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Exogenous NGF alters a critical motor period in rat striatum

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IN previous studies we found that there is a critical period during rat postnatal development when motor training starting at age 30 days (P30) but not before or after this age, induces a bilateral lifetime drop in B_{max} of the muscarinic radioligand [^{3}H]QNB in striatum. We examined the possibility that striatal NGF level would be a determining factor for the normal occurrence of this synaptic plasticity. With this aim, rats underwent training at P30–37 with or without simultaneous NGF perfusion into the left striatum. At P70, we found the expected bilateral enduring fall of striatal [^{3}H]QNB sites in trained controls. While the non-cannulated side of NGF-treated trained rats showed a similar drop in [^{3}H]QNB binding, the perfused striata from these animals were not affected by training. Thus, the findings add new evidence in favour of a major role of NGF in this critical period of activity-dependent permanent adjustment in the striatal muscarinic system. *NeuroReport* **10:2705–2709 © 1999 Lippincott Williams & Wilkins.**

Key words: Circling; Development; mAchR; NGF; Plasticity; Rat Striatum; Training

Introduction

During postnatal brain maturation the prototypic neurotrophin nerve growth factor (NGF) has demonstrated to act as a neuron survival factor and to be involved in phenotypic differentiation of sympathetic neurons, including those of striatal neurotransmission systems [1–3]. Brain development includes area-specific restricted critical periods of higher susceptibility to activity-dependent modulation of the synaptogenesis process, whereby permanent rearrangements in the neuronal net microstructure, organization and function occur. The description of this type of plasticity is mostly based on the visual pathway and other sensory-cortical models [4,5]. A proposed feature of these periods is the occurrence of synaptic competence facing a limiting neurotrophin support and a consequent pruning back of neurites [3]. Certainly, during postnatal visual system development *in vivo* NGF perfusion to input-deprived rats has been reported to maintain the synaptic sensitivity to the physiological activity until an age in which the respective deprived controls have permanently lost these plasticity properties [6].

In the case of the developing striatum, NGF immunoreactivity reaches a peak during postnatal days (P) 12–20 (about 0.3 ng/g wet tissue) but at P26–28, when the overall wiring and neurochemical maturation programmes are over [7], NGF is not detectable by ELISA [8]. By subjecting different aged developing rats to motor training we detected the occurrence in striatum of an activity-dependent permanent adjustment of the cholinergic muscarinic (mAchR) and dopaminergic type D2 (D2R) receptor systems exclusively between P30 and P37. The same physical activity schedule performed at P20–27 does not affect the striatal adult level of these receptors [9,10]. We hypothesize that the ontogenetic pattern of NGF expression and functional activity must interact in a determined way to allow the long-lasting changes in receptor expression triggered by the training. With this proposal in mind, we have recently shown the appearance of the enduring muscarinic plastic response in rats perfused intrastriatally with NGF during the plasticity period and trained after it [11]. Thus, the purpose of the present work has been to determine whether simultaneous NGF local perfusion and training during the critical period alter the natural enduring response of striatal mAchRs.

Materials and Methods

Animals and experimental groups: Male offspring from nine litters of Sprague–Dawley rats (local facilities, originally purchased from Holtzman Inst.) were randomly divided into four groups ($n = 10$ each) as follows: non-operated animals (P20 group), animals cannulated at age P25 and perfused with

mouse salivary gland 2,5S NGF (NGF group) or horse heart Cytochrome C (CC group) and a fourth group which received only sham surgical procedures (SH group). For each condition, six animals were appointed for training and four animals represented training controls. For all procedures of this work rats were taken care of in compliance with the NRC's guide for the care and use of laboratory animals.

Surgical procedures: Each animal was anesthetized with 3 ml/kg Equithesin and the left striatum cannulated with a brain infusion kit (in mm: A = 0.5; L = 5; V = 4.5). An osmotic minipump (model 2002) was placed s.c. and attached to the cannula via a vinyl tubing (minipump and infusion kit from Alza Corp, Palo Alto, CA). NGF (Alomone Lab., Israel) was perfused 0.4 μg/day. CC (from Sigma Co.) was selected to control the biological effect of NGF. At the end of the procedures, long-lasting penicillin was administered i.m., in order to prevent infections. After surgery, sham controls and CC and NGF animals were placed in individual 6 dm^3 stainless steel cages with food and water *ad lib*, except when indicated. Two hours after the last training session (at age P37), each pump was removed from the body using light ether anaesthesia (other details in [11]).

Motor activity induction before or during the critical period: Trained animals went through the circling training (CT) paradigm [12] starting at age P20 (group $P20_T$) or P30 (all operated trained groups). Briefly, animals learn to turn in a 1 m circle in session zero (S_0) and then they train daily for seven days (S_1–S_7), according to a continuous reinforcement schedule, earning as reward a drop of sucrose 10% per turn. Each session is finished when the animal has run for 30 min or 100 turns ($S_{0–3}$) or 150 turns ($S_{4–7}$). The initial sense of running is kept until the final training session. Speeds are automatically recorded. Four non-trained animals of each group (only habituated to the CT apparatus) were also subjected to water withdrawal receiving a sucrose supply equal to the trained rats average intake during CT sessions. Rats were killed at age P70.

Striata homogenization: Brains were quickly removed and left and right rostral parts of striatum were dissected. Ice-cold 20 mM Tris–HCl buffer, pH 7.4, containing 0.32 M sucrose, 1 mM EDTA and 0.5 mM PMSF protease inhibitor, was added to the tissues (10% w/v). Homogenization was performed at 1300 r.p.m. 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* (10 min at 4°C) the supernatants were saved and the pellets were washed twice with ice-cold homogenization buffer and then recentrifuged. The pooled supernatants were centrifuged again at 13 000 × *g* (30 min at 4°C) and the pellets (subcellular fraction P2) were resuspended in homogenization buffer containing 0.02% NaN_3 and stored at −70°C until binding assays were carried out.

[^{3}H]QNB binding assay: P2 membranes from each rat were separately analyzed through binding study. mAchRs were measured on 100 μg membrane protein using the saturating (2 nM) quinuclidilbenzilate (as [^{3}H]QNB from NEN Corp., 43.5 Ci/mmol) binding assay as reported previously [11] (originally reported in [13]). Each determination was made in triplicate and non-specifically bound [^{3}H]QNB was determined in a duplicate assay in the presence of 10 μM atropine (from 1% atropine sulfate solution, Alcon Lab., Buenos Aires). After 30 min incubation, samples were filtered through Whatman GF/B discs by vacuum. Liquid scintillation spectrometry (LKB Wallac) was used to assess radioactivity. Protein determination was made by the Lowry method [14].

Statistical analysis: Behavioral data were analyzed by a two-way ANOVA test with a repeated measures design. *Post hoc* Tukey-HSD was then applied for pairwise comparison of groups. The [^{3}H]QNB binding results from both hemistriata were studied *a priori* by two-way MANOVA analysis considering agent perfused and training condition as main effects, and *a posteriori* using the Scheffé F-test. The criterion for significance was $p < 0.05$. The software used was Statistica for Windows.

Results

We induced physiological activity and evaluated the functional response of the different groups subjecting rats to the CT test at ages P20–27 (control group $P20_T$) or P30–37 (all operated groups). No significant difference for any pairwise comparison was detected by ANOVA analysis (Table 1). Considering the fact that all the trained groups achieved roughly the same distance (900 m) up to the last training session, we believe that the circling behavior was not affected by surgical stress or acute effects of pharmacological treatments. Figure 1 shows the specific binding of [^{3}H]QNB to striatal membranes of rats killed at age P70. P2 membranes from both left and right striata of trained rats (groups $P20_T$, SH_T, CC_T and NGF_T) and corresponding non-trained control groups ($P20_C$, SH_C, CC_C and NGF_C) are displayed separately. Statistical significances for both factors, analysed by MANOVA

Table 1. Motor performance in the circling training (CT)

Age at training	Group	Session						
		1	2	3	4	5	6	7
P20–27	$P20_T$	3.2 (0.6)	5.6 (1.1)	8.4 (1.2)	9.8 (1.5)	10.9 (1.3)	11.9 (1.8)	11.7 (1.2)
	SH_T	3.9 (1.2)	5.8 (0.7)	6.7 (0.7)	8.9 (0.6)	9.9 (1.7)	11.0 (1.5)	11.3 (1.6)
P30–37	CC_T	3.8 (1.3)	6.8 (1.2)	7.9 (1.1)	9.0 (1.7)	10.0 (2.4)	11.0 (1.4)	11.2 (1.2)
	NGF_T	3.3 (1.0)	6.4 (1.1)	8.7 (0.7)	9.2 (1.1)	10.9 (1.2)	11.6 (1.4)	12.3 (1.7)

Data are the animals trained speeds in the CT apparatus. For each group $n=6$. Values are expressed as mean turns/min/session (s.d. in parentheses). Statistical analysis: two-way ANOVA, repeated measures design, $F(3,20)=0.2$, $p=0.89$.

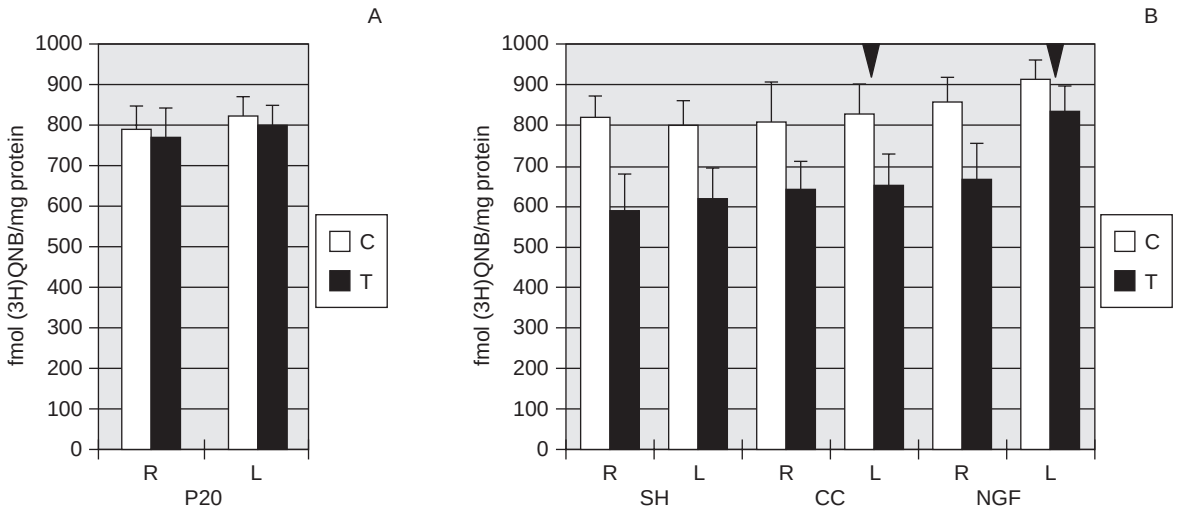

FIG. 1. [^{3}H]QNB binding in striata of 70 days old rats NGF perfused and trained during the critical period. (**A**) [^{3}H]QNB specific binding in samples from rats non-treated and subjected to training at age P20 (before the critical period) and the respective training controls (denoted as groups $P20_C$ and $P20_T$, respectively). (**B**) Results from sham-operated (SH_T) animals or those perfused with 0.4 μg/day NGF (NGF_T) or cytochrome C (CC_T) and simultaneously trained in CT during the critical period, and the corresponding non-trained controls, SH_C, NGF_C and CC_C. The striatal side perfused is marked by arrowheads. Statistical analysis: two-way MANOVA test. For agent infused as main effect, $R(6,62)=5.7$, $p<0.0001$; for training condition, $R(2,31)=16.0$, $p<0.00002$. By *post hoc* Scheffé F-test, for both hemistriata: CC_C *vs* CC_T, $p<0.05$, SH_C *vs* SH_T, $p<0.03$. For non-perfused side, NGF_C *vs* NGF_T, $p<0.05$. After Wilcoxon test on both left and right side binding data released from group NGF_T, $p<0.03$. R and L = right and left sides of striatum, respectively. C = animals only habituated to the CT apparatus; T = trained animals.

were as follows: with agent perfused as main effect, $R(6,62)=5.7$, $p<0.0001$; for training condition as main effect, $R(2,31)=16.0$, $p<0.00002$; interaction, $R(6,62)=1.68$, $p=0.14$. Further analysis showed no statistical difference between the [^{3}H]QNB B_{max} in trained animals from group $P20_T$ and the respective non-trained controls (Fig. 1A).

No significant difference was found in the neurochemical results between operated groups that represented positive controls for the training effect on mAchR system (groups SH and CC). However, by pairing the corresponding striatal membranes of trained and non-trained rats from these groups we detected a bilateral persistant fall of [^{3}H]QNB binding sites in trained animals (both for left and right striata, SH_T *vs* SH_C, $p<0.03$, CC_T *vs* CC_C, $p<0.05$, respectively).

From previous experiments we knew that the NGF dose administered (0.4 μg/day) is sufficient to affect only the plastic response of the cannulated side [11]. The non-cannulated right striata therefore represented individual internal controls for the local effect of the agent perfused. Comparing trained *vs* non-trained NGF treated animals, in group NGF_T a unilateral absence of the expected [^{3}H]QNB binding reduction in the striata ipsilateral to the side perfused was found: for the cannulated left striata, $p=0.81$; for the right side, a 23% drop in specific sites, $p<0.05$). Since the binding data obtained from each part of striatum fitted a two sample paired statistical design, we applied a non-parametric Wilcoxon test to examine side-dependent changes in group NGF_T, and a significant decrease was found in [^{3}H]QNB sites for intact striata in relation to the perfused side ($p<0.03$).

Discussion

Working on the postnatal mechanisms of synaptic plasticity in a subcortical model we found that only between ages P30–37 did circling motor activity produce a permanent decrease of mAchR and D2Rs

in striatum [9,10]. As in the visual system [15], this striatal critical period is prolonged in response to local administration of NGF [11].

In this study we trained NGF-supplied rats during the critical period. While sham and treated trained controls (groups SH_T and CC_T, respectively) at age P70 indicated similar bilateral enduring drops in [^{3}H]QNB sites to those described previously [9–11], the NGF-treated group only showed this type of decrease in the non-perfused side (Fig. 1B). Moreover, training at age P20 did not affect the striatal [^{3}H]QNB sites (Fig. 1A). Consequently, these findings would arise from specific effects depending on age of training and pharmacological treatments.

It is known that NGF influences developing cholinergic neurons via receptor coupled mechanisms involving a low affinity neurotrophin receptor (p75NGFR) and a trk-related proto-oncogene designated TrkA [2,16,17]. Thus, our findings suggest a role for the endogenous NGF-TrkA signalling system in the functional plasticity of the developing striatum. At this point there could be a substantial difference with the developing visual system. While some reports argue in favour of a TrkA-mediated pathway to explain the NGF plastic effects on the synaptic adjustment of the retina–cortex pathway [18], other groups have supported the principal involvement of brain derived neurotrophic factor (BDNF) and its specific trophin receptor system TrkB [19,20].

Regarding our experimental design, the simplest explanation for the results of this work is to state that the natural synaptic elimination programme due to competition by NGF would be reduced by NGF exogenous addition. A similar mechanism could be operating under physiological conditions in both hemistriata in the group trained at P20 (Fig. 1A). Nevertheless, additional mechanisms may be concurrent: down-regulation of cholinergic postsynaptic mAchRs may be present in our trained NGF-infused animals through the known NGF-induced activation of cholinergic neurons [21], and up-regulation of neurotrophin synthesis and release occurs during physical exercise [22]. On the other hand, we do not yet know the molecular pathways underlying this critical period for the permanent adjustment of receptor expression. However, Liu *et al.* [23] detected an increase in the phosphorylated form of GAP-43 as a consequence of NGF-induced restauration of activity-dependent plasticity in the adult visual cortex. This recognised marker of functional synaptic plasticity could be involved in the initial presynaptic changes associated with the long-lasting receptor setting in our striatal model. Circling exercise triggers in striatum a decrease in the phosphorylation of GAP-43 by C kinase 30 min after stopping circling activity when the training takes place during the critical period [24]. If changes in GAP-43 phosphorylation rate were part of the molecular events turned on during this window of striatal plasticity, the pharmacological NGF-induced increase of GAP-43 phosphorylation would modify the occurrence of long-term changes in striatal receptors. Indeed, NGF could affect PKC activity through an indirect pathway involving production of DAG, a C kinase activator, as was suggested for the visual model [23].

NGF has been already postulated as a therapeutical agent in terms of its direct pharmacological effects on cholinergic system from cortical and subcortical areas under physiological or pathological conditions [25]. The findings in the visual and striatal models of activity-dependent plasticity add initial evidence to show a new way in which NGF could be useful in neural syndromes treatment, modulating synaptic properties and preceding classical pharmacotherapeutics.

Conclusion

We have provided here new evidence supporting the role of NGF in the striatal activity-dependent synaptic adjustment period which physiologically occurs in rat at ages P30–37. Motor training in this period induces a long-lasting decrease in mAchR level. This effect was abolished by simultaneous intraparenchymal administration of NGF (0.4 μg/day). We stand for the explanation that available endogenous NGF is a determining factor in the regulation of the natural critical period of synaptic refinement. This is further supported by analogy with the results obtained from rats trained at age P20, when NGF level is almost the highest during striatal development and circling training does not induce changes in adult mAchRs as determined by [^{3}H]QNB binding assay [9,10].

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