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TYROSINE KINASE B AND C RECEPTORS IN THE NEOSTRIATUM AND NUCLEUS ACCUMBENS ARE CO-LOCALIZED IN ENKEPHALIN-POSITIVE AND ENKEPHALIN-NEGATIVE NEURONAL PROFILES AND THEIR EXPRESSION IS INFLUENCED BY COCAINE

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Abstract—Single- and double-label immunohistochemistry were used to determine the extent to which the tyrosine kinase B and C receptors, are expressed in enkephalin-immunopositive or enkephalin-immunonegative neuronal profiles in the rat neostriatum and nucleus accumbens. Results indicate that tyrosine kinase B and C receptors are co-localized in both enkephalin-positive and enkephalin-negative neurons in both of these nuclei, which suggests that these receptors influence both the striatal–pallidal (enkephalin) and striatal–ventral mesencephalic (substance P/dynorphin) pathways. We also examined the influence of acute or repeated injections of cocaine on the number of tyrosine kinase B and C receptors immunoreactive neuronal profiles in the rat neostriatum and nucleus accumbens. Following an acute injection of cocaine (15 mg/kg, i.p.), there were significant decreases in the number of tyrosine kinase B and C receptors immunoreactive profiles in specific regions of the neostriatum and nucleus accumbens relative to saline-pretreated rats. One or 14 days following the last of seven daily injections of 15 mg/kg cocaine or saline there were no differences in the numbers of tyrosine kinase B or C receptors immunoreactive neuronal profiles between these treatment groups.

Collectively, the present results indicate that tyrosine kinase B and C receptors in the neostriatum and nucleus accumbens are co-localized in enkephalin-positive and enkephalin-negative neuronal profiles, which suggests that the striatal medium spiny neurons expressing tyrosine kinase B and C receptors include those that project to the pallidum or the ventral mesencephalon. The current results also show that an acute injection of cocaine results in a decrease in the number of tyrosine kinase B and C receptors immunoreactive neuronal profiles in specific regions of the nucleus accumbens and neostriatum, indicating that cocaine-induced increases in extracellular dopamine in the striatal complex result in compensatory decreases in the expression of tyrosine kinase B and C receptors.

Key words: brain-derived neurotrophic factor, neurotrophin-3, tyrosine kinase, psychostimulant, sensitization.

Approximately 95% of the neurons in the striatal complex are GABAergic medium spiny-projection neurons (Groves, 1983). The medium spiny-projection neurons of the neostriatum and nucleus accumbens are differentiated based on their efferent targets and the co-expression of peptide neurotransmitters with GABA. Thus, projections from the neostriatum to the globus pallidus contain enkephalin, whereas the neostriatal efferents to the substantia nigra pars compacta express substance P and dynorphin (Mroz et al., 1977; Vincent et al., 1982; Beckstead and Kersey, 1985; Gerfen and Young, 1988; Le Moine and Bloch, 1995). The nucleus accumbens outputs to the ventral mesencephalon contain substance P and dynorphin, whereas accumbal projections to the ventral pallidum may include enkephalin or substance P/dynorphin (Curran and Watson, 1995; Le Moine and Bloch, 1995; Lu et al., 1997). Accumbal and neostriatal medium spiny-projection neurons also express tyrosine kinase receptor (Trk) B and TrkC mRNA and protein (Altar et al., 1994; Anderson et al., 1995; Fryer et al., 1996; Jung and Bennett, 1996; Numan and Seroogy, 1997; Yan et al., 1997; Canals et al., 1999; Costantini et al., 1999; Venero et al., 2000) and stimulation of these tyrosine kinase receptors by neurotrophins such as BDNF and NT-3 promote the survival and differentiation of striatal neurons (Mizuno et al., 1994; Ventimiglia et al., 1995; Nakao et al., 1996) and protect these cells from degeneration induced by metabolic stress or excitotoxins (Nakao et al., 1995; Martinez-Serrano and Bjorklund, 1996; Bemelmans et al., 1999). The present experiments, which were performed using sections of rat brain taken at the level of the neostriatum/nucleus accumbens, were designed to determine the extent to which (1) TrkB and TrkC receptors are co-localized in neuronal profiles, (2) TrkB and TrkC receptors are expressed in enkephalin-containing neuronal profiles and (3) TrkB and TrkC receptors are expressed in non-enkephalin-containing neuronal profiles.

A growing body of evidence indicates that neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) and their associated Trks play important roles in psycho-

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Abbreviations: ANOVA, analysis of variance, BDNF, brain-derived neurotrophic factor; DAB, diaminobenzidine tetrahydrocholoride, EDTA, ethylenediaminetetraacetic acid; HEPES, HCO(3-)-free *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, IEG, immediate early gene; IgG, immunoglobulin G, mRNA, messenger RNA; NT-3, neurotrophin-3, PBS, phosphate-buffered saline; TBS-T, Tris-buffered saline/Tween, Trk, tyrosine kinase receptor.

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stimulant-induced plasticity in the mesotelencephalic dopamine systems (Altar et al., 1992; Martin-Iverson et al., 1994; Martin-Iverson and Altar, 1996; Horger et al., 1999; Pierce et al., 1999). The mitogen activated protein kinase signal transduction cascade, which is stimulated following the activation of TrkB and TrkC receptors, is activated by psychostimulants (Berhow et al., 1995; Berhow et al., 1996; Valjent et al., 2000) and plays an important role in cocaine-induced behavioral sensitization (Pierce et al., 1999) and conditioned place preference to cocaine (Valjent et al., 2000). In order to further determine the extent to which the neurotrophins and their receptors contribute to cocaine-induced plasticity in dopaminergic projection areas, the present experiments also assessed the effect of acute and repeated cocaine injections on the number of TrkB and TrkC immunoreactive neurons in the nucleus accumbens and neostriatum.

EXPERIMENTAL PROCEDURES

Animal care

Adult male Sprague–Dawley rats (250–300 g) were obtained from Taconic Farms (Germantown, NY, USA) and housed individually in hanging stainless steel cages with food and water available *ad libitum*. A 12-h light/dark cycle (lights on at 7:00 a.m.) was used; all of the experiments were performed during the light cycle. All experimental protocols were consistent with the guidelines issued by the National Institutes of Health and were approved by the Boston University School of Medicine Institutional Animal Care and Use Committee. Every effort was made to minimize the pain and discomfort of the animals used in this study and the overall number of animals used.

Antibodies

The primary antibodies used in these experiments were goat polyclonal anti-TrkB (Santa Cruz Biotechnology, Santa Cruz, CA, USA; catalogue number sc-12), rabbit polyclonal anti-TrkC (Santa Cruz Biotechnology, catalogue number sc-117) and mouse monoclonal anti-enkephalin (Chemicon International, Temecula, CA, USA; catalogue number MAB350). The TrkB and TrkC antibodies were raised against epitopes adjacent to the carboxy termini of TrkB gp145 of mouse origin or TrkC gp140 of porcine origin. The enkephalin antibody does not distinguish between met- and leu-enkephalin but does not cross-react with β-endorphin or dynorphin. The TrkC and enkephalin antibodies were diluted at 1:25 in phosphate-buffered saline (PBS); the TrkB antibody was diluted at 1:50 in PBS.

Immunoblotting

One of the methods used to assess the specificity of the TrkB and TrkC antibodies used in the following experiments was immunoblotting. Striatal tissue was obtained from rats following rapid decapitation. The tissue was homogenized in lysis buffer using a mortar and pestle. The lysis buffer contained 10-mM HEPES, 1% Igepal, 10% glycerol, 2 mM phenylmethylsulfonylfluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mg/ml pepstatin-A, 2-mM EDTA, 0.2-mM EGTA, 5-mM dithiothreitol and 10-mM KCl. The homogenates were gently agitated at 4 °C for 25 min on a rotator and then centrifuged at 18,000 $\times g$ for 10 min. The protein concentration of the supernatants was subsequently determined using the Bradford assay. Protein samples were heated at 90 °C with 5% betamercaptoethanol for 3 min and then aliquots containing equal amounts of protein were separated electrophoretically on a 10% Novex Tris–glycine gel (Invitrogen, Carlsbad, CA, USA) and transferred electrophoretically onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Piscataway, NJ, USA). Nonspecific protein binding was blocked by incubation in Tris-buffered saline/Tween (TBS-T: 20-mM Tris base, pH 7.6, 137-mM NaCl, 0.2% Tween-20) containing 5% BSA for 45 min at room temperature. Membranes then were incubated overnight with either anti-TrkB or anti-TrkC at 4 °C. The membranes then were rinsed with TBS-T for 40 min and incubated with peroxidase-conjugated goat anti-rabbit or HRP-conjugated protein-A (Amersham Biosciences), dilution 1:5000, for 1 h at room temperature. The membrane then was washed in TBS-T and developed using an enhanced chemiluminescence system (Amersham Biosciences) and autoradiography.

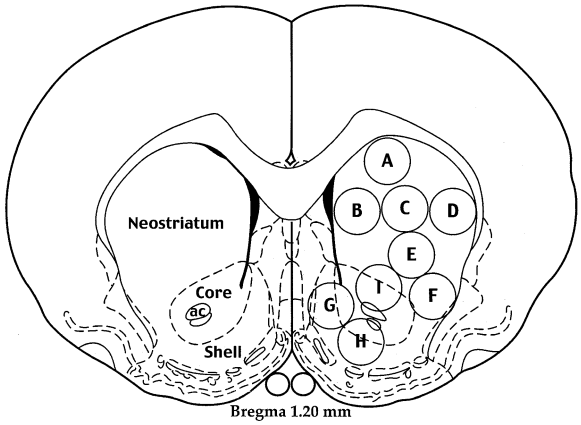

Fig. 1. Representation of a coronal section taken at the level of the anterior commisure including the nucleus accumbens and neostriatum (approximately 1.2 mm anterior to bregma from the atlas of Paxinos and Watson, 1997). Cell counts were obtained from circumscribed regions of the neostriatum and nucleus accumbens. The letters correspond to the regions sampled for quantification. A=dorsal-central neostriatum, B=medial neostriatum, C=central neostriatum, D=lateral neostriatum, E=ventral–central neostriatum, F=ventral–lateral neostriatum, G=medial nucleus accumbens, H=ventral–central nucleus accumbens, I=dorsal–central nucleus accumbens. The core and shell subregions of the nucleus accumbens are identified on the left side of the figure, as is the anterior commisure (ac).

Tissue collection and sectioning

For all immunohistochemical experiments, the rats were overdosed with 100 mg/kg pentobarbital (i.p.) and were perfused through the heart with 120 ml of 0.9% saline followed by 60 ml of 2% paraformaldehyde in 0.1-M PBS, pH 7.3. The brains were removed and placed in 2% paraformaldehyde for 48 h at 4 °C. Thirty-micron coronal serial sections were taken using a Vibratome (Energy Beam Sciences, Agawam, MA, USA) and placed in PBS until stained with Cresyl Violet or processed for single- or double-label immunohistochemistry. Sections used for all experiments were taken at the level of the anterior commisure [approximately 1.2 mm anterior to bregma according to the atlas of Paxinos and Watson (1997)] and included the nucleus accumbens and neostriatum (see Fig. 1).

Cresyl Violet staining

Brain sections were mounted on gelatin-coated microscope slides and air dried. The sections then were immersed in 100% xylene for 5 min followed by a series of decreasing concentrations of ethanol (100–50%, 3 min in each) and distilled water (3 min) before being immersed in Cresyl Violet (0.2%) for 8 min. The

sections then were rinsed in water and immersed in a series of increasing concentrations of ethanol (50–100%, 3 min in each) and, finally, xylene (100% for 5 min). The sections then were mounted with Permount and stored at 4 °C until quantification.

Single-label immunoperoxidase immunohistochemistry

All brain sections were incubated in 4% normal rabbit or goat serum (Jackson ImmunoResearch, West Grove, PA, USA) for 45 min to prevent nonspecific binding. The rabbit and goat sera were diluted in PBS. The sections were incubated in primary antibody overnight at 4 °C, followed by a 30-min wash in PBS. The sections then were immersed for 2 h in one of the following biotinylated secondary antibodies: rabbit anti-goat immunoglobulin G (IgG), rabbit anti-sheep IgG or goat anti-rabbit IgG (Vector, Burlingame, CA, USA), all of which were diluted at 1:500 in PBS. Next, the sections were rinsed for 30 min in PBS followed by a 30-min incubation in avidin–biotin–peroxidase reagent (ABC Elite; Vector). A final rinse in PBS preceded treatment with diaminobenzidine tetrahydrocholoride (DAB) solution containing H_2O_2 and nickel enhancing solution (Vector) for 10 min. Sections were mounted on slides using Slow Fade mounting media (Molecular Probes, Eugene, OR, USA). Control sections were treated as described above but with omission of the primary antibody. To demonstrate the specificity of the TrkB and TrkC receptor antibodies, some sections were preabsorbed with a five-fold excess of TrkB- or TrkC-blocking peptide (Santa Cruz Biotechnology), respectively, for 2 h prior to immunohistochemistry as described above.

Double-label immunofluoresence histochemistry

Double-label immunofluorescence histochemistry was performed to assess the extent of co-localization of TrkB–TrkC. Free-floating brain sections were incubated in 4% normal donkey serum (Jackson ImunoResearch) diluted in PBS for 45 min at room temperature to block nonspecific binding. The sections were incubated in anti-TrkB for 24 h at 4 °C. The sections then were rinsed with PBS for 30 min before incubation in FITC-conjugated donkey anti-goat IgG (1:200; Jackson ImmunoResearch) diluted in PBS for 2 h at room temperature. Sections then were washed in buffer for 30 min, blocked in 4% normal donkey serum (Jackson ImmunoResearch) for 30 min, and incubated in anti-TrkC for 24 h at 4 °C. Following a 30-min wash in PBS, the sections were incubated in Texas Red-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) for 2 h at room temperature. A final 30-min wash in PBS preceded the sections being mounted on slides with Slow Fade (Molecular Probes) and coverslipped. Control experiments followed the same protocol but exposure to the primary antibodies was omitted. The TrkB and TrkC immunoreactivity was visualized using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA), equipped with a xenon/krypton and an argon laser to detect FITC- and Texas Red-conjugated fluorochromes. Control experiments revealed no cross-recognition between the fluorochrome-labeled products.

Double-label immunoperoxidase histochemistry

Double-label immunoperoxidase histochemistry was performed to assess the extent of co-localization of TrkB–TrkC, enkephalin–TrkB and enkephalin–TrkC. Following a 45-min incubation in 4% normal horse serum (Jackson ImmunoResearch) to prevent nonspecific binding, the sections were incubated in anti-TrkC or anti-enkephalin overnight at 4 °C. The sections then were washed in PBS for 30 min, followed by incubation in biotinylated goat anti-rabbit IgG (1:500; Vector Laboratories) overnight at 4 °C. The next day, the sections were rinsed in buffer for 30 min, followed by immersion for 30 min in avidin–biotin–peroxidase reagent (ABC Elite; Vector Laboratories). A 15-min rinse in PBS preceded treatment with DAB solution containing H_2O_2 and nickel enhancing solution for 10 min, followed by a 15-min rinse in water. The sections then were incubated in 4% normal horse serum for 45 min and then incubated in the second primary antibody (anti-TrkB or anti-TrkC) overnight at 4 °C. The sections then were washed in PBS for 30 min, and incubated in biotinylated horse anti-goat IgG for 2 h at room temperature. Next, the sections were rinsed in PBS for 30 min and incubated in avidin–biotin–peroxidase reagent (ABC Elite; Vector) for 30 min. The sections were rinsed in PBS for 15 min and incubated in Vector Intensely Purple (Vector) for 10 min. The sections then were rinsed in PBS for 5 min, mounted on slides, air-dried, and mounted using Slow Fade (Molecular Probes). Control sections were treated as described above but with omission of the primary antibodies. These sections were photographed using a Zeiss axioscope equipped with a 35-mm camera.

Cocaine and saline injection protocols

Acute injections. Rats received a single i.p. injection of 15 mg/kg cocaine or 0.9% saline and were killed 24 h later.

Repeated injections, early withdrawal. Rats received seven once-daily injections of 15 mg/kg cocaine or saline (i.p.) and were killed 24 h after the last injection.

Repeated injections, late withdrawal. Rats received seven once-daily injections of 15 mg/kg cocaine or saline (i.p.) and were killed 14 days later, 24 h following a challenge injection of cocaine (15 mg/kg) or saline (i.p.).

With all acute and repeated cocaine or saline injections, tissue was collected 24 h after the terminal injection and tissue was collected, sectioned and stored prior to immunohistochemistry as described above. For these experiments, every set of reactions included brain sections from saline-control animals.

Microscopy and quantitative analysis

The numbers of cell profiles immunolabeled with the enkephalin, TrkB or TrkC antisera or that were stained with Cresyl Violet were determined in various sectors of the neostriatum and nucleus accumbens using a Leitz bright field microscope equipped with a camera lucida optic. A 25× objective was used to draw the labeled cell profiles on paper. All labeled cell profiles in a microscopic field corresponding to a diameter of 1 mm on the tissue section were drawn for each striatal sector examined. In most cases, two sections per rat and per striatal region were sampled and the average value of the two sections was calculated to determine the total number of labeled profiles in a particular sector. In a few instances, the quality of the tissue/labeling was less than optimal and the number of profiles was measured on only one section. Only profiles that had a visible nucleus were drawn and included in the quantification. For the Nissl-stained sections, glial cell profiles, which could be distinguished from neuronal cell profiles by their small size and/or intense staining were not recorded. In addition, nuclei in glial cell profiles are usually not distinguished from the cytoplasm. The number of neuronal profiles in different rats was measured at a comparable level of the striatum corresponding to 1.2 mm anterior to bregma according to the atlas of Paxinos and Watson (1997). The sectors to be analyzed were chosen in regions of the neostriatum and the core and shell of the nucleus accumbens. Drawing of labeled profiles was carried out by an experimenter who was blind to the experimental groups.

The number of labeled profiles for each experimental group and striatal region was calculated as the mean from four to 12 rats. Statistical differences between groups were determined with a one-way analysis of variance (ANOVA) followed by Fisher's pairwise comparisons with $P<0.05$ considered significant.

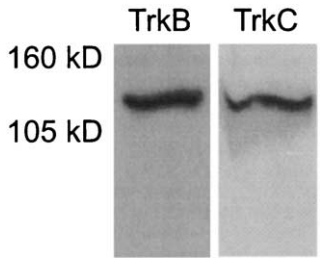

Fig. 2. Immunoblot demonstrating that the TrkB and TrkC antibodies used in this study produce a single band of the correct molecular weight (145 and 140 kD, respectively) using tissue obtained from the rat neostriatum.

Drugs

The cocaine hydrochloride (provided by the National Institute on Drug Abuse) was dissolved in 0.9% sterile saline. Pentobarbital was obtained from Sigma/RBI (St. Louis, MO, USA) and was dissolved in 20% ethanol.

RESULTS

Spatial and cellular distribution of TrkB, TrkC and enkephalin immunoreactivity in the neostriatum and nucleus accumbens

The specificity of the TrkB and TrkC antibodies was assessed in several ways. Sections in which the primary antibody was omitted showed no specific immunoreactivity (data not shown). Moreover, preabsorption with blocking peptides for TrkB and TrkC eliminated immunoreactivity in these sections (data not shown). Finally, electrophoresis/immunoblotting with the TrkB and TrkC antibodies was performed on tissue samples obtained from the neostriatum. These experiments revealed specific bands of the molecular weights appropriate for TrkB and TrkC (see Fig. 2). The anti-enkephalin antibody has been extensively characterized. This antibody recognizes both met and leu enkephalin but does not cross-react with β-endorphin or dynorphin (Cuello et al., 1984).

TrkB- and TrkC-immunopositive cell profiles were distributed throughout the neostriatum and nucleus accumbens, and included both large- and medium-sized neurons (see Figs. 3–5). Intense immunoreactivity was observed in the soma and proximal dendrites. However, many immunoreactive thin fibers and punctate profiles were observed in the neuropil. TrkB immunoreactivity frequently extended further into the dendrites than TrkC immunoreactivity (see Fig. 3A, B, respectively). Enkephalin-positive neuronal profiles also were diffusely present in the striatum, with immunoreactivity in the soma and proximal dendrites (Fig. 3C). The pattern of enkephalin immunoreactivity was somewhat patchy, with clusters of enkephalin-immunoreactive neuronal profiles in the neostriatum and nucleus accumbens.

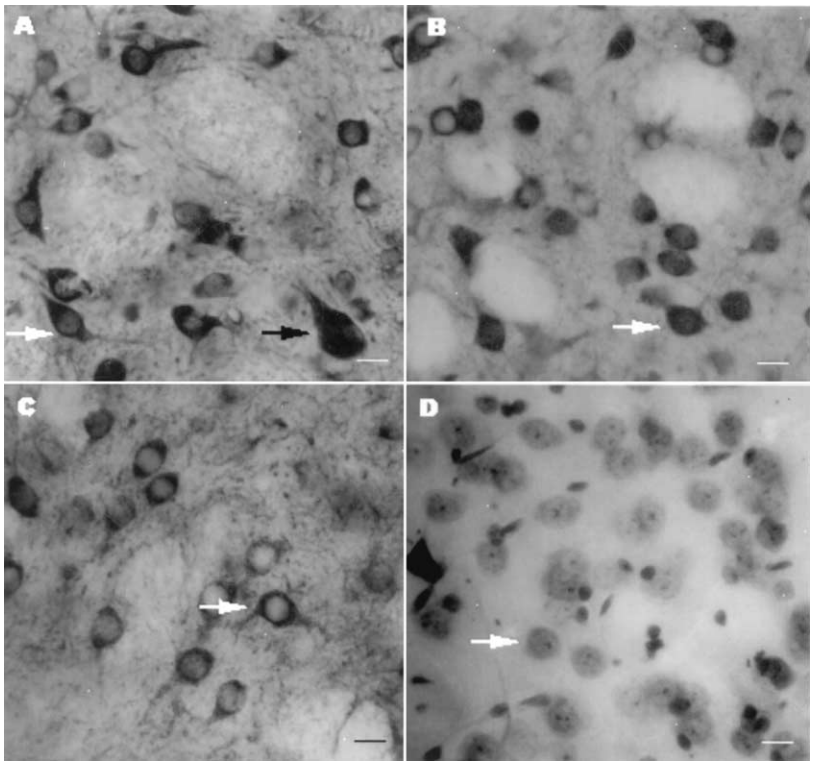

Fig. 3. Panels A–D show, respectively, TrkB-, TrkC- and enkephalin-immunoreactive neuronal profiles as well as Cresyl Violet-stained neuronal profiles in the neostriatum. The labeled cells include both medium and large (see lower left of panel A) profiles. Note that there are approximately one-third as many TrkB-, TrkC- and enkephalin-immunoreactive cells per field relative to the number of Cresyl Violet-stained neuronal profiles, which is representative of the cell counts presented in Table 1. The white arrows point to medium-sized neuronal profiles, which include medium spiny-projection neurons and spiny interneurons. The black arrow points to a neuronal profile with a relatively large soma, which presumably is a large aspiny cholinergic interneuron. Scale bars=11 μm.

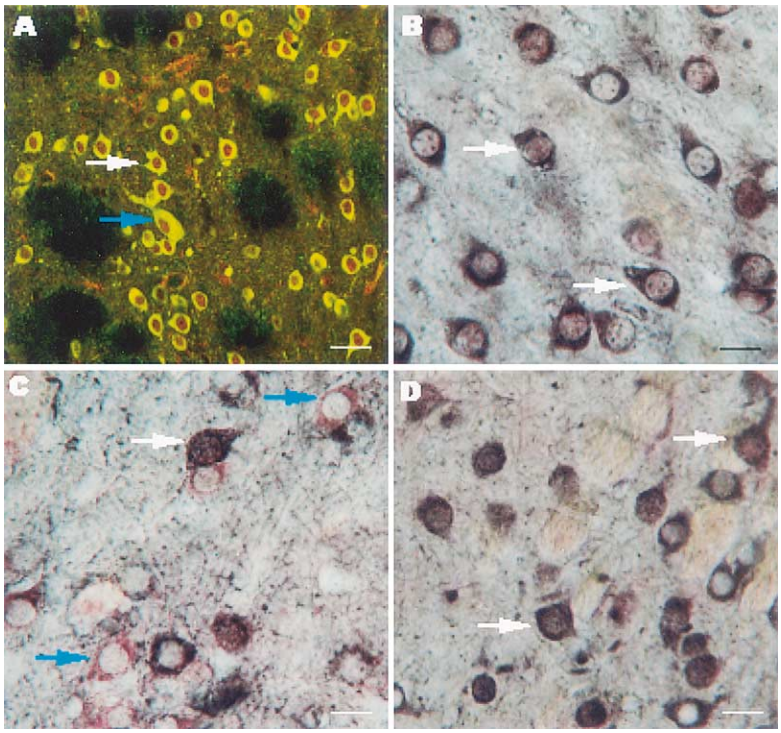

Fig. 4. Double immunolabeling of neuronal profiles in the rat neostriatum with antibodies raised against TrkB, TrkC and enkephalin. Panel A depicts double-fluorescence immunohistochemistry for TrkB (green) and TrkC (red). Note that all neuronal profiles in the field of view, which include both medium-sized (white arrow) and large (blue arrow), are double labeled. The same result was obtained with double-peroxidase immunohistochemistry for TrkB (black) and TrkC (pink), as shown in panel B. The arrowheads in panel B point to double-labeled neuronal profiles. Double-peroxidase immunohistochemistry was used to assess the extent to which neuronal profiles in the neostriatum are immunolabeled by enkephalin and/or TrkB or TrkC. In panel C, TrkB (pink) immunoreactivity was present in some enkephalin- (black) immunoreactive neuronal profiles (white arrow) but not others (blue arrow). In panel D, TrkC (pink) immunoreactivity was present in the majority of enkephalin- (black) immunoreactive neuronal profiles (white arrows). Scale bars: A=20 μm, B–D=11 μm.

Using double-label immunofluorescence and immunoperoxidase histochemistry we examined the extent of co-labeling with TrkB and TrkC antibodies. Virtually all labeled profiles in the neostriatum and nucleus accumbens exhibited dual staining with the TrkB and TrkC antisera. Labeled profiles included medium and large neurons (see Fig. 4A, B). Dual labeling with TrkB or TrkC and enkephalin yielded different results in that some profiles labeled with the enkephalin antiserum were co-labeled with the TrkB or TrkC antisera whereas others were not (see Fig. 4C, D). Visual examination of the slides indicated that the frequency of enkephalin-immunopositive profiles that were co-labeled and those that were not co-labeled with TrkB was comparable (see Fig. 4C). However, the frequency of profiles labeled with enkephalin and TrkC was higher than TrkC alone (see Fig. 4D).

In the double-label immunofluoresence experiments, there was no crossover between the neuronal profiles that were labeled with a FITC-conjugated fluorochrome and a rhodamine-conjugated fluorochrome. That is, the extent and pattern of labeling for a specific antiserum was similar when the tissue was single- or double-labeled.

In order to provide an estimate of the extent to which the antisera labeled the whole population of striatal neurons, the number of labeled profiles were measured in different regions of the neostriatum and nucleus accumbens (sectors B, D, G and I from Fig. 1) and these numbers were compared with the number of profiles Nissl-stained on adjacent sections (see Table 1). Quantification indicated that the numbers of profiles labeled with enkephalin, TrkB or TrkC were very similar and represented about one-third of the total number of neuronal profiles stained with Cresyl Violet. Enkephalin-positive neurons in the neostriatum and nucleus accumbens have been shown to represent between 40 and 60% of all neurons in these nuclei (Beckstead and Kersey, 1985; Curran and Watson, 1995; Lu et al., 1997); therefore, the numbers of neuronal profiles quantified in the present study may have been slightly underestimated.

An acute injection of cocaine decreases the number of neurons immunopositive for TrkB and TrkC in regions of the neostriatum and nucleus accumbens

The saline groups in Tables 2 and 3 are comprised of rats that were killed 24 h after an acute injection of saline, rats that were killed 24 h after the last of seven daily injections of saline and rats that were killed 24 h after a saline challenge injection administered 13 days following seven daily injections of saline. These three control groups were compared with a one-way ANOVA. Since there were no significant differences among the saline groups for any of

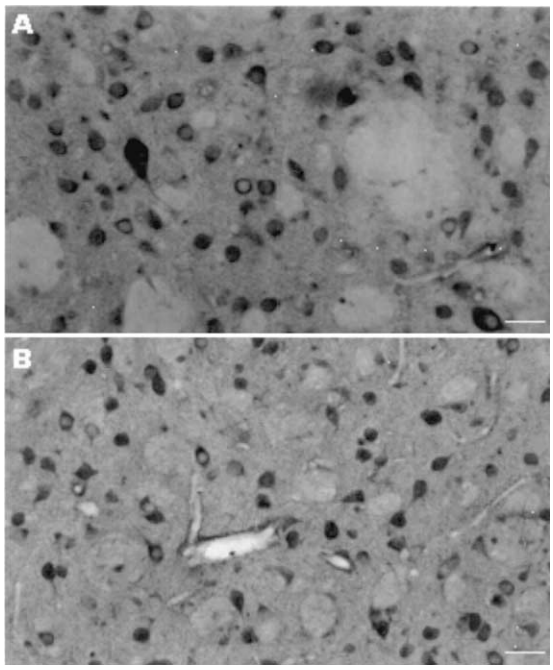

Fig. 5. Individual examples of TrkC-immunoreactive neuronal profiles in the ventral–central neostriatum of animals acutely pretreated with 0.9% saline (panel A) or 15 mg/kg cocaine (panel B). In both cases, the animals were killed 24 h after the saline or cocaine injection. Note that an acute injection of cocaine decreased the number of TrkC-immunolabeled neuronal profiles in the ventral–central neostriatum. Scale bars=30 μm.

the regions of the neostriatum or nucleus accumbens, these groups were combined into a single control group.

The number of neuronal profiles in regions of the neostriatum and nucleus accumbens that were immunoreactive for TrkB and TrkC 1 day after an acute injection of cocaine or saline was assessed. An acute injection of 15 mg/kg cocaine generally reduced the number of immunoreactive neurons throughout the neostriatum and nucleus accumbens, with significant decreases in the number of TrkB-immunoreactive neurons in the medial, central and ventral–lateral neostriatum as well as the medial nucleus accumbens (see Table 2) and a significant decrease in TrkC-immunoreactive neurons in the ventral–central neostriatum (see Table 3). Representative sections from the ventral–central neostriatum of animals injected with cocaine or saline acutely are shown in Fig. 5A and 5B, respectively. There were no statistically significant changes in the numbers of neuronal profiles immunoreactive for TrkB or TrkC 1 or 14 days after the last injection in the repeated-cocaine early group relative to the saline group (see Tables 2 and 3). Analysis of the size of medium and large neuronal profiles in the neostriatum and nucleus accumbens revealed no change in diameter following acute or repeated cocaine injections.

DISCUSSION

The present results indicate that TrkB and TrkC receptors in the neostriatum and nucleus accumbens are co-localized in enkephalin-positive and enkephalin-negative neuronal profiles. Moreover, an acute injection of cocaine decreased the number of TrkB- and TrkC-immunoreactive neuronal profiles in regions of the neostriatum and nucleus accumbens; tolerance to this effect of cocaine developed following repeated injections of cocaine.

TrkB and TrkC immunoreactivity in the neostriatum and nucleus accumbens

Although NT-3 and BDNF are not synthesized in the nucleus accumbens or neostriatum (Ernfors et al., 1990; Maisonpierre et al., 1990; Ceccatelli et al., 1991; Castren et al., 1995; Conner et al., 1997; Furukawa et al., 1998), axon terminals in these structures contain NT-3 and BDNF proteins (Conner et al., 1997; Furukawa et al., 1998; Katoh-Semba et al., 1998; Yurek and Fletcher-Turner, 2001), which are transported to the nucleus accumbens and neostriatum both retrogradely and anterogradely (Altar and DiStefano, 1998; Reynolds et al., 2000). These neurotrophins appear to act as neurotransmitters, being secreted from glutamatergic and dopaminergic terminals in the striatum and influencing striatal physiology by interacting with postsynaptic receptors, including TrkB and TrkC (Altar et al., 1997; Altar and DiStefano, 1998).

Approximately 95% of the neurons in the striatal complex are GABAergic medium spiny-projection neurons (Groves, 1983). The remaining neurons in the nucleus accumbens and neostriatum include large aspiny cholinergic interneurons and small GABAergic aspiny interneurons (Groves, 1983; Kawaguchi et al., 1995). The current results indicate that TrkB and TrkC are abundant and widely distributed throughout the neostriatum and nucleus accumbens. The TrkB- and TrkC-immunoreactive neuronal profiles included those with relatively large soma, presumably large aspiny interneurons, as well as neuronal

Table 1. Number of Cresyl Violet-stained as well as enkephalin TrkB- and TrkC-immunoreactive profiles in regions of the rat neostriatum and nucleus accumbens[a]

Structure	Cresyl Violet (*n*=5)	Enkephalin (*n*=5)	Enkephalin/ Cresyl	TrkB (*n*=5)	TrkB/ Cresyl	TrkC (*n*=5)	TrkC/ Cresyl
B Medial neostriatum	521 (13.2)	166 (7.9)	0.32	189 (5.7)	0.36	177 (8.3)	0.34
D Lateral neostriatum	417 (16.5)	137 (113)	0.33	175 (6.8)	0.42	142 (5.0)	0.34
G Medial accumbens	653 (14.9)	203 (9.3)	0.31	206 (8.4)	0.32	188 (10.6)	0.29
I Dorsal-central accumbens	567 (28.1)	194 (5.7)	0.34	230 (6.6)	0.41	183 (10.4)	0.32

[a] Also shown are the ratios of enkephalin, TrkB and TrkB immunoreactive to Cresyl Violet-stained neuronal profiles for each of the four brain regions examined. The letters preceding the structure name correspond to regions outlined in Fig. 1.

Table 2. Effect of acute and repeated cocaine on TrkB immunoreactivity in the rat striatum and nucleus accumbens[a]

Structure	Saline (n=10–12)	Acute cocaine (n=5–6)	Repeated cocaine, early (n=4)	Repeated cocaine, late (n=7)	Statistics**
Neostriatum					
A Dorsal-central	160.6 (5.0)	140.6 (5.9)	150.0 (11.2)	161.0 (8.7)	$F(3,24)=1.62$, $P<0.211$
B Medial	181.3 (6.0)	155.0 (5.3)*	199.0 (1.9)	169.4 (9.3)	$F(3,24)=4.41$, $P<0.013$
C Central	158.5 (3.1)	134.2 (17.0)*	176.3 (8.2)	168.6 (4.5)	$F(3,24)=4.52$, $P<0.012$
D Lateral	141.9 (6.7)	141.0 (10.2)	154.8 (6.0)	152.3 (7.4)	$F(3,24)=0.67$, $P<0.579$
E Ventral-central	179.6 (7.2)	167.8 (8.9)	190.5 (10.7)	192.6 (9.6)	$F(3,24)=1.26$, $P<0.312$
F Ventral-lateral	171.1 (9.8)	131.8 (3.6)*	165.3 (15.0)	148.6 (10.2)	$F(3,25)=2.88$, $P<0.056$
Nucleus accumbens					
G Medial	191.0 (10.7)	147.8 (21.4)*	222.8 (5.3)	199.0 (9.1)	$F(3,22)=4.33$, $P<0.015$
H Ventral-central	170.2 (10.6)	127.0 (17.9)	169.5 (19.1)	160.0 (5.5)	$F(3,22)=2.26$, $P<0.109$
I Dorsal-central	183.3 (10.5)	142.7 (10.8)	174.5 (38.5)	199.4 (9.9)	$F(3,25)=2.32$, $P<0.100$

[a] The letters preceding the structure name correspond to regions outlined in Fig. 1.
* Significantly different from the saline group ($P<0.05$, Fisher's least significant difference).
** One-way analysis of variance.

profiles with medium-sized soma, which include medium spiny-projection neurons and aspiny interneurons. These results are consistent with previous work indicating that TrkB and TrkC mRNA and protein are expressed in the striatal complex (Altar et al., 1994; Anderson et al., 1995; Fryer et al., 1996; Jung and Bennett, 1996; Numan and Seroogy, 1997; Yan et al., 1997; Costantini et al., 1999; Venero et al., 2000). In addition, the present results reveal that TrkB and TrkC are almost always found in the same neuronal profiles, which include those that were and were not immunoreactive for enkephalin. The peptide enkephalin is a marker for medium spiny neurons that express D2 dopamine receptors and project from the neostriatum to the globus pallidus or from the nucleus accumbens to the ventral pallidum (Mroz et al., 1977; Vincent et al., 1982; Beckstead and Kersey, 1985; Gerfen and Young, 1988; Gerfen et al., 1990; Le Moine et al., 1990, 1991; Curran and Watson, 1995; Le Moine and Bloch, 1995; Lu et al., 1997). Enkephalin-negative neuronal profiles include medium spiny output neurons that contain substance P and dynorphin as well as interneurons (Groves, 1983; Kawaguchi et al., 1995). However, medium-sized interneurons account for only 1–2% of all medium-sized striatal neurons (Groves, 1983; Kawaguchi et al., 1995). Therefore, the vast majority of medium-sized neuronal profiles in the present study that did not express enkephalin were accumbal projections to the ventral mesencephalon or ventral pallidum that express D1 dopamine receptors and substance P/dynorphin (Mroz et al., 1977; Vincent et al., 1982; Beckstead and Kersey, 1985; Gerfen and Young, 1988; Gerfen et al., 1990; Le Moine et al., 1990, 1991; Curran and Watson, 1995; Le Moine and Bloch, 1995; Lu et al., 1997).

The present results indicate that, in the neostriatum and nucleus accumbens, (1) TrkB and TrkC are almost always co-expressed in neurons, (2) both of these receptors are found in striatal interneurons and projection neurons and (3) TrkB and TrkC are expressed by neurons projecting from the striatal complex to the pallidum or ventral mesencephalon. Collectively, these results provide another example of the functional redundancy of neurotrophins and their receptors. For example, different neurotrophins can act through the same receptors and activate the same second messenger systems (Segal and Greenberg,

Table 3. Effect of acute and repeated cocaine on TrkC immunoreactivity in the rat striatum and nucleus accumbens[a]

Structure	Saline (n=11)	Acute cocaine (n=4)	Repeated cocaine, early (n=4)	Repeated cocaine, late (n=7)	Statistics**
Neostriatum					
A Dorsal–central	148.6 (8.7)	129.3 (15.7)	129.5 (11.5)	130.1 (12.3)	$F(3,22)=0.86$, $P<0.476$
B Medial	155.0 (8.3)	129.0 (5.3)	143.3 (14.1)	146.3 (14.5)	$F(3,22)=0.79$, $P<0.512$
C Central	148.9 (8.5)	125.0 (6.3)	136.0 (8.3)	139.2 (15.1)	$F(3,21)=0.80$, $P<0.510$
D Lateral	134.9 (10.3)	110.8 (8.4)	123.0 (13.4)	131.7 (11.0)	$F(3,22)=0.71$, $P<0.557$
E Ventral–central	166.6 (7.3)	129.0 (7.4)*	156.0 (2.5)	176.0 (10.0)	$F(3,22)=4.20$, $P<0.017$
F Ventral–lateral	143.5 (11.7)	112.3 (8.1)	143.3(22.2)	133.0 (13.3)	$F(3,22)=0.79$, $P<0.512$
Nucleus accumbens					
G Medial	164.4 (13.5)	169.8 (8.6)	165.3 (8.6)	173.3 (18.8)	$F(3,22)=0.08$, $P<0.972$
H Ventral–central	135.3 (7.2)	106.5 (11.3)	140.8 (18.0)	115.9 (16.3)	$F(3,22)=1.34$, $P<0.288$
I Dorsal–central	160.6 (7.6)	145.8 (5.7)	132.3 (9.5)	131.4 (11.1)	$F(3,22)=2.60$, $P<0.078$

[a] The letters preceding the structure name correspond to regions outlined in Fig. 1.
* Significantly different from the saline group ($P<0.05$, Fisher's least significant difference).
** One-way analysis of variance.

1996). Although BDNF and NT-3 preferentially activate TrkB and TrkC, respectively, these neurotrophins also bind to other Trks. Thus, NT-3 binds to TrkC with picomolar affinity and TrkA and TrkB with nanomolar affinity (Squinto et al., 1991; Soppet et al., 1991; Tsoulfas et al., 1993; Valenzuela et al., 1993; Shelton et al., 1995.). Moreover, BDNF and NT-3 bind the p75 receptor with equivalent affinity (Segal and Greenberg, 1996). Therefore, consistent with the functional overlap between the neurotrophins generally, functional differences between BDNF, NT-3 and their receptors in terms of influence on striatal output neurons are at present unclear.

Acute cocaine-induced decreases the number of TrkB- and TrkC-immunoreactive neuronal profiles in the nucleus accumbens and neostriatum

The current results indicate that although an acute injection of cocaine resulted in decreased numbers of TrkB- and TrkC-immunoreactive neurons in regions of the nucleus accumbens and neostriatum, these decreases did not persist. That is, in contrast to acute cocaine administration, seven daily cocaine injections had no detectable effect on the numbers of TrkB- or TrkC-immunoreactive neurons in the nucleus accumbens and neostriatum. These results suggest that the decreases in TrkB and TrkC observed following an acute cocaine injection are compensatory responses to the initial cocaine exposure. Interestingly, neurotrophins and their receptors are influenced by changes in extracellular dopamine in that dopamine depletion increases TrkB and BDNF mRNA and protein levels in the neostriatum (Dragunow et al., 1995; Numan and Seroogy, 1997; Venero et al., 2000; Yurek and Fletcher-Turner, 2001). Collectively, these results suggest that cocaine-induced increases in extracellular dopamine in the nucleus accumbens and neostriatum may have an inhibitory effect on the expression of neurotrophins and their receptors. A previous study reported that an acute injection of cocaine had no influence on TrkB or TrkC mRNA levels in the striatum (Jung and Bennett, 1996). While these results contrast with the cocaine-induced decreases in the number of TrkB- and TrkC-immunoreactive neurons in regions of the neostriatum reported here, there are significant differences between these studies, including the measurement of mRNA versus protein and the time after cocaine injection at which the measurements were made (1 versus 24 h), that likely account for these discrepancies.

The fact that acute cocaine-induced decreases in the number of TrkB- and TrkC-immunoreactive neuronal profiles in regions of the neostriatum and nucleus accumbens were not observed following repeated cocaine injections suggests that changes in the number of Trks do not contribute to the long-term expression of cocaine-induced neuronal and behavioral plasticity. The development of tolerance to the acute effect of cocaine on Trk expression in the striatal complex following repeated cocaine injections is reminiscent of the influence of cocaine on immediate early genes (IEGs). Thus, whereas an acute injection of cocaine increases mRNA levels of IEGs such as c-fos, c-jun and zif268 in the neostriatum and nucleus accumbens, these increases are not observed after repeated cocaine administrations (Hope et al., 1992; Mortalla et al., 1993; Daunais and McGinty, 1994; Hammer and Cooke, 1996). Collectively, these results raise the possibility that IEGs may negatively influence TrkB and TrkC expression. However, little is known about the TrkB and TrkC promoters and no studies to date have assessed the influence of IEGs on TrkB and TrkC expression.

CONCLUSIONS

The present findings indicate that medium and large neuronal profiles in the neostriatum that are immunoreactive for TrkB almost always also are immunoreactive for TrkC. Moreover, both enkephalin-positive and enkephalin-negative neuronal profiles in the neostriatum and nucleus accumbens are immunoreactive for TrkB and TrkC. These findings suggest that TrkB and TrkC are expressed by both interneurons and medium spiny-projection neurons in the striatal complex. The medium spiny neurons expressing TrkB and TrkC include those that project to the pallidum or the ventral mesencephalon. The current results also show that an acute injection of cocaine results in a decrease in the number of TrkB- and TrkC-immunoreactive neurons in regions of the nucleus accumbens and neostriatum, which suggests that cocaine-induced increases in extracellular dopamine in the striatal complex result in compensatory decreases in the expression of TrkB and TrkC in regions of the neostriatum and nucleus accumbens.

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