perfused with 250 ml of PBS followed by 300 ml of fixative which contained 4% paraformaldehyde and 1.5% picric acid in 0.1 M sodium phosphate buffer (PB; pH 7.4). All brains were postfixed overnight at 4°C and then cryoprotected in a solution of 30% sucrose in 0.1 M PB for 24 hours. All the brains were frozen by immersion in isopentane at -20°C for 5 minutes. 40 μm thick coronal sections were then collected sequentially in order to get five homogeneous section sets. All sections were preblocked with 3% Goat normal serum in PBS added with 0.3% H_2O_2 by 30 minutes, washed once in PBS containing 0.2% goat normal serum and 0.3% triton X-100 (PBS-GT) and later incubated at room temperature overnight with either anti-

Tyrosine Hydroxylase or anti-Glial fibrillary acidic protein, used at a dilution of 1:200 in PBS-GT. Sections were developed with Vector products, Elite anti-rabbit kit and DAB substrate.

All TH-positive cells were analyzed with an MCID, M4 imaging analyzer (Imaging Research Inc., Ontario, Canada).

Statistical Analysis

Behavioral differences among groups was determined by means of the Friedman test followed by the Wilcoxon Signed Ranks test (16,28). Although the percent of cells showing a particular frequency was graphed in order to determine tendencies according to age or grafted group, a two-tailed analysis of variance (ANOVA) followed by the Scheffeé contrast test based on the actual mean firing rates, was performed so as to determine significance levels of firing rate distribution (49). Similar tests were applied to the analysis of the number of TH-positive DAergic cells of the SN.

RESULTS

Motor Test

Comparison between naive young adults (3-5 mo) and naive aged rats (26 mo) as to the time it took them to cross the beams, showed significant differences at $p < 0.001$ for 3 and 6 mm beam widths and $p < 0.01$ at 12, 18 and 24 beam widths (see Fig. 1a). The motor performance of adult rats (13 mo) was equivalent to young and at 14

mo just after the transplant (see Fig. 1b and 1c). Although rats were tested every month, actually no significant changes were seen up until 26 mo. Thus only graphs of six months intervals after the 14th month are shown. At 20 mo of age a slight but non significant dispersion of motor performance could be seen (see Fig. 1d). Changes began to occur at ages 24 and 26 mo (only the latter is graphed, Fig. 1e), since it can be observed that only group 2, grafted with NGF treated chromaffin cells, retained beam crossing values (in sec) similar to those seen for young naive rats. In fact no significant differences could be found at any beam widths between these two groups. Motor improvements were also observed in groups 1 and 3, since a comparison between these two groups and old naive rats showed significant differences at each beam width ($p < 0.01$). However, at beam widths 3 and 6 a significant additional improvement was observed in group 2 with respect to groups 1 and 3 ($F=3.41$ $p < 0.01$ and $F=2.80$ $p < 0.01$ respectively).

INSERT FIGURE 1 ABOUT HERE

Electrophysiology

A total of 297 single units were recorded. Of these, 170 showed DA-like action potentials, while 127 showed non-DA like action potentials (see Figs. 2 and 3). In both cases, no action

/1potential morphological differences were found between young, aged or grafted rats. Of the 77 DA-like neurons recorded in young rats, 39% had a firing rate of 0-3 spikes/sec; 51% a firing rate of 3-6 spikes/sec and 10% a firing rate of 6-9 spikes/sec. This pattern of activity was quite different from the one observed in the 19 neurons recorded from aged rats, where 73.6% showed a firing rate of 0-3 spikes/sec; 15.78% a firing rate of 3-6 spikes/sec and 10.6% a firing rate of 6-9 spikes/sec. This change in the pattern distribution of activity was significant at $F=20.92$; $p < 0.001$. Of the 28 neurons recorded from Group 1 the firing rate was similar to that of aged naive rats, since 78% fired at 0-3 spikes/sec; 14.28% at 3-6 spikes/sec and 7.7% at 6-9 spikes/sec. Similarly of the 17 neurons recorded in Group 3, 82.3% fired at 0-3 spikes/sec, 11.7% at 3-6 spikes/sec and 6.8% at 6-9 spikes/sec. However, of the 29 neurons recorded from Group 2, only 51% fired at 0-3 spikes/sec; 41.3% at 3-6 spikes/sec and 7.6% at 6-9 spikes/sec. This firing rate was not significantly different from that observed in naive young rats, but was different from naive aged rats and Groups 1 and 3 ($F=12.26$, $p < 0.001$).

INSERT FIGURE 2 ABOUT HERE

Of the 47 non-DA like neurons recorded from naive young rats,

6.3% showed a firing rate of 0-5 spikes/sec, 38.3% of 5-10 spikes/sec, 32% of 10-15 spikes/sec and 23.4% of 15-20 spikes/sec. Of the 18 neurons recorded from naive aged rats, 15.3% showed a firing rate of 0-5 spikes/sec, 61.11% of 5-10 spikes/sec, 11.2% of 10-15 spikes/sec and 12.39% of 15-20 spikes/sec. The differences in spike frequency distribution between young and old naive rats was significant at $F=19.92$, $p < 0.001$. Of the 24 neurons recorded from Group 1, 12.5% showed firing rates of 0-5 spikes/sec, 62.5% of 5-10 spikes/sec, 17% of 10-15 spikes/sec and 8% of 15-20 spikes/sec. A similar distribution frequency of firing rates was seen in Group 3, whose 17 neurons recorded showed 15% firing rates of 0-5 spikes/sec, 67% of 5-10 spikes/sec, 10% of 10-15 spikes/sec and 8% of 15-20 spikes/sec. On the other hand, of the 21 cells recorded from Group 2, 9.5% had firing rates of 0-5 spikes/sec, 52.40% of 5-10 spikes/sec, 19% of 10-15 spikes/sec and 19.1% of 15-20 spikes/sec. The frequency distribution of Group 2 was significantly different from Groups 1 and 3 ($F=8.53$; $p < 0.001$).

In sum, the firing frequency distribution of both DA and non-DA neurons of rats transplanted with NGF treated cultured cells remained closer to the firing pattern observed in the young rats. This is clearly seen in Figs 2c and 3c.

INSERT FIGURE 3 ABOUT HERE

Histological analysis

GFAP immunostaining of striatal parenchyma of sham rats (Group 1) showed a small scar and in around the area of cannula tract, while the striatal parenchyma of rats transplanted with dissociated adrenal medullary cells (Group 3) showed a greater scar area additionally invaded by macrophages. In the latter animals TH-immunostaining showed no evidence of chromaffin cell survival.

In contrast rats transplanted with NGF cultured chromaffin cells (Group 2), showed a scar area similar to group 3, with astrocytes and macrophages around the debris of non-surviving cells within the transplant. However, in these animals there was some chromaffin survival, whose rate was close to 0.01% (or around 500 cells). Some of these cells migrated 0.1-0.3 mm into the host striatum showing a neuron-like phenotype. On the other hand, those which stayed within the area of the cannula tract kept the endocrine phenotype. Figure 4 Aa is an example of a differentiated TH positive chromaffin cell, located within the striatal parenchyma of a 26 month old rat. For comparison purposes a nigral cell from the same animal can be seen in Figure 4 Ac. In addition an example of a graft can be seen in Figure 4 Ab. In spite of the fact that some chromaffin cells developed short neurites it is difficult to establish whether they made synaptic contact with the host striatum. Moreover, no apparent evidence of host dopaminergic fiber sprouting could be observed.

Since at the end of the experiments each rat was recorded with

a micropipette, the tract left by the microelectrode was clearly detected in and below the region where positively labeled TH nigral neurons were observed, suggesting that cells recorded were in fact those from ventral mesencephalic neurons.

In addition a cell count of ventral mesencephalic dopaminergic cells was made with the imaging processor in order to determine whether age differences could be observed and whether transplants produced any modifications. The results are summarized in table 1, where it can be clearly seen that aged rats had a very significant drop in dopaminergic cell number $p<0.01$. Similar results were observed in sham grafted rats (Group 1, Fig. 4Ba) and in those which were transplanted with dissociated chromaffin cells (Group 3, Fig. 4Bc). A slight, but significant preservation in the number of dopaminergic cells could be seen in rats transplanted with NGF cultured chromaffin cells (Group 2, Fig. 4Bb) when compared to aged rats and groups 1 and 3 ($p < 0.01$). However, when compared to young animals there was a significant drop in cell numbers ($p < 0.01$).

INSERT FIGURE 4 AND TABLE 1 ABOUT HERE

DISCUSSION

The present study shows for the first time, that grafting tissue at midlife of rats can induce delays in some of the

manifestations of aging. In our study, by using a motor test which is sensitive to dopaminergic functions (16), it was clear that even 14 months after the transplants, 26 months old rats maintained a motor activity similar to that seen in much younger rats. More interestingly this occurred only in the group of rats who received transplants of chromaffin cells cultured in the presence of NGF, probably because they were the only ones which survived after 14 mo of being transplanted (see Fig. 4 Aa,b). It should be noted that the groups of rats which received either sham or simply dissociated chromaffin cells also showed retardation of motor detriments when compared to naive aged rats. This effect can be attributed to some kind of learning or practice effect due to the monthly evaluation on the beams, rather than on an effect produced by the transplant procedure, since naive aged rats performed very poorly on the motor task. This is supported by three facts. The first is related to observations which have shown that in aged rats behavioral improvement occurs as a result of repetitive evaluations (67). The second is that sham transplants per se in old naive rats does not improve motor performances in the task used in this study (28) or in others (26). The third is related to the fact that dissociated chromaffin cells of Group 3 did not survive after 14 months of being transplanted. Together, these latter two facts suggest that the improvement of motor behavior in Groups 1 and 3 cannot be attributed to a lesion effect or to viable chromaffin cells, but rather to the practice effect, caused by the repeated trials on the

beams. Despite the latter, these animals showed great difficulties in crossing the very narrow beams, while Group 2 animals did not.

An explanation as to the reason the viable transplanted cells, delayed motor deterioration, is evidently difficult. However, it should be pointed out that the Group 2 rats, with viable NGF cultured cells, not only retained good motor behavior, but also showed spike frequency discharges of both DA and non-DAergic cells closer to those seen in young rats. This not only confirms a previous study (49) which showed that grafts of adrenal medullary tissue into aged rats improves the firing rate of substantia nigra cells, but also suggests that the absence of motor detriments in aged rats receiving NGF cultured chromaffin cells may be due to the presence of neural firing frequencies within ranges closer to those seen in young rats. It should be additionally noted that although rats from Group 1 and 3 performed significantly better than aged naive rats, their firing rate of nigral cells was equal to the latter. This provides additional support for the idea that the improvement in motor performance in these animals was the result of a practice effect, rather than on an improvement in motor capabilities per se, although in the beam test it may be difficult to separate both components. Perhaps, the greater survival of nigral cells in combination with retention of firing rates comparable to those seen in younger rats, provided for Group 2 the background for the maintenance of motor performances over and above those induced by the practice effects seen in the other groups.

It is conceivable that the survival of nigral cells in Group 2 was related to effects of trophic factors, although we have no direct proof of this in the study. Several authors have suggested that the interaction of transplanted tissue with the host brain may lead to production of trophic factors (10,24,51,85,86) which may be involved in recovery of functions lost as a result of lesions or aging (6,19,28,31,37,47,48,73,87). Thus, there is a possibility that NGF cultured chromaffin cells favored trophic activity, which led to the delay of the aging manifestations examined in this study.

Finally, although cell survival of transplanted chromaffin or nigral cells has been reported to deteriorate after 6 to 8 months (14,31,45,61,73), the observation that cell survival occurred even 14 months after the graft, represents the longest time reported for transplanted chromaffin cell survival. Again conceivably, the fact that chromaffin cells were cultured with NGF, represents the possible determining factor leading to cell survival and differentiation, since it has long been shown that NGF is involved in both (61,79,84).

In summary, this is the first study showing that transplants in adulthood attenuate some of the manifestations of aging. It is also interesting to note from this study that in vivo survival time of grafted cells seems to increase as a result of their being cultured in the presence of trophic factors.

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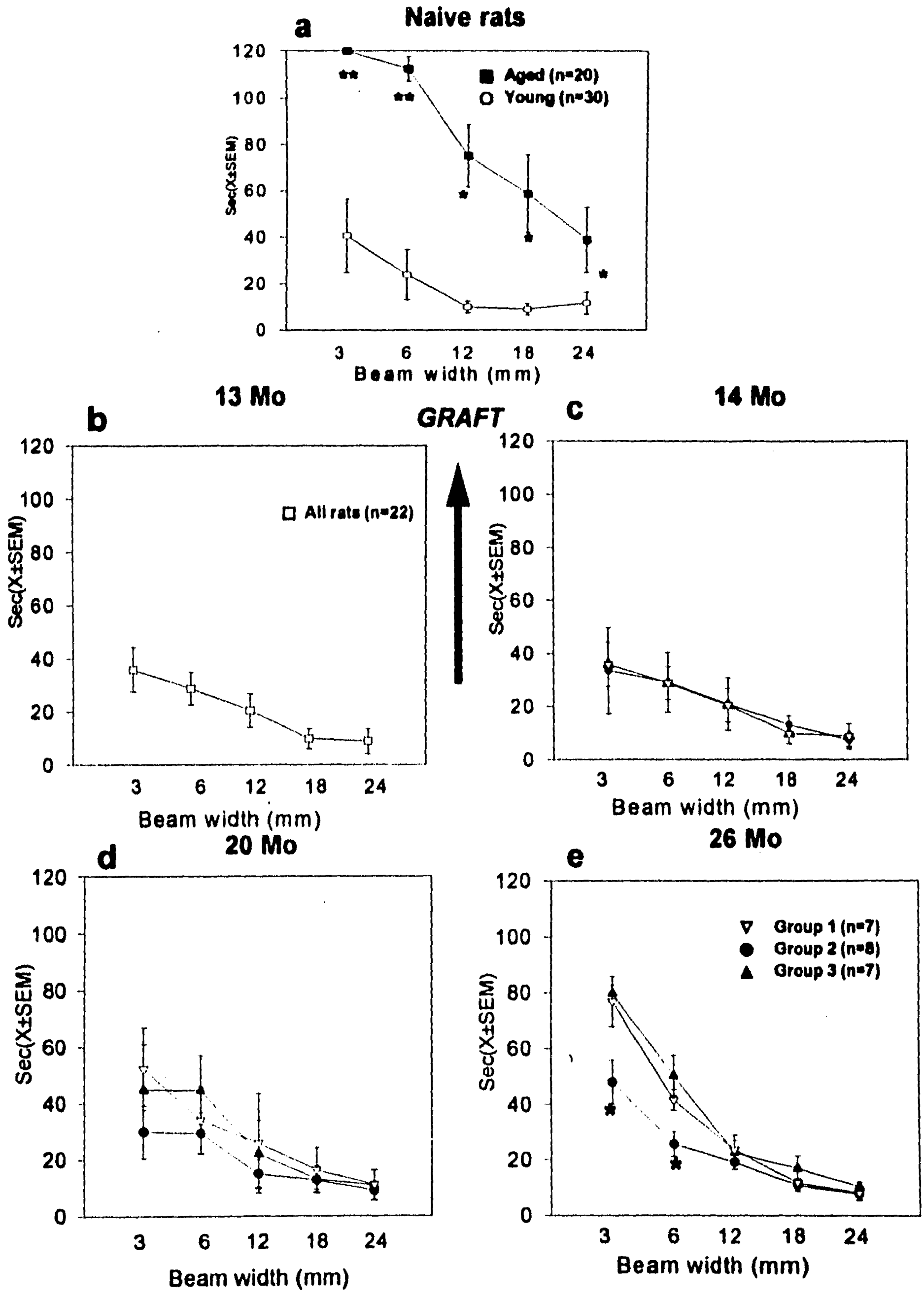

a
Naive rats
Aged (n=20)
Young (n=30)
Sec(X±SEM)
120
100
80
60
40
20
0
3 6 12 18 24
Beam width (mm)
13 Mo
b
All rats (n=22)
GRAFT
c
14 Mo
Beam width (mm)
d
20 Mo
e
26 Mo
Group 1 (n=7)
Group 2 (n=8)
Group 3 (n=7)

Fig 2

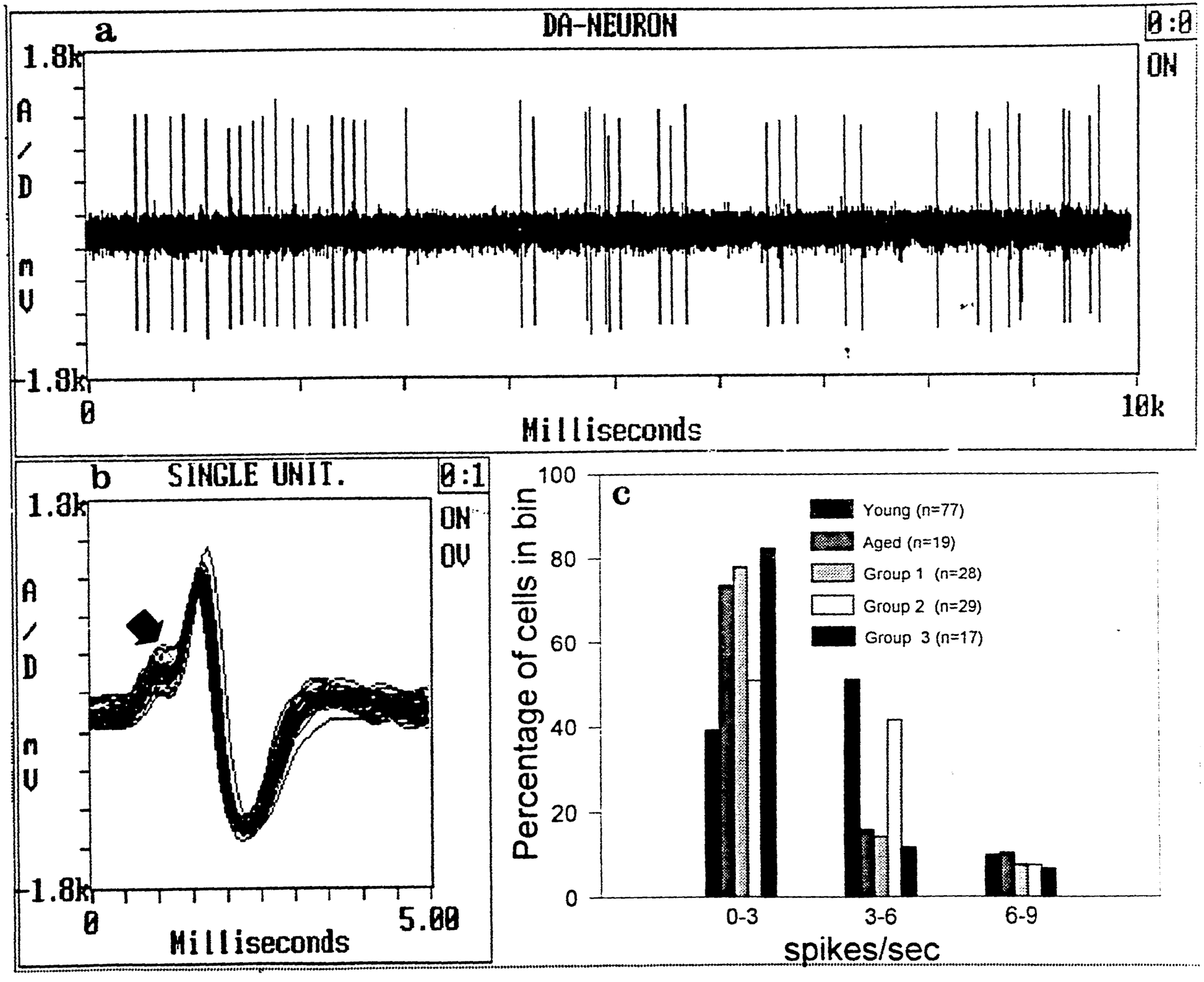

a
DA-NEURON
0:0
ON
1.8k
A/D mV
-1.8k
0
10k
Milliseconds
b
SINGLE UNIT.
0:1
ON
0V
1.8k
A/D mV
-1.8k
0
5.00
Milliseconds
c
Young (n=77)
Aged (n=19)
Group 1 (n=28)
Group 2 (n=29)
Group 3 (n=17)
Percentage of cells in bin
100
80
60
40
20
0
0-3
3-6
6-9
spikes/sec

Fig. 3

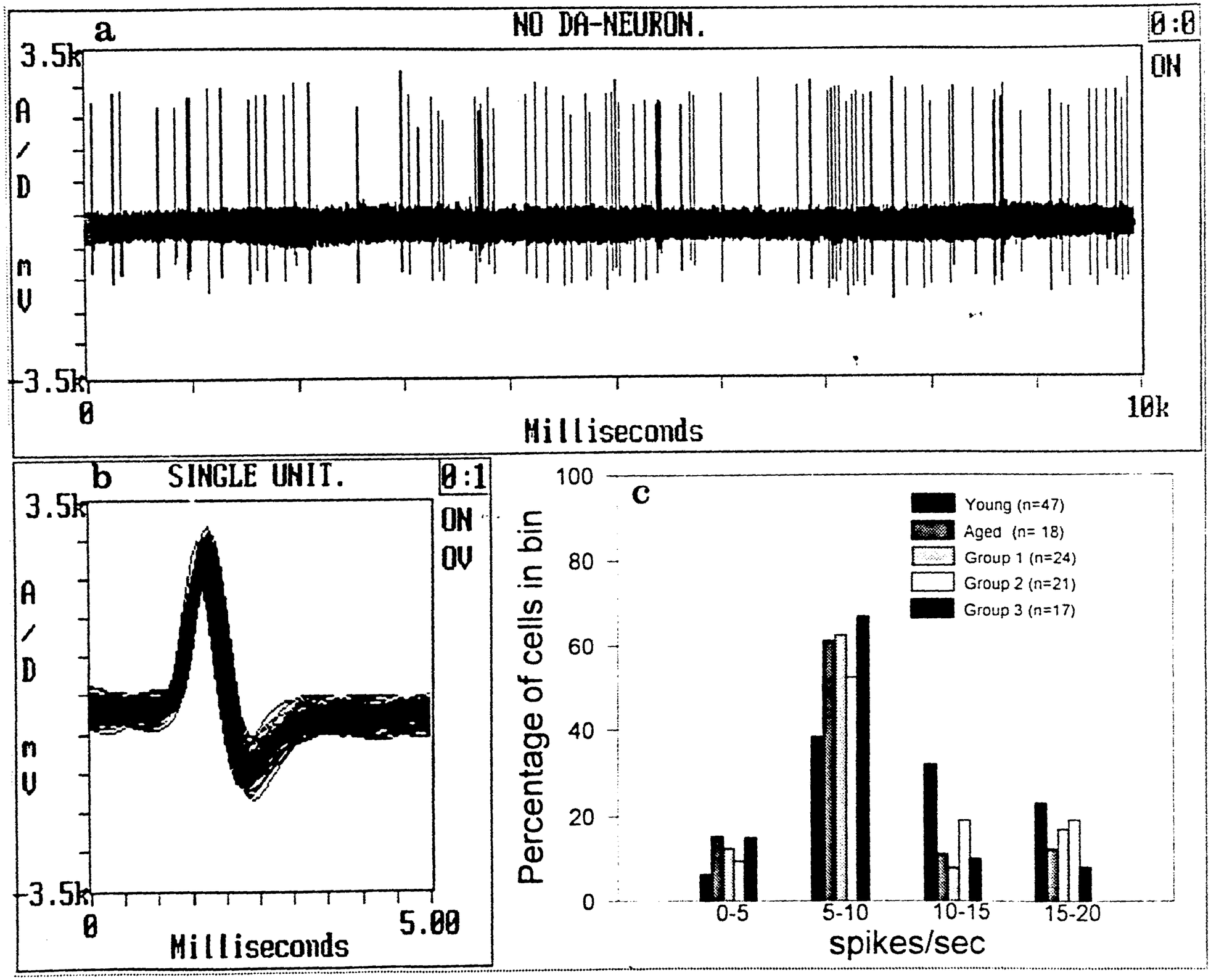

a
NO DA-NEURON.
0:0
ON
3.5k
A
/
D
m
V
-3.5k
0
10k
Milliseconds
b
SINGLE UNIT.
0:1
ON
OV
3.5k
A
/
D
m
V
-3.5k
0
5.00
Milliseconds
c
Young (n=47)
Aged (n= 18)
Group 1 (n=24)
Group 2 (n=21)
Group 3 (n=17)
Percentage of cells in bin
100
80
60
40
20
0
0-5
5-10
10-15
15-20
spikes/sec

Fig 4

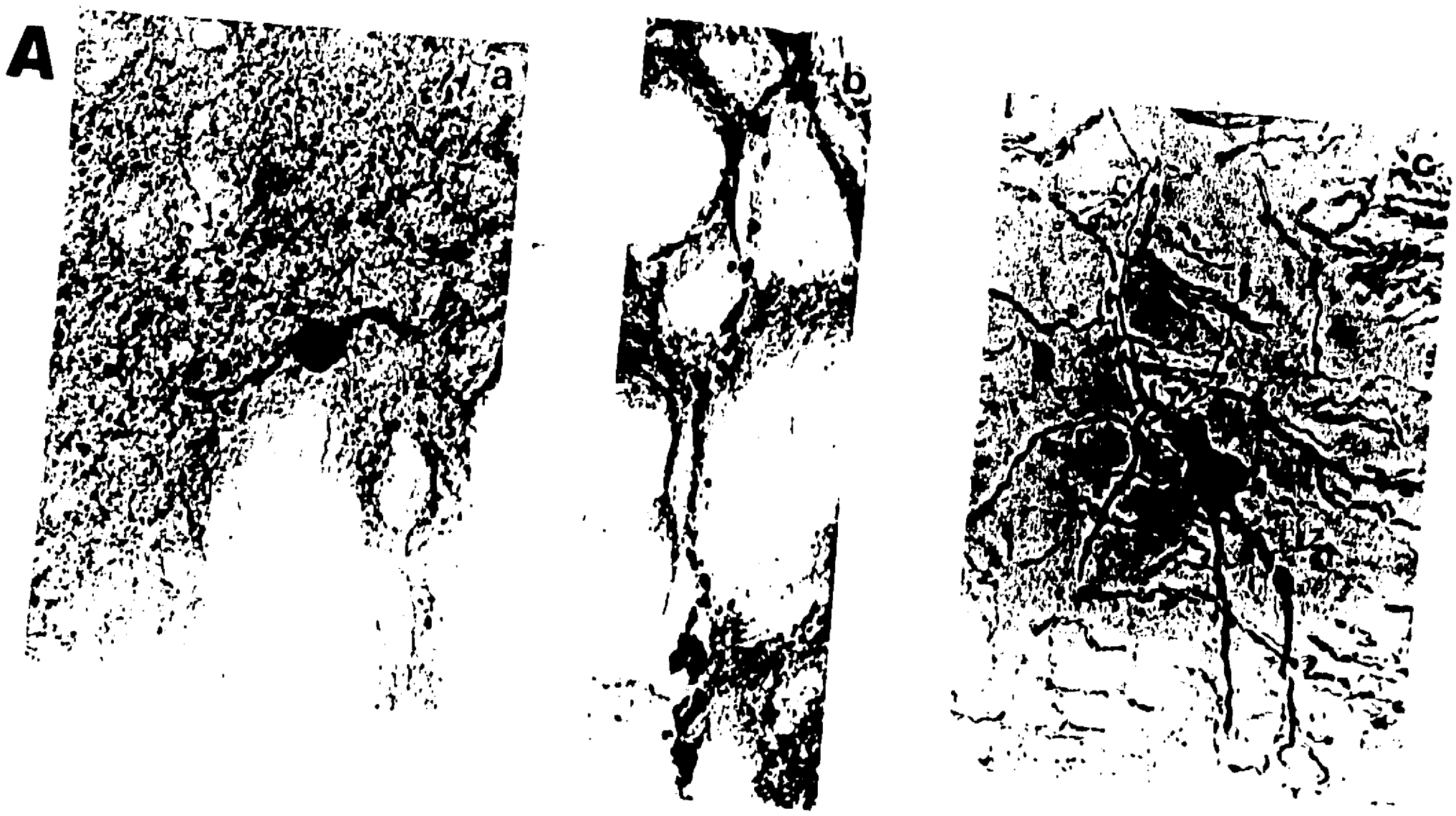


FIGURE LEGENDS

Figure 1. Graph illustrating the performance of rats of varying ages when traversing a 15° inclined 2 meter beam of varying widths. The graph (a) shows the difference between naive young rats of 3 to 5 mo of age versus naive old rats of 26 mo of age. The time it took them to traverse the beams was significantly different at all widths, meaning that young rats performed much better than aged ones. At 13 mo of age (b), no changes were seen with respect to 3 to 5 mo old rats. After the transplants neither at 14 (c) nor at 20 mo (d) of age a difference in motor performance could be observed. However, by age 26 mo (e) rats from Groups 1 and 3 performed significantly worse on 3 and 6 mm beam widths than rats from Group 2, who beared NGF cultured chromaffin cells and who continued to perform equally to 3-5 mo old rats at all beam widths. * $p < 0.01$; ** $p < 0.001$.

Figure 2. Graph showing in (a) a typical example of the actual firing rate of a DA neuron of the substantia nigra pars compacta from 26 mo old rat. The single unit in (b) shows the characteristic notch of DA neurons described in text

(see arrow) on the initial rising phase of the action potential (Amplitude cal is 1 cm = 200 μv). The bar graph (c) illustrates the spike frequency distribution according to an arbitrary division of spike frequencies. This bar graph clearly shows that the group grafted with NGF cultured chromaffin cells (Group 2) retained spike frequencies which were more comparable to those seen in young rats. Significance levels in text.

Figure 3. Graph showing in (a) a typical example of the actual firing rate of a non-DA neuron in a 26 mo old rat. The morphology of the single unit can easily be distinguished from the DA neuron (b). The bar graph (c) illustrates the spike frequency distribution according to an arbitrary division of spike frequencies. This bar graph clearly shows that the group grafted with NGF cultured chromaffin cells (Group 2) retained spike frequencies which were more comparable to those seen in young rats. Significance levels in text. Note the firing frequency difference with that seen in Fig. 2.Amplitude cal. is 1cm= 200 μv. Significance levels in text.

Figure 4. Photomicrographs of TH-immunoreactive cells. In Panel A is shown an example (a) of a neuron-like differentiated chromaffin cell, which survived 14 mo after being grafted; in (b) an example of the grafted area and in (c) an example of a nigral cell. All these micrographs come from the same 26 mo old rat. In a and c magnification x 250, in b magnification x 62.5. Panel B shows examples of the ventral mesencephalic region where DAergic nigral cells can be seen from (a) sham (b) NGF cultured chromaffin cells (c) dissociated grafted groups. All examples come from 26 mo old rats and the differences in number of cells are clearly evident. Note the greater survival rate in the rat exemplified in (b). In a, b and c magnification x 125.

Table 1. Number of TH-positive cells in ventral mesencephalic region in transplanted and control groups (X ± SEM)

YOUNG[1] n=5	AGED NAIVE n=5	SHAM n=5	NGF TREATED[2] CHROMAFFIN n=5	DISSOCIATED CHROMAFFIN n=7
4132 ± 199	2231 ± 197	2154 ± 126	3085 ± 131	3085 ± 131

[1] $p < 0.01$ (Young vs other groups).

[2] $p < 0.01$ (NGF treated vs other aged groups).

SEGUNDA PARTE.

Capítulo 4.

MECANISMOS DE ACCION DE LOS FACTORES NEUROTROFICOS.

Antecedentes.

En 1948 Bruecker mostró que el transplante de tejido tumoral de ratón a embrión de pollo es inervado por las neuronas sensoriales del hospedero. Esto dió lugar para que Levi-Moltancini y Hamburger (1953), postularan que el responsable de dicha respuesta es una substancia secretada por el tumor, la que purificaron y caracterizaron en 1954 junto con Cohen y cols. denominándola como "factor de crecimiento nervioso" (nerve growth factor, NGF).

Años más tarde se mostró que el NGF favorece la sobrevivencia de las neuronas colinérgicas del sistema nervioso central, ya que cuando se seccionar sus axones a nivel de la fimbria fornix hipocampica y se inyecta NGF intra-cerebro-ventricularmente, la degeneración de las neuronas colinérgicas se previene (Schwab y cols, 1979; Honegger y Lenoir, 1982; Gnahn y cols, 1983; Hefti, 1983; Hefti y cols, 1984). Al mismo tiempo, el gene que codifica para la síntesis del NGF se clonó y caracterizó (Scott y cols, 1983).

En el inicio de la década de 1980 los factores neurotróficos, derivado del cerebro (BDNF; Barde y cols, 1982) y ciliar (CNTF; Barbin y cols, 1984) fueron purificados y caracterizados. Se encontró que el BDNF y el NGF tienen semejanzas estructurales (Barde y cols, 1982), y utilizando como "primers" fragmentos de 10-20 pares de bases de estas zonas de homología en bibliotecas genómicas, se descubrió la neurotrofina 3 (NT-3;

Erfors y cols, 1990; Hohn y cols, 1990; Jones y Reichardt, 1990; Maisonpierre y cols, 1990; Rosenthal y cols, 1990) y las neurotrofinas 4 y 5 (NT-4; NT-5; Hallbook y cols, 1991; Berkemeier y cols, 1991). Estos factores constituyen hoy la familia de las neurotrofinas.

En los últimos diez años se han descubierto diversas familias de factores neurotróficos (Tabla 1), y dadas sus características de multifuncionalidad se les ha definido de un modo muy general. Una de las definiciones más aceptadas es la que sugieren Sporn y Roberts en 1988, que es aplicable a todos los factores citados en la tabla 1 y que excluye a compuestos tales como hormonas, esteroides y gangliósidos.

> *"Los factores neurotróficos son proteínas solubles endógenas que regulan la sobrevivencia,la plasticidad morfológica ó la síntesis de proteínas específicas en las neuronas".*

Especificidad de los factores neurotróficos.

La clasificación celular en relación a los efectos ponderados por los factores tróficos se estableció originalmente con las neurotrofinas y los miembros de la familia del FGF (Hebert y cols, 1990). Sin embargo, estos efectos dependen de la expresión funcional de receptores específicos, por lo que no es raro observar que varios factores producen los mismos efectos sobre una población celular (VER MÁS ADELANTE LA TRANSDUCCIÓN DE SEÑAL POR LA AUTOFOSFORILACIÓN DE TIROSINA EN EL RECEPTOR TRK.). Un

ejemplo de ésto es la posibilidad de inhibir el proceso de muerte de las neuronas colinérgicas utilizando NGF, BDNF ó FGF-2 (Alerson y cols, 1990; Knüsel y cols, 1991).

Se ha sugerido que el número de factores tróficos en realidad no es tan grande, argumentandose que los efectos neurotróficos están determinados por periodos críticos, en los que el factor cumple con una actividad distinta cada vez, es decir, en cada periódo o "ventana" espacio-temporal, la expresión de los receptor activan distintos mecanismos de transducción (Fallon y Loughlin, 1993). Esto es acorde al hecho de que algunos factores son determinates en momentos específicos del desarrollo y porqué algunos cambian sus efectos a lo largo de toda la vida (Leslie, 1993).

El concepto anterior se puede ilustrar con los resultados publicados por Sofroniew y col. (1990), este grupo mostró que después de la destrucción completa del hipocampo y sin que se hayan seccionado los axones de las neuronas colinérgicas telencefálicas, se produce atrofia celular pero no la muerte de éstas. Este resultado se puede explicar por el hecho de que los aportes de factores provenientes de las células de la glía hipocampal y septal, asi como de los de que las células que inervan a las neuronas colinérgicas, participan evitando la muerte celular. Esto se apoya además en el hecho de que después de un daño, el aporte de los factores tróficos se incrementa durante algunso días (Campenot, 1982a,b, 1987; Gall y Isackson, 1989; Zafra y cols, 1990; Hofer y cols, 1990; Maisonpierre y cols, 1990a).

Otro ejemplo de la ventana espacio-temporal de los receptores, se ilustra con los cambios que con la edad ocurren en el papel funcional de los factores neurotróficos en el sistema nervioso periférico. En estadíos fetales las neuronas simpáticas degeneran si se

les depriva de NGF, en tanto que en el estado adulto son menos vulnerables a dicha deprivación. Esto sugiere que el NGF en el estado adulto participa en la regulación de la inervación simpática, en tanto que en el estadio fetal favorece la sobrevivencia celular (Goedert y cols, 1978; Bjerre y cols, 1975a,b).

Por otro lado, en el cerebro de varios mamíferos incluyendo al hombre, algunas poblaciones neuronales son más susceptibles a la degeneración. Por ejemplo, las neuronas colinérgicas del telencéfalo, las cuales están involucradas en la memoria espacial de corto plazo y la discriminación de lugar, se encuentran deterioradas en la mayoría de las ratas viejas (Ingram y cols, 1981; Gage y cols, 1984, 1988; Rapp y cols, 1987; deToledo-Morell y cols, 1988; Aggleton y cols, 1989; Fischer y cols, 1991b). Estas deficiencias cognitivas pueden ser compensadas por la inyección intra-cerebro-ventricular de NGF (Fischer y cols, 1987, 1989, 1991a; Lärkfors y cols, 1987; Koh y cols, 1989; Hefti y Mash, 1989; Markowska y cols, 1994).

Con base a esta evidencia se ha propuesto que los factores tróficos podrían participar en el proceso de envejecimiento. Sin embargo y ya que las neuronas retienen la capacidad de responder a un aporte suplementario de factores (McNeill, 1983), es posible que su deficiencia sea generalmente consecuencia más que causa del envejecimiento (VER SECCION DE ENVEJECIMIENTO).

Por lo que toca a las neuronas dopaminérgicas del mesencéfalo ventral, la evidencia también apoya al concepto de la ventana espacio temporal de receptores. Se mostró que en condiciones de cultivo las neuronas dopaminérgicas fetales se benefician con la presencia de BDNF o NT-3, pues el número inicial sembrado no

Tabla 1. Ejemplos de proteínas que exhiben actividad neurotrófica.

FACTOR DE CRECIMIENTO	REFERENCIA
Proteínas caracterizadas como factor neurotrófico.	
FACTOR DE CRECIMIENTO NERVIOSO (NGF)	Thoenen y cols, 1987. Whittemore y Seiger, 1987. Hefti y cols, 1989.
F. NEUROTROFICO DERIVADO DEL CEREBRO (BDNF)	Barde y cols, 1982. Leibrock y cols, 1989.
NEUROTROFINA 3 (NT-3)	Ernfors y cols, 1990. Hohn y cols, 1990. Maisonpierre y cols, 1990 Rosenthal y cols, 1990.
NEUROTROFINA 4 (NT-4)	Hallbrook y cols, 1991.
NEUROTROFINA 5 (NT-5)	Berkemeier y cols, 1991.
F. NEUROTROFICO CILIAR (CNTF)	Lin y cols, 1989. Stöckli y cols, 1989.
F. N. DE PEGADO-HEPARINA	Kovesdi y cols, 1990.
F. BASICO DE CRECIMIENTO DE FIBROBLASTOS (FGF-2)	Morrison y cols, 1986. Walicke, 1988.
F. ACIDO DEL FGF (FGF-1).	Walicke, 1988.
F. DE CRECIMIENTO SEMEJANTE A LA INSULINA (IGF), INSULINA	Aizenman y cols, 1986. Baskin y cols, 1987.
F. DE CRECIMIENTO EPIDERMAL (EGF)	Fallon y cols, 1984. Morrison y cols, 1987.
F. DE CRECIMIENTO TRANSFORMANTE a (TGFa)	Derynck, 1988. Fallon y cols, 1990.
INTERLEUCINA 1	Spranger y cols,1990.
INTERLEUCINA 3	Kamegai, 1990.
INTERLEUCINA 6	Hama y cols, 1989.
PROTEASA NEXINA I Y II	Monard, 1987. Oltersdorf y cols, 1989. Whitson y cols, 1989.
F. DE DIFERENCIACION NEURONAL COLINERGICO	Yamamori y cols 1989.

disminuyó después de varios días. Este efecto fue más espectacular después de que se agrego en el medio de cultivo a las neurotoxinas 6-hidroxidopamina o 1-metil,4-fenil-1,3,4,6-tetrahidropiridina (MPTP; Hyman y cols, 1991; Knüsel y cols, 1991). Sin embargo y de manera contrastante, en la rata adulta dichos efectos no han sido observados.

Receptores de factores neurotróficos.

Generalidades.

Se ha demostrado que los receptores de los factores neurotróficos son en su mayoría proteínas transmembranales con actividad de tirosina cinasa. Estas proteínas son el producto de diversos tipos de proto-oncogenes y en general se les conoce como "receptores tirosina-cinasa" (Tyrosine kinase receptors). Los receptores presentan como característica general cuatro dominios, siendo el dominio extracelular el responsable de interactuar específicamente con un tipo particular de factor (complejo factor-receptor), esta interacción provoca la autofosforilación de una o varias tirosinas en el dominio intracelular. La región de tirosina fosforilada constituye sitios de alta afinidad para proteinas que contengan regiones conocidas como dominio SH2 (src homology 2 domains), siendo estas últimas las que transducen la señal a otras regiones celulares (Cadena y Gill, 1992; VER TRANSDUCCIÓN DE SEÑALES MÁS ADELANTE).

Clasificación de los receptores de los factores neurotróficos.

Las características de la secuencia primaria del dominio de cinasa han sido utilizadas para clasificar a los receptores en más de 100 miembros y tres categorias principales (Hanks y cols, 1988; Cadena y Gill, 1992). En la primera categoría se agrupa a los receptores diméricos que presentan en los dominios intra y extracelular regiones ricas en cisteínas, estos receptores caracteristicamente tienen la región catalítica intracelular continua. En la segunda categoría están los receptores tetraméricos formados por dos subunidades alfa y dos beta, los cuales además presentan características de inmunoglobulina y cuyo dominio intracelular es rico en cisteínas. En el tercer grupo se encuentran los receptores de la familia trk que tienen las cisteinas en la región cercana a la región del amino terminal del dominio extracelular y es posible que éstos funcionen a través de la formación de dímeros (VER DIMERIZACION DE RECEPTORES).

Transducción de señal por la autofosforilación de tirosina en el receptor trk.

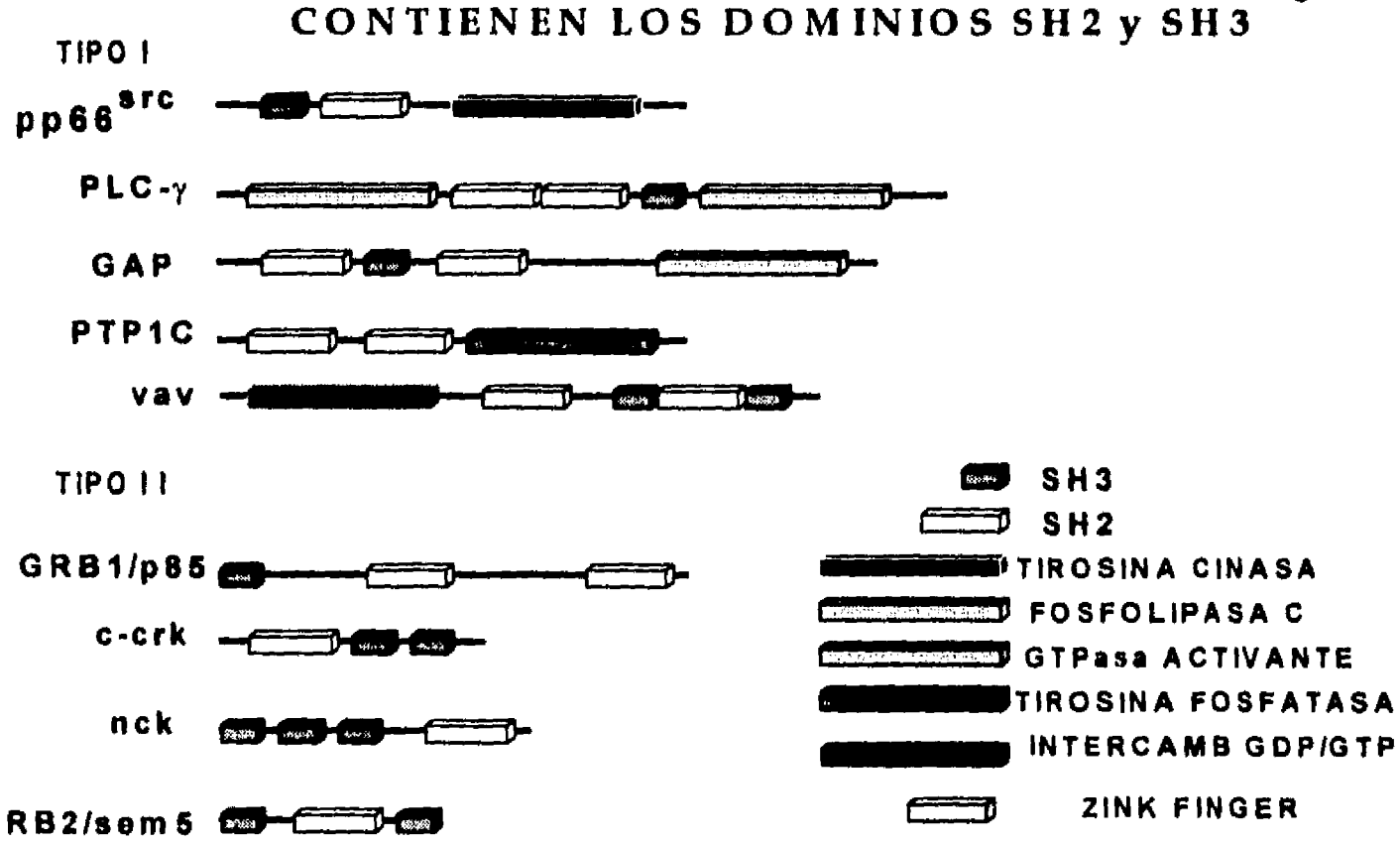
Después de que se forma el complejo factor-receptor la tirosina o tirosinas presentes en la región intracelular se auto-fosforilan comvirtiendose en sitios de alta afinidad para proteínas especificas que presentan el dominio SH2. Como resultado de ésto ocurren una serie de interacciones intracelulares que conducen a la proliferación, transformación ó diferenciación (Ullrich y Schlessinger, 1990; Schlessinger y Ullrich, 1992).

El dominio SH2 (src homology 2 domains) presente en las proteínas de transducción es una región de aproximadamente 100 residuos de aminoácidos. Este

dominio es característico en la fosfolipasa C-γ (FLC-γ), la tirosina cinasa ($pp60^{c\text{-}src}$), el intercambiador putativo GDP/GTP (vav), la GTPasa activante de ras, c-crk, nck y GRB2/sem5. Sin embargo, la manera en que estas proteínas reconocen a los distintos receptores es poco conocida (Suh y cols, 1988; Trahey y cols, 1988; Vogel y cols, 1988; Stahl y cols, 1988; Meisenhelder y cols, 1989; Margolis y cols, 1989, 1990a, 1990b; Wahl y cols, 1989; Burgess y cols, 1990).

El dominio SH2 generalmente se encuentra acompañado de otro denominado SH3 el cual consiiste de aproximadamente 50 aminoácidos que es importante en los procesos de la asociación de la proteína con el citoesqueleto, lo cual ayuda a la iteracción con los receptores fosforilados (Koch y cols, 1991; Heldin, 1991; Cantley y cols, 1991;Margolis, 1992). La figura 1 muestra un resumen de proteínas que presentan las regiones SH2 y SH3, que están involucradas en la transducción de señales acopladas a receptores que presentan actividad de tirosina cinasa.

FIGURA 1. PROTEINAS DE TRANSDUCCION QUE CONTIENEN LOS DOMINIOS SH2 y SH3



Estas proteínas se han clasificado en tipo I y tipo II. Las tipo I tienen actividad enzimática como la FLC-γ, $pp60^{c\text{-}src}$ y vav (Katzav y cols, 1989; Bustelo y cols, 1992; Margolis y cols, 1992; Adams y cols, 1992). Se ha mostrado que éstas se activan cuando el EGF, PDGF o FGF interactúan con sus receptores (Kim y cols, 1991; Kashishian y cols, 1992; Rotin y cols, 1992). Las tipo II lo constituyen c-crk, nck y GRB2/sem-5 que virtualmente sólo presentan las dominios SH2 y SH3, adjudicándoles una función de adaptadores o reguladores de otras subunidades catalíticas (Figura 1; Mayer y cols,1988; Lehmann y cols, 1990; Clark y cols, 1992; Lowenstein y cols, 1992).

Otras cinasas que se activan por la estimulación de factores de crecimiento incluyen a c-raf, $p21^{ras}$, proteína cinasa C y la proteína cinasa S6 del ribosoma, algunas de las cuales estan involucradas con la cascada de eventos asociados al crecimiento de procesos (Chao, 1992; Esto es tratado con detalle más adelante en la sección dedicada al receptor trkA).

Es necesario señalar que en el dominio intracelular de algunos de estos receptores, existen también secuencias regulatorias específicas involucradas en la fosforilación de serina y treonina, las que activan cinasas como MAP y ERK, ésto se ha detectado con los receptores del NGF, EGF o del factor de crecimiento derivado de plaquetas (PDGF; Schlessinger y Ullrich, 1992).

Dimerización de receptores.

Los factores EGF y PDGF son proteínas homodiméricos que al unirse con un receptor facilitan el pegado del segundo, esto ha sugiere que el receptor funcinal esta formado por un homodímero. Esta sugerencia se apoya en el análisis de la cinética de autofosforilación y fosforilación de substrato en relación con la cantidad de estos receptores, establece una reacción de segundo órden (Schlessinger, 1988; Ullrich y Schlessinger, 1990; Canals, 1992). Esto se ha comprobado con la co-expresión de receptores de EGF ó PDGF normales con mutantes carentes de la actividad de cinasa, lo que produjo receptores inactivos al establecerse diméros formados por un receptor normal con uno mutante (Honegger y cols, 1990; Kashles y cols, 1991; Ueno y cols, 1991).

Sin embargo, permanece por probar sí la dimerización de receptores es un mecanismo válido para la transducción de señales de otros receptores con actividad de tirosina cinasa, para los que presentan actividad de serina/treonina cinasa (entre los que se incluye al TGFß), para los que están asociados a guanilil ciclasa (como sería el receptor del péptido natriurético) y para aquellos con actividad de tirosina fosfatasa.

Ya que los estudios incluidos en la presente tesis están relacionados particularmente con la familia de las neurotrofinas, a continuación sólo se hara mención de las características de los receptores de las neurotofinas y de sus posibles mecanismos de acción.

Receptores de las neurotrofinas:

1.- Receptores del NGF, $P75^{NGFR}$ y trkA.

Los primeros estudios en torno a los efectos del NGF revelaron que los receptores de este factor están presentes en tejido nervioso y en líneas celulares tumorales. Se encontró que los receptores del NGF tienen dos estados de afinidad, 10-15% son de alta afinidad ($Kd=10^{-11}$ M) y el resto son de baja ($Kd=10^{-9}$ M; Sutter y cols, 1979; Landreth y Shooter, 1980; Schechter y Bothwell, 1981; Massagué y cols, 1981), y pese a la menor presencia de receptores de alta afinidad, la respuesta biológica es dependiente de la activación de éstos (Sonnenfeld e Ishii, 1985; Green y Greene, 1986). Sin embargo, queda por establecer que tipo de receptor participa en la internalización y transporte vesiculado al núcleo del complejo NGF-receptor (Hendry y cols, 1974; Thoenen y Barde, 1980; Escandón y Chao, 1990; Barde, 1989).

El peso molecular aproximado de los complejos NGF-receptor de alta y baja afinidad son de 158 y 100 Kd respectivamente (Sutter y cols, 1979; Rodriguez-Tébar y Barde, 1988; Rodriguez-Tébar y cols, 1992). En un principio se propuso que el receptor de alta afinidad es un heterodímero formado por $p75^{NGFR}$ y el producto del proto-oncogen $p140^{trkA}$, el receptor trkA, pues la cinética de estos componentes expresados de manera independiente en una línea celular, mostró sólo la presencia de receptores de baja afinidad, pero cuando se fusionaron las membranas de las células que expresan cada receptor, o cuando se co-expresaron en una misma célula, los estados de alta y baja afinidad del receptor se restablecieron (Hempstead y cols, 1989, 1991; VER FIGURA 2).

Sin embargo, el modelo del receptor de alta afinidad compuesto por $p75^{NGFR}$ y $p140^{trkA}$ podría no ser válido, al considerar que la expresión de $p140^{trkA}$ en oocitos de *Xenopus* ó en la línea celular de fibroblastos NIH3T3, es suficiente para establecer las características del receptor de alta afinidad con la capacidad de evocar respuestas morfológicas (Klein y cols, 1990; Cordon-Cardo y cols, 1991; Nebreda y cols, 1991; Birren y cols, 1992). Resultados similares se han reportado en células PC12 tratadas con un anticuerpo específico contra $p75^{NGFR}$ (anticuerpo 192-Ig; Chandler y cols, 1984; Weskamp y Reichardt, 1991).

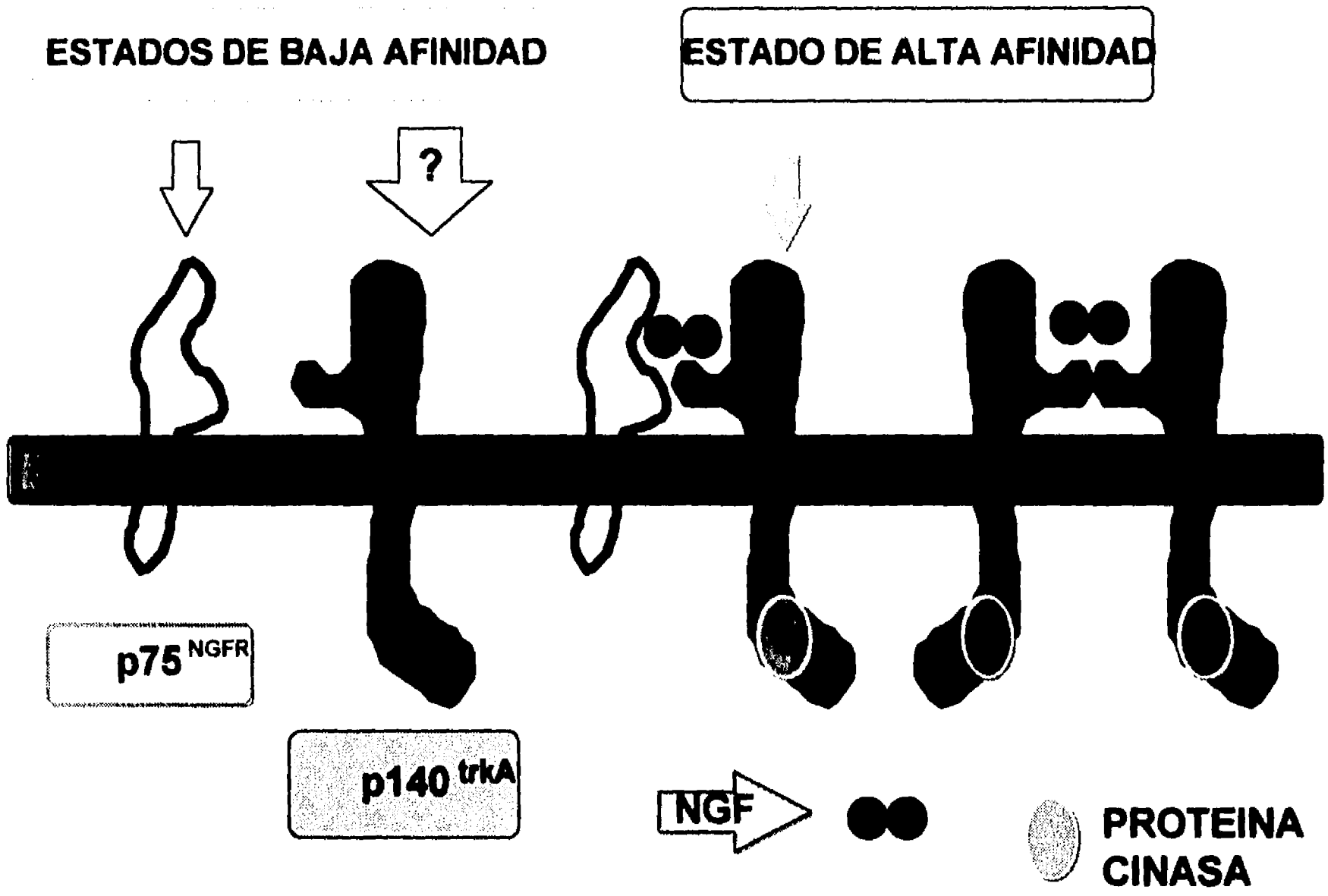
Receptor $P75^{NGFR}$.

El receptor $p75^{NGFR}$ debe su nombre al hecho de que su peso molecular en el ser humano es de aproximadamente 75 Kdn y curiosamente puede unirse con afinidad similar con las cuatro neurotrofinas (Rodriguez-Tébar y cols, 1990, 1992; Squinto y cols, 1991; Ernford y cols, 1990; Hallböök y cols, 1991).

Este receptor consiste de una cadena polipeptídica de 400 residuos de aminoácidos, cuyo dominio extracelular se caracteriza por la presencia de cuatro "loops" de seis residuos de cisteina y tres puentes disulfuro, en cuyo orden y estabilidad reside el sitio que reconoce al factor (Large y cols, 1989; Yan y cols, 1991). El dominio transmembranal es único y se continua con el dominio intracelular que presenta en la región del carboxilo terminal una secuencia semejante al mastoporan (región que interactua con proteínas G; Feinstein y Larhammer, 1990). Pese a esta característica, no

se ha demostrado que este receptor tenga un mecanismo de transducción a través de proteína G (Feinstein y Larhammer, 1990).

FIGURA 2. MODELO DEL RECEPTOR DE ALTA AFINIDAD PARA EL NGF



Es importante señalar que el dominio extracelular del receptor $p75^{NGFR}$ presenta homología con los receptores activadores de células B (CDw40) y células T, con el antígeno Fas y con los receptores del factor de necrosis tumoral TNFRI y TNFRII, junto con los que forma una superfamilia (Itoh y cols, 1991; Rabizadeh y cols, 1993). Sin embargo el dominio intracelular de $p75^{NGFR}$ es diferente del de los otros receptores (Stamenkovic y cols, 1989; Loetscher y cols, 1990; Schall y cols, 1990; Smith y cols, 1990; Mallet y cols, 1990; Hempstead y cols, 1991).

Se ha encontrado que en células termosensibles inmortalizadas y que no expresan al receptor trkA, la expresión del receptor $p75^{NGFR}$ esta asociada con la activación de la muerte celular sí se retira al suero del medio de cultivo. Sin embargo, si el suero se substituye por NGF, el fenómeno de apoptosis se evita complentamente (Rabizadeh y cols, 1993).

Otros estudios señalan que una fracción variable de $p75^{NGFR}$ forma homodímeros en la membrana celular (Grob y cols, 1985; Bothwell, 1995). Esta observación es interesante pues cuando el receptor esta en su estado de monómero se autofosforila en los residuos de serina en ausencia de NGF (Grob y cols, 1985).

Estos resultados sugieren que la autofosforilación inhibe la dimerización estimulando la apoptosis, o que la dimerización es estimulada por el NGF previniendo la autofosforilación y por ende la apoptosis (Bothwell, 1995).

Se ha observado que las células de Schwann que expresan $p75^{NGFR}$ migran en respuesta al NGF e incrementan la expresión del receptor después de un daño (Johnson y cols, 1988; Anton y cols, 1994). Esto sugiere que la sobreexpresión del receptor participe en los procesos de muerte celular en los sitios dañados, aunque también podría estar actuando como captador de factores, incrementando la concentración de las neurotrofinas en la vecindad de los sitios de alta afinidad del tejido dañado (Squinto y cols, 1991).

Receptor TrkA.

El producto del proto-oncogen $p140^{trkA}$ (trkA) se descubrió en un carcinoma de colon humano (Martin-Zanca y cols, 1986) y poco tiempo después se mostró que se expresa selectivamente en las neuronas colinérgicas del sistema nervioso (Kaplan y cols, 1991; Klein y cols, 1991; Hempstead y cols, 1991; Meakin y Shooter, 1991; Holtzman y col, 1992).

La caracterización de la cascada de eventos intracelulares asociados a la activación de trkA se ha realizado principalemente en líneas celulares, estas líneas celulares incluyen a las células PC12, COS, Rat-2, NIH3T3 y MAH (Saltiel y Decker, 1994).

El receptor TrkA consiste de una cadena de alrededor de 790 residuos de aminoácidos, la cual codifica para un dominio extracelular, un dominio transmembranal único y un dominio intracelular que presenta siete residuos de tirosina en la región con actividad de tirosina cinasa, además se han detectado dos residuos de tirosina flanqueando a dicha región, la autofosforilación tiene luagar por acción del NGF alcanzando su máxima activación a los 5 minutos y entonces decae progresivamente a lo largo de dos días (Kaplan y cols, 1991).

Cuando se autofosforilan los residuos de tirosina en el dominio intracelular del receptor, se activa la transducción de señal al propiciar la fosforilación de las proteínas que tengan los dominios SH2 y SH3 (Schlessinger, 1994). En el receptor trkA se ha detectado que el adaptador Shc (SH2 containing sequence) se une con el rersiduo de tirosina de la región yuxtamembranal (Tyr490), en tanto que la fosfolipasa Cγ1 (PLC-γ1)

se une al residuo cerceano al carboxilo terminal (Tyr785). Asimismo, la subunidad no catalítica de la cinasa 3 del fosfatidil inocitol (PI-3) se une con el residuo de tirosina 751 en el dominio de cinasa (Stephens y cols, 1994; Loeb y cols, 1994; Obermeier y cols, 1993a,b; Ohmichi y cols, 1991).

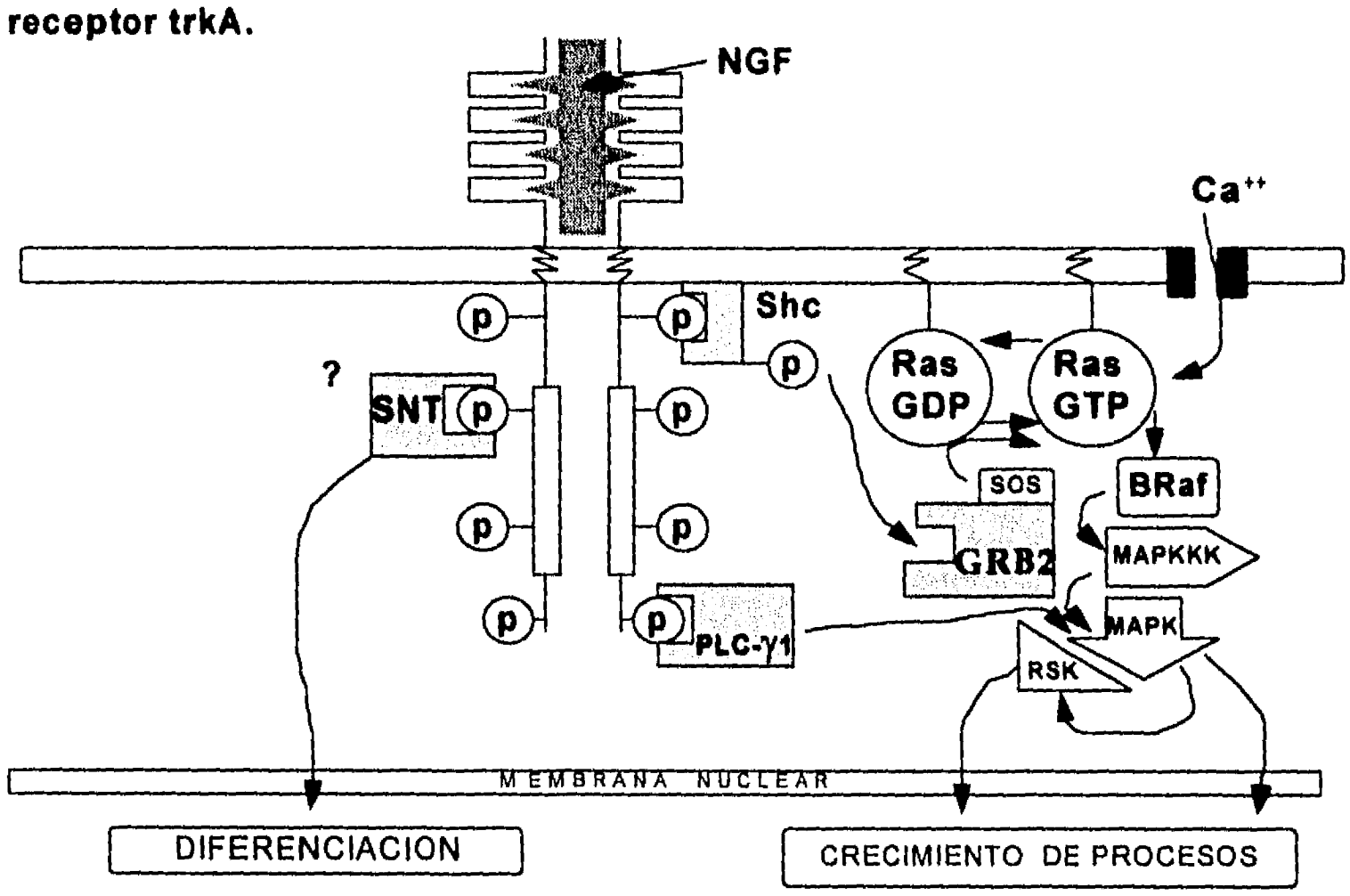
Cuando se activa al receptor trkA para estimular el crecimiento de procesos, el complejo citoplasmico GRB2-SOS formado por la proteína adaptadora GRB2 y la proteína SOS (son of sevenless) se acerca a la membrana plasmática para que la proteína Shc acoplada con el receptor fosforile a GRB2 (Stephens y cols, 1994). La proteína SOS por su parte, favorece la actividad de la proteina p21Ras que es una proteína G que oscila conformacionalmente entre los estados inactivo GDP-p21Ras y activo GTP-p21Ras (Burgering y cols, 1993; McCormick, 1994). Cabe señalar que la actividad de p21Ras también se puede inducir por la proteina GAP (Stephens y cols, 1994). El siguiente paso después de la activación de p21Ras es la activación del proto-oncogene B*Raf* con actividad de serina/trenina cinasa, que fosofrila las Serina 217 y 221 de la proteína mitogénica cinasa cinasa (MAPKK), que a su vez fosforila la treonina de otra proteína mitogenica cinasa (MAPK), que fosforila y activa a $p90^{Rsk}$, los dos últimos se translocan al núcleo para activar la transcripción de genes, por ejemplo el proto-oncogene *c-fos* (Janknecht y cols, 1993; Ver figura 3).

La fosfolipasa C-γ1 fosforilada participa también en la promoción del crecimiento de procesos activando directamente a la proteina MAPK (Obermeier y cols, 1994). Estos dos mecanismos de transducción se establecieron al mutar los residuos de tirosina 490 y 785

en el receptor trkA, pues sólo cuando se anularon ambos residuos se bloqueó el crecimiento de procesos (Obermeier y cols, 1994).

Otro mecanismo de transducción asociado a la activación del receptor trkA se establece con la fosforilación de la proteína SNT (*suc*-associated neurtrophic factor-induced tyrosine-phosphorilated target; Rabin y cols, 1993),. Este mecanismo parece ser una vía directa hacia el núcleo que participa en la diferenciación celular (Rabin y cols, 1993; Marsh y cols, 1993; Knüsel y cols, 1994; Ver figura 3).

Fiugura 3. Mecanismos de transducción activados por la estimulación del receptor trkA.



Finalmente se ha observado que en varios tipos celulares el receptor $p75^{NGFR}$ incrementa la sensibilidad del receptor trkA (Matsushima y Bogenmann, 1990; Henpstead y cols, 1989), ésto ha dado lugar a la sugerencia de que en las células que presentan una expresión baja del receptor trkA, el receptor $p75^{NGFR}$ facilita la formación de

homodímeros del receptor trkA, en tanto que en las células con una alta expresión del receptor trkA, la participación del receptor $p75^{NGFR}$ es menos relevante (Bothwell, 1995).

2.- Receptor de BDNF, NT-3 y NT-4/5: TrkB.

En 1982, Barde y cols purificaron al segundo miembro de la familia de las neurotrofinas, el factor neurotrófico derivado del cerebro (BDNF), esta proteína fue clonada por Leibrock y colaboradores en 1989, permitiendo un avance más rápido respecto del NGF, ya que la utilización de vectores de expresión para producir BDNF recombinante facilitó y aceleró su proceso de obtención. Al conocerse la secuencia también fue posible desarrollar sondas para establecer la presencia de este factor en diversos tejidos, tanto en extractos como *in situ* (Leikbrock y cols, 1989; Maisonopierre y cols, 1990a, 1990b; Ernfors y cols, 1990; Hofer y cols, 1990; Phillips y cols, 1990; Wetmore y cols, 1990).

Con el precedimiento de reacción en cadena de pilimerización con la enzima transcriptasa reversa (PCR-RT) y el uso de pequeños fragmentos de las homologías del NGF y BDNF, permitió el descubrimiento de la neurotrofina 3 (NT-3; Hohn y cols, 1990; Maisonpierre y cols, 1990; Rosenthal y cols, 1990), y la neurotrofina 4/5 (NT-4; Halböök y cols, 1991; Berkemeier y cols, 1991; Ip y cols, 1992).

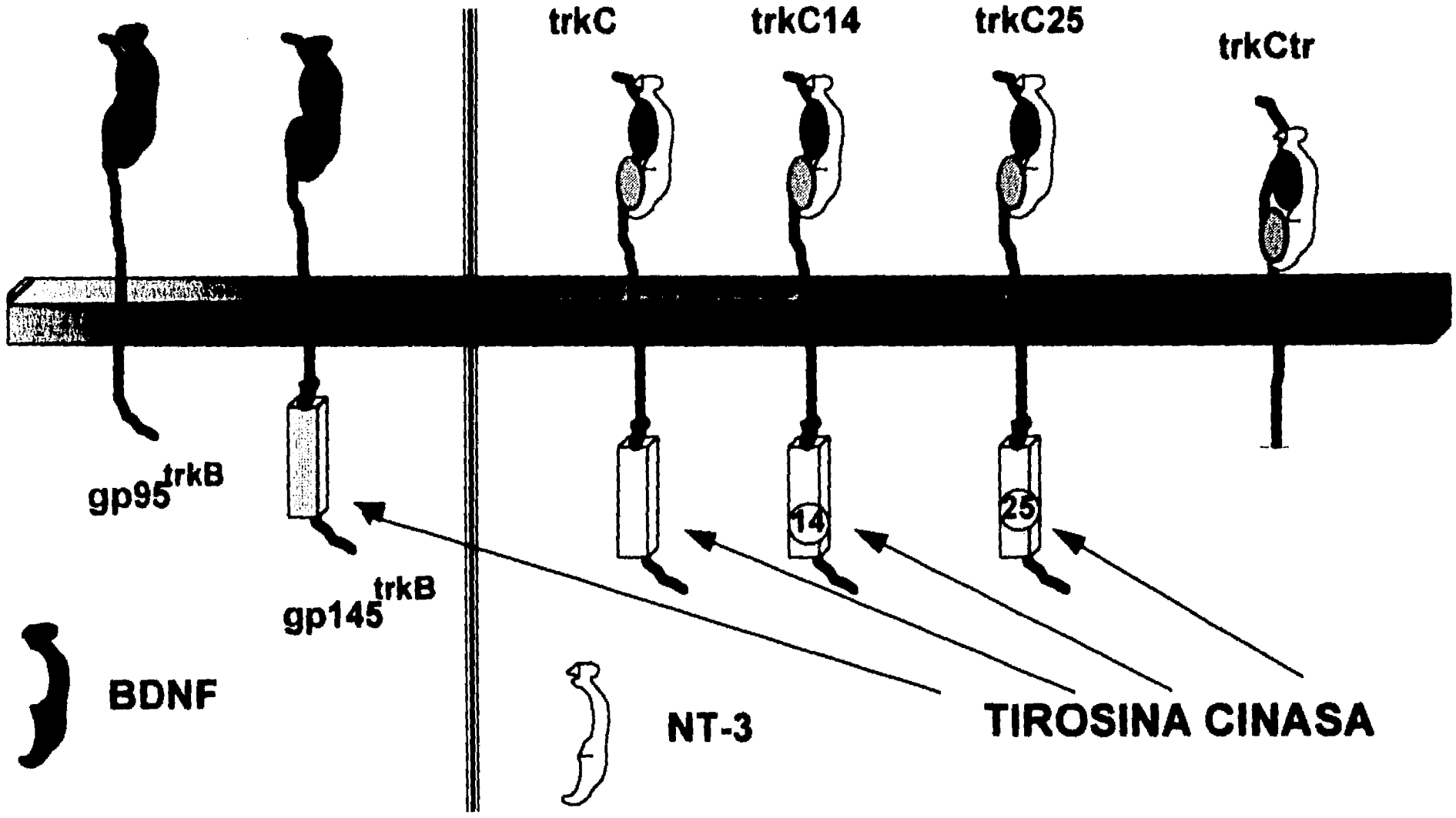
Los receptores sensiblea a estas neurotrofinas son el producto de los proto-oncogenes $gp145^{trkB}$ y $gp95^{trkB}$ (Klein y cols, 1990; Middlemas y cols, 1991). Estos receptores son idénticos de los aminoácidos 1 a 465, la cual codifica para los dominios extracelular y transmembranal, asi como los primeros doce animoácidos del dominio

yuxtamembranal, en este punto el receptor $gp95^{trkB}$ se continúa sólo con 11 aminoácidos que no definen alguna unidad catalítica. Por su parte el receptor $gp145^{trkB}$ se continua con 356 residuos de aminoácidos que incluye una unidad catalítica de tirosina cinasa. De este último existen al menos seis isoformas con variantes muy pequeñas que seguramente en el futuro cercano serán caracterizadas con mayor detalle (Figura 4; Klein y cols, 1990; Middlemans y cols, 1991). Ya que sólo el receptor $gp145^{trkB}$ presenta el sitio de transducción de señales, se le ha denominado también como receptor completo o "full-length" y al receptor $gp95^{trkB}$ como el receptor truncado (truncated; Klein y cols, 1990).

Se mostró el BDNF es incapaz de provocar cambios fenotípicos en las células PC12 que solamente expresan trkA. En contraparte el BDNF y NT-3 produjeron la fosforilación de trkB cuando éste se expreso en fibroblastos NIH3T3, cosa que no ocurrió con el NGF. En estos efectos no se requirió la presencia de $p75^{NGFR}$ (Klein y cols, 1991).

Estudios in vitro muestran que sólo cuando las neuronas colinérgicas se cultivan a baja densidad (66,000-130,300 células/cm^2), el BDNF (10-100 ng/ml) favorece su sobrevivencia hasta en tres ordenes de magnitud, incrementa la expresión del receptor $p75^{NGFR}$, de las enzimas Acetil-Colina Esterasa (AChE) y Colina-Acetil Transferasa (ChAT), también incrementa la eficiencia del sistema de recaptura de colina. En tanto que el NGF además de favorecer la sobrevivencia, sólo incrementa la expresión de la ChAT (Alderson y cols, 1990). El uso simultáneo de BDNF y NGF en estas células no produce efectos aditivos en la sobrevivencia, pero sí sobre los niveles de expresión de ChAT, lo que sugiere que algunos mecanismos de transducción son específicos y otros por vías independiente provocan la misma respuesta (Alderson y cols, 1990).

FIGURA 4. CARACTERISTICAS DE LOS RECEPTORES *trk*B Y *trk*C.



La concentración necesaria de la neurotrofina 3 (NT-3) para fosoforilar al receptor trkB, es 100-200 veces mayor que la del BDNF (Squinto y cols, 1991; Glass y cols, 1991; Klein y cols, 1991), en tanto que se requiere de cinco veces más de NT-4/5 para mimetizar los efectos del BDNF (Klein y cols, 1992; Ip y cols, 1992).

Localización anatómica del receptor trkB.

Existe evidencia de que durante el desarrollo embrionario, la expresión del receptor trkB coincide con los procesos de diferenciación neuronal en la corteza cerebral, el tálamo, el estrato piramidal del hipocampo, en las células de Purkinje y en la región

pedúnculo-caudal del cerebelo (Klein y cols, 1990), en tanto que el receptor truncado se encuentra en los plexos coroideos y en la pared ependimaria de algunas regiones del sistema ventricular (Klein y cols, 1990).

El BDNF favorece la sobrevivencia y diferenciación de las neuronas de los ganglios de la raíz dorsal (Lindsay y cols, 1985, Kalcheim y cols, 1987), las neuronas colinérgicas (Alderson y cols, 1990; Knüsel y cols, 1991) y las neuronas dopaminérgicas fetales del mesencéfalo ventral, evitando incluso en las últimas los efectos tóxicos del MPTP y 6-OHDA (Hyman y cols, 1991; Knüsel y cols, 1991).

3.- Receptor de la neurotrofina 3, TrkC.

En 1990 Rosenthal y colaboradores utilizando la técnica de reacción encadena de polimerización con la enzima transcriptasa reversa (RT-PCR), reportaron la existencia de un tercer factor con características similares a las del NGF y BDNF denominándolo neurotrofina 3 (NT-3). En 1991 Lamballe y colaboradores determinaron a partir de una biblioteca genómica de cerebro de cerdo, que el receptor $p145^{trkC}$ (trkC), un polipéptido de 825 residuos de aminoácidos presenta una homologia de 53% con el receptor trkB complento y de 55% con el receptor trkA. El receptor trkC comparte la disposición de los doce residuos de cisteína del dominio extracelular con trkB y de éstos, 10 corresponden a los presentes en trkA. La región catalítica del receptor trkC es similar a las de los receptores trkA y trkB, existiendo identidad entre los tres en los 8 residuos de la porción cercana al carboxilo terminal (Lamballe y cols, 1991).

De las cuatro neurotrofinas sólo NT-3 es capaz de provocar la autofosforilación del receptor trkC; determinándose que alrededor del 2% corresponde al receptor de alta afinidad con una Kd de 2.6 X 10^{11} M, en tanto que el receptor de baja afinidad tiene una Kd de 2-4x10^{-9}M, por lo que se considera hasta el momento que NT-3 es el ligando específico del receptor trkC (Lamballe y cols, 1991). Complementando lo anterior se mostró que NT-3 es más efectivo para provocar cambios morfológicos en células NIH3T3 que expresan a trkC, que aquellas que expresen ya sea trkA o trkB (Lamballe y cols, 1991).

El NT-3 promueve el crecimiento axonal de neuronas simpáticas, neuronas sensoriales derivadas de las placodas y en las del ganglio de la raíz dorsal. Sin embargo, en términos de concentración, estos efectos varian respecto de los provocados por el NGF; en las neuronas simpáticas los efectos de ambos factores son equivalentes, en las neuronas sensoriales NT-3 es más potente y las neuronas de la raíz dorsal lo es el NGF (Rosenthal y cols, 1990). El NT-3 parece no tener efectos sobre las neuronas colinérgicas y dopaminérgicas (Hohn y cols, 1990; Maisonpierre y cols, 1990a; Rosenthal y cols, 1990). Este resultado es paradojico por el hecho de que NT-3 es capaz de activar tanto a trkA como a trkB, por lo que podría esperarse que tuviera efectos.

Localización anatómica del receptor trkC.

El receptor trkC se localiza en el estrato de las células piramidales del hipocampo, en la capa granular del giro dentado, en algunas regiones de los estratos externos de la corteza cerebral telencefálica, en el estrato granular del cerebelo y en algunas neuronas

dopaminérgicas de la zona medial del mesencéfalo ventral. En estos sitios se han detectado que las neuronas que expresan al receptor trkC también secretan NT-3, por lo que se considera que los efectos de NT-3 son autocrinos (Lamballe y cols, 1991).

Isoformas del receptor trkC.

En una biblioteca genómica de cerebro de rata, Tsoulfas y colaboradores utilizando la misma técnica de RT-PCR (1993), detectaron que existen al menos cuatro isoformas del receptor trkC, la primera es la descrita por Lamballe y cols; la segunda y tercera denominados como trkC14 y trkC25 tienen entre los aminoácidos 711 y 712 inserciones de 14 y 25 aminoácidos respectivamente. La cuarta es un receptor truncado cuya diferencia de los anteriores ocurre a partir del aminoácido 528, este receptor al igual que el receptor trkB truncado, carece de unidad catalítica (ver figura 4).

El NT-3 provoca la fosforilación de tirosina de trkC14 y trkC25 y el tiempo en el que ocurre ésto es similar al reportado para trkA y trkB. La fosforilación trkC14 no está asociada con cambios morfológicos y aún no se conocen sus mecanísmos de transducción, en tanto que la fosforilación de trkC25 promueve el crecimiento de procesos (Tsulfas y cols, 1993). Al igual que el receptor trkB, las isoformas de trkC completo no requieren de la presencia de $p75^{NGFR}$ para la formación del receptor de alta afinidad, el cual constituye del 10-15% con una Kd de 5 pM para trkC25 y de 24 pM para trkC14, la Kd de baja afinidad es de 892 y 911 pM respectivamente (Tsulfas y cols, 1993).

Estas cuatro isoformas están presentes en el cuerpo estriado, cerebelo, corteza cerebral, hipocampo, timo, intestino, riñón y músculo estriado (Tsulfas y cols, 1993).

Las evidecias muestran que falta mucho por hacer en este campo, pues la caracterización de los factores tróficos ha sido básicamente de estudios in vitro y es necesario establecer cual es la función de éstos en el sistema nervioso completo y más aún a lo largo de la vida.

Durante este doctorado se realizaron tres trabajos enfocados a determinar cuál es el papel de las neurotrofinas a lo largo del desarrollo, en el primero se estableció la expresión de los receptores de las neurotrofinas durante el desarrollo embrionario y postnatal en diversos tejidos (VER ARTICULO "Regulation of neurotrophin receptor expression during embrionic and postnatal development", En el segundo el estudio se enfocó al análisis de la expresión ontogénica de trkB y trkC en el cuerpo estriado, tálamo y substancia nigra de la rata (VER ARTICULO "Functional neurotrophin receptors during embryionic and postnatal development of nigro-estriatal system in the rat". El tercer estudio se enfocó a determinar cual es el papel del BDNF y GDNF sobre el sistema nigro-estriatal (VER ESTUDIO PILOTO "Infusión crónica de BDNF en el parénquima del sistema nigro-estriatal").

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Regulation of Neurotrophin Receptor Expression during Embryonic and Postnatal Development

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Members of the NGF family of proteins act as neurotrophic agents for defined populations of peripheral and central neurons during embryonic and postnatal development. We have studied the presence of receptors for brain-derived neurotrophic factor (BDNF) and neurotrophin-3 and -4/5 (NT-3, NT-4/5) by cross-linking radioiodinated neurotrophins to specific cell surface receptors. We have identified neurotrophin receptors representing full-length TrkB and TrkC and their truncated forms (lacking a functional cytoplasmic kinase domain) in neuronal as well as in non-neuronal tissues. During chicken embryonic and early postnatal brain development, expression of full-length TrkB and TrkC proteins preceded the onset of the truncated forms of these receptors. A similar pattern was also observed in mouse embryonic and early postnatal brain. The relative levels of neurotrophin receptors in the basal forebrain and in the hippocampus did not change significantly with age in mice. High levels of receptors for the three neurotrophins were detected in the nigrostriatal system. Full-length TrkB and TrkC receptors were found in chicken and rat embryonic ventral spinal cord, as well as on purified motoneurons. Again, truncated TrkB appeared significantly later than the full-length form on spinal motoneurons. In chicken embryonic retina and optic tectum we detected full-length TrkB and TrkC; however, the optic tectum also expressed large amounts of the truncated form of TrkB. TrkC but not TrkB was detected in chicken embryonic skeletal muscle, suggesting that NT-3 may have a novel function in this tissue. The presence of neurotrophin receptors in a wide variety of embryonic and postnatal tissues underlines the significant role of BDNF, NT-3, and NT-4/5 in embryonic and postnatal development. The regulation of the ratio of full-length versus truncated neurotrophin receptors may play an important role in the development, maturation, and maintenance of various neuronal networks.

[Key words: brain-derived neurotrophic factor, neurotrophin-3, neurotrophin-4/5, neurotrophins, neurotrophin receptors, TrkB, TrkC, affinity cross-linking, development]

Neurotrophic factors regulate the proliferation, differentiation, process outgrowth, and survival of specific neuronal populations, and thus play a vital role in vertebrate neuronal development. Members of the "neurotrophin family"—NGF, brain-derived neurotrophic factor (BDNF), and neurotrophin-3 and -4/5 (NT-3, NT-4/5)—support the embryonic survival and regulate the transmitter phenotype of responsive neuronal populations. Among these are dorsal root ganglionic (DRG) sensory and sympathetic neurons in the PNS; and basal forebrain cholinergic, nigral dopaminergic, and spinal cord motor neurons and retinal ganglionic neurons in the CNS (Hefti et al., 1989; Chao, 1992; Davies et al., 1993; Persson and Ibañez, 1993). Identification of functional receptors of neurotrophins in different tissues may reveal potential unrecognized biological activities of these molecules. Studies of neurotrophin receptors in embryonic, adult, and aging tissues will lead to a better understanding of the roles of these agents in regulating the development and maintenance of neuronal and possibly non-neuronal systems.

Functional receptors for neurotrophins can be identified and characterized by chemical cross-linking, also known as affinity labeling, a method that can provide size information on the receptor–ligand complex, as well as an estimate of binding affinity (Escandón et al., 1993). All neurotrophins bind to the "low-affinity," or "fast," NGF receptor, also referred to as $p75^{NGFR}$ (Johnson et al., 1986; Radeke et al., 1987; Rodriguez-Tébar et al., 1990, 1992). The expression of $p75^{NGFR}$ is very widespread during embryonic development (Yan and Johnson, 1987; Escandón and Chao, 1989, 1990). In addition, neurotrophins bind to and stimulate tyrosine phosphorylation of members of the Trk protein family. The product of the *trk* proto-oncogene is a receptor tyrosine kinase that becomes activated upon NGF binding (Kaplan et al., 1991a,b; Klein et al., 1991a). BDNF is a ligand of the TrkB protein (Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991), whereas NT-3 preferentially binds to and stimulates the phosphorylation of the TrkC protein (at 0.01–0.1 nM concentrations) but is also able to bind to and phosphorylate the TrkB and Trk proteins at higher concentrations (0.2–1 nM) of the factor (Lamballe et al., 1991; Soppet et al., 1991; Tsoulfas et al., 1993). NT-4/5 stimulates phosphorylation of TrkB and, to a lesser degree, Trk (Berkemeier et al., 1991; Yp et al., 1992). However, detailed binding studies with

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NT-4/5 have not been reported yet. Several splice variants of the *trk*B and *trk*C genes have been described, some of which encode proteins that have an intact extracellular domain but lack the cytosolic catalytic tyrosine kinase domain (Klein et al., 1990a; Middlemas et al., 1991; Tsoulfas et al., 1993). Some of these transcripts encode unique C-terminal sequences.

In the CNS, the levels of $p140^{trk/trkB}$ (further referred to as Trk) are extremely low (Martin-Zanca et al., 1990) and are mainly localized to basal forebrain cholinergic nuclei that are dependent on NGF (Holtzman et al., 1992). Transcripts for *trk*B and *trk*C are abundant during development and in adult animals (Klein et al., 1990b; Lamballe et al., 1991; Tessarollo et al., 1993; Tsoulfas et al., 1993). BDNF and NT-4/5 are the primary ligands of TrkB and NT-3 of TrkC. We therefore focused our analysis using receptor cross-linking on these receptors. Due to the complexity of the multiple *trk*B and *trk*C transcripts, analysis of *trk* family mRNA expression in different tissues alone does not allow conclusions regarding functional receptors for the three neurotrophins. Here we report the identification by affinity labeling of functional neurotrophin receptors in embryonic and postnatal tissues as a more informative measure than previous mRNA expression studies. The results provide evidence for an active role of the neurotrophins in the formation and maturation of the nervous system and reveal non-neuronal tissues as possible novel targets for nonconventional neurotrophin effects. Based on our analysis described in this report, neurotrophin receptors appear to be developmentally regulated in a tissue-specific manner.

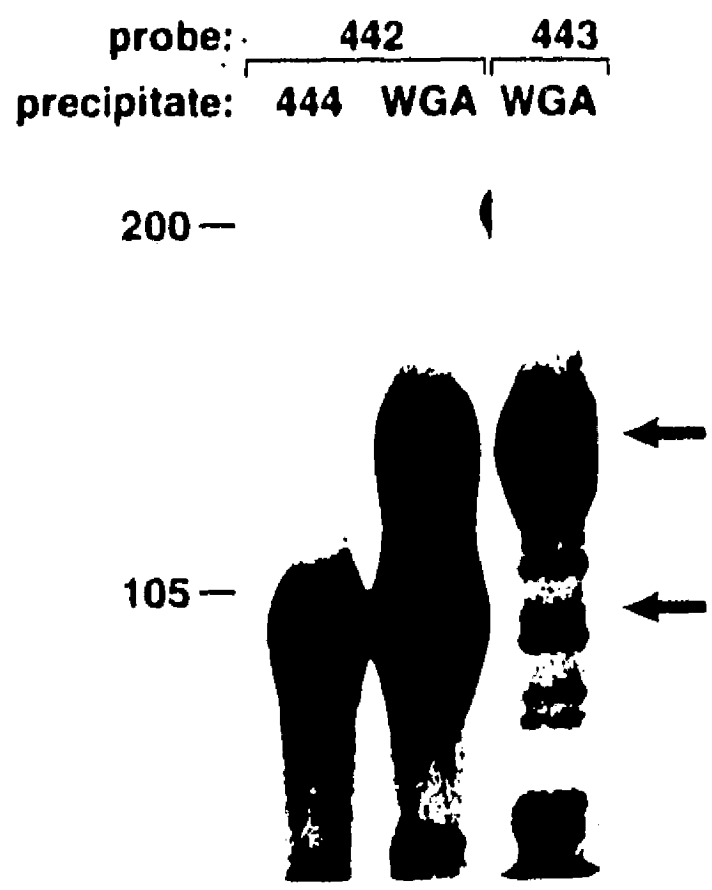

Figure 1. Western analysis of full-length and truncated TrkB receptors. NP-40 lysates of adult mouse cortex were precipitated with wheat germ agglutinin Sepharose (*WGA*) or rabbit polyclonal antiserum 444. The precipitates were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with TrkB-specific antiserum 442 or antiserum 443, which recognizes the full-length form of the *trk* family of receptors. The reactive bands were visualized with HRP-conjugated anti-rabbit IgG and the ECL reagent. The *lower arrow* indicates the position of truncated TrkB receptor; the *upper arrow* indicates the position of full-length TrkB receptor. Positions of prestained molecular size markers are indicated on the *left* in kilodaltons.

Materials and Methods

Neurotrophins and their radiolabeling. Recombinant NGF, BDNF, NT-3, and NT-4/5 were prepared essentially as described by Schmelzer et al. (1992), Rosenthal et al. (1990, 1991), and Berkemeier et al. (1992), respectively, with minor improvements. N-terminal sequence analysis indicated that fully intact, properly processed mature forms of each neurotrophin were present in the preparations with less than 5% internally processed sequences. The purity of the protein preparations was confirmed by SDS-PAGE, which indicated a >95% degree of purity as judged by Coomassie blue staining of the gels. Mass spectrometric analysis of the preparations was in agreement with >95% of the full-length, unprocessed form of each neurotrophin. The proteins were quantitated for cross-linking and binding studies by amino acid analysis employing norleucine as an internal standard.

Neurotrophins were iodinated using the Enzymobead reagent (Bio-Rad) as described (Escandón et al., 1993). Briefly, the beads were rehydrated in 2 ml of distilled water overnight at 4°C, centrifuged, and resuspended in 180 μl of distilled water. Fifty microliters of the Enzymobead suspension were added to the iodination reaction described by the manufacturer. The mixture was incubated for 2 hr at room temperature. An additional 40 μl of the resuspended beads was added and the reaction was incubated for another hour. Labeled neurotrophins were separated from free iodine using desalting columns (Pierce). Routinely 2 μg aliquots of the neurotrophins were incubated in the presence of 1.5 mCi of $Na^{125}I$ and preparations with specific activities ranging from 2000 to 3500 cpm/fmol were typically obtained. The ^{125}I-derivatized factors were stored at 4°C and used within 2 weeks of preparation. All of the iodinated proteins had full biological activities in neuronal survival assays (Escandón et al., 1993).

Preparation of tissues. Balb/c mice were killed, and different brain regions were dissected in cold (4°C) PBS containing 5 mg/ml glucose and sliced into smaller pieces. Cell suspensions from the different brain regions were prepared by gently passing the tissue through an 18 gauge needle until a homogeneous suspension was obtained. Large aggregates were removed from the cell preparation by allowing the samples to sediment for 1–2 min at 4°C and collecting the supernatant. No enzymatic treatment was used at any stage of these preparations in order to avoid possible modifications of cell surface receptor proteins. Brain, ventral spinal cord, retina, optic tectum, and various peripheral organs including heart, liver, kidney, gut, limbs, and, where indicated, skeletal muscle from chicken, mouse, and rat embryos and newborns were collected and treated similarly. Motoneurons were purified from chick [embryonic day 5 (E5)] or rat (E15) embryos as described (Bloch-Gallego et al., 1991; Camu and Henderson, 1992). Dissected non-neuronal tissues were mechanically triturated using a Brinkman homogenizer.

Preparation of antisera. Rabbit anti-peptide antisera were generated against keyhole limpet hemocyanin–conjugated peptides corresponding to mouse TrkB amino acids 45–60 (CTEPSPGIVAFPRLEP: peptide and antiserum 442) and 809–822 (NLAKASPVYLDILG: peptide and antiserum 443), and rat truncated TrkB tk-1 amino acids 454–465 (CGFVLFHKIPLDG: peptide and antiserum 444). Antiserum 443 recognizes Trk, TrkB, and TrkC expressed in NIH3T3 cells, whereas antiserum 442 is specific for full-length and truncated forms of TrkB (Soppet et al., 1991). Antiserum 444 recognize a truncated form of TrkB as shown by Western analysis of wheat germ agglutinin–extracted brain lysates (Fig. 1) and by Western analysis of cell lines expressing truncated TrkB (tk-1).

Affinity cross-linking procedures. For the receptor cross-linking experiments, the procedure of Escandón et al. (1993) was followed with some modifications. Cell triturations prepared from isolated tissues were incubated in PBS-glucose, pH 7.2, with ^{125}I-labeled neurotrophins (used in final concentrations of 0.5–1 nM, which corresponds to a maximally effective concentration of each neurotrophin). The samples were incubated at 4°C for 2 hr with gentle agitation. The cross-linking agent 1-ethyl-3-(3-dimethyl-aminopropyl carbodiimide)·HCl (EDAC; Pierce) was added to a final concentration of 6 mM, and the mixture rotated for 20 min at room temperature. The reaction was quenched with cold PBS containing 50 mM lysine, centrifuged, and washed three times with PBS. The cell pellet was resuspended in PBS, 5 mM $MgCl_2$, and 1% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride, and treated with DNase I (5–10 μg/sample) for 15 min on ice. After centrifugation the supernatants were either immunoprecipitated or mixed with SDS sample buffer and subjected to electrophoresis on a 6% polyacrylamide SDS gel unless otherwise indicated. For immunoprecipitation the resuspended samples (~600 μl) were mixed with 5 μl of each antiserum and incubated overnight at 4°C, followed by a 2 hr incubation in the presence of Protein A-Sepharose. The samples were washed three times

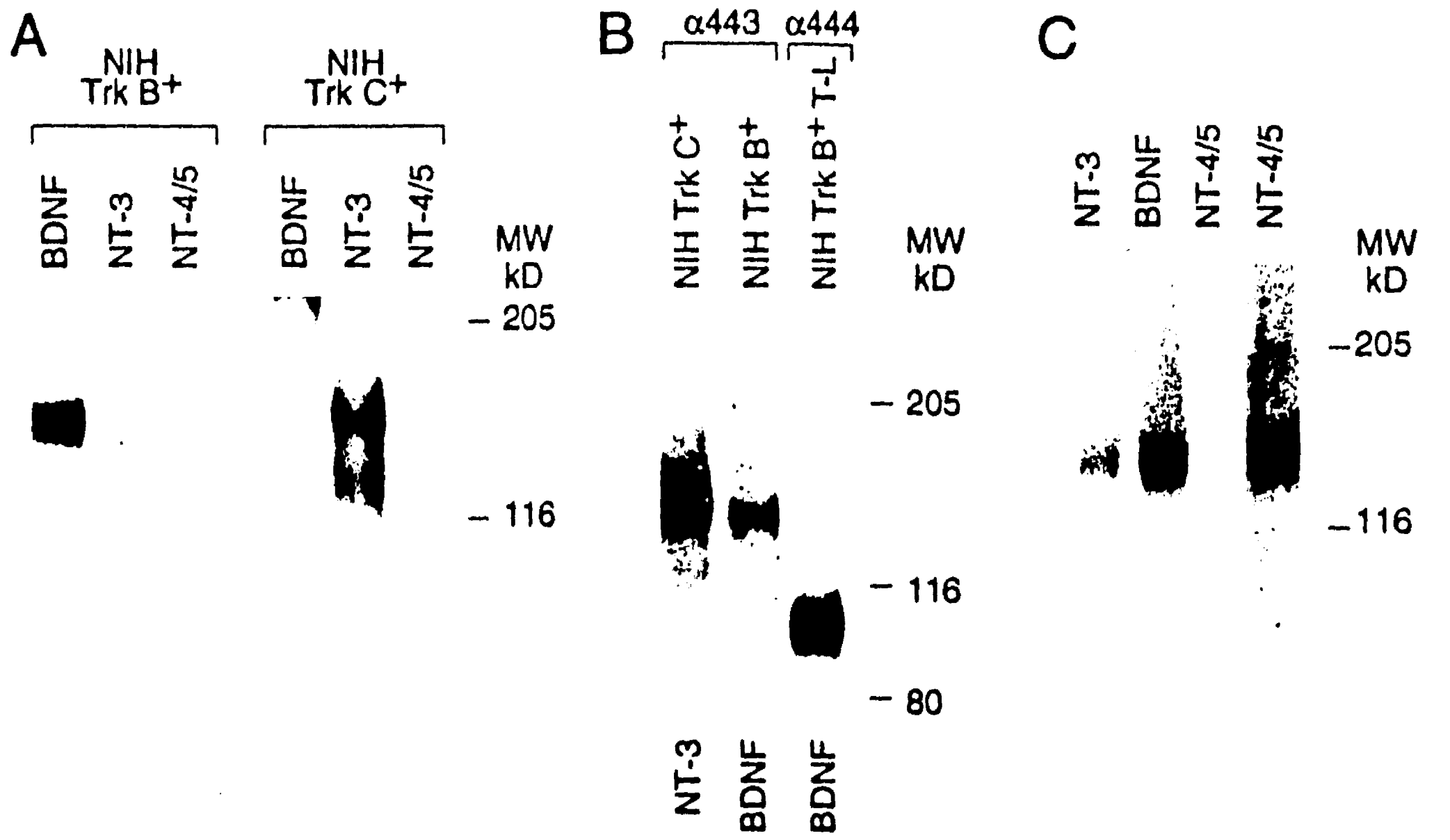

Figure 2. Specificity of neurotrophin affinity labeling and antibody reactivity with recombinant full-length TrkB and TrkC and truncated TrkB expressed in NIH3T3 cells (*A* and *B*) and with neuronal tissues (*C*). Cell suspensions of NIH3T3 fibroblasts expressing recombinant full-length TrkB, TrkC, and truncated TrkB receptors (*NIH TrkB' T-L*) or membranes prepared from 6-month-old mouse cerebral cortex were incubated with 0.5–1 nM of the indicated labeled neurotrophins. Following EDAC cross-linking the samples were either resuspended in SDS sample buffer and subjected to electrophoresis on a 6% polyacrylamide/SDS gel (*A*) or followed by immunoprecipitation with anti-443 or anti-444 antibodies as indicated. Samples in *C* were all immunoprecipitated with anti-443 antibodies and treated as above. The fixed and dried gels were exposed with autoradiographic film. The second NT-4/5 lane in *C* was exposed 10 times longer than all other lanes. Positions of molecular size markers are indicated in kilodaltons.

with the PBS, 1% Triton buffer and subjected to gel electrophoresis as described above. The fixed and dried gels were exposed with Kodak X-O-Mat film for different periods of time (as indicated in the figure captions) depending on the intensity of the label at −70°C with an intensifier screen.

Binding assays. Crude membrane preparations from stable NIH3T3 fibroblast cell lines expressing full-length TrkB protein (100–200 mg of total protein/sample) were resuspended in Leibowitz's L-15 media (GIBCO) supplemented with 5 mg/ml BSA and 0.1 mg/ml horse heart cytochrome C (Sigma) and 20 mM HEPES, pH 7.2. Incubation of the binding mixture (0.2 ml/assay) was carried out at 5°C with vigorous shaking overnight in 5 ml polypropylene test tubes. Typically 50–200 pM concentrations of iodinated BDNF or neurotrophin-4/5 were incubated with the membrane preparation together with increasing concentrations (1.56–6400 pM of BDNF or neurotrophin-3 or -4/5, or up to 100 nM for NGF) of unlabeled competitors. Membrane-bound radioactivity was determined after the samples were transferred to Millipore filters in a filtration unit and washed three times with 3 ml of ice-cold PBS containing 1 mg/ml BSA and protamine sulfate (Sigma). The filters were counted in an Iso-data series 100 gamma counter with a counting efficiency of 78%. Specific binding was calculated by subtracting the nonspecific binding from total binding. Nonspecific binding was measured by incubating the cell membranes with a 100-fold excess of unlabeled homologous ligand.

Results

Cross-linking of neurotrophins to TrkB and TrkC receptors

Radioiodinated neurotrophins were cross-linked to specific receptors in various embryonic and postnatal tissues that were mechanically dispersed. NGF and the neurotrophins used in this study were produced by recombinant expression (Rosenthal et al., 1990, 1991; Schmelzer et al., 1991; Berkemeier et al., 1992). The purified factors were iodinated by a procedure using immobilized lactoperoxidase and glucose oxidase as described in detail elsewhere (Escandón et al., 1993). The radioiodinated neurotrophins were homogeneous as analyzed by SDS-PAGE and retained full biological activity in peripheral sensory neuron survival assays (Escandón et al., 1993). EDAC was used as the cross-linking agent, as we had earlier reported that it is very efficient in cross-linking iodinated BDNF and NT-3 to their respective receptors, TrkB and TrkC (Soppet et al., 1991; Escandón et al., 1993; Tsoulfas et al., 1993). In this study, in addition to BDNF and NT-3, the cross-linking of NT-4/5 was also tested to members of the Trk family.

The efficiency of analyzing the cross-linked reaction products could be significantly enhanced by immunoprecipitation with specific TrkB/C antibodies. We used two antibodies for this purpose, anti-443 and anti-444 (Fig. 1). Anti-443 is an antibody specific for the C-terminus of full-length TrkB; however, it also recognizes TrkA and TrkC. Anti-444 is an antibody specific for the truncated form of TrkB raised against a unique region of the C-terminus of this protein. The antibodies did not cross-react with $p75^{NGFR}$ as tested by Western analysis and by immunoprecipitation of cross-linked BDNF–$p75^{NGFR}$ complexes (not shown; Escandón et al., 1993). Figure 1 shows Western analysis of full-length and truncated TrkB proteins; however,

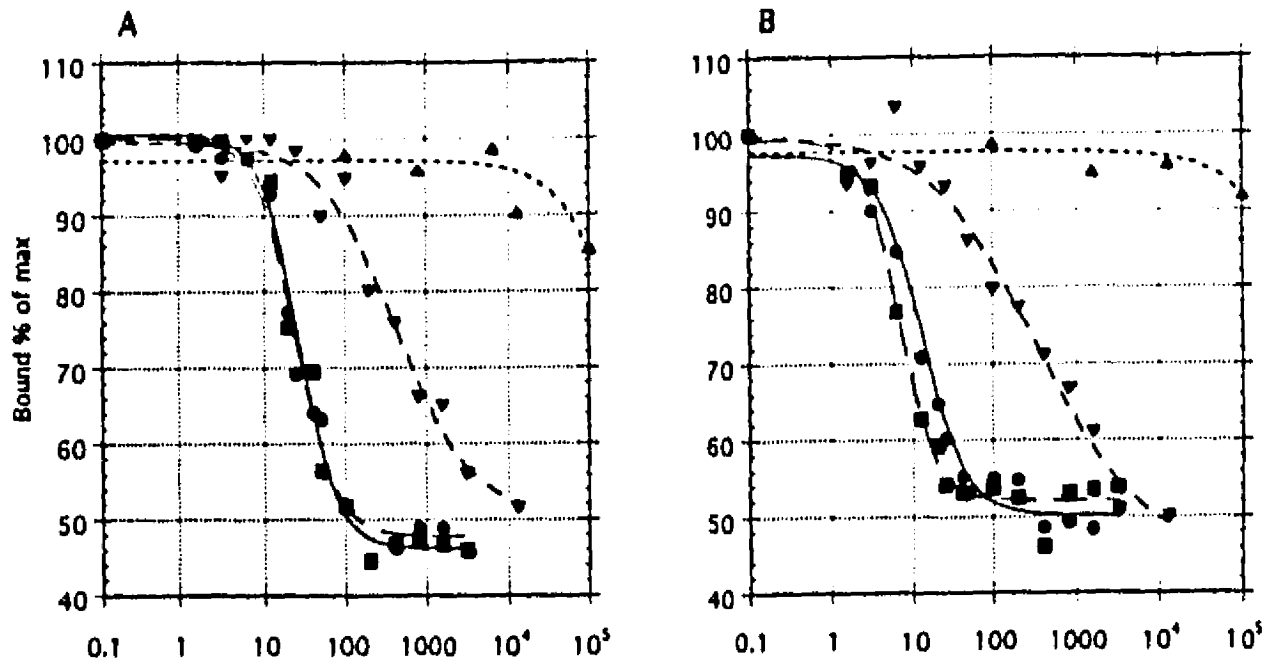

Figure 3. Self- and cross-competition analysis of BDNF (*A*) and NT-4/5 (*B*) binding to recombinant cells expressing p145^{trkB}. Crude membrane preparations (150 μg of total protein/tube) were incubated in the presence of 50–100 pM ^{125}I-labeled BDNF (*A*) or ^{125}I-labeled NT-4/5 (*B*) with increasing concentrations of homologous or heterologous unlabeled ligands. The symbols are as follows: ■, BDNF; ▼, NT-4/5; ●, NT-3; ▲, NGF. The binding assay conditions are described in Materials and Methods. The competitor neurotrophin concentrations (picomolar) are indicated.

both antibodies also efficiently precipitated cross-linked neurotrophin–receptor complexes (Fig. 2). As shown in Figure 2*A*, we were able to cross-link BDNF, NT-3, and NT-4/5 to TrkB; however, only NT-3 bound to TrkC. All neurotrophin–receptor complexes appeared as broad bands by SDS-PAGE analysis, probably reflecting the glycosylation of these receptor proteins. However, the cross-linked labeled products showed some differences. The M_r of the receptor–ligand complex when BDNF and NT-3 were cross-linked to TrkB or TrkC was approximately 150–160 kDa, whereas in the case of NT-4/5 it was about 160–170 kDa (Fig. 2*A*). A possible reason for this is that the stoichiometry of the reaction between NT-4/5 versus BDNF and NT-3 to their receptors may differ. It is unclear whether neurotrophins are cross-linked to TrkB as monomers or as dimers. Depending on this, cross-linking increases the size of the receptor band by either 13.5 or 27 kDa. Cross-linking followed by immunoprecipitation with these two antibodies, respectively, to full-length and truncated forms of TrkB, and the full-length form of TrkC (Fig. 2*B*) proved to be a very useful method to analyze such products in tissues. The two antibodies are specific for either the full-length or the truncated forms of TrkB and no nonspecific cross-reactivity was seen. In most experiments described below, except where indicated otherwise, we used a mixture of these two antibodies in order to capture both forms of TrkB and the full-length form of TrkC.

Specificity of neurotrophin binding and cross-linking to TrkB and TrkC receptors in vitro *and* in vivo

Using competition binding analysis we established that BDNF and NT-4/5 are equally efficient ligands to bind to TrkB (Fig. 3). Both agents bind to TrkB with high affinity (IC_{50} values of about 15–25 pM) and were able to displace each other at the same concentrations. NT-3 was a significantly less efficient competitor with an IC_{50} value of about 300 pM when either BDNF or NT-4/5 was used as the labeled ligands. The slope of the displacement curve with NT-3 significantly differed from those of BDNF and NT-4/5, suggesting that the molecular and kinetic details of the interaction between NT-3 and TrkB differ from those of BDNF and NT-4/5. NGF was inefficient in displacement of either BDNF or NT-4/5 from TrkB even at extremely high (10–100 mM) concentrations. These data indicate that both BDNF and NT-4/5 are specific high-affinity ligands of TrkB. The values obtained are similar to those reported for NGF–trk interactions (Vale and Shooter, 1985; Hempstead et al., 1991). In contrast to the displacement analysis, cross-linking of NT-4/5 to TrkB, although specific and detectable, provided a significantly less intense signal as compared to BDNF (Fig. 2*C*). A 10-fold longer exposure time was required for NT-4/5 to achieve a signal intensity comparable to that obtained with BDNF. We attribute this to a lower efficiency of cross-linking of NT-4/5 to TrkB with EDAC; fewer amino acid side chains between NT-4/5 and TrkB may be available for the reaction. Whereas BDNF and NT-3 have 11 and 10 lysines, respectively, NT-4/5 contains only three lysines that may be available for the cross-linking reaction (the number of Asp and Glu residues is approximately the same in all three NTs). In addition, cross-linking of NT-4/5 to chicken brain was not detectable under the same experimental conditions (not shown).

Using membranes from adult mouse cortex and E15 chicken brain, labeled BDNF, NT-3, and NT-4/5 were bound and cross-linked in the presence or absence of heterologous cold ligands followed by immunoprecipitation with a mixture of anti-443/444 (Fig. 4). The results indicated the presence of both full-length TrkB and TrkC, as well as truncated TrkB in CNS tissues of both rodent and avian species. As we have previously shown, both BDNF and NT-3 were able to bind to truncated TrkB (Soppet et al., 1991; Escandón et al., 1993). Binding of NT-3 to truncated TrkB was completely displaced in the presence of 100× cold BDNF. These experiments also demonstrated that truncated TrkB appears to be the most abundant receptor form in the adult mouse cortex, whereas in E15 brain the full-length and truncated forms are present at similar levels (compare Fig. 4*A*,*B*). NT-4/5 also bound to full-length and truncated TrkB in mouse cortex. This was demonstrated by the complete displacement of labeled NT-4/5 binding when cold BDNF was the competitor, in agreement with the binding displacement analysis (Fig. 3). A 100× excess of unlabeled NT-3 partially competed with NT-4/5 binding.

The binding and cross-linking data can be summarized as follows. (1) BDNF and NT-4/5 bind and cross-link to full-length and truncated TrkB receptors, a signal that is not displaceable by cold NGF; however, some displacement is found with a large excess (1–200×) of NT-3. In tissues where intense labeling is seen with BDNF and relatively weak labeling with NT-3 in which the latter is displaceable with BDNF, we believe that predominantly TrkB receptors are present. (2) NT-3 binds and cross-links predominantly to TrkC receptors to which BDNF and NT-4/5 do not bind. NT-3 binds to TrkB but with lower affinity than BDNF and NT-4/5, its native ligands. Therefore, in tissues where only NT-3 but no BDNF or NT-4/5 cross-

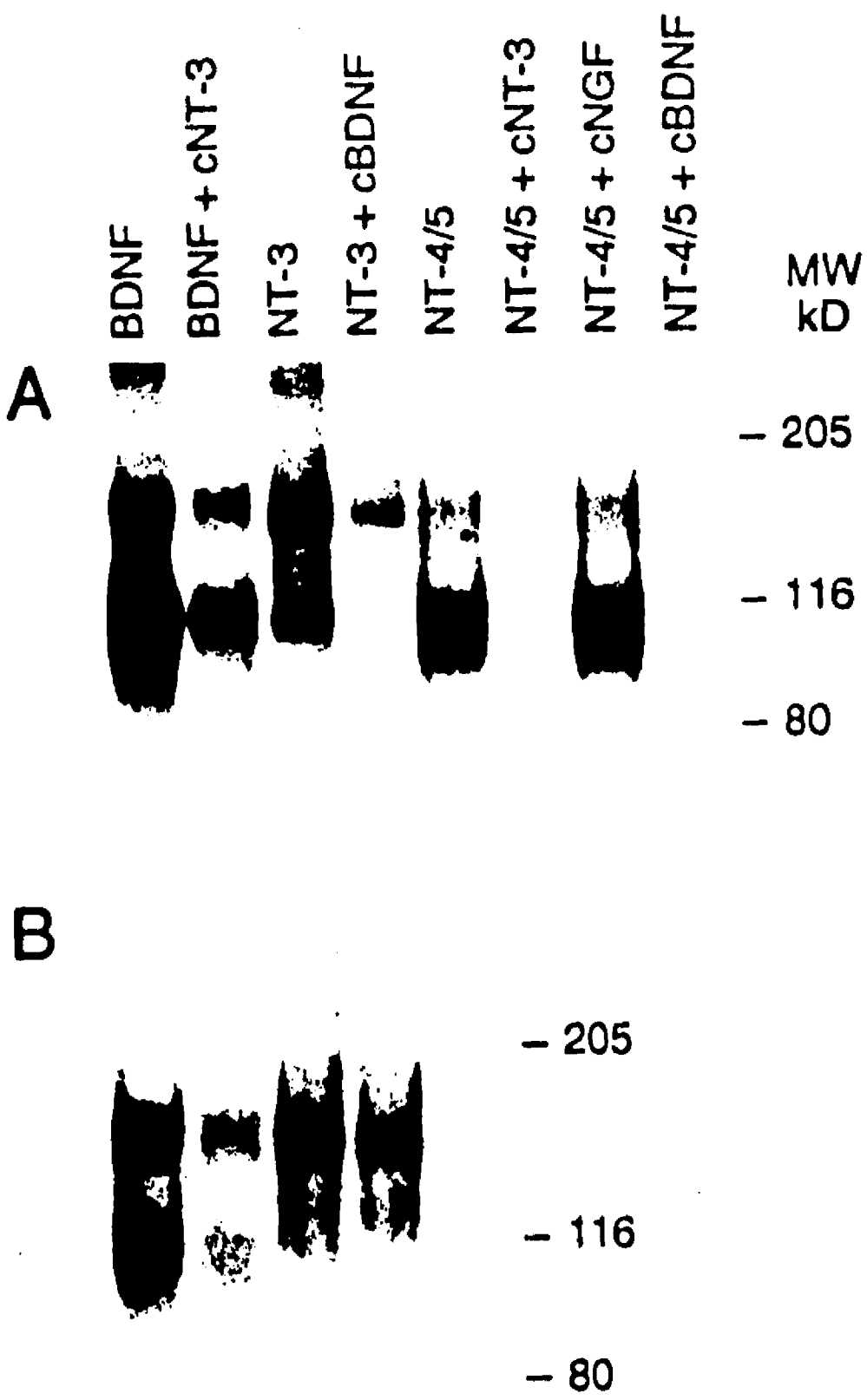

Figure 4. Effect of heterologous ligands on the affinity labeling of neurotrophin receptors *in vivo*: comparison between rodent and avian tissues. Equal amounts (5 mg of total protein/sample) of crude membranes prepared from 6-month-old cerebral cortex (*A*) or E15 chicken brain (*B*) were incubated with 1 nM labeled neurotrophins in the presence or absence of a 150-fold excess of heterologous ligand. Cold NGF was always used at a 1000-fold excess. *Lane 1*, BDNF; *2*, BDNF + cold NT-3; *3*, NT-3; *4*, NT-3 + cold BDNF; *5*, NT-4/5; *6*, NT-4/5 + cold NT-3; *7*, NT-4/5 + cold NGF; *8*, NT-4/5 + cold BDNF. After EDAC cross-linking the samples were immunoprecipitated with a mixture of anti-443/444 antibodies and analyzed by SDS-PAGE. The fixed and dried gels were exposed for 14 d. Positions of molecular weight markers are indicated in kilodaltons.

linking was found, we believe that predominantly TrkC receptors are present. (3) In a few instances (with no immunoprecipitation with anti-443/444) we found NT-3 cross-linking to a truncated receptor species not displaced by BDNF that most likely represents a truncated TrkC species. (4) As discussed in detail below, in many if not most tissues TrkB and TrkC and even their full-length and truncated forms are coexpressed; however, in some tissues at certain developmental times "pure" examples with either TrkB or TrkC receptors only have been found.

Neurotrophin receptors in the developing brain

To better understand the role of the neurotrophins in neuronal differentiation, maturation, and survival, it is important to identify the presence of their functional and specific receptors *in vivo* at different developmental and postnatal ages. Having established the cross-linking reaction conditions followed by immunoprecipitation with specific antibodies, and determined the ligand specificity of binding with recombinant receptors and brain tissue, we began to analyze functional receptors for the three neurotrophins in different tissues of different developmental and postnatal ages. Full-length and truncated forms of TrkB and TrkC are widely expressed in the adult CNS (Klein et al., 1990a,b; Escandón et al., 1993). Whereas full-length TrkB is the signal transducing receptor for BDNF and NT-4/5, it is still unclear what the role of truncated TrkB might be *in vivo*. We isolated chicken embryonic brains at different developmental times and subjected the tissues to affinity cross-linking using labeled BDNF and NT-3 followed by immunoprecipitation with anti-443 or -444 antibodies (Fig. 5*A*,*B*). We detected the presence of receptor complexes as early as E6 for the full-length forms. By E11, binding of BDNF and NT-3 to TrkB and TrkC, respectively, produces intense signals. In contrast, under the same binding and cross-linking conditions, truncated TrkB was not detectable until later developmental times (approximately E13). However, as development progresses truncated TrkB becomes the predominant form, very similar to that observed in the adult mouse brain, as described above. These two isoforms are developmentally regulated during maturation of the CNS.

Analysis of neurotrophin receptors in mouse embryonic brain showed a similar pattern to that found in avian brain (Fig. 5*C*). At early stages of rodent brain development the most abundant receptor forms are the full-length receptors, although even at the earliest time analyzed (E12), truncated TrkB was already present. At later developmental stages, full-length TrkB and TrkC decreased and by adulthood truncated TrkB was the most abundant form. The similarities in the relative profiles of BDNF and NT-3 receptor levels in two distinct species suggest an important role for these neurotrophins in brain development and maturation. In order to detect the cross-linked receptor products of NT-4/5 during mouse brain morphogenesis it was necessary to expose the gels for significantly longer periods of time as compared to the strong signals of BDNF and NT-3 cross-linked to TrkB and TrkC. The receptor profile for NT-4/5 follows the pattern obtained with BDNF. NT-4/5 binding to full-length TrkB reaches a peak around E17 and gradually decreases to low levels in the adult when binding to the truncated form of TrkB represents the majority of the NT-4/5 cross-linked receptors. Binding of NT-4/5 was completely displaced by the addition of cold BDNF but not NGF to the incubation reaction.

To characterize further the nature of the neurotrophin receptors identified by our affinity cross-linking procedure, we also studied the developmental appearance of the full-length and truncated TrkB proteins in mouse brain by Western blot analysis antisera using 442 and 444, specific against full-length TrkB and truncated TrkB, respectively. We prepared WGA-concentrated NP-40 extracts of whole brains from E13, E15, E17, E19, postnatal day 1 (P1), and adult mice. Equal protein loading of the extracts was resolved on a 7% SDS polyacrylamide gel and probed with antiserum 442 (Fig. 6, top). By E15 both truncated and full-length TrkB-encoded proteins are apparent at approximately equivalent levels. The levels of full-length TrkB increase between E15 and P1 and remain present in the adult. The levels of truncated receptor do not increase significantly until P1 and then rise dramatically in the adult. While not apparent from this exposure the truncated receptor in adult is severalfold higher

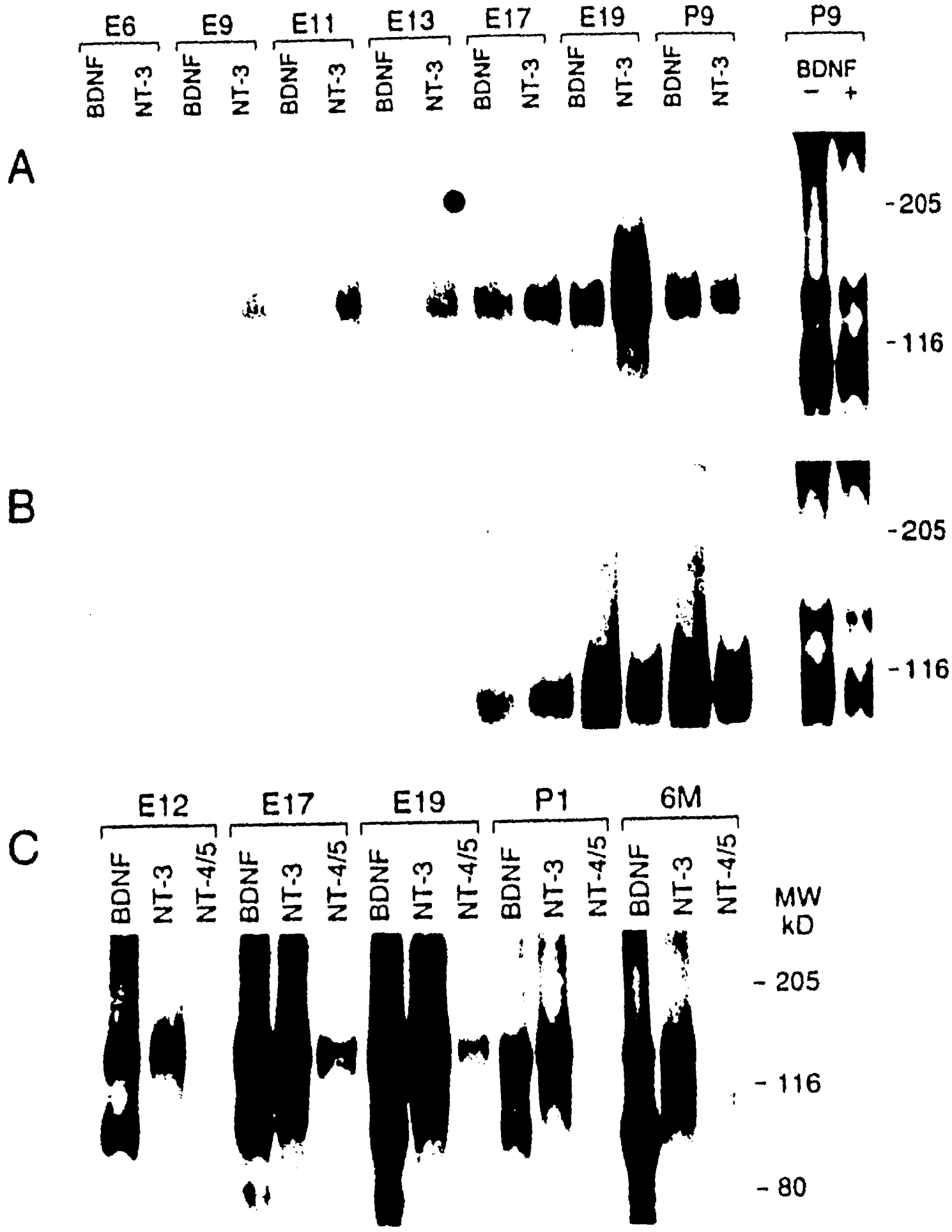

Figure 5. Binding of BDNF, NT-3, and NT-4/5 to full-length TrkB and TrkC and truncated TrkB receptors during brain morphogenesis. Crude membrane preparations derived from chicken (*A* and *B*) and mouse brains (*C*), isolated at different embryonic and postnatal stages, were incubated in the presence of 1 nM (*A* and *B*) or 0.5 nM (*C*) of the indicated labeled neurotrophins. After EDAC cross-linking the samples were immunoprecipitated with anti-443 (*A*), anti-444 (*B*), or a mixture of both (*C*), and analyzed by SDS-PAGE. The fixed and dried gels were exposed for 5 d (*A* and *B*) or 14 d (*C*). The *last two lanes in A* and *B* were not immunoprecipitated in order to serve as a control for total binding. − and + indicate the absence or presence of a 1000-fold excess of cold NGF. Positions of molecular size markers are indicated in kilodaltons. *6M*, 6 months.

than the full-length receptor. To verify the identity of the faster migrating form we stripped and reprobed the blot with antiserum 444 specific for the TrkB truncated form (Fig. 6, bottom). These results confirmed the identification of functional receptors for BDNF during development by affinity cross-linking.

Neurotrophin receptors in the postnatal cortex

The significant changes in full-length versus truncated forms of neurotrophin receptors seen in developing tissues prompted us to investigate receptor regulation at various postnatal times (Fig. 7). Cortical tissues from mice of 1, 6, and 18 months of age were used for cross-linking with BDNF and NT-3 in the presence and absence of heterologous ligands. The results demonstrate that after 1 month of age there were no significant changes in the levels and relative ratios of neurotrophin receptor forms. As already noted above, BDNF and NT-3 label overlapping receptors, that is, full-length and truncated forms of TrkB. When an excess (100×) of unlabeled NT-3 is present during the labeling with BDNF, a partial displacement of binding to a full-length receptor form (TrkB) can be observed and, conversely, labeling with NT-3 can be partially displaced with an excess of unlabeled BDNF. However, only BDNF can displace labeling of the truncated TrkB form, whereas NT-3 does not eliminate BDNF binding to full-length and truncated TrkB even at a 100× excess (compare Fig. 7*A*,*B*).

Neurotrophin receptors in the basal forebrain and hippocampus

Neurotrophins exert trophic effects on neurons of the basal forebrain that project to the hippocampus (Hefti et al., 1989; Knüsel et al., 1991, 1992). To begin to understand the differences in target responsiveness to different neurotrophins we analyzed their receptors in both neuronal tissues using BDNF, NT-3, and NT-4/5 (Fig. 8). We analyzed mouse basal forebrain and hip-

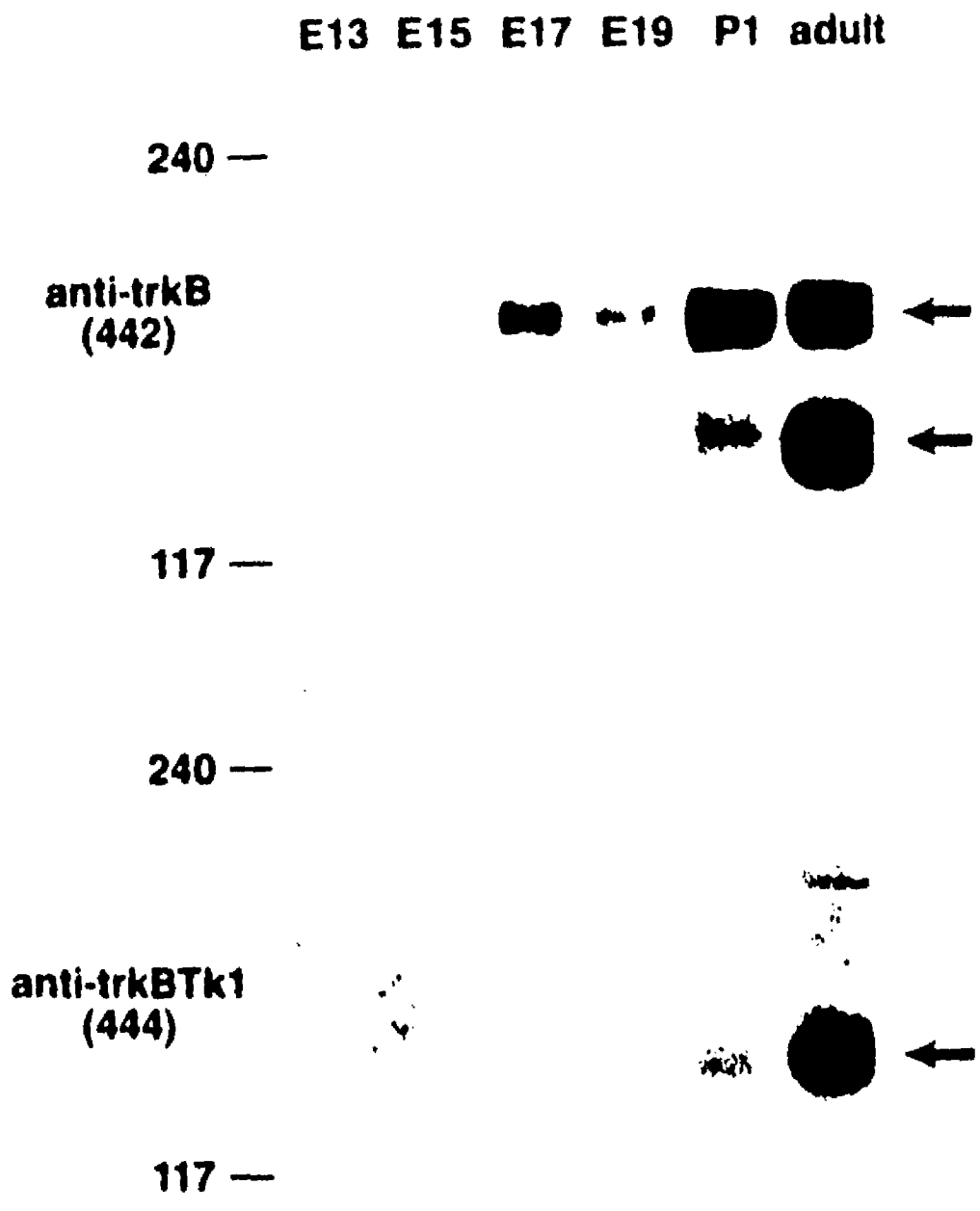

Figure 6. Developmental expression of TrkB receptors in the mouse by Western analysis. NP-40 lysates of cortex obtained from embryonic (E13, E15, E17, E19), neonatal (P1), or adult mice were precipitated with WGA–Sepharose; the precipitates were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with TrkB-specific antiserum 442 (*top*), and then stripped and reprobed with truncated TrkB-specific antiserum 444 (*bottom*). The reactive bands were visualized with HRP-conjugated anti-rabbit IgG and the ECL reagent. Positions of molecular size markers are indicated in kilodaltons.

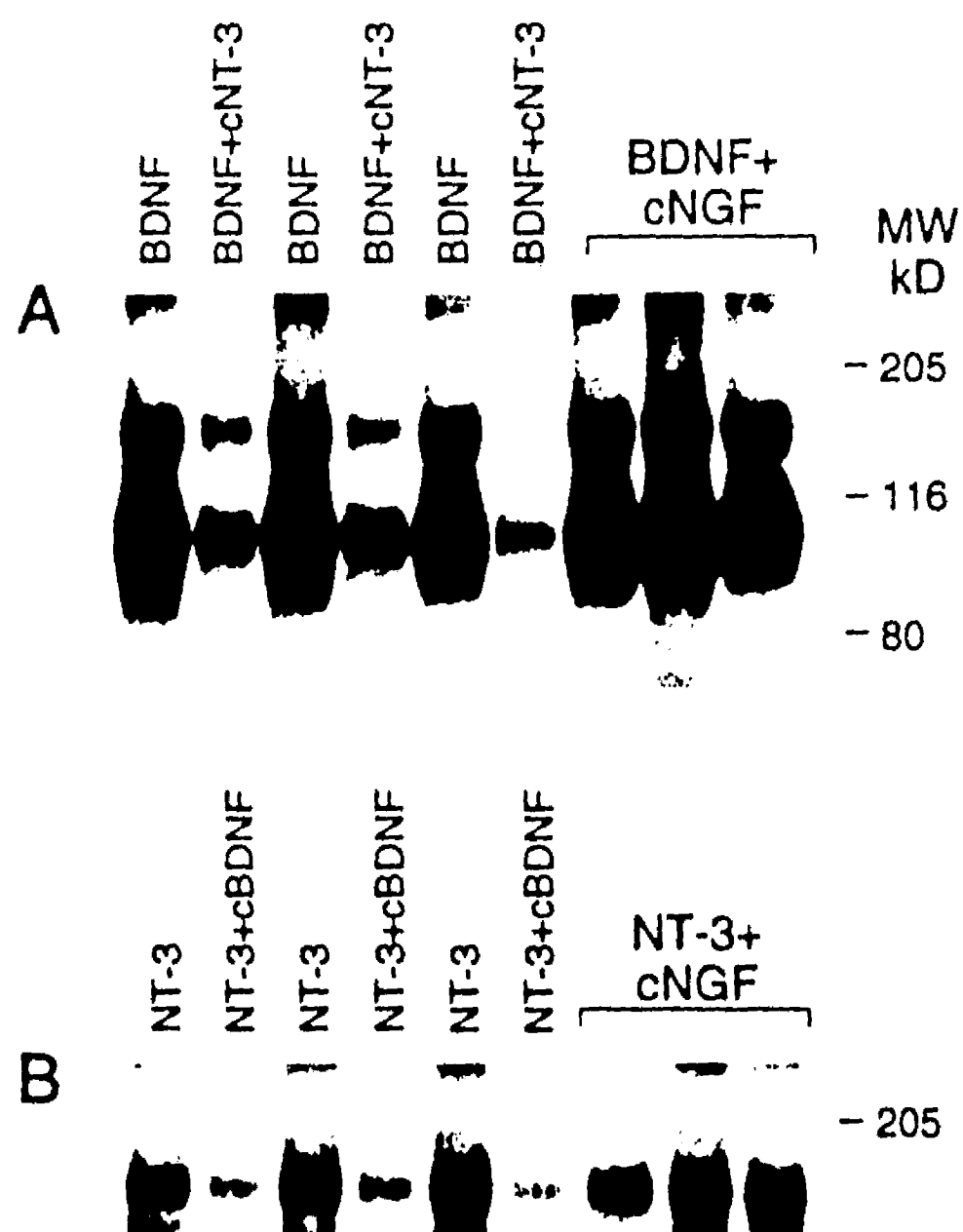

Figure 7. Expression profile and ligand specificity of neurotrophin receptors in the postnatal cerebral cortex. Crude membrane preparations from mouse cortex were dissected at progressive postnatal ages (1, 6, and 18 months). Equal amounts of membranes (5 mg of total protein/assay) were incubated with 1 nM labeled BDNF (*A*) or NT-3 (*B*) in the presence or absence of unlabeled heterologous ligands. Following EDAC cross-linking and immunoprecipitation with a mixture of anti-443/444 antibodies, the samples were subjected to gel electrophoresis on a 6% SDS/polyacrylamide gel. Unlabeled BDNF and NT-3 were used at 150-fold excess, and NGF at 1000-fold excess. The fixed and dried gel was exposed for 7 d. The positions of the molecular size markers are indicated in kilodaltons.

pocampal tissues obtained from animals of different ages in order to see if age-related changes could be detected. All three neurotrophins bound to receptors in both tissues; however, clearly detectable differences were found. Both basal forebrain and hippocampus are rich in full-length and truncated TrkB as demonstrated by the intense labeling with BDNF. A significant although lower degree of NT-3 labeling was also found in both tissues. In the basal forebrain (Fig. 8*B*) the amount of full-length receptors cross-linked with NT-3 was substantially lower in comparison to the extensive labeling with BDNF. These results are in direct agreement with the relative efficacies of the two neurotrophins in mediating survival effects of cholinergic neurons in the basal forebrain. In addition, NT-3 cross-linking showed unique differences in the relative binding to full-length TrkC versus truncated TrkB receptors in these tissues. NT-3 labeling of full-length TrkC was higher in the hippocampus where poor cross-linking to truncated TrkB was found. In contrast, in the basal forebrain full-length TrkC was diminished together with a substantial increase in the binding of NT-3 to truncated TrkB, which was very abundant in both tissues as judged by BDNF binding. Although additional studies are necessary to understand the biological implications of this differential regulation of neurotrophin receptor expression and binding activity, our results suggest a complex functional interaction between the different full-length and truncated Trk receptors with their respective ligands *in vivo*. NT-4/5 cross-linked receptor products showed a weak signal in both tissues similar to that observed and discussed above in whole embryonic brain. Only basal forebrain cross-linking of NT-4/5 is shown in a longer exposure time.

With the exception of a subtle decrease in full-length and truncated TrkB, we did not detect significant age-dependent alterations in the relative ratios or total amounts of the receptor complexes identified by affinity labeling with BDNF, NT-3, and NT-4/5. A similar analysis of whole-brain tissue in the presence of the heterologous cold ligand (150×) provided similar results.

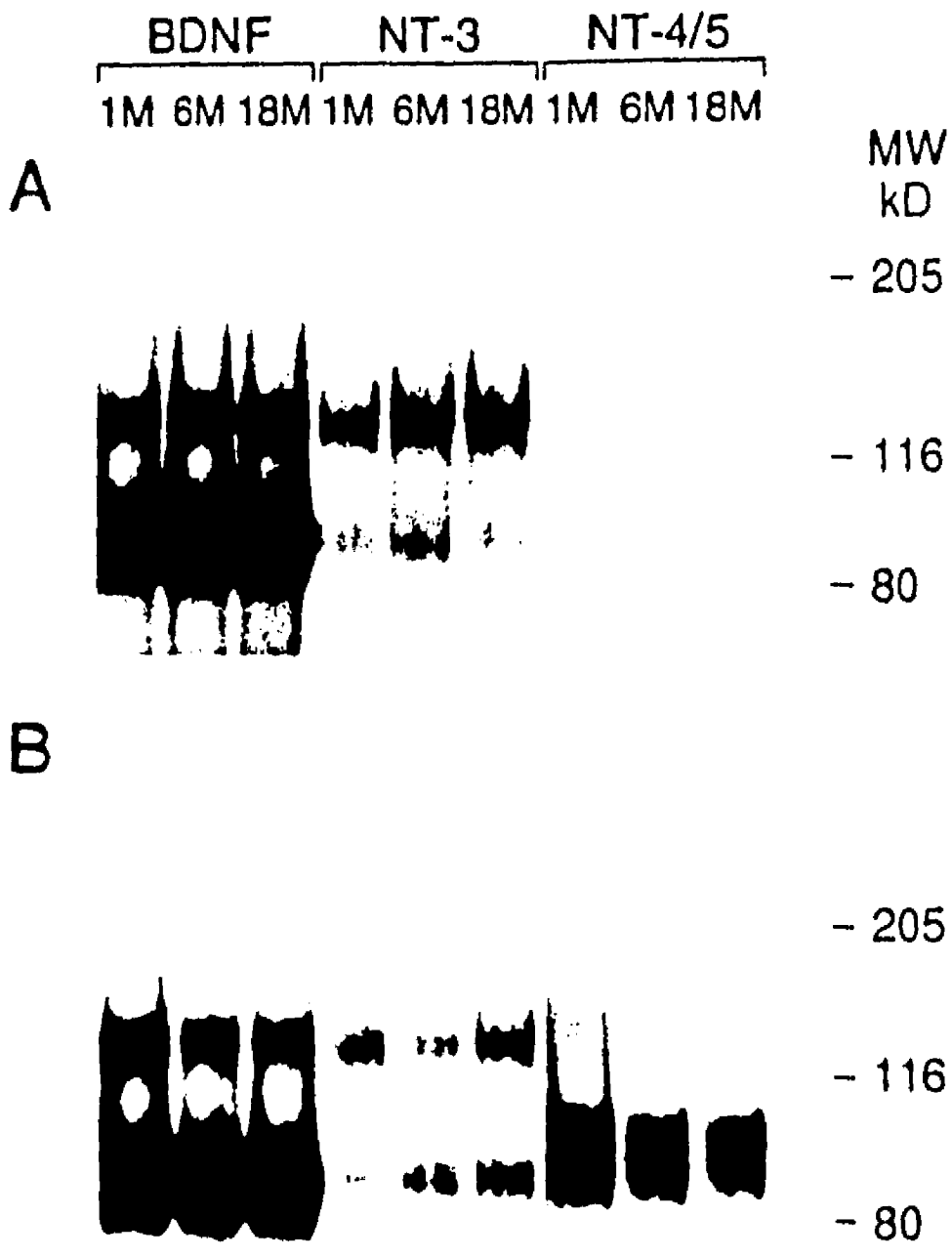

Figure 8. Comparison of functional neurotrophin receptors identified in basal forebrain (*A*) and hippocampus (*B*) of young, adult, and aged mice. Dissected tissues were incubated in the presence of 1 nM labeled BDNF, NT-3, or NT-4/5. After EDAC cross-linking the samples were immunoprecipitated with a mixture of anti-443/444 antibodies and subjected to electrophoresis on a 6% polyacrylamide/SDS gel. The fixed and dried gels were exposed for 5 d, with the exception of NT-4/5 cross-linking in *B*, which was exposed for 14 d. The positions of the molecular size markers are indicated in kilodaltons.

The abundant NT-3 binding to truncated TrkB was completely displaced by cold BDNF. On the other hand, cold NT-3 at 200× excess was unable to compete completely with the binding of labeled BDNF to full-length or truncated TrkB under these experimental conditions. In contrast, unlabeled NGF even at a 1000× excess failed to displace any noticeable level of either BDNF or NT-3 binding to trk receptors *in vivo* measured by affinity labeling.

Substantia nigra and corpus striatum

BDNF and NT-4/5 have been shown to act as trophic factors for dopaminergic neurons in the substantia nigra *in vitro* but not *in vivo* and both factors also protect this population of neurons from neurotoxic damage *in vitro* (Hyman et al., 1991; Knüsel et al., 1991, 1992; Hynes and Rosenthal, 1993). We therefore analyzed the presence of neurotrophin receptors in the substantia nigra, as well as the corpus striatum, the target area of the nigral neurons. Based on histochemical analysis the dissection procedure yielded tissue from newborn rat brain that was greater than 50% dopaminergic neurons (J.-L. Mendoza-Ramirez, unpublished observations). Interestingly, as shown in Figure 9, both tissues showed significant receptor levels when cross-linking with either BDNF, NT-3, or NT-4/5. The relative amounts

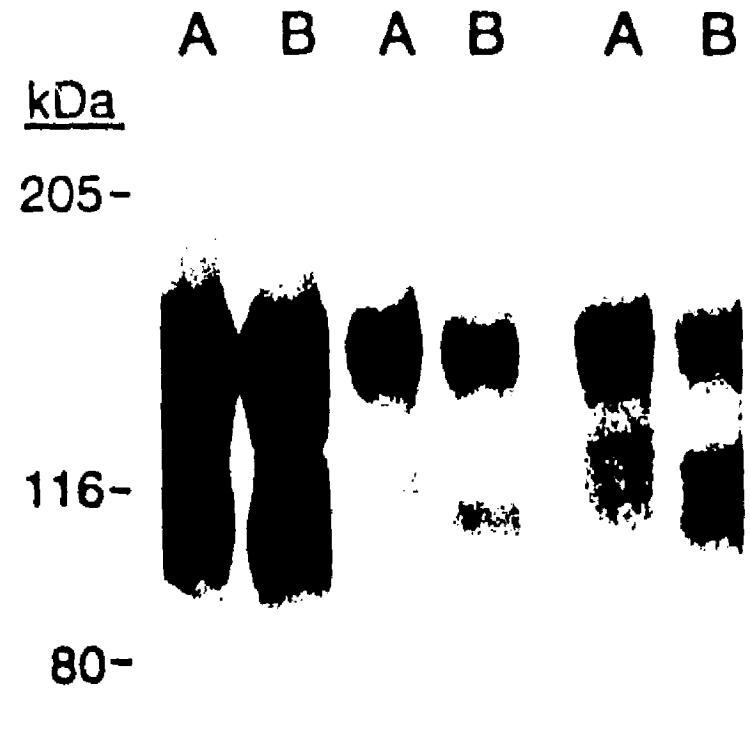

Figure 9. Receptors for BDNF, NT-3, and NT-4/5 in the neonatal nigrostriatal system. Corpus striatum (*A*) and substantia nigra (*B*) tissues were isolated from P1 rat brains and subjected to EDAC cross-linking (0.75 mg total protein/sample) in the presence of 1 nM radioiodinated neurotrophins, as indicated. Following affinity labeling, the samples were immunoprecipitated with a mixture of anti-443/444 antibodies and electrophoresed on a 6% SDS/polyacrylamide gel. The fixed and dried gel was exposed for 6 d. The positions of the molecular size markers are indicated in kilodaltons.

of full-length and truncated receptors were not significantly different in the two tissues at this developmental stage. There was relatively more intense labeling with NT-4/5 as compared to that observed with other tissues.

Neurotrophin receptors in developing spinal motoneurons

Neurotrophin receptors in the spinal cord were analyzed with particular focus on the motoneuron population (Fig. 10). In embryonic chick ventral spinal cord, products corresponding to p75, full-length TrkB, and TrkC could be identified during the phase of naturally occurring motoneuron cell death (E5.5, E6.5, and E7.5). A dramatic increase in full-length TrkB and TrkC expression from E5.5 to E6.5 was observed while the relatively high levels of p75 remained constant. In purified motoneurons from E5 chick spinal cord (Bloch-Gallego et al., 1991) we were able to identify a very weak cross-linked product with NT-3 but not with BDNF or NT-4/5, suggesting that only low levels of full-length TrkC receptors were present at this early developmental age (Fig. 11). This is in agreement with the lack of survival effect of BDNF, NT-3, and NT-4/5 on motoneurons purified from E5 chick embryos (Arakawa et al., 1990; Henderson et al., 1993).

BDNF (Oppenheim et al., 1992; Sendtner et al., 1992; Yan et al., 1992) and more recently NT-3 and NT-4/5 (Henderson et al., 1993) have been reported to exert trophic effects on mammalian motoneurons *in vivo* and *in vitro*. In addition, mRNA for BDNF and NT-3 has been detected in rodent skeletal muscle at the time developing motoneurons undergo programmed cell death, strongly suggesting that neurotrophins play active roles in regulating motoneuron survival *in vivo*. We have identified functional receptors for BDNF, NT-3, and NT-4/5 in isolated rat ventral spinal cord tissues at E15, E17, and E20 (Fig. 12). Full-length forms of TrkB and TrkC are abundantly expressed during this developmental time, while truncated TrkB expres-

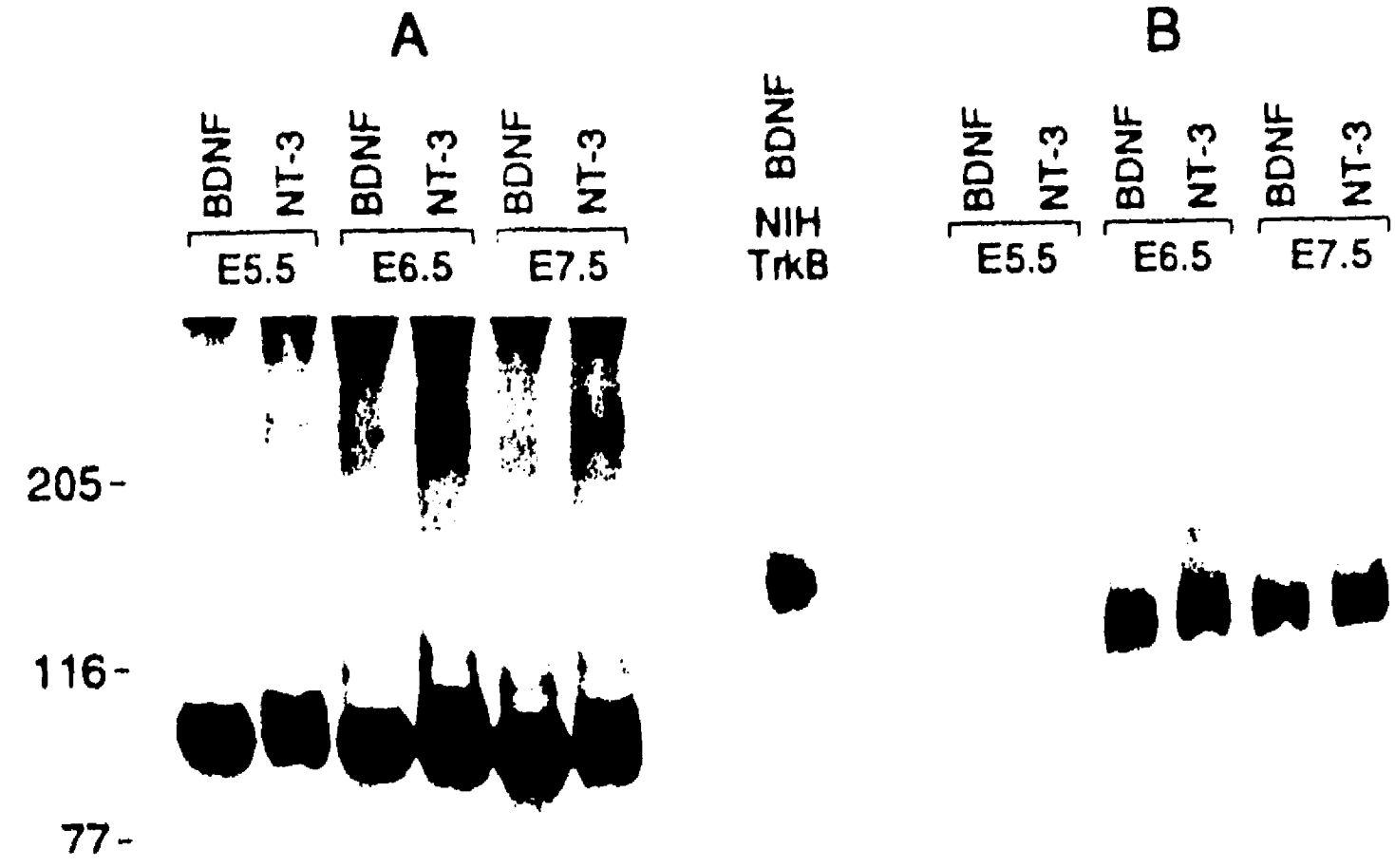

Figure 10. Coexpression of p75 and *trk* receptors in developing avian ventral spinal cord. Viable cells (1×10^6) were dissociated from isolated chicken ventral spinal cord at E5.5, E6.5, and E7.5. The cells were incubated in the presence of 1 nM labeled BDNF or NT-3. After EDAC cross-linking half of each sample was resuspended in SDS sample buffer and subjected to electrophoresis on a 6% polyacrylamide/SDS gel (*A*). The other half of each sample was immunoprecipitated with anti-443 antibodies and then electrophoresed (*B*). Cross-linking of labeled BDNF to NIH3T3 cells expressing full-length TrkB receptors was used as a positive control. *A* was exposed overnight. The other samples were exposed for 12 d. Positions of molecular size markers are indicated in kilodaltons.

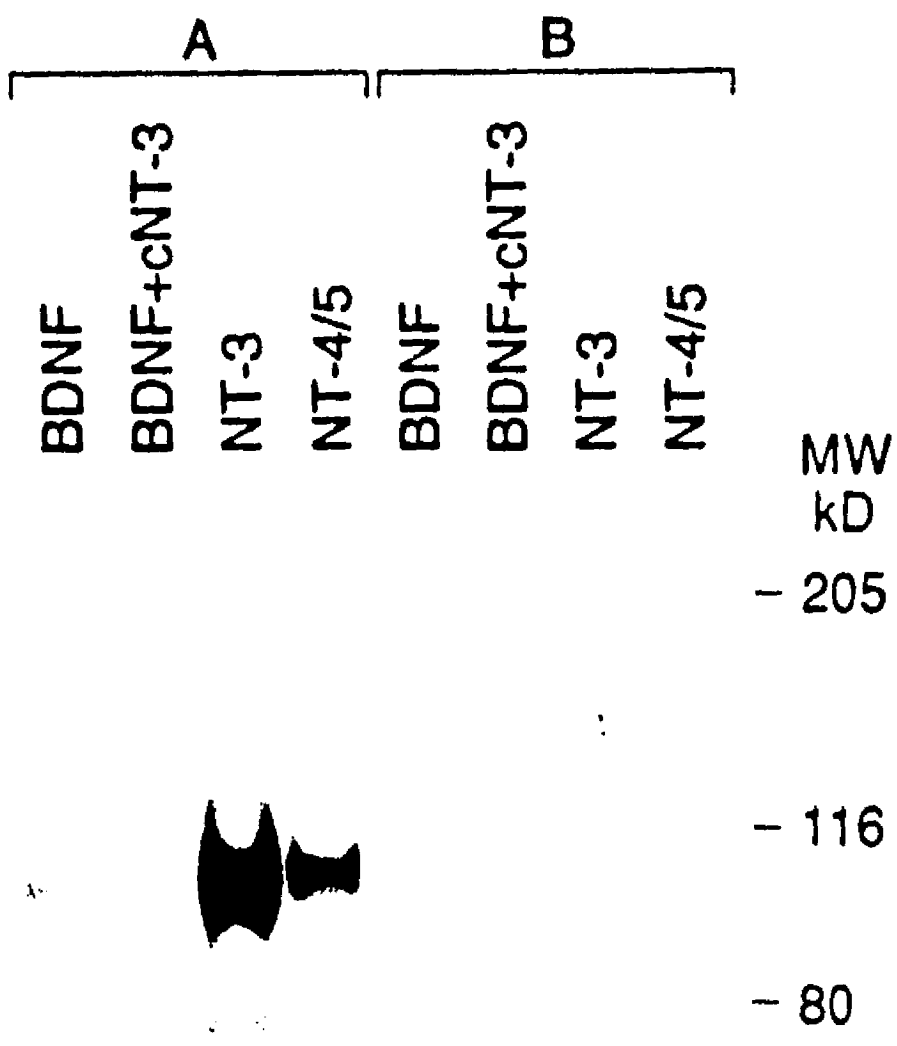

Figure 11. Binding of neurotrophins to purified avian motoneurons. Chicken E5 motoneurons were purified as described in Materials and Methods. Equal amounts of cells (~1×10^5/assay) were incubated in the presence of the indicated labeled neurotrophins with or without a 20-fold excess of unlabeled NT-3. After EDAC cross-linking the samples were resuspended in sample buffer and subjected to electrophoresis on a 6% polyacrylamide/SDS gel (*A*), or immunoprecipitated with anti-443/444 antibodies and treated as above (*B*). The fixed and dried gels were exposed for 3 d (*A*) or 12 d (*B*). Positions of molecular size markers are indicated in kilodaltons.

sion increases significantly by E20. This overall pattern is analogous to that seen in brain tissue. NT-4/5 cross-linked receptor complexes, as we have shown above, require longer exposure times and the receptor species identified are similar to the ones identified with BDNF (not shown). To characterize the specificities of the Trk receptors expressed in E15 rat ventral spinal cord we performed receptor cross-linking in the presence of heterologous unlabeled ligands (Fig. 13). Our results demonstrate the presence of highly specific functional receptor complexes for BDNF and NT-3. Interestingly, NT-4/5 was cross-linked only to full-length TrkB, as shown by full displacement with excess (20×) unlabeled BDNF.

We then analyzed the specificity of neurotrophin cross-linking to purified motoneurons obtained from E15 rat embryos by the method described earlier (Camu and Henderson, 1992) in the presence or absence of heterologous ligands (Fig. 13). The results, in agreement with our observations in ventral spinal cord tissue, demonstrate the presence of considerable levels of full-length TrkB and TrkC receptors. A lower level of truncated TrkB was also present in E15 motoneurons although the full-length isoform was the most abundant one. With E15 rat motoneurons under the cross-linking conditions used for BDNF and NT-3 we found very low to undetectable levels of NT-4/5 binding to a *trk* family receptor identified by the anti-443/444 antibodies, which most probably was due to the lower efficiency of EDAC to link NT-4/5 covalently to the TrkB receptor.

Neurotrophin receptors in the retinotectal system

Retinal ganglionic neurons have been reported to be responsive to BDNF (Johnson et al., 1986), and therefore we analyzed retinal tissue and the optic tectum, which contains the terminals of these neurons projecting to the brain. In E15 chicken neural retina only full-length TrkB could be detected, whereas in the optic tectum very high levels of both truncated and full-length TrkB were found (Fig. 14). In the case of NT-4/5, again we found no detectable binding/cross-linking to chicken tissues, neuronal or non-neuronal, when immunoprecipitation with anti-443/444 antibodies was used. In this study only chicken tissues were analyzed at one developmental age. A detailed analysis of neurotrophin receptors in developing tissues (embryonic and postnatal) of the mammalian visual system using the same cross-linking methodology applied here has recently been described (Allendoerfer et al., 1993).

Peripheral nerves

DRG-derived neurons from E9 chick embryos have previously been shown to respond to BDNF and NT-3. However, sym-

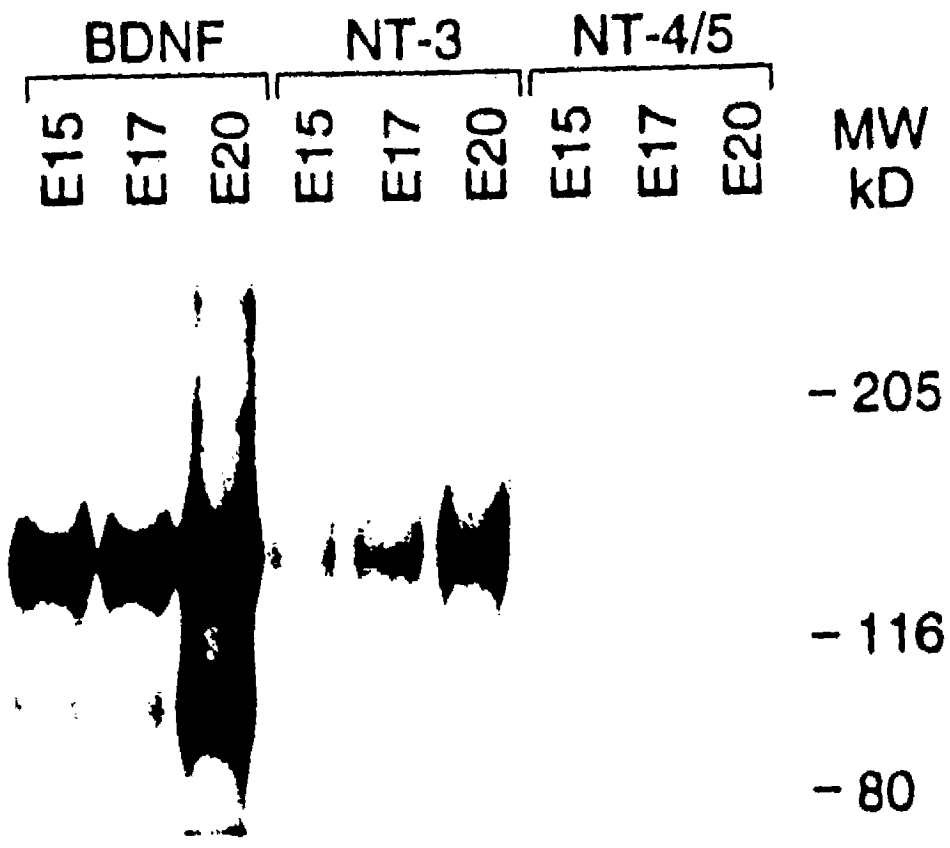

Figure 12. Developmental profile of affinity-labeled BDNF, NT-3, and NT-4/5 receptors in rat ventral spinal cord. Dissociated cells (1×10^6/assay) were incubated in the presence of 1 nM of the indicated labeled neurotrophins. Following EDAC cross-linking and immunoprecipitation with a mixture of anti-443/444 antibodies, the samples were treated as described in Figure 9. The fixed and dried gel was exposed for 10 d. Positions of molecular size markers are indicated in kilodaltons.

pathetic neurons are not responsive to BDNF. Therefore, we analyzed neurotrophin receptors in DRG, and compared them with sympathetic ganglia at two different embryonic times. The E9 time point precedes and E14 follows the phase of naturally occurring cell death of these neural crest–derived neurons. DRG at E9 displayed functional receptors for BDNF and NT-3 that corresponded to the full-length forms of TrkB and TrkC (Fig. 15). The signal obtained with NT-3 was particularly intense compared to BDNF. By E14 in DRG neurons there was a clear decrease in neurotrophin receptor expression that appears to coincide with the onset of dependence on neurotrophins of DRG neurons. On the other hand, purified sympathetic neurons showed abundant truncated TrkB on E9 that decreased to undetectable levels by E14. Specific NT-3 cross-linking indicative of full-length TrkC was detectable on both E9 and E14. These findings support that E14 sympathetic neurons survive when treated with NT-3, but not with BDNF.

Neurotrophin receptors in non-neuronal tissues

Surprisingly, in addition to neuronal tissues we also detected functional neurotrophin receptors in some embryonic avian non-neuronal tissues: liver, skeletal muscle, gut, and kidney (Fig. 14). The pattern of TrkB and TrkC expression in these tissues appears to differ significantly from those found in neuronal tissues. High levels of a product that most likely represents truncated TrkC were found in several embryonic chicken tissues, while truncated TrkB was undetectable under identical experimental conditions (Fig. 14, top). We also found full-length TrkC receptors in dissected skeletal muscle and to a lesser degree in gut and kidney, whereas full-length TrkB was undetectable.

Several mouse non-neuronal embryonic tissues were also analyzed by neurotrophin cross-linking (Fig. 16). Similar to developing avian tissues, full-length TrkB was not detectable in isolated E13.5 tissues (limbs, heart, gut, and liver); however, receptors were present in whole-body samples. Only the truncated form of TrkB could be detected in E12 whole body. Interestingly, by E13.5 there was a significant increase in truncated TrkB expression and full-length TrkB was first detected. In whole body, the BDNF cross-linked receptors may represent in part binding to neuronal tissues like spinal cord, sympathetic ganglia, and DRG because non-neuronal tissues isolated at the same embryonic time (E13.5) expressed very low amounts of TrkB binding. We also detected binding of BDNF to truncated TrkB in the placenta. Adult brain was used as a positive control.

In the mouse, expression of full-length TrkC was already detectable by E12 whole body and was considerably increased by E13.5. However, in contrast to avian non-neuronal embryonic

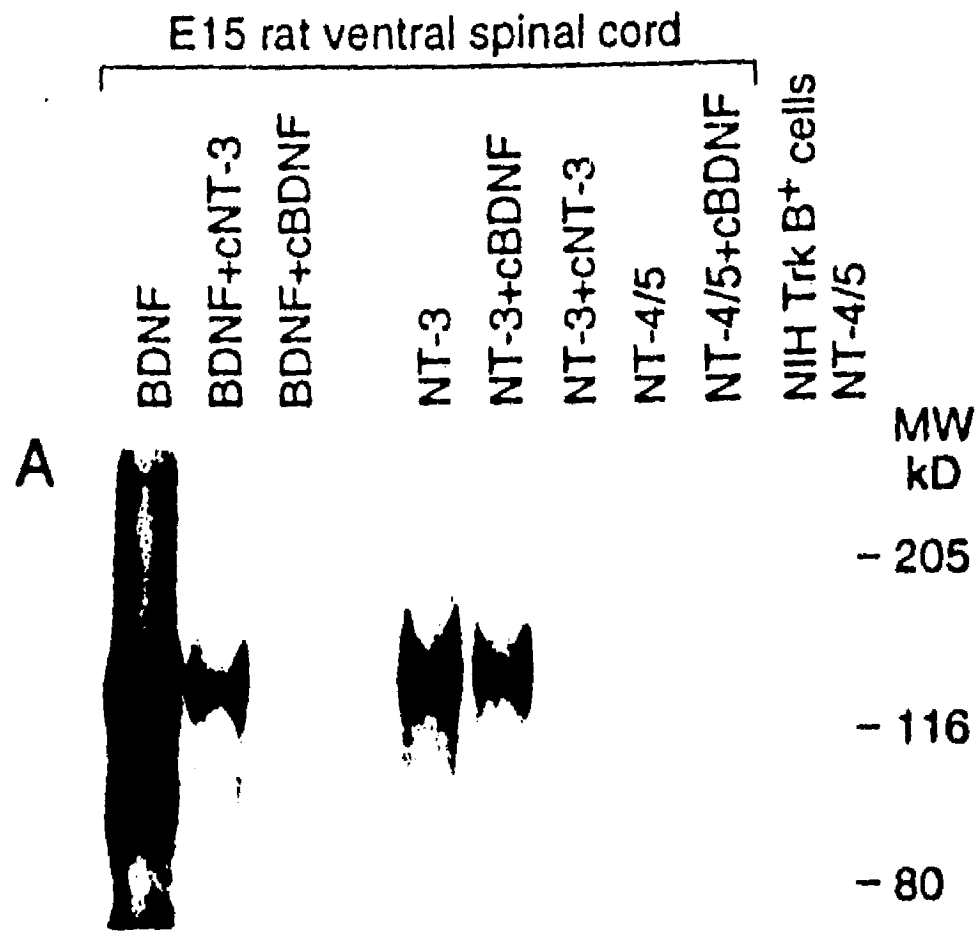

Figure 13. Specificity of neurotrophin binding to E15 rat ventral spinal cord and purified motoneurons. Dissociated cells (~5×10^5 and 1×10^5/assay, *A* and *B*, respectively) were incubated in the presence of 1 nM labeled neurotrophins with or without an excess of unlabeled BDNF, NT-3 (150-fold), or NGF (1000-fold). After EDAC cross-linking the samples were immunoprecipitated with a mixture of anti-443/444 antibodies and treated as described under Materials and Methods. The fixed and dried gels were exposed for 14 d. Positions of molecular size markers are indicated in kilodaltons.

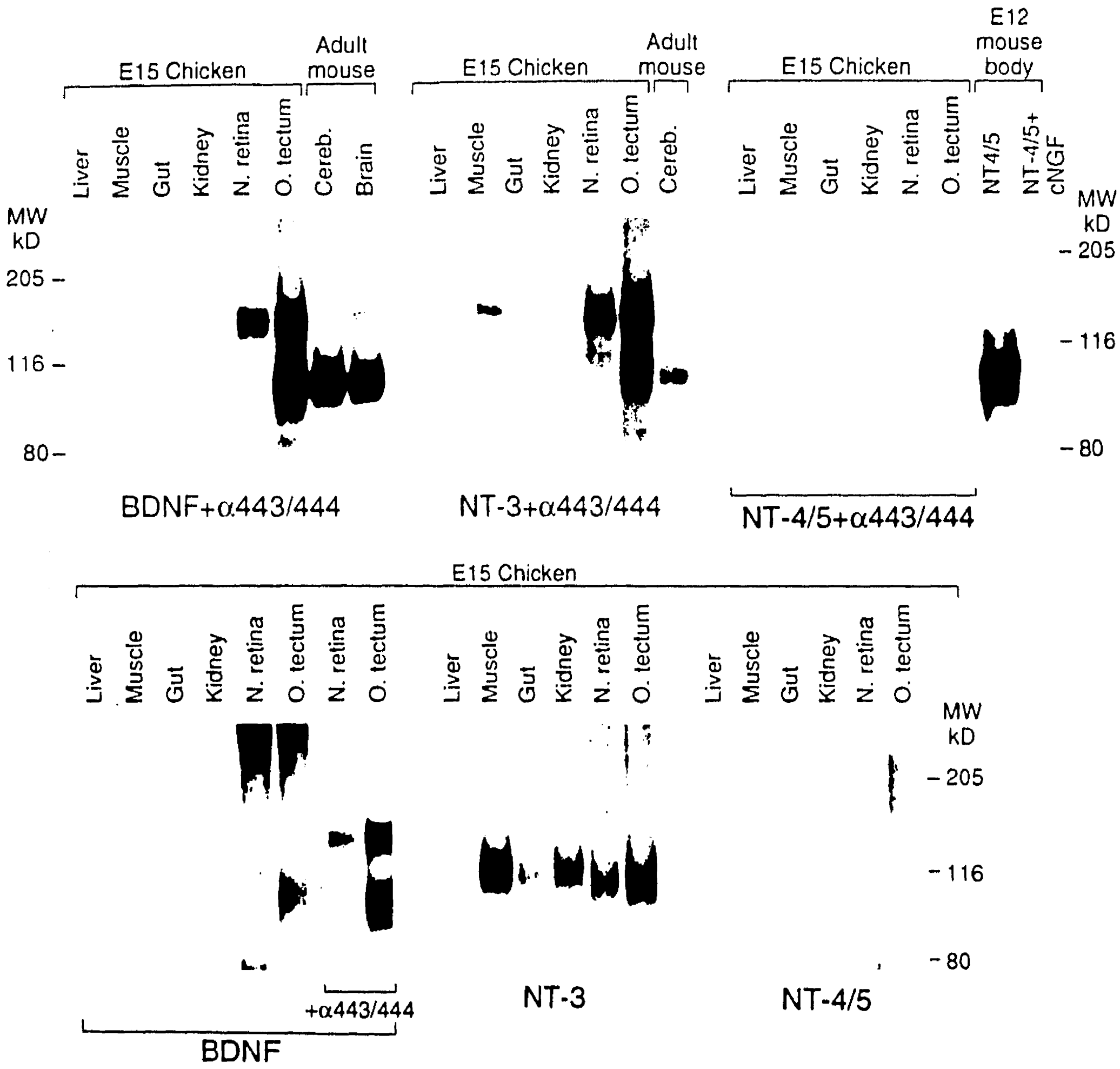

Figure 14. Identification of neurotrophin receptors in chicken non-neuronal tissues and in the developing visual system. Crude membranes were prepared from several E15 chicken tissues. The samples (3 mg of total protein/assay) were incubated in the presence of 1 nM labeled BDNF, NT-3, or NT-4/5. After EDAC cross-linking the tissues were either resuspended in lysis buffer and analyzed with 6% SDS-PAGE, or immunoprecipitated with a mixture of anti-443/444 antibodies and treated as described in Materials and Methods. All the incubation reactions, with the exception of NT-4/5 cross-linking to E12 mouse body, were done in the presence of a 1000-fold excess unlabeled NGF. In addition to E12 mouse body, adult mouse brain and cerebellum were used as positive controls. The fixed and dried gels were exposed for 5 d.

tissues, we did not identify specific binding of NT-3 in isolated non-neuronal tissues (limbs, heart, gut, and liver) at these developmental stages in the mouse. Cross-linking of labeled NT-4/5 followed, again with lower intensity, the BDNF binding profile when the reaction was immunoprecipitated with anti-443/444 antibodies (Fig. 16). Control samples not immunoprecipitated clearly showed intense binding of NT-4/5 to $p75^{NGFR}$, the low-affinity NGF/neurotrophin receptor. The addition of cold NGF completely displaced the binding of NT-4/5 under these conditions. Interestingly, placental tissue expressed detectable levels of $p75^{NGFR}$.

Discussion

We have analyzed various tissues from different embryonic and postnatal ages associated with neurogenesis, migration, differentiation, development of connections, cell death, remodeling, and aging. Neurotrophins, which act as survival factors, have also been reported to stimulate processes of cell proliferation, differentiation, as well as axonal and dendritic arborization (Cattaneo and McKay, 1990; Sieber-Blum, 1991; Kalcheim et al., 1992; Wright et al., 1992). A complete understanding of the complex and multiple functions neurotrophins play temporally

and spatially in the various processes from neurogenesis to long-term survival of terminally differentiated neurons will require studies of neurotrophin and neurotrophin receptor gene expression, as well as studies of functional proteins encoded by these genes. Initial studies of neurotrophin gene expression have shown complex patterns during embryonic development (Ernfors et al., 1990; Hofer et al., 1990; Phillips et al., 1990; Rosenthal et al., 1990; Maisonpierre et al., 1991; Timmusk et al., 1993). Unfortunately, with the exception of NGF, the lack of specific and sensitive antibodies has not permitted a detailed analysis of tissue distribution of neurotrophin proteins. Reports of neurotrophin receptor (*trk* family members and p75) gene expression have underlined and extended our view of the complexity of potential roles neurotrophins play in development. *Trk*, *trk*B, and *trk*C genes were found (by Northern methods or by *in situ* hybridization) to be actively transcribed during development (Klein et al., 1990b; Lamballe et al., 1991; Middlemas et al., 1991; Merlio et al., 1992; Persson and Ibañez, 1993; Tessarollo et al., 1993; Tsoulfas et al., 1993). A direct analysis of functional neurotrophin receptors either by analysis of binding or by immunohistochemical identification of receptor proteins during embryonic development has not been reported. In the present study we addressed this question by a specific and sensitive methodology developed for this purpose.

The results described here demonstrate that BDNF, NT-3, and NT-4/5 receptors corresponding to full-length and truncated forms of TrkB and TrkC proteins are present in neuronal and non-neuronal tissues during embryonic and postnatal development. BDNF and NT-4/5 bind to full-length and truncated forms of TrkB, whereas NT-3 binds to full-length and truncated forms of TrkC, and to a lesser degree to the two forms of TrkB. Earlier reports established that BDNF, NT-3, and NT-4/5 were ligands of TrkB and TrkC (Klein et al., 1991b; Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Berkemeier et al., 1991; Yp et al., 1992; Tsoulfas et al., 1993). BDNF and NT-4/5 bind to TrkB equally well, as determined in this study; however, NT-3's interaction with TrkB differs from the two other factors in affinity, kinetics, and probably structural details. Cross-linking of NT-4/5 to TrkB was not as efficient as BDNF or NT-3, most likely due to a limited availability of reactive side chains in NT-4/5 as compared with the other two factors. Furthermore, NT-4/5 could not be cross-linked to chicken receptors whereas mammalian BDNF and NT-3 showed no differences between mammalian and avian receptor binding. This finding may suggest that NT-4/5 differs more substantially between mammalian and avian forms (the latter have not been identified yet), which may support the proposal that NT-4 and NT-4/5 are species variants of the same molecular entity (Yp et al., 1992), and therefore we used the designation NT-4/5 in this article.

Radioiodinated ligand cross-linking is a convenient method to obtain information on the presence and some biochemical characteristics of functional receptors. The method provides very useful complementary information to *trk*, *trk*B, and *trk*C mRNA expression regarding the appearance and size of the molecular forms of neurotrophin receptor proteins (Escandón et al., 1993). By extending the cross-linking technique using immunoprecipitation with two different antibodies, we were able to improve substantially the sensitivity and specificity of analyzing tissues. Using this method, we have identified full-length forms of TrkB and TrkC, as well as the dominant truncated form of TrkB, which lacks the intracellular kinase domain.

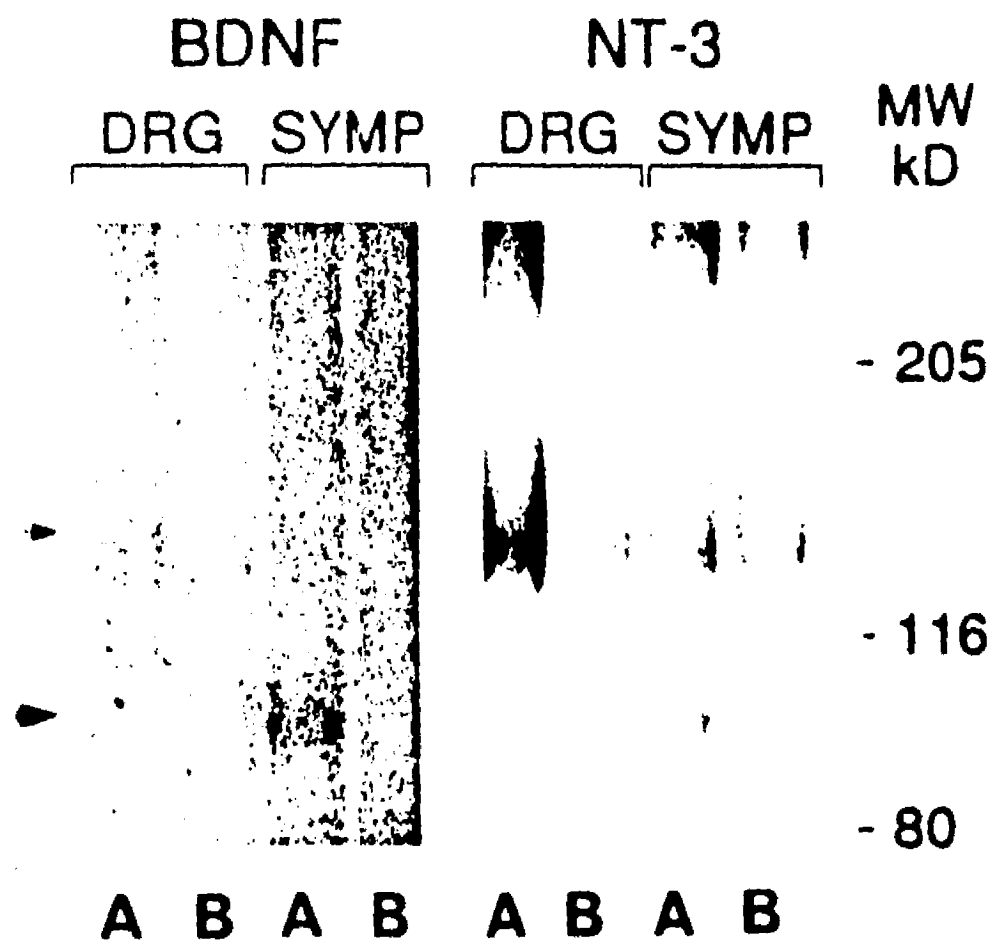

Figure 15. Functional receptors for BDNF and NT-3 in sensory and sympathetic neurons. Cell suspensions (200 μg of total protein/sample) were prepared from E9 (*A*) or E14 (*B*) isolated chicken dorsal root or sympathetic ganglia. The samples were incubated in the presence of 1 nM iodinated BDNF or NT-3. After EDAC cross-linking the samples were immunoprecipitated with a mixture of anti-443/444 antibodies. The tissues were resuspended in sample buffer and subjected to electrophoresis on a 6% SDS/polyacrylamide gel. The fixed and dried gels were exposed for 18 d (BDNF) or 10 d (NT-3). Positions of molecular size markers are indicated in kilodaltons.

in several developing tissues. By subtracting signals obtained with the two antibodies used, we also identified a receptor species that most likely represents truncated TrkC in some tissues.

One of the most important observations we have made is that during embryonic development of different tissues the first form of TrkB and TrkC expressed is their full-length form containing the tyrosine kinase domain. At the earliest time points investigated, very low or no truncated receptor forms (lacking a functional kinase domain) are present. In every case this is followed by a major upregulation of the expression of truncated receptor forms, whereas the full-length receptor remains either unchanged or only slightly decreased. This appears to be a quite general phenomenon that we observed in most tissues. Although we do not have a clear explanation for this developmental shift, several possibilities exist that have already been raised in reports describing truncated *trk* forms (Middlemas et al., 1991; Schneider and Schweiger, 1991). One possibility is that truncated *trk* receptor forms may downregulate the activity of the full-length forms by forming inactive dimers. It will be important to complement our findings with receptor phosphorylation data to test this hypothesis. Another possibility is that the truncated forms act as adhesion molecules and they function to maintain connections established with cells producing full-length receptor molecules. Furthermore, truncated Trks may act as localizing molecules to provide trophic support for specific neurons in a spatially defined manner (Jessell and Melton, 1992). Most major changes in receptor levels and the shift from full-length to truncated forms observed in our analysis take place during embryonic and early postnatal development, but subsequently tissue-specific patterns are established that appear to be maintained throughout adulthood.

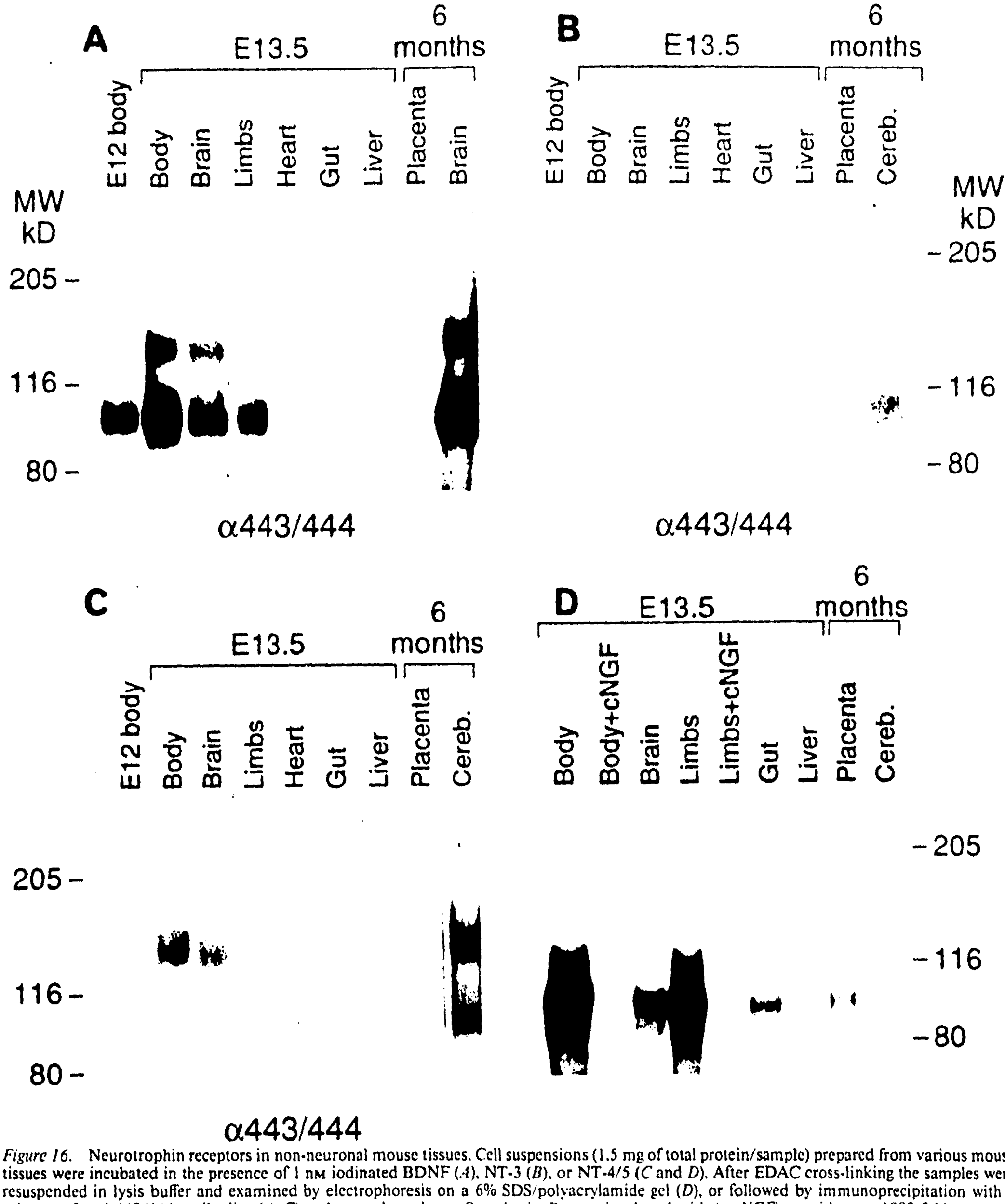

Figure 16. Neurotrophin receptors in non-neuronal mouse tissues. Cell suspensions (1.5 mg of total protein/sample) prepared from various mouse tissues were incubated in the presence of 1 nM iodinated BDNF (*A*), NT-3 (*B*), or NT-4/5 (*C* and *D*). After EDAC cross-linking the samples were resuspended in lysis buffer and examined by electrophoresis on a 6% SDS/polyacrylamide gel (*D*), or followed by immunoprecipitation with a mixture of anti-443/444 antibodies (*A–C*) and treated as above. Samples in *D* were incubated with (*+cNGF*) or without a 1000-fold excess of unlabeled NGF.

NGF has been shown to act as a trophic agent for magnocellular cholinergic neurons of the basal forebrain (Hefti et al., 1989). It has recently been shown that BDNF like NGF but not NT-3 can rescue cholinergic neurons in the basal forebrain of rodents (Alderson et al., 1990; Knüsel et al., 1991, 1992). In addition, both BDNF and NT-3 have been reported to have effects on hippocampal neurons: both neurotrophins upregulate c-*fos*, but only NT-3 stimulates calbindin levels (Collazo et al.,

1992). In postnatal and aging brains we did not detect significant shifts of cross-linked receptor forms between the basal forebrain and the hippocampus. BDNF and more recently NT-4/5 have been shown to act as trophic factors *in vitro* for dopaminergic neurons of the substantia nigra (Hyman et al., 1991; Knüsel et al., 1991; Hynes and Rosenthal, 1993). However, the concentrations of both factors required to elicit the effect are at least 2 orders of magnitude greater than those required for other neurons. Furthermore, BDNF was found to be ineffective *in vivo* on dopaminergic neurons in a lesion model (Knüsel et al., 1992). Using our method of affinity cross-linking, we were able to detect receptors for all three neurotrophins in neonatal substantia nigra and corpus striatum. The relative tissue levels of these receptors were approximately the same as in the basal forebrain or in spinal cord, which are tissues responsive to neurotrophins (Knüsel et al., 1991, 1992; Henderson et al., 1993). This suggests that the low degree of neurotrophin responsiveness and the lack of *in vivo* response are not due to a lack of functional receptors.

In the ventral spinal cord obtained from chick embryos both BDNF and NT-3 showed no or barely detectable signals at E5.5, but there was intense labeling of full-length receptor forms by E6.5, suggesting that within this embryonic day motoneurons become neurotrophin responsive. In motoneurons purified from E5.5 embryos, again a very weak signal was found with BDNF and NT-3. This finding is in agreement with the lack of survival effects of neurotrophins on chick motoneurons from E5–E5.5 embryos (Arakawa et al., 1990; Bloch-Gallego et al., 1991). In contrast, in E14–E20 rat ventral spinal cord intense labeling with BDNF, NT-3, and NT-4/5 was found. In motoneurons purified from E15 rat spinal cord, all three neurotrophins bound to TrkB and TrkC receptors. This is also in good agreement with cell culture studies showing that all three neurotrophins act as potent survival factors for these neurons (Henderson et al., 1993). The timing of motoneuron cultures from chick embryonic spinal cord thus may be critical for the analysis of neurotrophin effects in this system.

Tsoulfas et al. (1993), using a sensitive RNase protection assay, have recently shown the presence of full-length and truncated forms of the *trk*C gene in newborn mouse skeletal muscle, intestine, and kidney. However, using our affinity labeling procedure we were unable to detect functional receptors for NT-3 in those tissues. This may reflect differences in the sensitivity of these assays, or indicate a discrepancy in the relative levels of *trk*C-specific transcripts and the amount of functional NT-3 receptor proteins. We were surprised to find significant levels of full-length TrkC in E13.5 chicken skeletal muscle and somewhat lower levels in embryonic kidney and gut. We also identified large amounts of NT-3 cross-linking to what most probably represents truncated TrkC in embryonic avian skeletal muscle, gut, and kidney, but not liver. In comparison, iodinated BDNF failed to affinity label any *trk* receptors in the same avian embryonic tissues. On the other hand, we have detected binding of BDNF to truncated TrkB in embryonic mouse limbs and developing skin (data not shown). *In situ* hybridization studies of *trk*B expression in non-neuronal tissues have not been published to date. The presence of functional receptors identified by affinity labeling in non-neuronal tissues suggests the possibility of nonconventional developmental roles for the neurotrophins. An elaborate hypothesis for a role of neurotrophins in non-neuronal tissues has recently been described by Scheeterson and Bothwell (1992). Our results warrant further investigation to identify novel physiological effects elicited by neurotrophins in those potentially responsive receptor-positive non-neuronal tissues.

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Functional Neurotrophin Receptors During Embryonic and Postnatal Development of the Nigrostriatal System in the Rat

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) have been suggested to act as trophic agents for dopaminergic neurons of the substantia nigra during embryonic development, and adult life. We have studied the presence of receptors for BDNF, and NT-3 by crosslinking these radioiodinated neurotrophins to specific cell-surface receptors in tissue preparations of the substantia nigra (SN) and corpus striatum (CS) at different embryonic and postnatal ages in rats. Both areas contained functional, ligand binding receptors for the two neurotrophins at all ages beginning at embryonic day 14 (E14). A significant increase in the relative levels of neurotrophin receptors in the SN and CS was observed with increasing age. At early embryonic ages, mostly full-length TrkB and TrkC receptors were found in the SN, whereas a significant amount of truncated TrkB could be detected in the CS. In the SN, truncated TrkB appeared significantly later (by E19) than the full-length form. In both tissues truncated TrkB became the predominant receptor form by adult age. The presence of BDNF, NT-3 and NT-5 receptors in the embryonic and postnatal nigrostriatal system underlines the possible role of BDNF, NT-3 and NT-4/5 in the differentiation and/or survival of neurons in this area. The regulation of the ratio of full-length versus truncated neurotrophin receptors may play an important role in the development, maturation and maintenance of this system.

INTRODUCTION

The neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins -3, and -4/5 (NT-3, NT-4/5) support the embryonic survival and regulate the transmitter phenotype of specific peripheral and central neurons (Barde, 1989; Thoenen, 1991; Snider, 1994). BDNF, NT-3, and NT-4/5 have been found to act as trophic factors for dopaminergic neurons of the substantia nigra (SN). *In vitro* BDNF and NT-4/5 and to a lesser degree NT-3 stimulate the survival of embryonic nigral dopaminergic neurons, and BDNF and NT-4/5 attenuate the neurotoxic effects of MPP^+ and 6-hydroxydopamine on these cells although at relatively high concentrations of the factors (Knüsel et al, 1991; Hyman et al, 1991; Spina et al, 1992; Hynes et al, 1994). BDNF and NT-3 are retrogradely transported from the CS to the SN in dopaminergic neurons, a phenomenon generally associated with neurotrophic effects (DiStefano et al, 1992). In contrast, *in vivo* BDNF does not protect adult, lesioned dopaminergic neurons of the SN (Knüsel et al, 1992; Altar et al, 1992; Lapchak et al, 1993; Sauer et al, 1993). NT-3 has no protective effect on dopaminergic neurons of the SN *in vivo* (Knüsel et al, unpublished).

The neurotrophins bind to specific, high-affinity receptors, members of the Trk family of protein tyrosine kinases and to a low affinity receptor, $p75^{NGFR}$ (Chao, 1992; Dechant et al, 1994). Here we report the expression and relative quantities of functional BDNF and NT-3 receptors using an specific and sensitive affinity labeling technique in the nigrostriatal system of the rat during early and late embryonic and postnatal development.

MATERIALS AND METHODS

Tissue preparations

Based on histochemical analysis using affinity purified anti-tyrosine hydroxylase antibodies (Chemicon), the tissue blocks designated substantia nigra from embryonic and newborn brains represented highly enriched dopaminergic neurons (Mendoza-Ramirez and Escandón, not shown). The embryonic time points were selected based on the establishment of dopaminergic cells in the rat by E13 - E14; and the period of programmed cell death being between E15 and E18 (Voorn et al, 1988). Specific brain regions, designated substantia nigra, striatum, and thalamus, from the embryos were dissected in cold (4°C) PBS containing 5mg/ml glucose and sliced into smaller pieces. Cell suspensions from the different tissues were prepared by gently passing the tissue through an 18-gauge needle until a homogenous suspension was obtained. Large aggregates were removed from the cell preparation by allowing the samples to sediment for 1-2 minutes at 4°C and collecting the supernatant. No enzymatic treatment was used at any stage of these preparations in order to avoid possible modifications of cell surface receptor proteins.

Affinity Crosslinking Procedures

For the receptor crosslinking experiments, the procedure of Escandón et al, 1993) was followed with minor modifications. Briefly, cell triturations prepared from equal amounts of isolated tissues were incubated in PBS-glucose, pH 7.2 with ^{125}I-labeled neurotrophins (used in final concentrations of 0.5 to 1 nM which corresponds to a maximally

effective concentration of each neurotrophin). The neurotrophins were labeled with the Enzymobead procedure as described (Escandón et al, 1993). The samples were incubated at 4°C for 2 hours with gentle agitation. The crosslinking agent 1-ethyl-3- (3-dimethyl- aminopropyl carbodiimide).HCl (EDAC, Pierce) was added in a final concentration of 6mM, and the mixture rotated for 20 minutes at room temperature. The reaction was quenched with cold PBS containing 50 mM lysine and washed three times with PBS. The cell pellet was resuspended in PBS, 5mM $MgCl_2$ and 1% triton X-100 containing 1mM phenyl-ethylsulphonyl fluoride, and treated with DNase I (5-10 mg/sample) for 15 minutes on ice. After centrifugation the reaction products were immunoprecipitated with a mix of two antisera, anti-443 and anti-444. Antiserum 443 recognizes the extracellular domain (amino acids 45-60) of full-length TrkB, whereas antiserum 444 recognizes the C-terminus (amino acids 454-465) of tk-1 truncated TrkB (Escandón et al, 1994). The supernatants were mixed with 5µl aliquots of the two antisera and incubated overnight at 4°C, followed by a 2-hour incubation in the presence of Protein A-Sepharose. The samples were washed 3 times with PBS, 1% triton X-100 buffer and subjected to SDS-polyacrylamide gel electrophoresis. The fixed and dried gels were exposed with Kodak X-O-Mat film for different periods of time at -70°C with an intensifier screen. The specificity of this combined crosslinking and immunoprecipitation procedure has been established previously (Escandón et al, 1994).

RESULTS

BDNF and NT-3 receptors in the substantia nigra, striatum, and thalamus at different developmental times were analyzed by an affinity crosslinking procedure using radiolabeled ligands followed by immunoprecipitation with TrkB and TrkC antibodies (Fig. 1A-F). This procedure allows the identification of functional, ligand binding neurotrophin receptors. Full-length and truncated forms of TrkB and full-length TrkC were found to be present in all three tissues analyzed. We detected the presence of receptor complexes as early as embryonic day 14 for the full-length forms.

In the substantia nigra (Fig 1A, D) there was a significant increase of full-length TrkB protein between E14 and E17. After this stage, the level of full-length TrkB remained constant through the newborn. Truncated TrkB was undetectable or very low before E19 and showed a very modest increase until early postnatal age. By adult (P90) age, the level of full-length TrkB showed a significant decline in the SN and the truncated form of this receptor became the predominant form. NT-3 receptor, full-length TrkC appeared at E16, and remained constant through postnatal times. At P90, we found a low level of crosslinking of NT-3 to truncated TrkB (Fig 1D) which appeared to be the major receptor form at this age. The possible presence of truncated TrkC has not been analyzed due to the lack of a specific antibody.

In the corpus striatum (Fig 1B, E) the major increase of full-length TrkB expression occurred by E19, which followed the one observed in the SN by at least two embryonic days. Subsequently, the level of full-length TrkB showed a further slight increase up to adult ages. In contrast to the SN and Th, in the CS there was a significant level of truncated TrkB

already at E14 and E16. Subsequently, however, truncated TrkB decreased until the newborn stage, followed by a sharp increase by adult age. From the three tissues analyzed, the CS had the highest levels of TrkC, which exceeded the level of TrkB at E14 and E16. Full-length TrkC was already present at E14, and E16 at relatively high levels. After a decline at E17, this receptor form increased repeatedly by postnatal ages.

In the thalamus a very similar pattern to that seen in the SN could be observed (Fig 1C, F). Full-length TrkB protein increased substantially between E14 and E17. Subsequently, the level of full-length TrkB remained about the same through newborn age and then decreased by adult age. Truncated TrkB was undetectable until E19 and then increased slightly by early postnatal stage but by adult age, it became the predominant receptor form. Full-length TrkC in the thalamus increased from E14 to E16 and remained at relatively constant levels through adult ages.

DISCUSSION

The neurotrophins initiate their effects by binding to specific, high-affinity receptors, members of the Trk family of receptor tyrosine kinases and stimulating their autophosphorylation (Chao, 1992; Dechant et al, 1994). NGF is the primary ligand of Trk, BDNF and NT-4/5 are the primary ligands of TrkB whereas NT-3 is the preferred ligand of TrkC but it is also able to bind to the TrkB and Trk proteins. Several splice variants of the *trk*B gene have been described, some of which encode proteins that have an intact extracellular domain but are intracellularly

truncated with unique C-terminal sequences, and they lack the signal transducing tyrosine kinase domain (Klein et al, 1990; Middlemas et al, 1991). TrkC variants have also been identified which contain short inserts in their kinase domains or lack a functional kinase domain (Tsoulfas et al, 1993; Valenzuela et al, 1993).

In the central nervous system, the expression of *trk* is very low (Martin-Zanca et al, 1990) and appears to be localized to basal forebrain cholinergic nuclei and to GABAergic neurons (Holtzman et al, 1992). However, transcripts for *trk*B and *trk*C are abundant during development and in adult animals (Klein et al, 1990; Lamballe et al, 1991; Tessarollo et al, 1993; Tsoulfas et al, 1993; Allendoerfer et al, 1994; Escandón et al, 1994). BDNF, NT-4/5, and NT-3, respectively, are the primary ligands of these proteins, and therefore, we focused our analysis using receptor crosslinking on these receptors. Due to the complexity of the multiple *trk*B and *trk*C transcripts, analysis of mRNA expression in different tissues alone does not allow conclusions regarding functional receptors for the neurotrophins.

The results presented here demonstrate that BDNF, and NT-3 receptors corresponding to full-length and truncated forms of TrkB and to TrkC are present in tissues of the nigrostriatal system during embryonic and postnatal development. At the early embryonic time points analyzed, in the substantia nigra and in the thalamus, the first form of TrkB is its full-length form containing the tyrosine kinase domain. This is later followed by an abundant expression of truncated receptor which lacks a functional kinase. In the striatum, however, even at the earliest times,

both full-length and truncated TrkB was present, which later shifted to a pattern similar to that observed in the other two tissues.

Despite the presence of functional neurotrophin receptors in the developing nigrostriatal system, it is unclear what exact roles the neurotrophins may play in this distinct population of neurons. BDNF, NT-3, and NT-4/5 have been reported to act as trophic factors for mesencephalic dopaminergic neurons prepared from E14-E15 rat *in vitro* (Knüsel et al, 1991; Hyman et al, 1991; Hyman et al, 1994; Hynes et al, 1994). In addition, BDNF and NT-4/5 protect these neurons from toxic agents in culture (Spina et al, 1992; Hynes et al, 1994). However, the concentrations required of BDNF and NT-4/5 to exert their trophic effects on nigral dopaminergic neurons *in vitro* far exceed (20 to 100-fold) the concentrations required of the same factors for other types of neurons such as motoneurons, or sensory peripheral neurons although they involve the same receptors (Hyman et al, 1991; Henderson et al, 1993; Buchman and Davies, 1993; Hynes et al, 1994). In experiments analyzing the concentration-dependence of the crosslinked signals as described earlier (Escandón et al, 1993), we found no difference between the SN, Th, and CS, as compared with other tissues, including cortex, spinal motoneurons, and retinal neurons. This suggests that the binding affinity of TrkB in the SN is not different from TrkB in other tissues. As another possibility, mesencephalic cultures may produce an inhibitory agent which necessitates high concentrations of neurotrophins. From our current data we can exclude the possibility that excessive truncated TrkB downregulates the activity of the full-length receptor. In mice lacking the BDNF or NT-3, or the *trk*B or *trk*C genes, no gross abnormalities of

nigral dopaminergic neurons have been found (Klein et al, 1993 and 1994; Ernfors et al, 1994a,b; Fariñas et al, 1994; Jones et al, 1994). This would suggest that the neurotrophins do not contribute to the survival and/or to the establishment of the dopaminergic phenotype of nigral neurons. Independently, other factors have been discovered which act as highly potent trophic agents for these neurons, such as GDNF, and TGF-ß3 (Lin et al, 1993; Poulsen et al, 1994). It is possible therefore, that this system requires multiple agents which regulate various functions, and the neurotrophins could play a more subtle role rather than regulating survival in this system. One possibility could be a role in regulating synaptic transmission, as e.g. in the case of the neuromuscular synapse (Lohof et al, 1994).

An important observation of our study is the developmental shift from predominantly full-length to predominantly truncated TrkB receptors. This pattern appears to be characteristic to most neuronal systems, as the developing visual system, and spinal motoneurons (Allendoerfer et al, 1994; Escandón et al, 1994). It is still unclear what the role of truncated TrkB might be *in vivo*. The truncated form of TrkB may inhibit the full-length receptor. It may act as an adhesion molecule and help maintain connections established with cells producing full-length receptor molecules. Furthermore, truncated TrkB may act as presentation molecules to localize trophic factors. The cellular source of the truncated receptor could be glial, as glial proliferation parallels the major increase in truncated TrkB levels.

According to the neurotrophic theory, trophic factors are secreted in limited quantities by innervated tissue. Trophic factors are internalized

and retrogradely transported by the innervating neurons. Although our procedure allows the temporal analysis of neurotrophin receptors in specific, well defined tissues, it does not provide cellular resolution which will be required to put our current findings in perspective.

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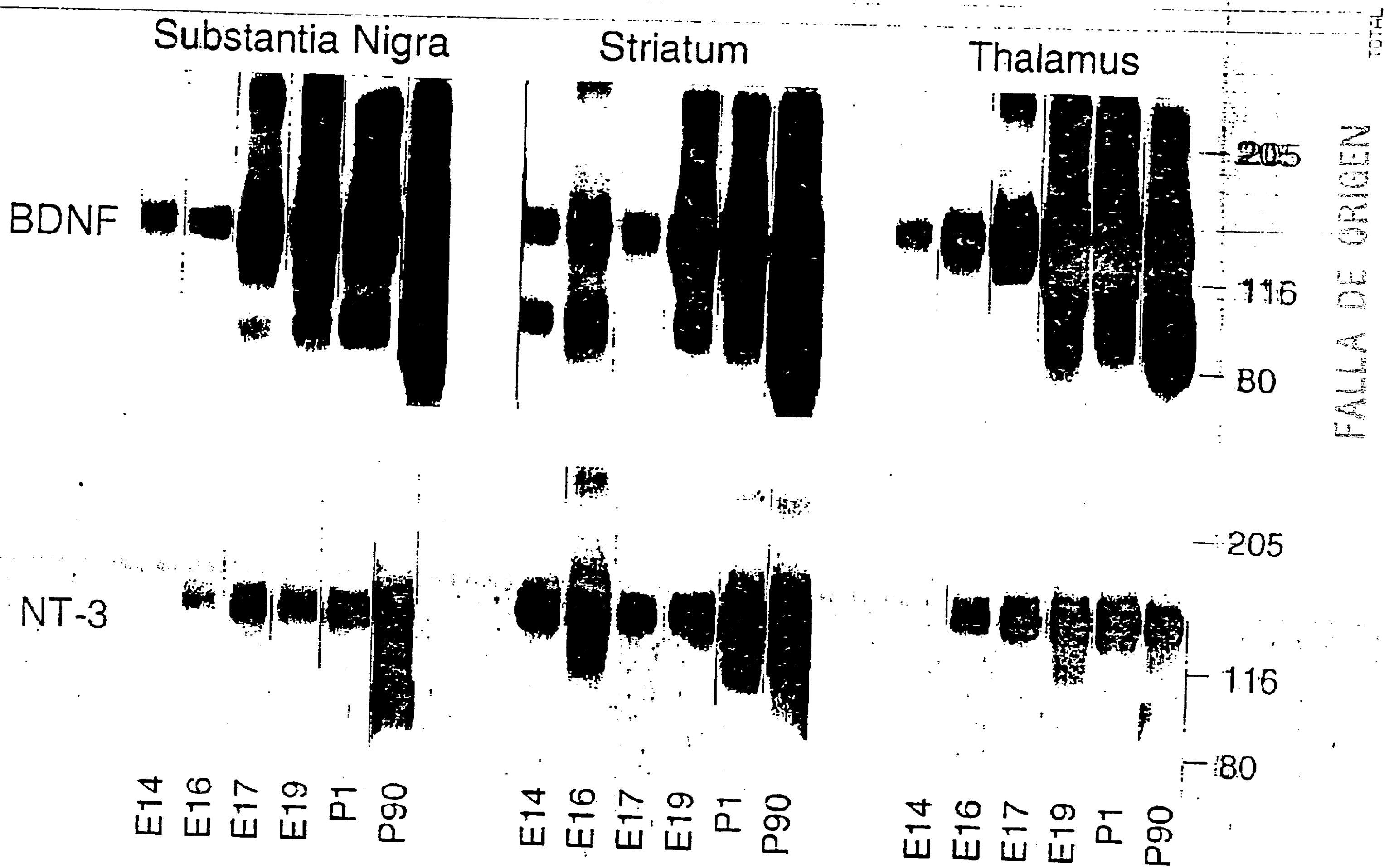

Figure 1. Developmental profiles of functional BDNF and NT-3 receptors in the embryonic and postnatal nigro-striatal system. SN, CS, and Th tissues were isolated form embryonic (E) and postnatal (P) rat brains of the indicated ages (numbers refer to days), and subjected to crosslinking in the presence of radioiodinated neurotrophins. Subsequently, the samples were immunoprecipitated with a mixture of anti-443/444 antibodies as described under Materials and Methods and electrophoresed on a 6% SDS/polyacrylamide gel. The fixed and dried gels were exposed with Kodak -ray film for 6 days. The positions of the molecular size markers are indicated in kilodaltons.

ESTUDIO PILOTO.

"INFUSION CRONICA DE BDNF EN EL PARENQUIMA DEL SISTEMA NIGRO-ESTRIATAL".

Introducción.

Se ha demostrado que en condiciones de cultivo, las neurotrofinas BDNF y NT-3 favorecen la sobrevivencia de las neuronas dopaminérgicas fetales, previniendo los efectos neurotóxicos del MPTP y la 6-OHDA (Hyman y cols, 1991; Knüsel y cols, 1991; Hynes y Rosenthal, 1993). También se ha observado que el BDNF se transporta retrógradamente hacia los somas dopaminérgicos cuando se inyecta en el parénquima estriatal (Lindsay, 1992). No obstante, la inyección intracerebral de BDNF es incapaz de evitar la muerte celular producida por la lesión del sistema nigro-estriatal (Knüsel y cols, 1992).

Mas recientemente se ha demostrado que tanto el BDNF como el NT-3 se expresan en algunas de las neuronas dopaminérgicas en la región medial del mesencéfalo, argumentandose que éstas pueden corresponder con aquellas que generalmente se preservan en la enfermedad de Parkinson (Seroogy y Gall, 1993), por lo que estos factores podrían estar actuando de manera autocrina (Escandón y cols, 1995).

Altar (1991) y Lapchak y cols (1993) han mostrado que en animales intactos, el BDNF inyectado intranigralmente favorece la conducta giratoria orientada hacia el lado ipsilateral, después de la estimulación con anfetamina.

Esto lo adjudicaron a una disminución funcional del sistema nigro-estriatal del lado inyectado.

Por otro lado, se ha observado que el factor neurotrófico derivado de una línea celular de glia, favorece la sobrevivencia de las neuronas dopaminérgicas y parece evitar los efectos tóxicos de la 6-OHDA in vivo (Lin y cols, 1993, 1994; Kearns y Gash, 1995). Sin embargo, existen muy pocas evidencias de los efectos de este factor en condiciones in vivo.

En el presente estudio se muestran los resultados preliminares de los efectos que tiene la perfusión crónica de BDNF, NGF, citocromo c, y anti-GDNF sobre las neuronas dopaminérgicas del mesencéfalo ventral de animales intactos.

Material y método.

Se utlizaron ratas macho de la cepa wistar con un peso corporal de 180±5gr al momento de la cirugia y minibombas osmóticas (Alzet No. 2002) con el kit de perfusión de la misma compañia. Los animales fueron agrupados de la siguiente manera:

Grupo 1. Diez ratas perfundidas con BDNF (0.4μg/0.5μl/hora).

Grupo 2. Cinco animales perfundidos con NGF (0.4μg/0.5μl/hora).

Grupo 3. Cinco fueron perfundidos con citocromo c (Citc; 0.4μg/0.5μl/hora).

Grupo 4. Cinco perfundidos con α-GDNF (0.4μg/0.5μl/hora).

Cada minibomba se implantó en una cavidad subdérmica en la región dorsal del cuello de la rata. La minibomba se conectó a través de un cateter de teflón a una cánula implantada en el parénquima del cuerpo estriado del lado izquierdo de la rata. En cinco ratas que se perfundieron con BDNF el cateter se colocó a 0.5-1 mm por encima de la substancia nigra del lado izquierdo de la rata.

Histología.

Los tiempos de sobrevivencia fueron 5 y 10 días después de iniciada la perfusión. Entonces fueron perfundidos intracardialmente con ayuda de una bomba peristáltica, primero con 300 ml de PBS-heparina (buffer de fosfatos 0.01 M, pH 7.4; NaCl 8.5%; 3000 unidade de heparina/litro) y después con 300 ml de fijador de Somogy (PB 0.1M, pH 7.4; paraformaldehido 4% y ácido pícrico 1.5%). Los cerebros fueron criopreservados en sacarosa al 20% durante 48hrs y siguiendo el plano horizontal se les corto en un criostato a 30 μm. Subsecuentemente se procesaron inmunohistoquícamente por el método de ABC para la enzima tirosina-hidroxilasa.

Resultados.

Durante los 10 días de perfusión los animales que recibieron Citc no mostraron alteraciones conductuales aparentes. De éstos, tres se procesaron para histología a los cinco días y los otros dos se procesaron después de 10. Los

5 animales tratados con NGF mostraron una ligera perdida de peso en relación al peso con el que inició el experimento (10-15%), pero tampoco desarrollaron alteración motora, estos se procesaron de igual manera que los tratados con Citc.

Los animales tratados intraestratalmente con BDNF desarrollaron un estado de adipsia y afagia crónicos que causo que bajaran de peso (50-60%), se observo una asimetria postural orientada hacia el lado de la perfusión, la cual se transformó en un estado de catalepsia bilateral a partir de los 7 y 8 días. De éstos murieron 3 y se procesaron dos. El estado de acinesia se revirtió por unos cuantos minutos cuando los animales eran manipulados o cuando se les colocó en un ambiente nuevo. Sin embargo, durante estos periodos de motilidad debida al estres, los animales no comieron ni bebieron.

De los 5 animales infundidos en el mesencéfalo ventral murieron dos en un lapso de 4 días por lo que los tres restantes se perfundieron al quinto día (en uno de éstos la perfusión ocurrió en la unión del cateter con la minibomba y no mostró las alteraciones descritas).

Por lo que toca a los animales infundidos con anti-GDNF, se encontró que con la dosis utilizada se desarrolla una asimetría postural y motora orientada hacia el lado contralateral de la perfusión a los tres días. Estos animales no mostraron pérdida de peso y de éllos tres fueron procesados para histologia al quinto día. En los dos restantes la asimetría se revirtió entre el septimo y octavo día, siendo sacrificados al décimo día del tratamiento.

Resultados histológicos.

El análisis morfológico del sistema nigro-estriatal en los animales infundidos ya sea con Citc o con NGF mostró que las neuronas dopaminérgicas presentan una apariencia normal (ver figuras 1 y 2; este resultado se ha observado previamente por otros investigadores).

En los cuatro animales infundidos con BDNF que desarrollaron el estado de acinecia y la pérdida de peso, el análisis cualitativo del sistema nigro-estriatal mostró una disminución en la inmunoreactividad de las neuronas dopaminérgicas, aunque algunas neuronas mantuvieron un nivel de inmunoreactividad similar al observado en los animales de los grupos tratados con Citc y NGF (ver figura 3). Es importante señalar que a pesar de que la perfusión fue unilateral los efectos fueron bilaterales, aunque se puede aprecia un efecto mayor en el lado ipsilateral de la perfusión (ver figura 3A). En estos animales no se observaron neuronas muertas (crenadas o pignóticas).

Los resultados histológicos de los animales infundidos durante cinco días con α-GDNF mostraron un incremento evidente en la densidad de fibras TH+ (comparar figura 4 con las figuras anteriores). Estos efectos parecieron revertirse después de 10 días de perfusión (comparar figura 5 con la figura 4). Comparando las muestras de los animales de 5 y 10 días de perfusión, pareciera como que existe un ligero decremento en el número de neuronas inmunoreactivas (figura 5B). La figura 6A muestra los efectos de la perfusión

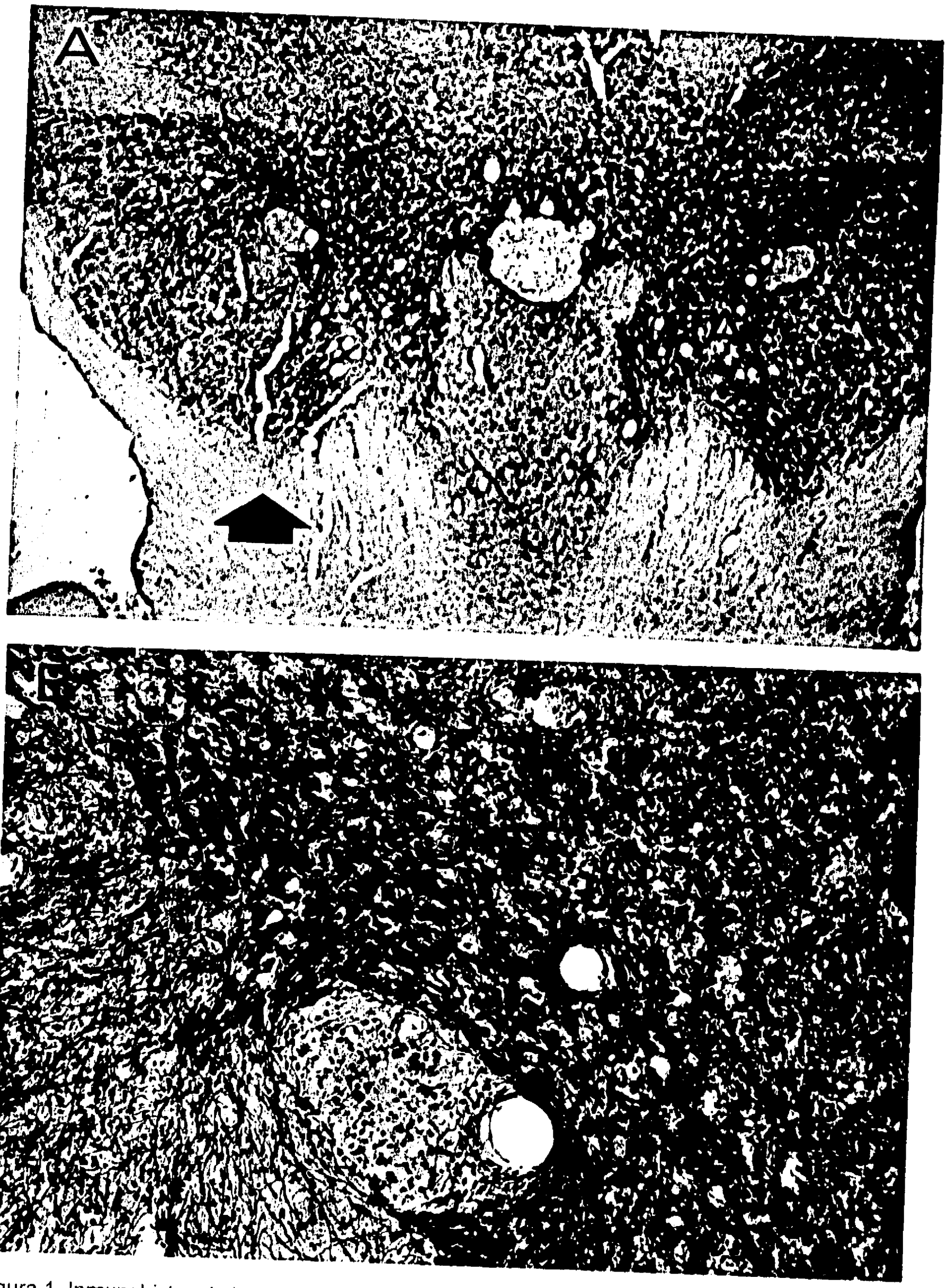

Figura 1. Inmunohistoquímica para TH en el plano horizontal del mesencéfalo ventral de una rata con infusión crónica de CroC. En A se señala a la substancia nigra pars compacta (snpc), área ventral tegmental (VTA) y la flecha señala el lado izquierdo donde se realizó la infusión (20X). B es un acercamiento de A (200X), donde se puede apreciar que los somas y las fibras no fueron afectados por la infusión.

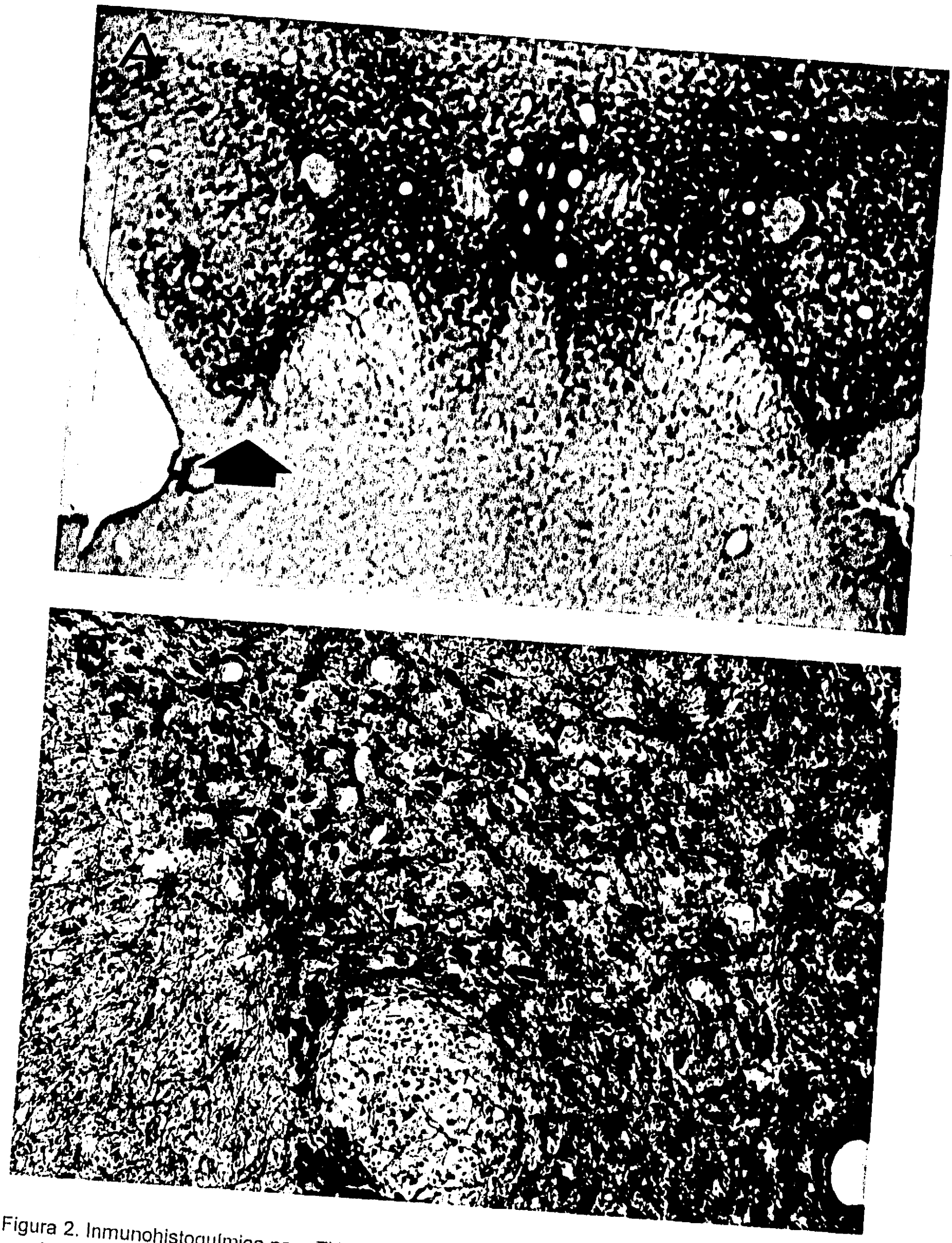

Figura 2. Inmunohistoquímica para TH en el plano horizontal del mesencéfalo ventral de una rata con infusión crónica de NGF, no se observan diferencias respecto de las infundidas con CroC. Los planos A y B son homólogos a los de la figura 1.

Figura 3. Inmunohistoquímica para TH del mesencéfalo ventral de una rata con infusión crónica de BDNF. En comparación con las figuras 1 y 2, los animales de este grupo mostraron una reducción significativa en la tinción para la TH. Como puede verse en A y aunque la infusión fue unilateral ambos lados se afectan, siendo el lado izquierdo el que muestra la mayor disminución (20X). En "B" se puede observar un gradiente de inmunoreactividad en los somas, las flechas abiertas señalan casos en los que la tinción es mayor y las cerradas donde la señal es mínima (200X).

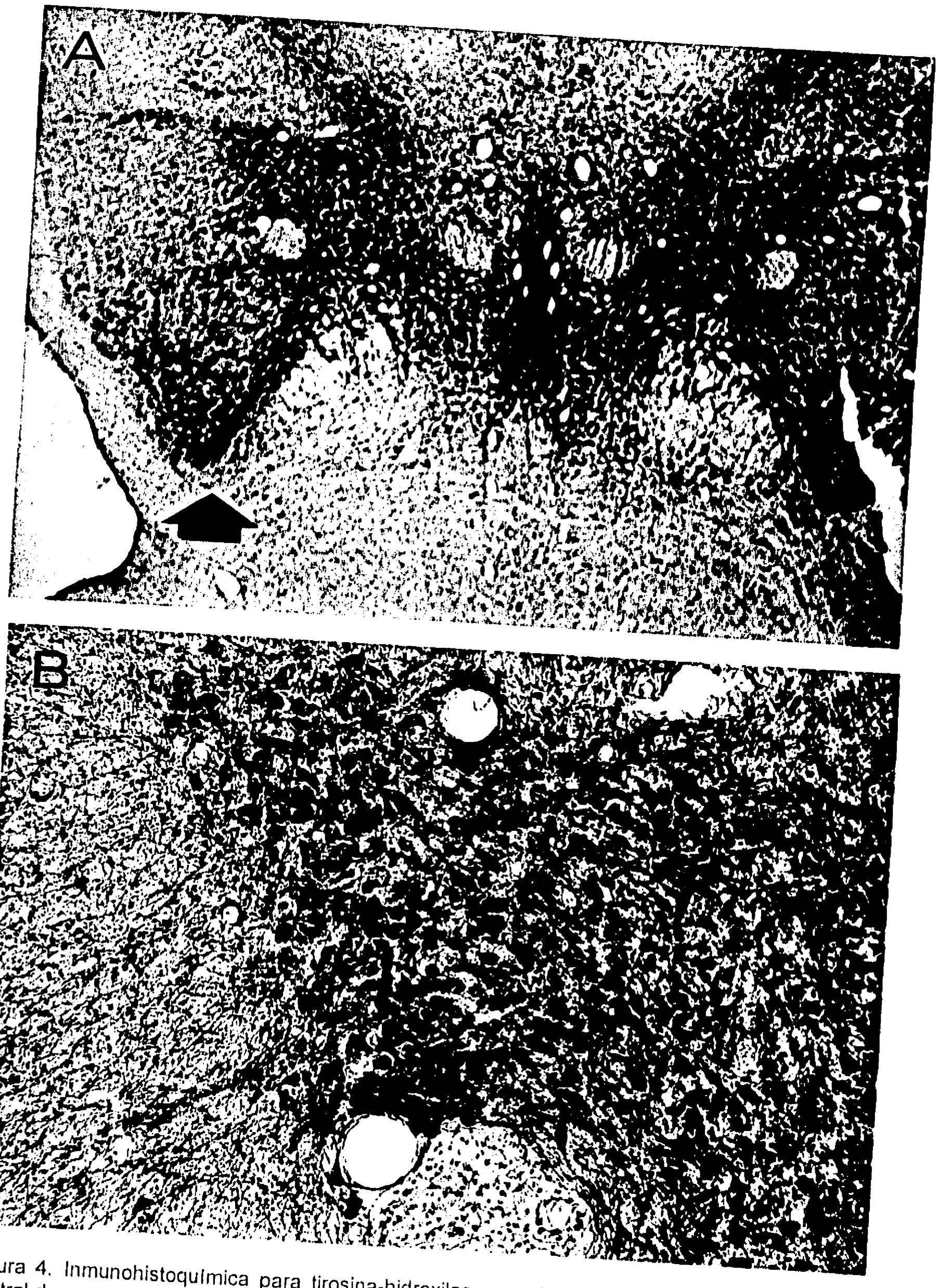

Figura 4. Inmunohistoquímica para tirosina-hidroxilasa en el plano horizontal del mesencéfalo ventral de una rata con infusión crónica de α-GDNF durante 5 días. Como puede observarse los efectos producidos en este caso muestran incremento en la inmunoreactividad para la TH, este efecto es evidente en B, donde la proliferación de fibras en las zonas intercelulares también es evidente (A, 20X; B, 200X).

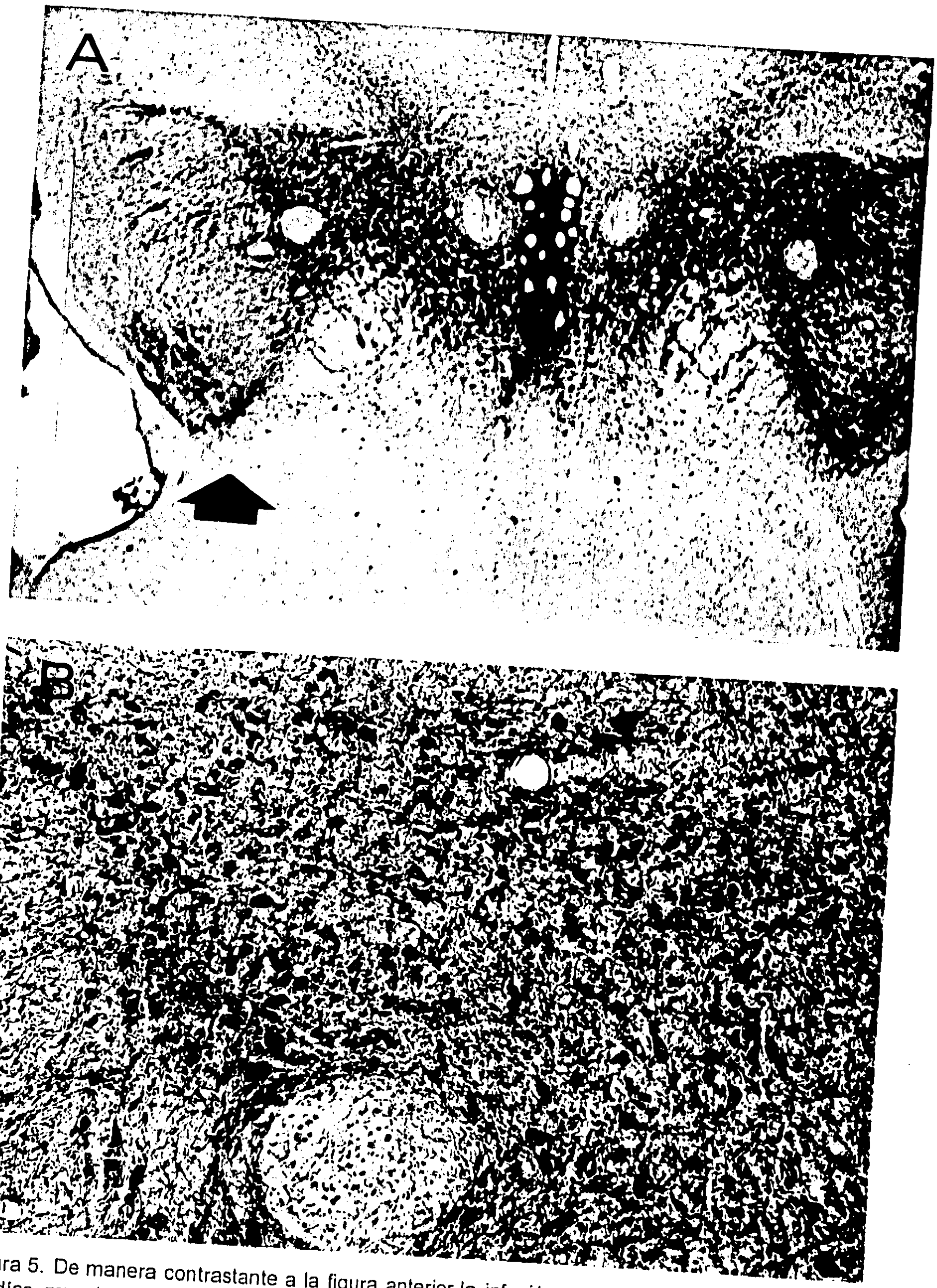

Figura 5. De manera contrastante a la figura anterior la infusión crónica de α-GDNF después de 10 días muestra que los efectos sobre las neuronas dopaminérgicas dismunuyen a un nivel similar al observado en animales infundidos con CroC o NGF. (A, 20X; B, 200X).

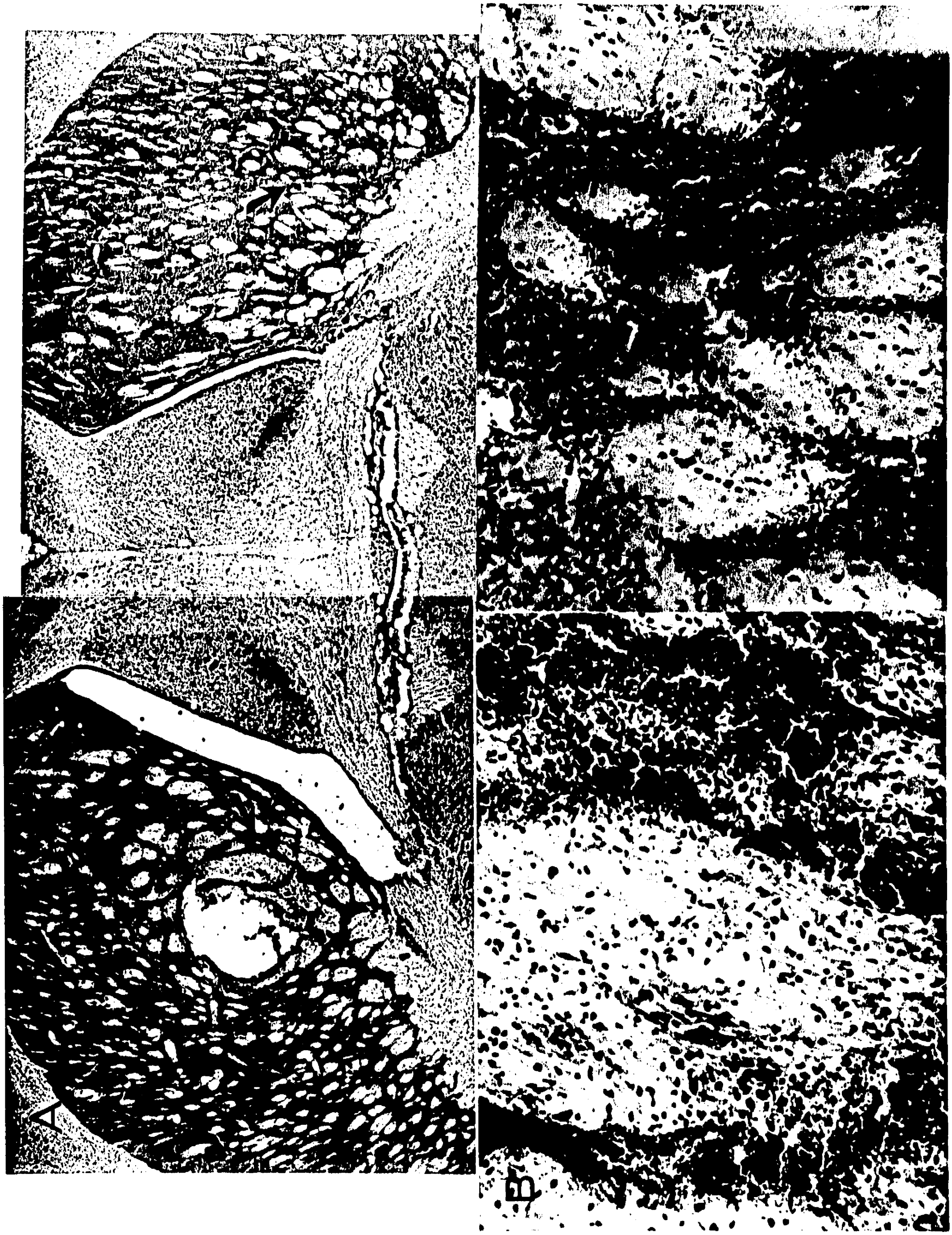

Figura 6. Inmunohistoquímica de tirosina-hidroxilasa en el plano horizontal a nivel del cuerpo estriado de una rata con infusión crónica de α-GDNF durante 5 días. Como puede observarse en A (20X), el lado infundido muestra mayor inmunoreactividad que el lado contralateral. Aparentemente ésto ocurre por un incremento en el número de las fibras provenientes del mesencéfalo (B, 200X).

intraestriatal de α-GDNF, en la que se puede observar un incremento en la inmunoreactividad debida a la proliferación de fibras TH+ como puede constatarse en la figura 6B.

Discusión.

Los resultados del presente estudio sugieren que la perfusión crónica de BDNF en animales adultos produce un estado de acinecia generalizada durante el reposo. Esto parece deberse a una disminución en la expresión de la enzima tirosina hidroxilasa en las neuronas dopaminérgicas del mesencéfalo ventral.

Estos resultados son acordes a los resultados reportados Lapchak y cols (1993), quienes han observado que la inyección intranigral de 1μg de BDNF cada segundo día a lo largo de 19 días favorece la conducta de giro ipsilateral al lado inyectado, observando una disminución del 30% en la eficiencia del sistema de recapatura de dopamina, la expresión de receptores dopaminérgicos estriatales del tipo DA2 disminuyo en un 40%, la cantidad de RNAm de la enzima tirosina hidroxilasa y la actividad de la enzima presente también disminuyen en un 30%.

En el citado estudio no se observaron los efectos conductuales que se describen en el presente estudio y es posible que ésto puede deberse al hecho de que Lapchak y cols. aplicaron el factor de manera aguda cada segundo día, en tanto que en el presente estudio el tratamiento fuecrónico.

Estos efectos son acordes a la teoría de la ventana espacio temporal de los factores de crecimiento, pues las neuronas dopaminérgicas en los estadios

fetales se venefician con las neurotrofinas, en tanto que en el estado adulto, el mismo factor parece conducir a una disfunción dopaminérgica.

Entonces la aparente disminución en la inmunoreactividad de la tirosina podría observada en el presente estudio puede correlacionarse con el estado de catalepsia generalizada observado en los animales tratados con BDNF.

Por otro lado, los resultados preliminares observados en los animales tratados con α-GDNF, sugieren que el factor glial podría ser importante para la sobrevivencia de las neuronas dopaminérgicas del mesencéfalo. Esta sugerencia parace válida ya que recientemente se publicó que la inyección intracerebral de GDNF antes de la lesión del sistema nigro-estriatal, proteje a un 50% a las neuronas dopaminérgicas del mesencéfalo ventral (kearns y Gash, 1995).

La asimetria postural y motora orientada hacia el lado contralateral de la perfusión observada durante los primeros cinco días de perfusión, parece correlacionar con un incremento en el número de fibras TH+ en el cuerpo estriado del lado tratado. Es posible que esta cualitativa proliferación de fibras dopaminérgicas en el cuerpo estriado, se deba a que el anticuerpo anuló los efectos del GDNF endógeno, estimulando a las fibras a buscar una fuente alternativa del factor. En estos animales el fenómeno de proliferación aparentemente también ocurrió en los somas dopaminérgicos del mesencéfalo ventral. Sin embargo, es necesario considerar estos resultados con cierta reserva hasta que se cuantifiquen los efectos.

El segundo efecto se observó a los 10 días de perfusión, cuando las asimetrias postural y motora se habían revertido. Esto parece correlacionarse con el hecho de que la proliferación observada a los cinco días, se había también revertido. Estos resultados sugieren que durante la fase inicial del tratamiento las fibras dopaminérgicas proliferaron para buscar el poco factor que normalmente existe en el neuropilo, esto parece coincidir conductualmente con la asimetria. Sin embargo, el constante bloqueo del factor provocó que este mecanismo se abortara, lo que coincide con la reversión de la asimetría corporal.

Estos resultados plantean la posibilidad de que las neuronas dopaminérgicas son dependientes del GDNF y si ésto es cierto, podría esperarse que el proceso de muerte neuronal se produciría si la perfusión del anticuerpo se prolonga o si se incrementa su concentración. Si la hipótesis es correcta es posible que la enfermedad de Parkinson pueda deberse a una disminución en el aporte de GDNF. Sin embargo, esta hipótesis requiere ser evaluada en el futuro cercano.

DISCUSION:

Los estudios que conforman la presente tesis muestran:

TRABAJO 1. "*Delay in manifestations of aging by grafting NGF cultured chromaffin cells in adulthood.*"

RESUMEN.

Se ha mostrado que las deficiencias motoras observadas en las ratas viejas se relacionan con una disminución en la transmisión dopaminérgica, y pueden ser revertidas con agonistas dopaminérgicos o con transplantes intracerebrales de tejido catecolaminérgico. Sin embargo, a la fecha no se ha determinado sí el tejido catecolaminérgico transplantado en ratas adultas, puede retrazar dichas alteraciones cuando el animal sea viejo.

En este estudio se utilizo el paradigma de la secuencia aleatoria de barras inclinadas en el que se utilian cinco barras de madera con un grosor de 3, 6, 12, 18, 24mm, dos metros de longitud y una inclinación de 15°; la prueba consiste en cuantificar el tiempo que le toma a cada animal cruzar cada barra desde el extremo inferior al superior, con un tiempo límite de 120 segundos.

Se evaluó la eficiencia motora de ratas macho de 13 meses de edad y una vez aprendida la tarea las ratas fueron agrupadas como sigue: El grupo 1 fue el de los animales con transplante falso; el grupo 2 fue transplantado con células cromafines cultivadas en presencia de NGF; y en el grupo 3 el transplante consistió de células cromafines disociadas. Subsecuentemente se evaluó cada mes la eficiencia motora hasta que los animales cumplieron 26 meses. Se

incluyeron dos grupos más de animales, el grupo 4 se formo con animales de 26 meses de edad y el grupo 5 se formo con ratas macho de 3-5 meses de edad, estos dos grupos se evaluaron sólamente una vez. La actividad electrica basal de algunas neuronas del mesencefalo ventral se registro en todos los animales.

Los resultados muestran que los transplantes intraestriatales de células cromafines cultivadas en presencia de NGF favorecen la eficiencia motora de la rata vieja. Asimismo, la tasa basal de disparo de las neuronas registradas fue comparable a la observada en animales jovenes y la pérdida de neuronas dopaminérgicas en el mesencéfalo fue menor a la de los otrs grupos de animales viejos.

Discusión: Este estudio establece que las células cromafines cultivadas en presencia de NGF son capaces de sobrevivir por largos periodos (14 meses) después de que se les trasplanta en el parénquima estriatal. También se muestra por primera vez que los transplantes implantados meses antes del envejecimiento, se puede producir un retardo significativo en las alteraciones motoras propias del envejecimiento. Esto establece que las posibilidades de sobrevivencia de las células cromafines transplantadas se incrementan significativamente, si antes de ser implantadas se les mantiene en presencia de NGF.

Por otro lado se encontro que en los animales con compensación motora asociada a la presenica del transplante, la actividad eléctrica de las neuronas del mesencéfalo ventral se encuentra en un estado intermedio entre animales

jovenes y viejos intactos o viejos, en los que no sobrevivió el transplante. Además de que la pérdida neuronal fue menor.

Cabe señalar que los efectos observados a nivel de actividad eléctrica de los animales con transplante no es exclusivo de las neuronas dopaminérgicas delmesencéfalo ventral, ya que la actividad eléctrica de neuronas no dopaminérgicas que coexisten con las anteriores, tambien mostraron una activadad eléctrica intermedia entre animales jovenes y viejos.

Con base en lo anterior y considerando las evidencias citadas en la sección de transplantes de tejido catecolaminérgico y modelos de la enfermedad de Parkinson, se puede argumentar que los efectos conductuales son el resultado de cambios a nivel global en el cerebro, lo cual apoya el modelo funcional propuesto en dicha sección.

Ahora bien es posible que el retraso en el desarrollo de las alteraciones motoras también se deba a la participación de factores tróficos, ya que como se señaló en la sección de los mecanismos de acción de los factores troficos y en la sección de transplantes, algunos factores que son secretados por las células cromafines, estan involucrados en la sobrevivencia celular y las compensaciones motoras.

Estas evidencias apoyan la hipótesis de que los efectos compensatorios de los transplantes en la actividad motora, se deben a la presencia de las células trasplantadas, las cuales cooperan con los mecanismos neurotróficos

compenatorios propios del sistema nervioso. Sin embargo, no se descarta que parte del efecto compensatorio también se deba a un efecto de práctica.

Trabajo 2: *"Regulation of neurotrophin receptor expression during embryonic and postnatal development"*

RESUMEN.

Los miembros de la familia de las neurotrofinas actuan como agentes neurotróficos en distintas poblaciones celulares del sitema nervioso central y periférico. El presente estudio se avocó a determinar la presencia de los receptores del factor neurotrófico derivado del cerebro (BDNF) y las neurotrofinas 3 y 4/5 (NT-3; NT-4/5) al utilizar el metodo de "cross-linking" con las neurotrofimas radio-macadas con [^{125}-I].

Se identificaron receptores trkB y trkC completos y truncados tanto en tejido neurvioso como en tejido periférico. Durante el desarrollo embrionario y post-natal temprano del pollo la expresión de las formas completas de los receptores trkB y trkC preceden a la expresión de las formas truncadas. Un patrón similar se detectó en el ratón. Se encontró que la expresión relativa de los receptrores tanto en el telencéfalo basal y el hipocampo no cambia significativamente en el ratón con la edad. En el sistema nigro-estriatal se detectaron los mayores niveles de expresión para las tres neurotrofinas.

En la médula espinal ventral y en motoneuronas aisladas de pollo y de ratón se detectaron receptores completos de trkB y trkC. Encontrandose nuevamente que las formas truncadas aparecen significativamente después de las formas completas. Se En la retina embrionaria y tectum óptico de pollo se detectaron los receptores completos de trkB y trkC; sin embargo, el tectum óptico también se detectaron los recetores truncados de trkB.

En el músculo estriado se detectó únicamente la expresión de los receptores trkC, sugiriendo que el NT-3 puede tener una función novedosa en éste tejido. La presencia de los receptores de las neurotrofinas en diversos tejidos tanto embrionarios como post-natales, establecen la participación significativa de las neurotrofinas BDNF, NT-3 y NT-4/5 en el desarrollo embrionario y post-natal. La regulación del radio de los receptores completo versus receptor puede jugar un papel importante en el desarrollo, maduración y mantenimiento de las distintas redes neuronales.

Discusión. El análisis de la expresión de los receptores de las neurotrofinas en varios tejidos, muestra por primera vez que las isoformas completas y truncadas de los receptores trkB y trkC, se expresan a lo largo del desarrollo embrionario y post-natal de distintos tejidos, confirmandose que el BDNFy NT-4/5 se unen a trkB completo y truncado, mientras que el NT-3 se une preferencialmente con las isoformas de trkC. De manera curiosa se encontró que el NT-4/5 no interactua con trkB y trkC de las aves.

Una de las aportaciones más importantes de este trabajo señala que durante el desarrollo embrionario y en los primeros días después del nacimiento, en la mayoría de los tejidos estudiados la expresión de receptores es asincrónica, es decir, las formas completas de los receptores trkB y trkC se expresan primero y subsecuentemente se expresan las formas truncadas, siendo estas últimas las formas predominantes en el estado adulto.

Esto sugiere nuevamente que las isoformas truncadas podrían estar regulando la funcionalidad de las formas completas al establecer dímeros inactivos (ver dimerisación de receptores en la sección de mecanismos de acción de los factores tróficos). Alternativamente los receptores truncados podrían estar actuando como moléculas de adesión celular, pués se ha mostrado que en el dominio extracelular los receptores trk presentan algunas características similares a las moléculas de adesión celular, en consecuencia estos receptores podrían participar en el mantenimiento espacial de las interacciones celulares (Merlio y cols, 1992).

Sin embargo, no se debe descartar la posibilidad de que que los receptores truncados tengan un mecanismo de transducción distinto a los mecanismos de transducción establecidos (ver mecanismos de transducción en la sección de mecanismos de acción de los factores tróficos).

Por lo que toca a la comparación de la expresión de ambas isoformas de receptores en jovenes y viejos no se detectaron cambios significativos en el hipocampo y la región donde residen las neuronas colinérgicas telencefalicas.

Como ya se cito el BDNF y más recientemente el NT-4/5 son capaces de tener actividad trófica en las neuronas dopaminérgicas fetales, sin embargo dicha actividad no se produce cuando el tratamiento se realiza en animales adultos. En este contexto se mostró que el BDNF, NT-3 y NT-4/5 son capaces de interactuar con los receptores trkB y trkC de la substancia nigra y cuerpo estriado neonatal, por lo que a primera vista la aparente falta de efectos de las neurotrofinas no se debe a la falta de receptores.

En el presente estudio también se detectó la expresión de trkC completo y truncado en el músculo esqueletico, riñón y en el intestino de embriones de pollo de 13.5 días.

El receptor trkB truncado se expresó en las extremidades y en la piel de fetos de ratón. La presencia de receptores de las neurotrofinas en órganos periféricos sugiere que las neurotrofinas juegan un papel importante en los mecanismos de inervación de dichos órganos. Sin embargo, la reciente demostración de que en animales transgénicos incapaces de expresar los receptores o la síntesis de las neurotrofinas establece que el papel funcional de de las neurotrofinas permanece como un campo muy amplio de posibilidades funcionales para las neurotrofinas en el desarrollo.

TRABAJO 3. "*Functional neurotrophin receptors during embryonic and postnatal development of the nigrostriatal system in the rat.*"

RESUMEN.

Se ha sugerido que el factor neurotrófico derivado del cerebro (BDNF) y la neurotrofina 3 (NT-3) tienen efectos neurotróficos sobre las neuronas dopaminérgicas de la substancia nigra durante el desarrollo embrionario y en la vida adulta. La presencia de los receptores para el BDNF y NT-3 se evaluó por el método de "crosslinking" con los factores radiomarcados con [^{125}-I] y con muestras de tejido de la substncia nigra y cuerpo estriado de distintas edades embrionarias y post-natales.

Se detectaron receptores funcionales en los dos nucleos para ambas neurotrofinas comenzando al día embrionario 14 (E14). Se encontro un incremento en la expresión relativa de los recetores conforme se incrementó la edad. En los estadios tempranos del desarrollo, la substancia nigra mostró la mayor proporción de receptores completos de trkB y trkC, mientras un aporte significativo del receptor trkB truncado se detectó en el cuerpo estriado. En la substancia nigra el receptor truncado de trkB se detectó significativamente después que el receptor completo (E19). En abos tejidos se encontró que en el adulto predomina la forma truncada de ambos receptores.

La presencia de los receptores para BDNF, NT-3 y NT-4/5 a lo largo de la vida del sistema nigro-estriatal sugiere la posible participación del BDNF, NT-3 y

NT-4/5 en la diferenciación, maduración y mantenimiento del sistema nigro-estriatal.

Discusión. Con base en la evidencia de que los receptores trkB y trkC se expresan en el cuerpo estriado y substancia nigra, parece paradojico que las neurotrofinas sean incapaces de inducir efectos neurotróficos en las neuronas dopaminérgicas adultas.

Sorpresivamente se detectó que en el cuerpo estriado las isoformas completa y truncada de trkB se expresan simultáneamente, para establecerse después del nacimiento el patrón predominante de los receptores truncados.

Se estableció que la concentración requerida de BDNF para la activación de trkB, es simialr a la determinada en otros tejidos en los que las neurotrofinas ejercen efectos tróficos, como la corteza cerebral, motoneuronas y neuronas de la retina. En este contexto se puede descartar la posibilidad de que el receptor trkB truncado sea el responsable de la falta de efectos tróficos en el modelo de la lesión de la vía nigro-estriatal, ya que en ratones transgénicos carentes de la expresión de BDNF, NT-3, trkB o trkC, no muestran anormalidades aparentes en el desarrollo de las neuronas dopaminérgicas. Esto plantea que las neurotrofinas pueden no ser los factores escenciales durante el desarrollo y establecimiento del sistema dopaminérgico nigro-estriatal.

Esta evidencia nos lleva a retomar la propuesta que se planteó en la seccion de los mecanismos de accion de los factores neurotróficos, que plantea que las neuronas requiere de multiples factores.

Trabajo 4 PILOTO "*Infusión crónica de BDNF en el parénquima del sistema nigro-estriatal*"

RESUMEN.

Se ha mostrado que el factor neurotrófico derivado del cerebro ejerce efectos funcionales en el sistema nigro-estriatal cuando se inyecta tópicamente en la substancia nigra. Sin embargo, existen resultados controversiales en torno a si el factor es capaz o no de evitar la muerte de las neuronas dopaminérgicas cuando se destruye al sistema dopaminérgico nigro-estriatal. Asimismo, se propuesto que el factor neurotrófico derivado de una línea celular de glía (GDNF) favorece la sobrevivencia de las neuronas dopaminérgicas, evitando incluso los efectos de la tóxina 6-hidroxidopamina.

En este estudio se evaluaron los efectos de la perfusión crónica de BDNF y α-GDBF en el sistema dopaminérgico nigro-estriatal, encontrandose que el BDNF causa un estado de acinecia generalizada cuando se perfunde en el parénquima del cuerpo estriado o substancia nigra, esto parece asociarse con un decremento en la expresión de la enzima tirosina-hidroxilasa de las neuronas dopaminérgicas.

En contraparte, la perfusión de α-GDNF produjo una aparente proliferación de fibras dopaminérgicas durante los primeros días del tratamiento, lo cual corresponde con una asimetría postural orientada hacia el lado contralateral del lado tratado. Este efecto se fué desvaneciendo conforme transcurrian los días dejando de ser eidente a los 8 días de tratamineto.

Estos resultados prilimares sugieren que el BDNF en el animal adulto puede inactivar los mecanismos de expresión de la enzima tirosina hidroxilasa, fenómeno que ya habia sido observado previamente. Asimismo, apoya la hipótesis que se habia sugerido en torno a la dependencia que las neurona dopaminérgicas tienen por el GDNF.

Discusión. Si bien en este estudio no se cuantificó los niveles de expresión de la enzima tirosina hidroxilasa, la propuesta de que una disminución en su expresión es uno de los mecanismos que conducen al estado de acinecia observado se apoya en los resultados reportados por Lapchak y cols, (1993), quienes al inyectar 1μg de BDNF en el parénquima de la substancia nigra cada segundo día durante 19 días, cuantificaron una disminución en la cantidad de la enzima así como de su RNA mensajero. Estos investigadores también establecen una disminución en la cantidad de receptores dopaminérgicos del tipo DA2 en el cuepo estriado.

Es posible, que dichos investigadores no encontraron el estado de acinecia que se observó en el presente estudio, por el hecho de que en su estudio utilizaron la aplicación discontínua del factor , en tanto que en el estudio que se presenta en esta tésis, la inyección del factor tuvo una tasa constante de 0.4μg/0.5μl/hora durante todo el tiempo que duro el estudio. Cabe señalar que esta dosis es equivalente a la utilizada en cultivo durante los estudios en los que se estableción que las neuronas dopaminérgicas del mesencéfalo ventral fetal

pueden resistir los efectos de la 6-OHDA o MPTP, por lo que se consideró que no debería ser tóxica para las neuronas.

Por otro lado, la evidencia de que el BDNF y NT-3 así como los receptores trkB y trkC se co-expresan en 25-50% de las neuronas dopaminérgicas del área ventral tegmental y en 10-30% de las de la substancia nigra, planteó la posibilidad de que las neuronas dopaminérgicas existentes en los estadios más avanzados de la enfermedad de Parkinson ideopática, son aquellas que co-expresan las neurotrofinas y sus receptores (Seroogy y cols, 1992, 1994). Otra posibilidad es que las neuronas dopaminérgicas secreten al BDNF y al NT-3 en el cuerpo estriado, estimulando la incorporación de GABA en las neuronas GABAérgicas (Serrogy y cols, 1994). Esta sugerencia parece válida pués el BDNF detectado en el cuerpo estriado no se produce en este núcleo (Merlio y cols, 1992).

En contraparte, la perfusión del αGDNF provocó la proliferación de las fibras dopaminérgicas en la zona del cuerpo estriado donde se infundió el anticuerpo. Estos resultados a reserva de que se corroboren a nivel bioquímico parecen apoyar la hipótesis de que el GDNF podría ser el factor que podría evitar la muerte neuronal en el sistema nigro-estriatal en animales adultos. En este sentido se ha mostrado que el GDNF promueve la sobrevivencia y mantenimiento del fenotipo dopaminérgico de las neuronas mesencefálicas.

CONCLUSIONES:

Como conclusión de los estudios mostrados en la presente tesis se puede decir que:

1. Los trasplantes de células cromafines ejercen un efecto trófico sobre la funcionalidad del cerebro provocando un enlentecimiento de los procesos degenerativos propios de la vejez.
2. Que la expresión ontogénica de las neurotrofinas no es exclusiva del sistema nervioso, lo que indicaría que tienen distintas funciones en el organimo que aún no han sido establecidas.
3. Se muestra que el BDNF y el NT-3 aún cuando ejercen efectos tróficos en las neuronas dopaminérgicas fetales, sus efectos podrían ser mediados transcelularmente, es decir que las neurotrofinas podrían estar activando poblaciones celulares que activaran la secreción de otro factor, como por ejemplo el GDNF, sin embargo el efecto transcelular en animales adultos es diferente y produce efectos contrarios.
4. El GDNF puede ser el factor potencialmente más efectivo desde el punto de vista terapéutico, ya que recientemente se ha mostrado que su efectividad se observa aún cuando se lesionan los axones dopaminérgicos en el paradigma de la lesión del sistema nigro-estriatal.
5. El siguiente paso en la serie de estos estudios será el establecer si los transplantes de células cromafines cultivadas estimulan la expresión de

GDNF en el cerebro de ratas viejas, si ésto es cierto podría esperarse un efecto similar en los humanos, lo que apoyaría el uso de dicho factor en el tratamiento de la enfermedad de Parkinson. Sin embargo, en tanto no sepamos cuales son todos los agentes causantes de esta enfermedad, no podremos establecer una terápia efectiva. No obstante, los resultados de la presente tesis podrían ser importantes para favorecer la postergación del envejecimento y los procesos provocados por la enfermedad de Parkinson..

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