ELSEVIER

Brain Research 705 (1995) 39–44

BRAIN RESEARCH

Research report

Permanent alteration of muscarinic acetylcholine receptor binding in rat striatum after circling training during development

Gustavo R. Ibarra *, Jorge A. Rodriguez, Gustavo Ch. Paratcha, Julio M. Azcurra

Laboratorio de Biología Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 4° Piso, Pabellón II, Ciudad Universitaria, 1428 Buenos Aires, Argentina

Accepted 8 August 1995

Abstract

We evaluated the effect of circling training (CT) in the expression of muscarinic acetylcholine receptor (mAchR) in developing rat striatum. For this, male and female rats were subjected to CT at 20, 30, 40 and 60 days of age during 7 days. Animals trained at 30 days but not at other ages showed an average decreased binding to mAchR of 33% in males and 24% in females, representing a significant difference with respect to control non-trained animals (males $P < 0.001$, females $P < 0.005$), and showing also a differential response between sex ($P < 0.01$). mAchR drop was found invariably either 2 months or 1 year after training indicating a long term plastic change due to circling training. Scatchard analysis showed that altered binding represents a variation of the total receptor number instead of its binding affinity, with no significant differences found among K_d ($P > 0.1$). mAchR variation was correlated with the motor performance accomplished in the test. Regarding total distance run, male rats trained for 3 days (300 meters run), for 5 days (600 meters) and for 7 days (900 meters) showed a drop of 19, 28 and 33% respectively ($r^2 = 0.91$, $P < 0.001$), while female changes were of 21, 23 and 24% ($r^2 = 0.78$, $P < 0.001$). Nevertheless, no correlation with running speed was found ($r^2 = 0.13$ male, $r^2 = 0.02$ female; $P > 0.1$). In summary, these results demonstrate the presence of a limited sensitivity period during striatum development where mAchR expression may be affected by the activity performed during CT, representing a permanent alteration of the receptor levels.

Keywords: Muscarinic receptor; Receptor binding; Circling training; Plasticity; Development; Rat striatum

1. Introduction

The striatum is viewed as the major component of the basal ganglia exerting a fundamental role in motor, motivational and learning process by means of a highly functional organization [12]. As a representative brain area of motor function, the striatum has been well studied in terms of its input and output systems, internal architectural organization, and neuronal and neurotransmitter types involved in normal activity [13]. Moreover, it is a valuable model for the study of the CNS development since the general rules that govern the formation of central neuronal connections are represented in this area [14]. Through the wide spectrum of concerns, the striatum seems to be a valuable biological model for the combined studies of behavioral and neurochemical process involved in motor activity. At a molecular level, neuroreceptors play an important role in these processes not only by signal reception and transduction during motor activity but also by the participation in various neuronal plasticity mechanisms where variation in the number and in the receptor response have been found in different physiological conditions [18,19,27]. Furthermore, through the neuronal activity, neuroreceptors mediate in the pattern formation of synaptic connectivity [10,15,24]. Accordingly, the influence of the receptor number expressed in synapses and the intensity of neural firing during brain activity are crucial to neural pattern generation during development. Nonetheless, correlation of these two variables is not clearly understood. On the other hand, we have recently pointed out the role of the circling training (CT) in the study of neurochemical mechanisms underlying motor activity [5]. With this in mind, we made use of this model to study the effect of the enhanced motor activity performed by submitting animals to this test on neuroreceptor expression during brain development, focusing on the neostriatum as a representative area for these studies and on the muscarinic cholinergic receptor (mAchR) as the molecular target. mAchR presents in the striatum

* Corresponding author.

SSDI 0006-8993(95)01076-9

one of the highest concentration of this receptor in the Central Nervous System (CNS) exhibiting a central role in striatal modulatory functions [8,13], and mediating probably in learning and memory mechanisms of the CNS where acetylcholine is involved [1,22,9]. Thus, this receptor seems to be an appropriate target to our evaluation. In the present report, we have investigated the effect circling training exerts during development in the pattern of expression of rat striatal mAchR.

2. Materials and methods

2.1. Circling training

Male and female Sprague-Dawley rats (local facilities, originally purchased from Holtzman Inst.) were placed at 20, 30, 40 and 60 days old in the Circling Training Test (CT) performed as we described previously [4], adapted from Yamamoto and Freed [28]. Briefly, animals deprived of water for 24 h were trained to turn in circles for a reward (50 μl of 10% sucrose/water solution per turn). The first training session consisted in rewarding each rat by successive approximation to a full turn in the prescribed direction (selected randomly) during 30 min: the first ten min a quarter turn in the appropriate direction were rewarded, the next 10 min a half turn and the last 10 min a full turn in the appropriate direction were rewarded. Then training was conducted daily for 3, 5 or 7 days depending on the experiment and according to a continuous reinforcement schedule. Each rat was required to perform 100 complete turns in each of the first three sessions, and 150 complete turns on each of four subsequent days. If this condition was not fulfilled within 30 min, the session was ended (not frequent). Turns detection, reinforcer delivery and time counting were automatically executed. The left or right-hand turning direction was randomly assigned to each rat in the first session and this selected direction was kept on during the rest of the training. Non-trained control animals were also subject to water withdrawal receiving a water supply equal to the average ingestion of trained animals during training days. Control animals were daily placed in the CT apparatus for 30 min but no training was performed (habituation controls). In all the cases, the body weight was daily recorded and animals with more than a 10% decreased variation in weight discarded.

2.2. Tissue preparation

Rats were killed by decapitation at 90 days old (unless other age indicated on experiment), brains were quickly removed and left and right striatum were dissected separately on ice and weighted. Tissue was added to ice cold 20 mM Tris-HCl buffer, pH 7.4 (5% w/v solution), containing 0.32 M sucrose, 1 mM EDTA and 0.5 mM PMSF. Homogenization was performed at 1300 rpm in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. Following centrifugation at $1090 \times g$ for 10 min, the pellets were washed twice and then discarded. Supernatants were centrifuged again at $13{,}000 \times g$ for 30 min. Following two washes, the pellets were resuspended in the same buffer containing 0.02% NaN_3, and stored at -70°C until assays performed.

2.3. mAchR measurement

mAChR level was measured by saturating ligand binding assay as described elsewhere [29]. Each determination was performed by triplicate and non-specifically bound [^{3}H]QNB was determined in a duplicate experiment in the presence of 10.0 μM atropine. Samples (150 μg protein) were incubated 30 min. in 1.0 ml 50 mM Tris-HCl, pH 7.4, at 37°C in the presence of saturating concentration of [^{3}H]QNB (New England Nuclear Corp.) (2 nM). Following incubation, 3.0 ml ice-cold buffer was added and the content was passed through a Whatman GF/B filter under vacuum at flow speed of 3.0 ml/min. Following three washes with 5.0 ml of the same buffer, the filters were placed in vials and dried overnight at 42°C. A 10 ml toluene-POPOP-PPO solution (0.25% POPOP, 4.0% PPO w/v) was added to each vial and after 30 min darkness incubation at room temperature, radioactivity was assayed by liquid scintillation spectrometry at a counting efficiency of 40%. Scatchard analysis of data obtained with various concentrations of [^{3}H]QNB was also carried out as previously described [25]. Protein was determined by the method of Lowry et al. [20] using bovine serum albumin as the standard.

2.4. Statistical analysis

Statistical analysis was performed by a two-way ANOVA test unless a one-way ANOVA test is indicated in the text. The criterion for significance was $P < 0.01$. Linear regression coefficients were determined when necessary by the method of least squares.

3. Results

3.1. Tissue processing data

Dissected striata were evaluated for mAchR binding in trained and non-trained animals. Previous to biochemical measurements, each striatum was grouped according to the following conditions: trained vs. non-trained, male vs. female and for each animal, right vs. left striatum and ipsi- vs. contralateral to turn direction. After recording several variables from tissue fractionation such as weight tissue and protein concentration of final pellet, statistical evaluation was performed in each group described according to a one-way ANOVA test showing no differences in the com-

Table 1
mAchR binding in rat striatum after CT

Training age (days)	mAchR binding (fmol/mg protein)			
	Males		Females	
	Trained	Controls	Trained	Controls
20	751 ± 94	712 ± 107	802 ± 156	786 ± 133
30	462 ± 52	688 ± 99	560 ± 66	736 ± 102
40	809 ± 109	715 ± 101	753 ± 128	741 ± 117
60	836 ± 96	775 ± 103	881 ± 180	822 ± 166

Comparison of different training ages. Animals were sacrificed at 90 days of age, n of each average value = 6.

pared ratios (average tissue weight 38.3 ± 6.6 mg; protein concentration 66.8 ± 14.1 μg/mg of tissue; $P > 0.1$, $n = 16$).

3.2. mAchR measurement after circling training

To study the effect of motor activity stimulation during CT test in mAChR levels, male and female rats were trained for seven consecutive days starting at 20, 30, 40 or 60 days of age, sacrificed at day 90 and receptor binding assay performed immediately. For each animal, statistical analysis of [^{3}H]QNB binding showed no significant differences in left vs. right (that is also ipsi- vs. contralateral) striatum analysis ($P > 0.1$), data not shown. For this reason, data from right and left striatum were averaged and presented as the single receptor value of each animal. Further comparison was performed among animals trained at 20, 30, 40 and 60 days (Table 1). Ligand binding (B_{max}) to mAchR in striata of animals trained at 30 days showed a drop of 35% in male and 24% in female respect to the control group ($P < 0.001$ and $P < 0.005$, respectively). No differences were found in animals trained at other ages ($P > 0.1$). When Scatchard analyses were performed, results showed that K_d variation was not found in 30 days trained animals (trained male: 0.60 ± 0.11, control male: 0.52 ± 0.08; trained female: 0.49 ± 0.10, control female: 0.59 ± 0.06; $P > 0.05$, $n = 16$), indicating that the differences of binding is due to a modification of the total receptor number instead of its binding affinity.

3.3. Duration of receptor binding alteration

Providing that receptor level variation given above was found when animals were sacrificed at 90 days old, new experiments were performed in order to determine if the same results could be found in more aged animals. For this reason, animals trained starting at 30 days old were sacrificed at different ages (Fig. 1). Results show that no end-point was found for mAchR variation at least for evaluation performed up to one year after training. Differences among trained and control animals were not shortened although time elapsed, 37% decrement in trained males and 21% in trained females at 360 days of age against 33% trained males and 24% trained females at 90 days of age.

3.4. mAchR alteration related to the total turns performed during CT

Each turn in the CT represents a distance of 1.0 meter. Thus, after seven day sessions each animal had covered a final length of 900 meters. To demonstrate if there is a correlation between the mAchR variation and the distance run, the following experiment was performed. Male and

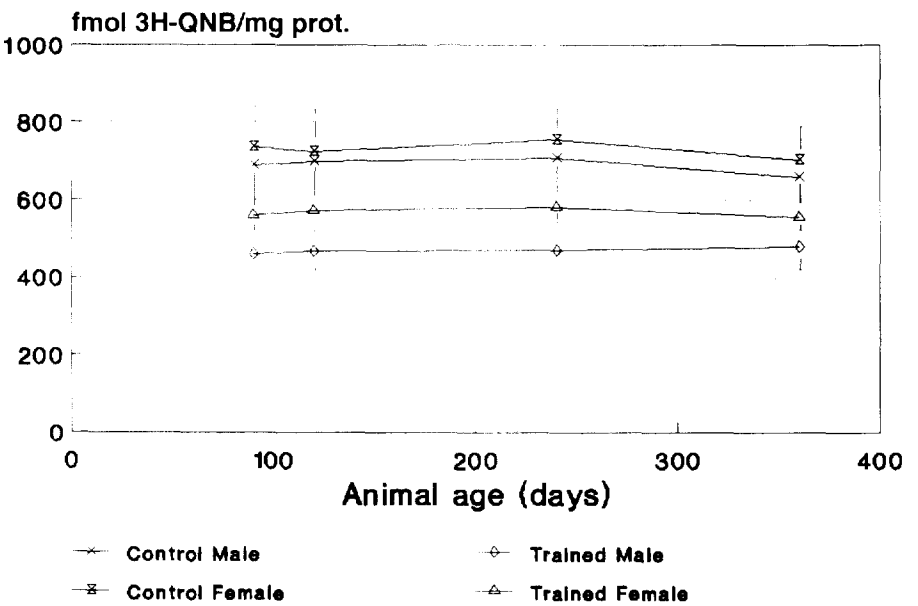

Fig. 1. Duration of receptor binding alteration after CT on 30 days old rats. Measures were performed at 90, 120, 240 and 360 days of age. n per point plotted = 6.

female animals (30 days old) were trained for 3, 5 or 7 days. According to our training schedule, this represents 300, 600 and 900 meters respectively. Striatum mAchR level was assayed and compared to non-trained animals. Male data were collected (Fig. 2) showing a correlation between these two variables (r^2: 0.91, $P < 0.001$, $n = 16$) where mAchR binding decreased 19, 28 and 33% after 3, 5 and 7 days of training respectively. Females also showed a drop in receptor variation with training, being of 21%–3 days, 23%–5 days and 24%–7 days (r^2: 0.78, $P < 0.001$, $n = 16$).

3.5. mAchR alteration related to the motor speed performed during CT

Even though animals have run 900 turns after the seventh session in CT, each one achieved a particular final time for this goal where speed performed during training represents a new identified variable. Plotting the rate of responses in turns per minute (V) vs. the number of reinforcers received up to that moment (accumulated reinforcers) (R_a), and after a transformation using a Scatchard plot, it is possible to achieve the theoretical maximum response of the animal (V_m, maximum running speed) [5]. Thus, although the distance covered after CT is exactly the same, the time necessary to achieve this goal clearly not.

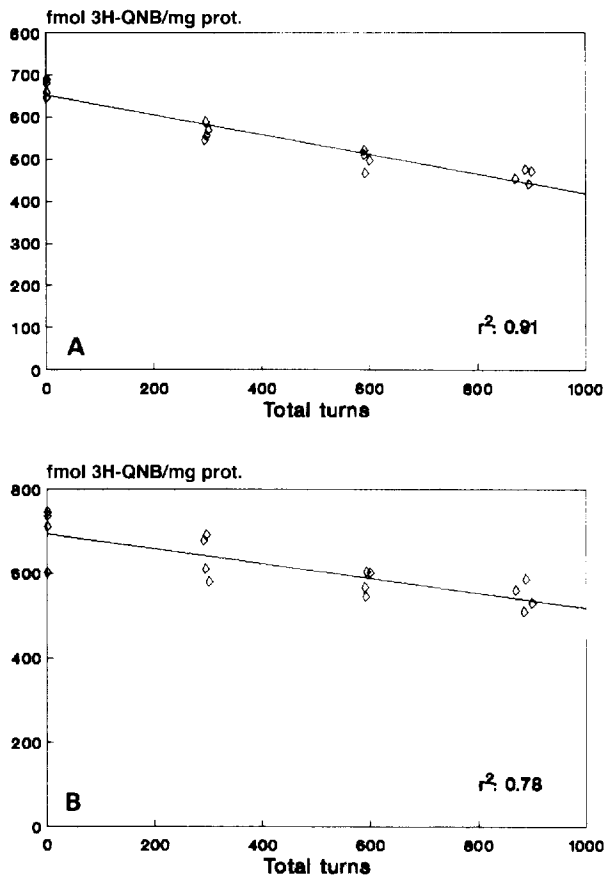

Fig. 2. Correlation between B_{max} and total turns run. A: male data. B: female data. $P < 0.001$, $n = 16$.

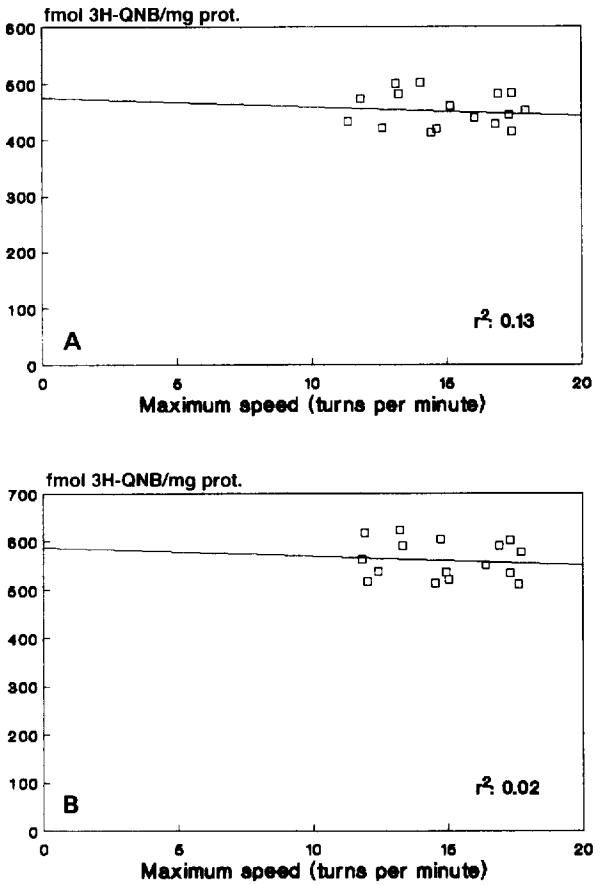

Fig. 3. Correlation between B_{max} and motor endpoint (maximum speed). A: male data. B: female data. Not significant, $n = 16$.

Hence, receptor variation could be affected not only by the total activity (distance) but also by the intensity (speed) with which it was performed. For this reason, we have correlated the mAChR level with the end-point motor performance (V_m) representing a measure of the intensity of the motor activity. Fig. 3 shows no significant correlation between Vm and receptor measurement (male r^2: 0.13, female r^2: 0.02; $P > 0.1$; $n = 16$). That means that receptor variation after CT was related to the total turns run rather than to the running speed.

4. Discussion

We studied the role of circling training in the pattern of expression of mAchR in rat striatum. By means of this, we found that motor activity during development elicit a fall in the total number of muscarinic receptor binding sites (35% in males, 24% in females) when animals were trained during 7 days starting at 30 days of age, representing a long term alteration since it was found 30 days after training was concluded. Similar results were not reached when CT performed at other ages, neither before (20 days old group) nor immediately after that period (40–60 days old groups). These results suggest the presence of a sensi-

tivity period during postnatal development where mAchR level could be modified and coinciding with the period of progressive increase of mAchR content and other cholinergic markers toward the normal adult level in striatum [6,7]. At the same time, studies on the dopamine (DA) receptors reported similar results, where drugs administered perinatally alter the receptor number and a limited sensitivity period was found for this effect [11,21]. However, one difference of our work is based on the fact that variation in the receptor number and the presence of a sensitivity period is related to a physiological event (motor activity) rather than to an extrinsic factor (drug administration) elicited one. In this respect, we believe that our findings are complementary to those studies supporting the existence of plasticity periods during neural development [3,17]. Furthermore, the fact that receptor variation is only found during a limited period supports that some plastic changes are tightly correlated to a particular time of development. In this case, final mAchR level in striatum could be set in that specific period (between 30 and 40 days old) where the presence of an unexpected overactivity can alter this setting in some extent, while modifications performed before or after this time have no consequence in final mAchR levels. Our experiments further support the combination of physiological mechanisms (response to CT) and a genetic basis (sensitivity period) for plasticity event to occur. In this order, the interactions during CNS development are reasons of what constitute the basal level of this receptor in the adult brain where plastic change turns out to be transient. A point of concern is related to which is the reason of receptor variation during training since CT has not only a motor but also a learning component. From the point of view of motor activity, both total turns and speed reflect this situation. Nevertheless, there is also a learning component implicated, since the animal is rewarded by performing a correct turn (a turn in the preestablished direction), and the errors (turns in non-rewarding direction) decrease throughout the test. Consequently, the animal learns to turn in one direction and not in the other with high efficiency. At least with this test, the cause of receptor variation cannot be distinguished between the motor and the learning component, where both components could even be combined. Therefore, when referring to motor activity, we also include the associated learning processes not discarding this influence in biochemical changes observed, although the motor component seems to be more related to this changes. In fact, a role of the learning component is weakly supported by the negative results found at other ages where learning processes are supposed to be the same. Nevertheless, since molecular learning mechanisms are not well understood, its implications in the plasticity of mAchR can by no means be discharged, particularly when alterations of the mAchR support the putative role of this receptor in learning processes [16,26]. Further characterization of receptor variation showed that this change was inversely related to the quantity of motor activity evaluated by total turns/meters run during CT but no significant relation was found to the intensity measured as animal running speed. This implies that such two variables, although both measuring motor activity, did not represent a common indicator and that its influence on motor related-receptor plasticity should be regarded separately. Furthermore, differences between sex were found in receptor variation. In fact, male mAchR level variation was more clearly evident while female levels were affected to a lesser extent. Although not expected, sex differences were not surprising. Different studies have been reported correlating estradiol level in rat striatum with receptor activity, function and sensitivity, and also with overall motor performance [2,23]. Probably hormone mechanisms could also underlie the sex differences found in mAchR binding after CT. Even this may provide an explanation; female minor differences could also be related to the constantly greater dispersion coefficient in receptor measurement among individuals in female experiments. A final finding of our experiments is the fact that mAchR variation is still present one year after CT was performed. Regarding animal life span, we have interpreted these findings as a permanent alteration of receptor expression. Although alteration of receptor level has been described in literature, we have to date no knowledge of similar evidence of long term plasticity related to motor activity in mammal central nervous system. In the light of this issue, these data may provide a rational basis for the development of a model for the clinical application of early motor stimulations. Finally, taking into account the wide distribution of mAchR among striatal neurons and its modulatory effects, it is probably implicated in the multiple functions of neostriatum. Hence, mAchR level and the balance with other neuroreceptors strongly regulate the cellular and molecular mechanisms by which the neostriatum exerts its function in specific motor behaviors. For this reason, we are presently evaluating whether mAchR variation is correlated with other biochemical changes within the neostriatum.

Acknowledgements

The authors are indebted to Cinthia Rossi who kindly agreed to revise the English version of the manuscript. This work was supported by grants from Consejo Nacional de Investigaciones Cientificas y Tecnológicas (CONICET), Argentina. G.R.I., J.A.R. and G.Ch.P. are recipients of a research fellowship from this institution. Presented in part at the 26th Annual Meeting of the American Association for Neurochemistry, Santa Mónica, CA, March 5–9, 1995.

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