Nerve Growth Factor Preserves a Critical Motor Period in Rat Striatum

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ABSTRACT: **We previously found the occurrence of a critical motor period during rat postnatal development where circling training starting the 7-day schedule at 30 days—but not before or after—induces a lifetime drop in the binding to cholinergic muscarinic receptors (mAChRs) in striatum. Here, we studied whether nerve growth factor (NGF) participates in this restricted period of muscarinic sensitivity. For this purpose, we administered mouse salival gland 2.5S NGF (1.4 or 0.4 μg/day, infused by means of ALZA minipumps) by intrastriatal unilateral route between days 25 and 39, and then trained rats starting at 40 days. Under these conditions, NGF induced a long-term reduction in the striatal [^{3}H] quinuclidilbenzylate (QNB) binding sites despite the fact that motor training was carried out beyond the natural critical period. Thus, at day 70, measurement of specific QNB binding in infused striata of trained rats showed decreases of 42% ($p < .0004$) and 33% ($p < .02$) after administration of the higher and lower NGF doses, respectively, with respect to trained rats treated with cytochrome C, for control. Noncannulated striata of the NGF-treated rats also showed a decrease in QNB binding sites (44%; $p < .0001$) only at the higher infusion rate. This effect was not found in the respective control groups. Our observations show that NGF modulates the critical period in which activity-dependent mAChR setting takes place during rat striatal maturation.** J Neurobiol 38: 129–136, 1999

Keywords: **NGF; plasticity; mAChR; rat striatum; development; circling training**

During normal development, there are restricted periods when the functional activity allows synaptic and neurochemical rearrangements to occur with lifetime consequences (Bonhoeffer, 1996). Nowadays, our knowledge of these critical periods of plasticity is almost exclusively derived from the mammalian visual pathway (Wiesel, 1982) and other sensory cortical models (Udin and Scherer, 1990; Fox, 1994; Uylings, 1994; Bottjer and Arnold, 1997).

In the striatum, a basal ganglia with tight structural and functional relations to the cortex (Calabresi, 1996), the cholinergic and dopaminergic systems reach 70–80% maturation on about postnatal day (P) 28 (Coyle and Campochiaro, 1976). Afterward, adjustments are produced before reaching the adult values. For example, we have reported that training of developing rats to run in a circular path per se decreases maximal binding (B_{max}) of [^{3}H]quinuclidilbenzilate ([^{3}H]QNB) to striatal muscarinic receptors (mAChRs) up to 1 year of life (Ibarra et al., 1995). Interestingly, this activity-dependent effect is induced when the 7-day circling training program is started at P30, but not at P20, P40, or P60. The permanent effect of training in this period is not restricted to the cholinergic system, since at least dopamine D2 receptor (D2R) covariates with mAChR under the same experimental conditions (Azcurra et al., 1995; Ibarra et al.,

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1996). In addition, we found a decrease in the phosphorylation of protein kinase C (PKC)-specific substrate GAP-43 30 min after finishing turning activity in a rotary platform performed at these ages (Azcurra et al., 1998; Paratcha et al., 1998). Considering that GAP-43 is a marker of processes of synaptic modeling (Pfenninger et al., 1991; Levin and Dunn-Meynell, 1993), all these results strongly suggest the presence of a period of activity-dependent synaptic refinement in the muscarinic system of rat striatum. The above striatal markers have shown to be direct or indirect neurochemical targets of the prototypic neurotrophin nerve growth factor (NGF) (Levi-Montalcini and Angeletti, 1968; Cuello and Thoenen, 1995) in different models (Jumblatt and Tischler, 1982; Lapchak et al., 1993; Eva et al., 1994; Liberini et al., 1995; Liu et al., 1996; Kobayashi et al., 1997). NGF is induced by physiological activity (Lindholm et al., 1994; Neeper et al., 1996) and the role of NGF in activity-dependent synaptic modeling during development is a point of concern (Black, 1993; Calamandrei et al., 1993; Bonhoeffer, 1996). In the visual system model, NGF administration extends or reinduces the critical period of experience-dependent plasticity, which normally takes place only early in life (Domeneci et al., 1991; Carmignoto et al., 1993; Gu et al., 1994; Liu et al., 1996).

Having in mind these previous results, we hypothethized that the level of NGF could be a determinant of the sensitivity window of striatal receptors to the circling activity. It could be thus expected that the alteration of the natural pattern of NGF expression during this restricted period of development could modify the natural pattern of the muscarinic sensitivity to circling motor activity.

MATERIALS AND METHODS

Animals and Experimental Groups

The male offprings from 10 litters of Sprague–Dawley rats (local facilities, originally purchased from the Holtzman Institute) were divided at random into 13 groups used in three experiments.

For Experiment 1, the four groups were two nonoperated groups trained at ages P30–37 (T_{30}, $n = 6$) or P40–47 (T_{40}, $n = 6$), and their two nontrained controls (C_{30}, $n = 6$, and C_{40}, $n = 6$, respectively).

Experiments 2 and 3 included a total of nine groups. In these experiments, groups were intrastriatally perfused by 2 weeks starting at age P25 through implanted cannula and then trained at ages P40–47, as follow: Experiment 2 involved sham-operated and trained animals (group SH_T, $n = 6$), cytochrome C (CC)-infused and trained animals (group CC_T, $n = 6$), NGF-infused and trained animals (group NGF_T, $n = 5$), and the respective nontrained controls, groups SH_C, CC_C, and NGF_C ($n = 3$ each). Experiment 3 involved a similar schedule as Experiment 2, with a lower perfusion rate (three groups denoted as: sh_T, $n = 6$; cc_T, $n = 6$; and ngf_T, $n = 5$).

In all procedures in these experiments, animals were cared for in compliance with the National Research Council's guide for the care and use of laboratory animals.

Surgical Procedures

Intrastriatal cannulation (coordenates in millimeters: A = 0.5; L = 5; V = 4.5) and minipump implantation were performed at age P25 on operated groups under sterile procedures (White and Schwartz, 1994). Each animal was anesthetized with 3 mL/kg of Equithesin, intraperitoneally (i.p.), which contained pentobarbital (10 mg/mL) and chloral hydrate (40 mg/mL), and cannulated into left striatum with a brain infusion kit. Then, an osmotic minipump [Model 2002, 14 days of continued perfusion, rate (0.5 μL/h) was immediately placed into a subcutaneous pocket in the intrascapular region (minipump and brain infusion kit from Alza Corp., Palo Alto, CA) and then attached to the cannula via a vinyl tubing. Mouse salival gland NGF 2.5S (1.4 or 0.4 μg/day for Experiments 2 and 3, respectively) was perfused to NGF-treated groups. CC (Sigma) was used as a biological control, since it has similar physical and chemical features but no neurotrophin activity. The vehicle for dilutions of NGF and CC was artificial cerebrospinal fluid (CSF). After surgery, sham controls (SH and sh groups) and CC and NGF animals were allocated to individual 6-dm^3 stainless-steel cages with food and water ad libitum except when indicated for training procedure. The pumps were extracted in the morning of P39 under light ether anaesthesia.

Circling Training

Trained animals were submitted to the circling training (CT) paradigm starting at age P30 (for group T_{30}) or P40 (for T_{40} and the operated groups). The procedures of this behavioral testing were already described (Brusés et al., 1991; Brusés and Azcurra, 1993). Briefly, animals learn to turn in a 1-m circle in session 0 and then train daily for 7 days (S_1 up to S_7, 1 session/day), according to a continuous reinforcement schedule with a drop of sucrose 10% per turn as the reward. Each session was finished when the animal ran for 30 min or 100 turns (in S_{0-3}) or 150 turns (in S_{4-7}). The sense of running (clockwise) was maintained until the final training session. Turn detection, reinforcement delivery, and time counting were automatically executed. Three nontrained animals in each group were also subjected to water withdrawal, receiving a sucrose supply equal to the average ingestion of trained rats during CT sessions. In addition, these nontrained groups were placed daily in the CT apparatus for 30 min, but no training was performed. Before each training session, the body weight was recorded. After

training, animals were returned to their individual cages. At decapitation (P70) no difference in the body weight of the rats was found.

Striata Homogenization

Brains were quickly removed and left and right rostral parts of striatum were dissected. Ice-cold 20 m*M* Tris-ClH buffer, pH 7.4, containing 0.32 *M* sucrose, 1 m*M* ethylenediaminetetraacetic acid (EDTA) and 0.5 m*M* PMSF protease inhibitor was added to tissues (10% w/v). Homogenization was performed at 1300 rpm in a Potter-Elvehjem glass homogenizer by means of three series of 20 strokes with a Teflon pestle (clearance = 0.25 mm). After centrifugation at 1090 × *g* for 10 min at 4°C, supernatants were saved and the pellets were washed twice with ice-cold homogenization buffer and then recentrifuged. The pooled supernatants were centrifuged again to 13,000 × *g* for 30 min at 4°C, and pellets were resuspended in the homogenization buffer containing 0.02% NaN_3 and stored at −70°C until binding assays were performed.

[^{3}H]QNB Binding Assay

Membrane samples from each rat were analyzed separately by binding study. mAChRs were measured on 100 μg membrane protein by saturating 2 n*M* quinuclidilbenzilate (as [^{3}H]QNB; NEN Corp., 43.5 Ci/mmol) binding assay as originally reported by Yamamura and Snyder (1974). Each determination was made in triplicate, and nonspecifically bound [^{3}H]QNB was determined in duplicate assay in the presence of 10 μM atropine (from 1% atropine sulfate solution; Alcon Laboratory, Buenos Aires). After 30 min incubation, samples were filtered through Whatman GF/B discs under vacuum. The filters were washed and then dried, and the radioactivity was counted by liquid scintillation spectrometry (LKB Wallac). Protein was determined by the Lowry method (Lowry et al., 1951).

Statistical Analysis

In each of the three experiments, statistical analysis of motor behavior was performed by two-way analysis of variance (ANOVA) with a repeated measures design in the session factor. The behavioral data were further analyzed by post hoc Tukey-HSD test. For operated groups, post hoc Tukey-HSD for unequal *N* (Spjotvoll/Stoline test) was applied. The QNB binding results were studied a priori by one-way MANOVA test with treatment as the principal factor and two dependent variables (left and right striata), and a posteriori, applying the Scheffé *F* test. In addition, for Experiment 2, two-way MANOVA analysis was carried out with training condition as a second factor to detect the statistical significance of the interaction between agent infused and circling activity. The criterion for significance was $p < .05$. Statistica for Windows software was used.

RESULTS

We previously found that the permanent decrease of the binding of [^{3}H]QNB to striatal membranes from rats trained during the critical motor period is dependent on the number of meters run by the animals during CT (Ibarra et al., 1995). Thus, we evaluated the behavioral response of the different groups for an appropiate analysis of the long-term effects of NGF on QNB binding (Fig. 1). No significant difference for any pairwise comparison of treatment factor was detected by ANOVA analysis [including all trained groups: $F(7, 38) = 1.21$; $p = .32$]. Also, no change in speed comparing nonoperated [Fig. 1(A)] and operated [Fig.1(B)] rats was found [$F(1, 44) = 2.36$; $p = .13$]. Because all groups ran roughly the same distance (900 m) after the last training session, we believe the circling motor behavior was not affected by surgical stress or pharmacological treatments.

In all cases, nontrained animals explorated the circular path of the CT apparatus, but no one reached more than 10 turns per session (data not shown).

As positive and negative controls for the muscarinic plastic response induced by the CT, we trained infant rats during or after the critical motor period of striatum, respectively. Figure 2 shows the binding of [^{3}H]QNB to striatal membranes from young adult rats whose seven-session training program was started at age P30 or P40 (groups T_{30} and T_{40}). An enduring effect of training per se on [^{3}H]QNB binding sites if nontreated animals started training at P30 (but not at P40) was evident when they reached the age of P70 [MANOVA results for Experiment 1: $R(6, 38) = 5.63$; $p < .0003$]. Post hoc Scheffé test rendered significant drops of 32% ($p < .001$) and 28% ($p < .008$) in [^{3}H]QNB binding sites of left and right striata, respectively, with respect to nontrained controls.

In Figure 3, we determined at age P70 [^{3}H]QNB binding sites in both perfused groups submitted to training starting at P40 and the respective training controls. Data from Experiments 2 and 3 (infusion rates: 1.4 and 0.4 μg/day, respectively) are shown separately. Since in all pairwise comparisons by statistical analysis between sham and CC groups of both experiments no difference was found, NGF-induced effects were analyzed comparing NGF-perfused groups to CC control ones.

Membranes from cannulated striata from trained rats treated with NGF for 2 weeks, including the natural window of sensitivity, showed a decrease of

specific [^{3}H]QNB binding sites of 42% [Fig. 3(A)] (1.4 μg NGF/day; $p < .0004$) or 33% [Fig. 3(B)] (0.4 μg NGF/day; $p < .02$). Noncannulated (right) striata of operated groups were studied as individual internal controls when paired with the perfused ones. Only the biggest dose of NGF produced a significant fall (-44%; $p < 2.10^{-5}$) in mAChR sites [Fig. 3(A)] in noncannulated striata. To detect possible nonactivity-related effects of NGF on mAChR system, striata from nontrained animals perfused with the higher dose of NGF or CC were tested in Experiment 2 [Fig. 3(C)]. Only a nonsignificant augmentation of 15% in the [^{3}H]QNB binding sites of left striata in NGF_C with respect to the CC_C group was found. An additional two-way MANOVA analysis of data from Experiment 2 showed slight effects for agent [$R(4, 38) = 3.85$; $p < .01$] and training [$R(2, 19) = 3.24$; $p < .06$] as principal factors, but a high statistical significance for the interaction was found [$R(4, 38) = 6.93$; $p < .0003$].

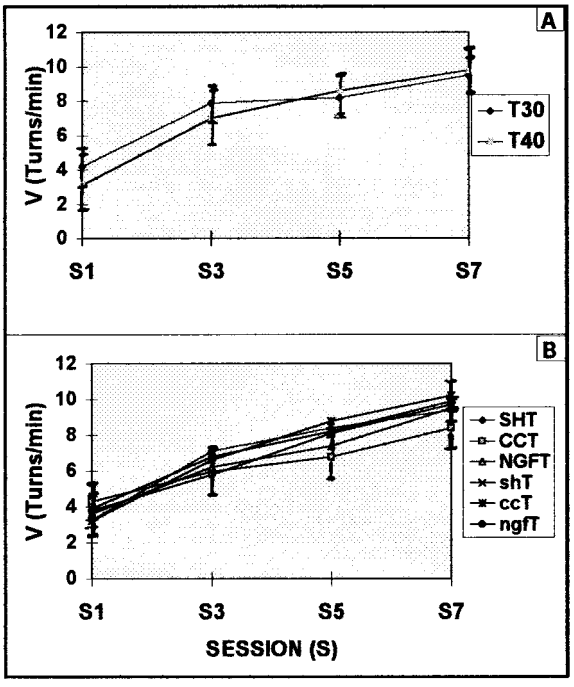

Figure 1 Motor performance in the circling training (CT). (A) Speeds of nonoperated animals (Experiment 1) trained in the CT apparatus between ages P30 and P37 (group T30; $n = 6$) or P40 and P47 (group T40; $n = 6$) are presented. (B) Speed of groups sham operated (SHT and shT; $n = 6$ each) or perfused in left striata with cytochrome C (CCT and ccT; $n = 6$ each) or NGF (NGFT and ngfT; $n = 5$ each) during ages P25–39, and then trained starting the CT schedule at age P40. Two doses of NGF or CC were assayed: 1.4 μg/day for groups NGF_T and CC_T (Experiment 2), and 0.4 μg/day for groups ngf_T and cc_T (Experiment 3). Speed (V) values are expressed as turns per minute per session, in mean ± S.D. Statistical analysis: two-way ANOVA using a repeated measures design in the factor session, followed by post hoc Tukey test. Taking all eight groups as levels for the ANOVA and comparing nonoperated (grouping $T_{30} + T_{40}$) versus all operated groups, no significant difference was found. For the sake of clarity, only the results from four representative sessions (S1, S3, S5, and S7) are shown (see Materials and Methods for more details).

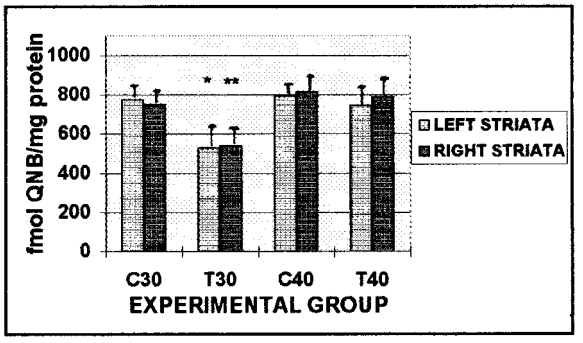

Figure 2 Experiment 1: [^{3}H]QNB binding assay in striata of 70-day-old rats after circling training performed at ages P30–37 (critical period) or P40–47 (nonsensitive period). Values of [^{3}H]QNB specific binding to mAChRs are expressed as fmol · mg^{-1} protein, mean ± S.D. on bar top. Groups T_{30} and T_{40} are trained animals starting the 7-day CT schedule at ages P30 and P40, respectively. Groups C_{30} and C_{40} are the nontrained respective controls. Since left and right frontal striata were not pooled, their binding results were evaluated separately. Statistical analysis: one-way MANOVA test [$R(6, 38) = 5.63$; $p < .0003$], followed by post hoc Scheffé F test comparing trained ($n = 6$) versus control ($n = 6$) animals for each training age. *$p < .001$; **$p < .008$.

DISCUSSION

During nervous system maturation, NGF has been shown to act on the survival, growth, and differentiation of sensory and simpathetic neurons (Levi-Montalcini and Angeletti, 1968, Cuello and Thoenen, 1995). Indeed, afferent neurons converging in distinct zones or the same target depend upon NGF to survive or differentiate in different stages (Black, 1986; Mendell, 1995).

Application of NGF during the critical period of visual system maturation retains the capacity of this pathway for activity-dependent plasticity response beyond the chronological age at which this faculty has disappeared in nontreated animals (Domeneci et al., 1991; Carmignoto et al., 1993). More recently, changes in GAP-43 phosphorylation have been reported as a consequence of NGF-induced restoration

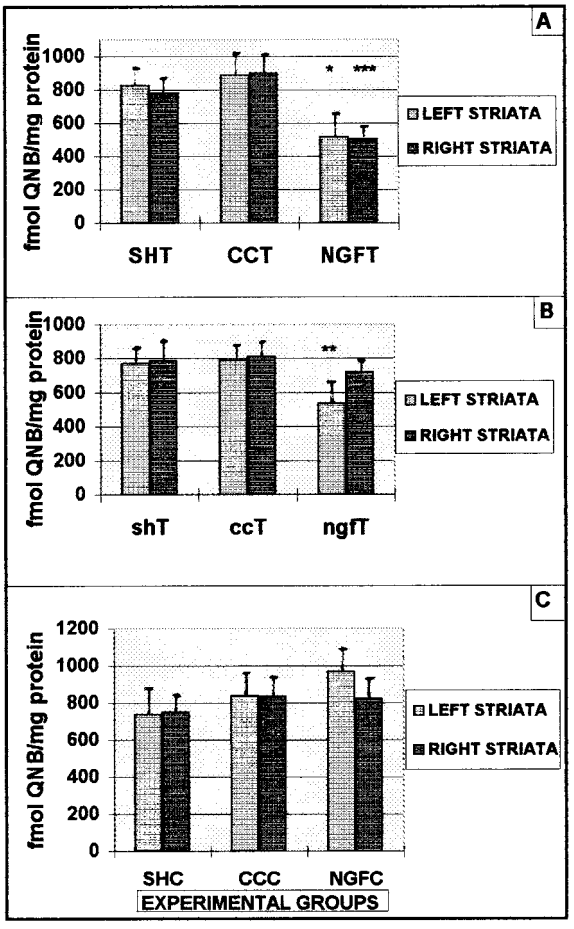

Figure 3 Experiments 2 and 3: [^{3}H]QNB binding assay in striata of 70-day-old rats perfused with NGF and then trained at ages P40–47. [^{3}H]QNB specific binding in striata at age P70 from the perfused groups trained in CT at ages P40–47 as presented in Figure 1 (B), and perfused nontrained ones. (A) Groups of Experiment 2 perfused by 2 weeks (1.4 μg/day, between ages P25 and P39) with NGF (NGF_T) or cytochrome C (CC_T). (B) Groups in Experiment 3, perfused with a lower dose (0.4 μg/day) of NGF (ngf_T) or cytochrome C (cc_T). SH_T and sh_T are sham-operated control groups. (C) Binding to synaptic membranes from nontrained rats of each operated group, as training controls for Experiment 2 (groups SH_C, CC_C, and NGF_C). Expression and statistical analysis of results as in Figure 2. For results in (A,B), $R(10, 54) = 8.43$; $p < .0001$. For nontrained rats in (C), $R(4, 10) = 1.01$; $p = .45$. Pairwise comparison of differences among means using Scheffé F test were significant for NGF-infused groups compared to CC, ones except for right striata of group ngf_T. *$p < .0004$; **$p < .02$; ***$p < .0001$.

of activity-dependent plasticity in the adult visual cortex (Liu et al., 1996).

In the above experiments, the permanent drop of [^{3}H]QNB sites detected in striatum is critically dependent on the ontogenetic period when the activity is induced. Only between ages P30 and P37 does circling motor activity produce a permanent decrease in mAChRs (Azcurra et al., 1995; Ibarra et al., 1996). We also found at age P32 a change in the phosphorylation of GAP-43 by PKC kinase 30 min after stopping circling motor activity (Paratcha et al., 1998). Thus, these facts pose the question of whether NGF could participate in this restricted ontogenetic period of activity-dependent muscarinic receptor setting. A change in the same nature of mAChRs observed only in the positive control group T_{30} and group NGF_T [Fig. 2, −32%/−28%; and Fig. 3(A), −42%/−44%, for left/right striata, respectively] seems to support this possibility. Even more, the data from group T_{40} (negative control) and the controls for surgery and the biological specificity of infused agents indicate that the effects observed in the NGF-perfused trained groups (NGF_T and ngf_T) were not a consequence of handling, anesthesia, or cannulation. An effect of NGF per se may be precluded a priori because no significant change in the mAChR level of group NGF_C with respect to CC_C was found [Fig. 3(C)]. Besides, it was possible to further analyze the interaction of the effects of NGF perfusion and the CT test for Experiment 2. Applying a two-way MANOVA test and comparing by post hoc Scheffé F test (NGF × Training) and (NGF × No training) conditions, the p values were $<.003/<.007$ for left/right striata, respectively.

A point of concern was raised in this work by the significant mAChR drop detected on the right side despite the fact that NGF was perfused in left striata [see group NGF_T in Fig. 3(A)]. *In vivo* infusion of neurotrophins shows that at a distance of 3 mm from the cannula tip, the real rate is roughly a 500× dilution of the original dose (White and Schwartz, 1994), and the intrastriatal infusion of a few nanograms of NGF is sufficient to affect specific markers of cholinergic neurons in striatum (Kordower et al., 1996). Thus, a biologically effective distribution of NGF between both brain hemisferes could result in a rate of 1.4 μg/day but not a lower perfusion rate (Venero et al., 1996).

It is known that NGF influences cholinergic neurons via receptor-coupled mechanisms involving a low-affinity neurotrophin receptor ($p75^{NGFR}$) and a trk-related proto-oncogene designated TrkA (Chao, 1992; Chao and Hempstead, 1995). The expression of these receptors in cholinergic interneurons of striatum and other cholinergic pathways has specific spatial and temporal patterns from late embryonic ages to adulthood (Yan and Johnson, 1988; Ringstedt et al., 1993; Steininger et al., 1993; Gibbs nd Pfaff, 1994; Yu et al., 1995). During rat striatal development, NGF immunoreactivity reachs a peak at P12–20 (about 0.3

ng/g wet tissue), but at age P26 it is not detectable (Mobley et al., 1989). In addition, the TrkA product increases its tyrosin-kinase activity up to P30. For other members of the neurotrophin family, such as brain-derived neurotrophic factor and neurotrophins 3 and 4/5, scarce trophin-induced receptor responsiveness beyond age P14 in the striatum was observed (Knüsel et al., 1994). These findings support a role for an endogenous NGF-TrkA signaling system in the maturation of striatal cholinergic neurons during the period of synaptic modeling, as was already suggested by Altar (1991) and Fagan et al. (1997). This is a substantial difference with the visual model, since in the visual cortex and lateral geniculate nucleus, TrkA receptor was not detected (Schoups et al., 1995; Cabelli et al., 1997).

However, how striatal neurons primed by pharmacological NGF and physiologically elicited neural activity interact to induce a persistent drop of mAChR levels is not clear. A difference between the plastic phenomenon occurring in striatum during the natural period of synaptic refinement and the present pharmacological NGF effects is that in the former case, NGF levels gradually decrease in the striatum from age P18 to undetectable levels at P26 (Mobley et al., 1989). In perfusion conditions, cessation of NGF supply is more abrupt. One week after intraparenchymal 2-week infusion of NGF, cholinergic hyperinnervation, reduction of spontaneous neuronal discharge rate, and induction of messenger RNAs for low- and high-affinity NGF receptors were found in striatum by Forander et al. (1996). Moreover, an increase of acetylcholine occurs after NGF treatment (Maysinger et al., 1992). Thus, we can hypothetize that a down-regulation of cholinergic postsynaptic mAChRs may be present in our NGF-infused groups at age P40. This could be further enhanced by presynaptic hyperstimulation in response to the activation of cholinergic neurons and the up-regulation of NGF synthesis via acetylcholine muscarinic receptors (Lindholm et al., 1994) developed during the training paradigm. These neurons, in turn, will take up more NGF, generating a positive feedback. Although it cannot be rejected on an experimental basis, a direct effect of NGF on postsynapsis is not supported on the known distribution of TrkA receptors. Cholinergic neurons constitute the predominant compartment of NGF binding to the TrkA system in striatum, and the described mechanisms by which NGF affects cholinergic pathways have focused on presynapsis (Riopelle et al., 1987; Lapchak et al., 1993; Berninger and Poo, 1996). Another possibility is an indirect effect of NGF through neuropeptides involved with activity-dependent synaptic modeling during postnatal development, as the neuronal secretory-peptide precursor VGF which is present in striatum (Snyder et al., 1998). The VGF gene transcription is rapidly induced by NGF in PC12 cells (Baybis and Salton, 1992). Besides, VGF is modulated in lateral geniculate nucleus by retinal activity during the critical period of visual pathway maturation. In addition, a persistent induction of VGF messenger RNA in striatum after ipsilateral deafferentiation by cortical lesion was reported by Snyder et al. (1998). How these proposed midterm effects are stabilized in the long term and whether the trophin effects are causally related to the permanence of the experience-dependent synaptic modification have to be determined (Berninger and Poo, 1996). Synaptic elimination due to acute competition when trophin is a limitant factor during development and the maintainance of previously active terminals in the adulthood is the most supported mechanism (Black, 1986; Mendell, 1995). Further studies will be necessary to elucidate the mechanisms underlying NGF-induced plasticity in the visual pathway as well as in the case of striatum.

Preliminary results were presented at the 29th annual meeting of the American Society of Neurochemistry show that simultaneous intrastriatal NGF infusion and circling training, with both procedures starting at P30, attenuate the reported mAChR drop as detected at age P70 in the striatum of these animals (Azcurra et al., 1998).

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