Characterization and Topography of High-Affinity ^{125}I -Neurotrophin-3 Binding to Mammalian Brain

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The binding of biologically active and ^{125}I -labeled neurotrophin-3 (NT-3) was studied with both dry film and emulsion autoradiography to compare with NGF binding and discover areas where NT-3 may function *in vivo*. The equilibrium binding of 300 pM ^{125}I -NT-3 to rat brain sections was reversible and inhibited by unlabeled NT-3 (IC_{50} , 420 pM). ^{125}I -NT-3 bound in a saturable manner, with high affinity (K_d , 227–269 pM), and with a capacity (B_{max} , 26 fmol/mg protein) that exceeded that of NGF by threefold. As with NGF, ^{125}I -NT-3 also bound to a second population of sites with lower affinity (K_d , 2.8 nM) and higher capacity (B_{max} , 170 fmol/mg protein). ^{125}I -NT-3 binding was not blocked by NGF, or serum proteins, and brain-derived neurotrophic factor (BDNF) competed for it in a distinctly biphasic manner (IC_{50} values of 230 pM and 37 nM). Microdensitometry confirmed graphically and by Hill analysis the monophasic displacement of ^{125}I -NT-3 and the biphasic displacement of ^{125}I -NT-3 binding by BDNF in hippocampus, caudate-putamen, neocortex, and olfactory tubercle. In rat or cat, the topography of ^{125}I -NT-3 binding differed from that reported for ^{125}I -NGF binding or for the low-affinity NGF receptor. The highest binding densities were found in neocortical layers 1 and 2, the stratum oriens and radiatum of hippocampus, molecular layer of the dentate gyrus, nucleus of the lateral olfactory tract, entorhinal cortex, anterior olfactory nucleus, anteromedial thalamic nucleus, and amygdala. Moderate densities were found in neocortical layers 4–6, the neostriatum, amygdala, the dorsal root ganglia, and the central gray of spinal cord. Emulsion autoradiography also revealed binding in nerve terminal-rich regions of superficial neocortex and hippocampus but not on neural cell bodies. Binding was absent in many other brain regions, including cholinergic nuclei, and in all peripheral organs studied including liver, kidney, pancreas, heart, and skeletal muscle. ^{125}I -NT-3 binding to sections of human basal ganglia resembled that seen in rat or cat, including high densities in the caudate, putamen, and superficial neocortex. The unique distribution and pharmacology of ^{125}I -NT-3 binding to BDNF-sensitive and -insensitive sites in brain predict predominantly neuronal actions for these factors that

are likely to be more widespread and distinct from those of NGF.

[Key words: neurotrophin-3, receptor autoradiography, neurotrophic factor, brain-derived neurotrophic factor, growth factors]

Neurotrophin-3 (NT-3) is a member of the NGF family of molecules termed neurotrophins that support the survival and outgrowth of brain and peripheral neurons (Hohn et al., 1990; Maisonpierre et al., 1990a,b; Rosenthal et al., 1990; see Thoenen, 1991, for review). The localization of NT-3 mRNA by Northern analysis and *in situ* hybridization reveals a widespread distribution in both brain and periphery, with high levels not only in cerebellum, hippocampus, neocortex, and midbrain but also in kidney, heart, spleen, skin, gut, lung, liver, and muscle (Enfors et al., 1990; Hohn et al., 1990; Maisonpierre et al., 1990a,b). Predictions of the brain regions or peripheral targets upon which NT-3 is likely to act *in vivo* or *in vitro* would be facilitated by the use of NT-3 binding assays. However, NT-3 binding has been studied only with cultured fibroblasts (Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991) and in homogenates of hippocampus from 2-week-old rats (Soppet et al., 1991). In contrast, many studies over the last decade have characterized ^{125}I -NGF binding to a variety of tissues, including analysis by receptor autoradiography (Richardson et al., 1986; Raivich and Kreutzberg, 1987; Altar et al., 1991a,b). These autoradiographic studies show that ^{125}I -NGF binding is found mostly in the brain, spinal cord, and dorsal root ganglia (DRG). Within brain, almost all ^{125}I -NGF binding is found in a subset of forebrain cholinergic neurons of the medial septum, diagonal band, substantia innominata, hippocampus, and neostriatum. Of these areas, all but the neostriatum and hippocampus are labeled by monoclonal antibody 192 (Chandler et al., 1984), which recognizes the low-affinity neurotrophin receptor (LNGFR; e.g., Pioro and Cuellar, 1988, 1990; Batchelor et al., 1989; Gibbs et al., 1989; Woolf et al., 1989). Interestingly, many areas that are immunopositive for LNGFR contain little, if any, high-affinity binding of NGF (Yan and Johnson, 1989; Koh et al., 1989; Pioro and Cuellar, 1990). Other brain areas, such as thalamus, hypothalamus, and neocortex, are nearly devoid of both LNGFR immunostaining and high-affinity NGF binding. These observations raise the issue as to whether another neurotrophin family member, such as NT-3 or brain-derived neurotrophic factor (BDNF), may bind to these or other brain areas, and whether binding will predominate on cell bodies, as with ^{125}I -NGF, or in nerve terminal regions. Through the use of biologically active ^{125}I -NT-3 (DiStefano et al., 1992) and receptor autoradiography, this article identifies high-affinity ^{125}I -NT-3 binding in rat, cat, and human brain. Our results demonstrate

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that the distribution of ^{125}I -NT-3 binding to brain is distinct, more dense, and more widely distributed than that of ^{125}I -NGF binding to brain. Under high-affinity binding conditions, ^{125}I -NT-3 binds to BDNF-sensitive and -insensitive sites, demonstrating likely CNS roles for both neurotrophins in areas that do not contain high- or low-affinity NGF receptors.

Materials and Methods

Production and purification of NT-3. Recombinant human NT-3 (119 amino acid residues) was produced in chinese hamster ovary cells as previously described (Radziejewski et al., 1992). The purity of the preparation was >95%; 50% was comprised of intact protein, and 50%, of cleaved product in which the first five N-terminal amino acids were missing. The NT-3 was monitored for biological activity using dissociated lumbar dorsal root ganglia (DRG) cells from embryonic day 8 (E8) chicken embryos maintained 2 d *in vitro* (Lindsay and Peters, 1984). Murine NGF (muNGF; generously provided by Dr. Ralph Alderson, Regeneron Pharmaceuticals), BDNF, and NT-3 each mediated maximal survival of 50%, 30%, and 35%, respectively, of these DRG neurons, each with EC_{50} values of approximately 0.01 ng/ml.

Iodination of NT-3. NT-3 was iodinated by the lactoperoxidase method (Marchalonis, 1969) with several modifications (DiStefano et al., 1992) that resulted in a specific activity of 2800–4400 cpm/fmol of NT-3 (1414–2222 Ci/mmol of NT-3). ^{125}I -NT-3 was stored at 80–100 nM at 2–8°C and used within 1–5 d after preparation. The biological activity of ^{125}I -NT-3 was assessed by monitoring its ability to stimulate survival and neurite outgrowth of dissociated E8 chicken DRG neurons, and this activity was compared to unlabeled NT-3. ^{125}I -NT-3 was as potent as unlabeled NT-3 in the DRG bioassay over the entire 0.001–1 ng/ml standard concentration range (not shown). ^{125}I -NT-3 was also retrogradely transported from crushed sciatic nerve to the rat DRG, and this transport was specifically blocked by unlabeled NT-3 (DiStefano et al., 1992). The retrograde transport, the indistinguishable potencies of NT-3 and ^{125}I -NT-3 in the DRG bioassay, and the inhibition of ^{125}I -NT-3 binding by equal concentrations of NT-3 (see Results) indicate that the radioiodinated NT-3 used here was biologically equivalent to NT-3.

Tissues. Male Sprague–Dawley rats (200–250 gm; Zivic-Miller) were maintained on a 12:12 hr light: dark cycle and given food and water *ad libitum*. The brain of each rat was frozen in isopentane at –15°C within 5 min of death. Serial 12- μm -thick coronal and horizontal sections of these tissues were collected at 0.30–0.50 mm intervals from the frontal cortex to the medulla at the level of the dorsal motor nucleus of the vagus (coronal sections) or through the olfactory bulbs, caudate-putamen, and cerebellum (horizontal sections) and were used for association, dissociation, and equilibrium binding analyses. Sections were thaw-mounted onto 1% gelatin-coated 1 $\times$ 3 inch glass microscope slides and stored frozen for up to 1 month at –70°C. The brain of an adult male cat (provided by Dr. Max Cynader, University of British Columbia) was obtained within 0.5 hr post-mortem and frozen in isopentane as described for the rat brain. Coronal sections were prepared through the midbrain as described for the rat. Two male and two female human brains (generously provided by Drs. Satya Kar and Remi Quirion, Douglas Research Hospital, McGill University) were obtained 18–48 hr following death. Two patients aged 61 and 67 years died from myocardial infarction or ventricular fibrillation. Two patients aged 87 and 81 years had been diagnosed with Alzheimer's dementia and died following a brief coma or from complications of pneumonia. Coronal blocks of tissue through the basal ganglia including the caudate, putamen, and globus pallidus were dissected from each brain and frozen in isopentane as described for the rat. Twelve micron thick sections were obtained from the blocks and thaw-mounted onto 2 $\times$ 3 inch gelatin-coated microscope slides.

^{125}I -NT-3 binding assay. Binding assays using brain sections were based on the ^{125}I -muNGF binding procedure of Richardson et al. (1986) with modifications (Altar et al., 1991a). After thawing, each section was preincubated for 1 hr at 22°C in 100 mM phosphate-buffered saline (pH 7.4) containing 0.5 mM MgCl_2 and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Prior studies showed equivalent binding for sections that were preincubated in buffer for 1, 3, or 24 hr (data not shown). Sections were then incubated in Dulbecco's modified Eagle's medium tissue culture medium containing high glucose, 10% heat-inactivated fetal calf serum (60°C for 0.5 hr), 25 mM HEPES buffer, 4 $\mu\text{g}/\text{ml}$ leupeptin, 0.5 mM PMSF (Bethesda Research Labs, Gaithersburg, MD; first dissolved to 0.1 mg/ml isopropyl alcohol), 0.5 mM MgCl_2 , and 10 pM to 9 nM

^{125}I -NT-3 (equilibrium saturation analysis) or 200–300 pM ^{125}I -NT-3 (all other assays) based on a dimeric molecular weight of 26,000. Slides containing adjacent brain sections were incubated in the same solutions with the addition of 300 nM unlabeled NT-3 to define nonspecific binding. Recombinant human ciliary neurotrophic factor (hCNTF; Masia-kowski et al., 1991) and BDNF (Leibrock et al., 1989), provided by Drs. Nikos Panayotatos (Regeneron) and James Miller (Amgen), respectively, and muNGF (Suda et al., 1978), were prepared by established methods. Various concentrations of each agent were used to compete with ^{125}I -NT-3 binding in several experiments. As with BDNF, NT-3, and NGF, hCNTF was shown to be biologically active in the 0.2 ng/ml range as determined by the survival of 40% of E8 ciliary ganglion neurons after 24 hr of bioassay. Following the incubation, the sections were washed for 0.5 hr in the phosphate buffer. The association and dissociation of 300 pM ^{125}I -NT-3 binding after a 1 hr wash (association experiment) or 3.5 hr incubation (dissociation experiment) was also determined. After washing, sections were fixed for 10 min in 4% para-formaldehyde at 22°C, rinsed for 2 sec in water at 22°C, and dried within 5 min by a stream of room temperature air.

The labeled sections and ^{125}I -containing radioactivity standards (Amersham, Inc.) were exposed at room temperature for 2–5 d to ^{125}I -sensitive film (Hyperfilm, Amersham, Inc.). Additional slides containing ^{125}I -NT-3-labeled rat brain sections were dipped in Kodak NTB-2 photographic emulsion diluted 1:1 with 1% glycerol. The films and slides were developed 2–4 d (films) or 1–2 weeks (slides) later in Kodak D-19 developer at 15°C. Computer-assisted image analysis using a Loats RAS-CAL system was used to quantitate the amount of ^{125}I -NT-3 bound in brain subregions using a mouse-driven cursor (Altar et al., 1984).

Data analysis. After generation of autoradiographs, the horizontal brain sections were scraped from the slides with a razor blade, placed into 12 $\times$ 75 mm borosilicate glass tubes, and assayed for radioactive decay using a Packard gamma counter (Cobra model). K_d values were calculated for four brains under nonequilibrium association and dissociation conditions (Weiland and Molinoff, 1981). Equilibrium dissociation and association rate constants were obtained under pseudo-first order conditions, since far less than 10% of free ligand was bound to sections after 3 hr of incubation. K_d and B_{max} values were calculated by equilibrium saturation analysis with each of four brains according to the best fit to a parabola by iterative, nonlinear regression analysis (Rodbard and Lewald, 1970). IC_{50} values were calculated by the method of Bliss and James (1966). Statistical analyses for between-group comparisons were made with Dunnett's *t* test following a one-way analysis of variance.

Results

In preliminary studies, 40–400 pM concentrations of ^{125}I -NT-3 were found to bind in a dense and heterogeneous manner to horizontal and coronal sections of rat brain. Most (70–90%) of the binding was displaced by a 1000-fold greater concentration of NT-3. Little or no ^{125}I -NT-3 binding was lost by alcohol–xylene defatting procedures. Because displaceable binding was robust in the caudate-putamen, neocortex, and hippocampus, horizontal sections that contained these areas were used for subsequent quantitative studies.

Kinetic studies showed that ^{125}I -NT-3 associated to and dissociated from its displaceable binding to horizontal sections in a consistent manner across brain regions. The amount of binding varied by 4–20% (SEM) at association time points and by 10–25% at dissociation time points. The association of 200 pM ^{125}I -NT-3 increased until 3 hr, at which time equilibrium was achieved and no additional binding was obtained during an additional 5 hr incubation (Fig. 1, top panel). The association rate constant for binding to these sections was $0.0074 \times 10^9 \text{ min}^{-1} \text{ mole}^{-1}$. A highly significant linear fit ($p < 0.001$) was obtained for each association curve plotted as a function of log time by log amount bound, with *F* (model, error degrees of freedom) values of 481 (1, 49) for total bound, 136 (1, 49), nonspecific bound, and 239 (1, 49), specific bound. Sections incubated for 3.5 hr with 200 pM ^{125}I -NT-3 were also washed in buffer containing 200 nM NT-3 from 2 min to 18 hr. This

Figure 1. Association (top) and dissociation (bottom) of 200 pM ^{125}I -NT-3 binding to horizontal rat brain sections. Each value is the mean $\pm$ SEM fmol of NT-3 bound per milligram protein calculated from the dpm per section from four animals. Following association experiments, sections were washed for 1 hr. After 3.5 hr of equilibrium binding, the labeled sections were placed for various times in 70 ml PBS that contained 200 nM NT-3. NSB, nonspecific binding; SB, specific binding.

Table 1. Capacity and affinity of low- and high-affinity ^{125}I -NT-3 and ^{125}I -rhNGF binding to brain

Concentration range	K_d	$B_{\max}$ (fmol/mg protein)
^{125}I -NT-3		
10–750 pM	269 ± 64 pM	26 ± 3
0.75–10 nM	2.8 ± 0.34 nM*	$170 \pm 15^*$
10 pM–10 nM	3.0 ± 0.28 nM*	$200 \pm 6^*$
^{125}I -rhNGF*		
4–294 pM	69 ± 9.7 pM	9.9 ± 1.8
Kinetic values		
	^{125}I -NT-3	^{125}I -rhNGF ^a
Association (k_1)	$0.0074 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$	$0.066 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$
Dissociation (k_{-1})	$19.4 \times 10^{-4} \text{ min}^{-1}$	$8.9 \times 10^{-4} \text{ min}^{-1}$
K_d (k_{-1}/k_1)	227 ± 28 pM	28 ± 11 pM

Horizontal sections of rat forebrain ($n = 4/\text{group}$) as shown in Figure 4 were incubated in 10 pM to 10 nM ^{125}I -NT-3 with or without 300 nM NT-3 to define nonspecific binding. The K_d and $B_{\max}$ values are calculated as mean $\pm$ SEM.

^a Average values from Altar et al. (1991a).

* $p < 0.01$ versus high-affinity (10–750 pM) ^{125}I -NT-3 binding. Student's t test.

Table 2. Relative distribution of specific ^{125}I -NT-3 binding sites in rat brain

Area	Figure 5 label
High binding	
Molecular layer, hippocampal dentate gyrus	J1–L1
Stratum oriens and radiatum, hippocampus	J2–L2
Nucleus of lateral olfactory tract	H4, I1
Entorhinal cortex	L7
Layers 1 and 2, frontal neocortex	A2, B1–D1
Layers 1 and 2, parietal neocortex	D2–H2
Layers 1 and 2, cingulate neocortex	B2–D2, E3–H3
Anterior olfactory nucleus	A1
Anterior ventral and medial thalamus	H1, I2
Basolateral amygdala	J3
Medium binding	
Layers 4–6, neocortex	B–L
Nucleus accumbens	E1, F1
Caudate-putamen	E–J
Interpeduncular nucleus, lateral and dorsomedial	ns
Tenia tecta	E5
Dorsal raphe nucleus	ns
Superior colliculus	L3
Spinal gray	ns
Olfactory tubercle	E4, F4
Central gray	L5
Low binding	
Pars compacta of substantia nigra	L4
Cerebellum	ns
Pars reticulata of substantia nigra	L6
Hypothalamus	J5
Thalamus	J6, K3, L8
No detectable binding	
Pyramidal or granule cells, hippocampus	
Skeletal muscle, liver, kidney, pancreas, heart	
Globus pallidus	
Medial lemniscus	
Internal capsule	
Corpus callosum	
Ependymal layer	
Choroid plexus	
Anterior commissure	

The relative amounts of NT-3 binding were evaluated from visual evaluation of autoradiographs generated with 300 pM ^{125}I -NT-3. The nonspecific binding with 300 nM NT-3 appeared uniform throughout the brain sections and accounted for only 10–20% of the total amount of bound ligand. Abbreviations after each area refer to the labels in Figure 5. ns, the area is not shown in Figure 5.

revealed a dissociation of specific ^{125}I -NT-3 binding at a rate of $19.4 \times 10^{-4} \text{ min}^{-1}$ (Fig. 1, bottom panel). The ratio of the kinetically derived dissociation and association rate constants (k_{-1}/k_1 ; Weiland and Molinoff, 1981) gave an average equilibrium dissociation constant (K_d) value of 227 ± 28 pM (Table 1).

Additional horizontal brain sections equilibrated with 10 pM to 9 nM ^{125}I -NT-3 revealed a saturation isotherm that was clearly biphasic and displaceable (Fig. 2, left). Scatchard analysis (B/F vs. B) confirmed the biphasic nature of binding (Fig. 2, right). The saturation of displaceable binding from 10–750 pM was used to calculate the fit for high-affinity K_d and $B_{\max}$ values by nonlinear regression analysis. The K_d was 269 ± 64 pM and the capacity of binding was 26 ± 3 fmol/mg protein (Table 2). A higher range of concentrations, from 0.75 to 9 nM, revealed a second plateau of specific binding of lower affinity ($K_d = 2.8 \pm 0.34$ nM) and higher capacity ($B_{\max} = 170 \pm 14$ fmol/mg protein);

Figure 2. Left, total, nonspecific, and specific binding of 10 pM to 9 nM concentrations of ^{125}I -NT-3 to horizontal brain sections ($n = 4$ sections/point). Right, B/F (bound/free) versus bound ligand plot of the specific binding data in the adjacent figure. The two populations of specific ^{125}I -NT-3 binding sites are represented by the two linear functions. Affinity and capacity values for these populations are given in Table 2. NSB, nonspecific binding; SB, specific binding.

Table 2). The B_{max} value calculated for the entire range of 10 pM to 9 nM was an additive function of the separate B_{max} values for the high- and low-affinity sites. The high-affinity component of ^{125}I -labeled recombinant human NGF (^{125}I -rhNGF) binding associated more quickly and dissociated more slowly than did 300 pM ^{125}I -NT-3 binding and bound with about 4- to 10-fold greater affinity and at least 3-fold lower density than did ^{125}I -NT-3 (Table 1).

To address the pharmacology of ^{125}I -NT-3 binding, sections that were still wet from the final rinse were swabbed from the slides and counted after being incubated in 300 pM ^{125}I -NT-3

with or without various concentrations of competing molecules (Fig. 3). ^{125}I -NT-3 binding was not prevented by coincubation with up to 100 nM of either muNGF or hCNTF. NT-3 competed with 300 pM ^{125}I -NT-3 on an apparent mole-for-mole basis (IC_{50} of 420 ± 60 pM) and noncooperatively for a homogeneous population of binding sites, as Hill coefficients (nH) did not significantly differ from unity ($\text{nH} = 1.2 \pm 0.26$). A replicate experiment with brains from four additional animals used image analysis to measure the inhibition of ^{125}I -NT-3 binding over eight concentrations of NT-3 (10 pM to 200 nM) that were more concentrated over the IC_{50} value of 420 pM (Fig. 4). The amounts

Figure 3. Inhibition of ^{125}I -NT-3 binding to swabbed horizontal brain sections by NT-3 ($\text{IC}_{50} = 420 \pm 60$ pM; $\text{nH} = 1.2 \pm 0.26$) and BDNF ($\text{IC}_{50} = 800 \pm 108$ pM) but not by muNGF or hCNTF ($\text{IC}_{50} > 100$ nM; $n = 4$ brains/point; SEM = ± 5 –20%). The inhibition by BDNF was resolved into two separate curves for each animal, with $\text{IC}_{50} = 230 \pm 100$ pM for the 10–1000 pM range of BDNF ($\text{nH} = 0.76 \pm 0.18$) and $\text{IC}_{50} = 37 \pm 2.9$ nM for the 0.2–100 nM range of BDNF.

Figure 4. Quantitation of 300 pM ^{125}I -NT-3 binding to layers I and II of neocortex (I-II CX), layers III-VI of neocortex (III-VI CX), the molecular layer of hippocampus (Mol), or caudate-putamen (CP) over increasing concentrations of NT-3 (left) or BDNF (right). In Figures 4–7, greater image darkness reflects a greater amount of bound ^{125}I -NT-3. Representative sections illustrating the distribution of residual binding are shown at several concentrations of either displacer. The image of ^{125}I -NT-3 binding in the presence of the highest concentration of BDNF represents the greatest amount of residual binding observed in several experiments.

of ^{125}I -NT-3 binding in several brain areas were measured directly from digitized autoradiographic films and quantitative image analysis techniques (Altar et al., 1984). As expected, the amounts of ^{125}I -NT-3 binding in several of these more densely labeled areas exceeded the average amount of binding to the swabbed horizontal sections (compare Figs. 3 and 4). NT-3 inhibited ^{125}I -NT-3 binding with an apparent slight shoulder at approximately 0.2 nM, which was midway along the steepest portion of the curve. ^{125}I -NT-3 binding was again potently inhibited in the dentate molecular layer and superficial cortex, giving IC_{50} values of 200 ± 18 and 544 ± 101 pM, respectively, and Hill coefficients that, again, did not differ from unity (0.95 ± 0.05 and 0.93 ± 0.22 , respectively).

BDNF also competed for most of the binding of 300 pM ^{125}I -NT-3 binding ($\text{IC}_{50} = 800 \pm 108$ pM; $n = 4$) but did so in a distinctly biphasic manner over the 50 pM to 100 nM concentration range using swabbed brain sections (Fig. 3). The Hill coefficient for inhibition was 0.33 ± 0.019 , consistent with ligand inhibition to multiple sites (Wieland and Molinoff, 1981). Inhibition by BDNF could be resolved into two separate curves by determining IC_{50} values from competition with 10 pM to 1 nM BDNF and from 200 pM to 100 nM BDNF. IC_{50} and Hill coefficient values for these two ranges of unlabeled BDNF were 230 ± 100 pM ($nH = 0.76 \pm 0.18$) and 37 ± 2.9 nM ($nH = 0.94 \pm 0.05$), respectively. As with the swabbed brain sections,

microdensitometry of the autoradiographs showed that BDNF displaced ^{125}I -NT-3 binding in a biphasic manner, and at about the same concentrations as in the swabbed sections (compare Figs. 3, 4).

Autoradiographs of either coronal or horizontal rat brain sections revealed a topography of specific ^{125}I -NT-3 binding (Fig. 5) that was quite distinct from that previously shown for NGF. These patterns are summarized in Table 2, and the letter-number pairs identify the labels in Figure 5. The highest levels of binding were found in the anterior olfactory nucleus, nucleus of the lateral olfactory tract, and the hippocampus, including the dentate gyrus, stratum oriens and radiatum, the amygdala, anterior thalamus, and the neocortex. In particular, the first and second layers of neocortex were prominently labeled in the frontal, parietal, and cingulate regions. Layers 1 and 2 were also labeled in the entorhinal cortex. Intermediate binding was also found throughout nucleus accumbens, caudate-putamen, interpeduncular nucleus, ventral and dorsal horns of the spinal cord, superior colliculus, and other areas (Table 2). Lower amounts of specific binding were present in the pars compacta, medial and lateral geniculate nuclei, medial septum, thalamic nuclei, hypothalamus, pars reticulata, and cerebellum. Little or no displaceable binding was found in the globus pallidus, or in other regions of the pons, medulla, or other amygdaloid nuclei. No displaceable binding was detected in myelinated fiber bundles

Figure 5. Coronal rat brain sections were incubated in 300 pM 125 I-NT-3 alone or with 300 nM NT-3 to define nondisplaceable binding in adjacent sections. The numbers in each panel refer to the labels for each anatomical region listed in Table 2. Note the high levels of binding in the molecular layer of dentate gyrus and stratum oriens and radiatum of hippocampus (I–L), caudate-putamen (E–L), and superficial neocortex (A–L).

including the corpus callosum, anterior commissure, internal capsule, or the interbulbar internal capsule, or in circumventricular organs such as the choroid plexus or ependymal cell layers. Specific binding was also absent from peripheral organs, including liver, muscle, kidney, pancreas, lung, stomach, intestine, and heart (data not shown).

Ten days of exposure to photographic emulsion and cresyl violet counterstaining for Nissl substance revealed intense, displaceable labeling in the almost neuron-free first layer of neocortex and the second neocortical layer (Fig. 6*A*), the stratum oriens, radiatum, and molecular layers of hippocampus (Fig. 6*C*) and throughout the caudate-putamen (Fig. 6*B*). No labeling was seen in pyramidal or granule cell layers of hippocampus (Fig. 6*C*), or in the fiber bundles of the internal capsule (Fig. 6*E*). 125 I-NT-3 did not accumulate silver grains in cell bodies or nuclei of any brain area in the hundreds of sections analyzed. Instead, the labeling by 125 I-NT-3 was reminiscent of binding to nerve terminals, in terms of both the diffuse pattern of labeling and its predominance in areas with dense nerve terminal innervation patterns (e.g., molecular layer of hippocampus, layer 1 of neocortex).

To address species similarities or differences in binding, sections from cat and human brains were also used. A pattern of binding in cat brain was obtained that closely resembled that observed for rat, particularly as evidenced by intense labeling of striatum, neocortex, hippocampus, and olfactory areas (Fig. 7). Sections from the human basal ganglia (Fig. 8) revealed a similar distribution for 125 I-NT-3 binding to the caudate-putamen, neocortex, superficial and deeper neocortex, nucleus accumbens, fundus of striatum, and septum, but also in claustrum,

which was not labeled in the rat. 125 I-NT-3 showed no specific labeling of myelinated fiber paths, globus pallidus, or hypothalamus, also as seen in coronal rat brain sections (Fig. 5). No obvious differences in 125 I-NT-3 binding were noted between the brains obtained from patients diagnosed with or without Alzheimer's disease.

Discussion

Receptor autoradiography with biologically active 125 I-NT-3 has revealed that this neurotrophin binds with high affinity in a saturable, reversible, and pharmacologically specific manner to rat brain and with a very different distribution from that observed for 125 I-NGF binding. These findings, and the high affinity of BDNF for about half of 125 I-NT-3 binding, indicates that endogenous NT-3 and BDNF may have neurotrophic or other physiological effects in a variety of brain areas and provide a basis for both *in vitro* and *in vivo* studies to assess potential sites of action for these neurotrophins.

Kinetics and pharmacology of high-affinity

125 I-NT-3 binding

As determined by Scatchard analysis, 125 I-NT-3 specifically bound to rat brain sections with high (269 pM) and low (2.8 nM) affinities. Thus, like the binding of BDNF (Rodriguez-Tébar et al., 1990) or NGF (Sutter et al., 1979) to sensory neurons, and 125 I-NT-3 binding to trkB-expressing fibroblasts (Squinto et al., 1991), NT-3 binds to high- and low-affinity sites in brain. Because the concentration of 125 I-NT-3 required to half-saturate high-affinity binding was 2- to 10-fold higher than that observed for 125 I-BDNF or 125 I-NGF binding to sensory neurons, the high-

Figure 6. Emulsion autoradiography of coronal brain sections incubated with 300 pM ^{125}I -NT-3 alone (*A*, *C*, *E*) or in the presence of 300 nM unlabeled NT-3 (*B*, *D*, *F*). Darkfield illumination reveals intense accumulations of silver grains in the first two layers (*1,2*) of cingulate cortex (*A*), in the stratum oriens (*O*), stratum radiatum (*R*), and molecular layers (*MOL*) of hippocampus (*C*), and throughout the caudate-putamen (*E*). Bright-field illumination of these cresyl violet sections revealed a low density of nondisplaceable silver grains in the molecular (*LM*), pyramidal (*P*), and granule (*G*) cell layers of hippocampus, in the corpus callosum, and in the fiber bundles of the internal capsule.

Figure 7. Coronal sections of an adult cat brain were incubated with 300 pM ^{125}I -NT-3. The binding of 300 pM ^{125}I -NT-3 throughout adjacent sections in the presence of 300 nM NT-3 (to define nondisplaceable binding) resembled the level of gray observed in the corpus callosum in *A–D*. Note the similarity of binding to rat sections in Figure 5.

affinity nature of this binding was also evaluated with non-equilibrium analysis. The K_d value of 227 pM determined by the ratio of the dissociation and association rate constants agreed closely with the 269 pM value obtained under equilibrium conditions. In the dissociation experiments, ^{125}I -NT-3 dissociation proceeded over several hours but only in the presence of a 1000-fold excess concentration of NT-3. This requirement for excess homologous ligand to promote the dissociation of high-affinity neurotrophin ligand binding has also been reported for the high- but not low-affinity binding of BDNF (Rodríguez-Tébar et al., 1990) and NGF (Sutter et al., 1979; Altar et al., 1991a), and is again consistent with the labeling by ^{125}I -NT-3 of a high-affinity site.

Pharmacological analysis of the high-affinity binding of 300 pM ^{125}I -NT-3 to brain sections revealed that NT-3 and BDNF, but neither NGF nor hCNTF competed for binding. Recent observations with PC12 cells and fibroblasts have shown that the trk family of proto-oncogenes represents specific and biologically relevant tyrosine kinases that can function as receptors for the neurotrophins. Specifically, trkA, trkB, and trkC bind NGF, BDNF and NT-3, respectively, with high affinity (Klein et al., 1989, 1991; Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991). NT-3 can also bind to trkA and trkB as well as trkC in these cells (Rodríguez-Tébar et al., 1990) and can mediate signal transduction through either receptor (Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991). trkA does not appear to have been recognized in brain sections by ^{125}I -NT-3, since NGF did not inhibit ^{125}I -NT-3 binding to any region of horizontal brain sections. Also, the topography of ^{125}I -NT-3 binding did not resemble that of ^{125}I -NGF (Richardson

et al., 1986; Raivich and Kruetzberg, 1987; Altar et al., 1991a,b). Unlabeled NT-3 prevented ^{125}I -NT-3 binding at a concentration about equal to the ^{125}I -NT-3 concentration and with a Hill coefficient near unity, consistent with a noncooperative inhibition of binding from independent sites (Wieland and Molinoff, 1981). In contrast, BDNF inhibited NT-3 binding but did so in a clearly biphasic manner, with IC_{50} values of 230 pM and 37 nM. It is of considerable interest then that the pattern of trkB localization by *in situ* hybridization (Vasquez and Ebendal, 1991) resembles the distribution of ^{125}I -NT-3 binding that was most displaced by 300–400 pM BDNF in the neocortex, septum, and caudate-putamen.

The residual ^{125}I -NT-3 binding in the presence of 1 nM BDNF is presumably to trkC sites. Although no trkC-specific antibody yet exists, *in situ* hybridization with an RNA probe for the extracellular domain of trkC resembles this binding in many brain regions. Thus, ^{125}I -NT-3 appears to bind equally well to both trkC and trkB, whereas BDNF recognizes the trkB and trkC components of ^{125}I -NT-3 binding with high and low potency, respectively. The subtle shoulder in the curve for NT-3 inhibition of labeled NT-3 binding may reflect slightly different potencies for NT-3 competition at trkB and trkC sites. Alternatively, ^{125}I -NT-3 may also bind to truncated forms of trkB (Klein et al., 1989) or trkC (Lamballe et al., 1991) in brain. Significantly different affinities of BDNF but not NT-3 for these putative truncated trk proteins (Lamballe et al., 1991) or trkB and trkC receptors might explain the clearly heterogeneous displacement of ^{125}I -NT-3 by BDNF but not by NT-3. In addition, the trk family of gene products shares a high level of homology not only among themselves but also with other tyrosine kinases.

Figure 8. Frozen sections from four adult humans (A–D) were incubated with 300 pM 125 I-NT-3. Intense, highly displaceable labeling was present in the caudate nucleus (CN), putamen (P), nucleus accumbens (NA), fundus of striatum (fSt), claustrum (CLAUI), bilateral septum (SEPTA), amygdala (AMYG), subcallosal cortex (SC Cx), insular cortex (InCx), frontal cortex (FCx), and first layer of cortex (I Cx). The low binding in the globus pallidus (GP), fornix (Fornix), anterior commissure (ac), internal capsule (ic), corpus callosum (cc), external or internal globus pallidus (GPe, GPi), or lateral hypothalamus (LHA) were not displaced by excess NT-3. LV, lateral ventricle.

Therefore, it is possible that NT-3 also recognizes other, as yet undiscovered, tyrosine kinase receptors with complex pharmacological profiles, and these may include BDNF-sensitive sites.

Distribution of NT-3 binding in mammalian brain

125 I-NT-3 bound in a regionally heterogeneous manner throughout coronal or horizontal sections. These binding patterns were conserved across species and were consistently found between experiments. In contrast, the 10–30% nondisplaceable component of 125 I-NT-3 binding in the presence of excess NT-3 was homogeneously distributed at both the dry film and emulsion levels of resolution. The uniform topography of this nondisplaceable binding may represent receptor-independent association of 125 I-NT-3 to diverse tissue components, including myelinated fiber bundles and nonspecific protein binding within brain parenchyma. A larger proportion of nondisplaceable binding has been reported for 125 I-muNGF binding to tissue sections (Richardson et al., 1986; Raivich and Kreutzberg, 1987). A decrease in nondisplaceable 125 I-rhNGF binding to brain and spinal cord sections (Jakeman et al., 1990; Altar et al., 1991a,b) has been obtained with the protein carriers used by the present

methods, and these conditions probably contributed to the high specificity of the NT-3 signal obtained here. Nevertheless, slightly more (70–90%) 125 I-NT-3 binding was displaced by excess homologous ligand than is 125 I-rhNGF (60–80%; Altar et al., 1991a,b) under identical binding assay conditions.

The amount of high-affinity 125 I-NT-3 binding in swabbed brain sections exceeded by at least threefold the amount reported even within areas with the densest NGF binding (Altar et al., 1991a,b). Using microdensitometry, 125 I-NT-3 binding was yet again three- to fourfold greater in hippocampus and superficial neocortex than the average amount of 125 I-NT-3 binding in swabbed horizontal sections. Thus, 125 I-NT-3 binding exceeds by about 10-fold the amount of 125 I-NGF binding when areas with the greatest densities are compared. The topography of 125 I-NT-3 binding to rat brain sections also clearly differed from 125 I-NGF binding (Richardson et al., 1986; Raivich and Kreutzberg, 1987; Altar et al., 1991a,b). None of the cholinergic nuclei in the diagonal band, nucleus basalis, or seventh cranial nerve nucleus were labeled by 125 I-NT-3, whereas these areas show the greatest amounts of high-affinity labeling with 125 I-NGF (Richardson et al., 1986; Raivich and Kreutzberg, 1987; Altar et al., 1990a,b). Unlike 125 I-NGF (Richardson et al., 1986; Raivich

and Kreutzberg, 1987), emulsion autoradiography with ^{125}I -NT-3 failed to find neuronal cell body binding in any brain region in sections at 25 coronal levels. Instead, ^{125}I -NT-3 binding appeared to predominate in brain regions that are replete with nerve terminals. For example, the neuron-free first layer of neocortex and layer 2 bound ^{125}I -NT-3, and more so in rostral cortex. The absence of labeling on neuronal somata, and the diffuse pattern of labeling in most brain regions suggest that terminal rather than cell body labeling is a distinct property of high-affinity ^{125}I -NT-3 binding. This property of ^{125}I -NT-3 binding may also explain the relatively low binding of ^{125}I -NT-3 in the molecular layer of cerebellum. The granule cell layer is labeled with *trkC* mRNA using *in situ* hybridization techniques (Lamballe et al., 1991). A relatively diminished translation of *trkC* message into functional receptor in cerebellum may explain the presence of *trkC* mRNA but not ^{125}I -NT-3 binding in the cerebellar granule layer, and the presence of functional NT-3 receptors may result from the transport of this binding site to terminals within the molecular layer. Clearly, selective denervation techniques are required to identify neuronal terminals upon which these ^{125}I -NT-3 binding sites may reside.

The present results allow some comparisons to be made between the patterns of ^{125}I -NT-3 binding and LNGFR immunostaining in several rat and human brain areas. This comparison is of interest in part because LNGFR recognizes many rat brain regions that are not labeled by high-affinity ^{125}I -NGF binding (Pioro and Cuello, 1988, 1990; Batchelor et al., 1989; Cohen-Corey et al., 1989; Gibbs et al., 1989; Koh et al., 1989; Woolf et al., 1989; Yan and Johnson, 1989; Cuello et al., 1990). Some of these areas include hippocampus, neocortex, raphe nuclei, and olfactory bulb. Unlike NGF, ^{125}I -NT-3 binding is present in these areas of rat brain, and thus may result from an interaction with LNGFR. Of these areas, the hippocampus, neocortex, and olfactory pathways also express high levels of NT-3 and *trkC* mRNA (Ernfors et al., 1990; Lamballe et al., 1991). ^{125}I -NT-3 binding was also clearly evident in cat and human brain and resembled that seen in rat brain sections, including binding to septum, amygdala, caudate-putamen, nucleus accumbens, and superficial neocortex. LNGFR immunostaining has been found in the medial septum, diagonal band, nucleus basalis, and cerebellar Purkinje cell of adult human and monkey (Hefti et al., 1986; Kordower et al., 1988; Hefti and Mash, 1989; Mufson et al., 1991) and in primate hippocampus, dentate gyrus, olfactory tubercle, and layers 1, 2, and 4 of neocortex (Kordower et al., 1988; Mufson et al., 1991). Thus, as in rat, some of the high-affinity ^{125}I -NT-3 binding is found in primate species in brain regions that also contain the LNGFR. However, ^{125}I -NT-3 binding also appears in a number of rodent or primate brain regions in which LNGFR is either absent or barely detectable, including claustrum, nucleus accumbens, fundus of striatum, neostriatum, superficial neocortex, substantia nigra, dorsal raphe nucleus, central gray, and basolateral amygdala. It thus appears unlikely that LNGFR is required in these areas for high-affinity NT-3 or BDNF binding, since either ligand potentially displaced ^{125}I -NT-3 in each brain area where LNGFR is not detected. This conclusion is consistent with observations of LNGFR-independent binding of NGF or BDNF for *trkA* (Klein et al., 1991) or *trkB*-transfected cells, respectively (Glass et al., 1991; Klein et al., 1991; Soppet et al., 1991; Squinto et al., 1991).

The high-affinity component of ^{125}I -NGF binding of prior studies reflects the predominant if not exclusive physiological

effects of NGF on a subset of brain cholinergic neurons. In a similar manner, the demonstration of high-affinity ^{125}I -NT-3 binding in rat, cat, and human brain supports a role for this neurotrophin in adult brain function, and provides an anatomical basis for investigations of brain functions for BDNF as well as NT-3. *In vitro* bioassays have revealed that BDNF and NT-3 can each promote the survival and phenotypic expression of dopaminergic, GABAergic, and cholinergic neurons derived from embryonic rat brain (Alderson et al., 1990; Knüsel et al., 1990; Hyman et al., 1991a,b). More recent *in vivo* studies have shown that BDNF enhances the survival of septohippocampal cholinergic neurons following axotomy (Morse et al., 1992), elevates dopamine turnover in the caudate-putamen, and augments behavioral functions of nigrostriatal neurons (Altar et al., 1992). These functional studies, and the localization of high-affinity ^{125}I -NT-3 binding in brain, indicate that BDNF and NT-3 are likely to have more widespread effects than NGF on the developing, adult, or aging brain, or following neuronal injury and subsequent repair.

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