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Research report

Decreased GAP-43/B-50 phosphorylation in striatal synaptic plasma membranes after circling motor behavior during development

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Abstract

We evaluated the in vitro phosphorylation of the presynaptic substrate of protein kinase C (PKC), GAP-43/B-50 and the PKC activity in the striatum of rats submitted to a circling training (CT) test during postnatal development. Motor activity at 30 days of age, but not at other ages, produced a unilateral reduction (-29.5%; $p < 0.001$) in the level of GAP-43/B-50 endogenous phosphorylation in the contralateral striatum with respect to the ipsilateral side, while non-trained control animals did not show asymmetric differences. Compared to controls, the contralateral striatum of trained animals also showed a significant reduction (-29.3%; $p < 0.001$) in the incorporation of ^{32}P-phosphate into GAP-43. This decreased in vitro GAP-43 phosphorylation was seen at 30 min, but not immediately after circling motor behavior. This contralateral change in GAP-43 phosphorylation correlated with the running speed developed by the animals [($r = 0.9443$, $p = 0.0046$, $n = 6$, relative to control group) and ($r = 0.8813$, $p = 0.0203$, $n = 6$, with respect to the ipsilateral side of the exercised animals)]. On the contrary, GAP-43/B-50 immunoblots did not show changes in the amount of this phosphoprotein among the different experimental groups. Back phosphorylation assays, performed in the presence of bovine purified PKC, increased the level of GAP-43/B-50 phosphorylation in the striatum contralateral to the sense of turning [($+22\%$; $p < 0.05$, with respect to ipsilateral side of the same trained group) and ($+21\%$; $p < 0.05$, relative to control group)]. Taken together, these results demonstrate that the activity developed in the CT test induces a reduction in the phosphorylation state of GAP-43/B-50 in the specific site for PKC. We conclude that general markers of activity-dependent neuronal plasticity are also altered in the same period that long-lasting changes in striatal neuroreceptors are triggered by circling motor behavior.

Keywords: GAP-43/B-50; PKC; SPM; Circling training; Development; Rat striatum

1. Introduction

Circling training (CT) is a conditioned behavioral test where motor activity induces neurochemical modifications in rat striatum [33,56,57]. Recently, we have shown a correlated reduction in the expression of striatal dopamine (DA) D2 (D2R) and muscarinic acetylcholine (mAChR) receptors after CT activity [32]. Noteworthy, this modification is induced only when the animals are trained during a limited period of postnatal development (30–37 days of age) and represents a long-lasting change of the receptor levels. Furthermore, the limit of this period of plasticity can be modified by the intrastriatal infusion of nerve growth factor (NGF) [58].

This period coincides with a postnatal stage of the striatal development when maturation of cholinergic and dopaminergic systems are bearing toward formation of mature neuronal connections. In rodent, the cholinergic system undergoes rapid development during the first 3–4 weeks after birth. Previous work indicated that tyrosine hydroxylase and choline acetyltransferase activities reach 70–75% of the activity present in the adult striatum around 28 days of age [14]. Moreover, the quantity of dopaminergic and cholinergic receptors achieve the adult values by the end of the first month of life [15,44]. Dopaminergic and cholinergic-dependent neuropeptide activity also develop in this period, and contribute to synaptic maturation, affecting function and plasticity [45,52].

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Although neurotransmitter receptors are key molecules in these events, distinct signal transduction pathways should be implicated in the amplification and stabilization of the initial molecular steps that induce long-term alteration of mAChR and DA D2R levels. In this regard, we have focused our interest in two molecular species widely involved in neuronal plasticity: the growth associated protein GAP-43/B-50 and protein kinase C (PKC).

GAP-43/B-50 is a calmodulin-binding protein substrate for PKC, localized in growth cones, axons and presynaptic nerve endings [1,3]. This phosphoprotein has been implicated in neuronal differentiation and axon regeneration, neurotransmission, long-term potentiation (LTP), associative learning and memory formation for passive avoidance in chicks and rats [2,10,24,46,47].

It was involved in morphological and functional neuroplasticity by way of its participation in plasma membrane signal transduction and neurotransmitter release [8,18].

On the other hand, PKC is also involved in several neuronal functions such as excitability, transmitter release, cell growth and survival and regulation of gene expression [5,17,31,41]. Besides its presynaptic role in GAP-43 phosphorylation, PKC has also been implicated in mAChR phosphorylation, although this has not been probed in vivo yet [29].

Previous studies have also implicated PKC as a secondary messenger in the release of DA in the nigrostriatal pathway, and have shown that D1 and D2 agonists exert a direct inhibitory effect on particulate PKC activity in striatal synaptoneurosomes [22,23]. Moreover, inhibitors of PKC activity inhibit the potassium-evoked release of DA from striatal synaptosomes [23].

Together, these findings support a role for DA receptor-mediated modulation of PKC activity.

Recently, we have found that endogenous phosphorylation of GAP-43/B-50 is reduced by physical exercise in rat striatum of 30-day old animals [43]. Based upon these observations, we proposed that the GAP-43/PKC system could participate in the initial steps that trigger CT-induced long-lasting neuroreceptor alterations.

In the present report, we evaluate the participation of PKC-dependent phosphorylation of GAP-43/B-50 and PKC activity in the initial mechanisms that underlie circling motor behavior during striatal development.

2. Materials and methods

2.1. Circling training

Male Sprague–Dawley rats were subjected to the CT test at 20, 30, 40 and 60 days old, as described previously [32,33]. Briefly, animals deprived of water for 24 h were trained to turn in a circle for a water reward in three consecutive sessions. In the first training session, each rat was rewarded for an approximation of a full turn in the prescribed direction (selected randomly) over a 30-min period: in the first 10 min, a quarter turn in the appropriate direction was rewarded, in the next 10 min, a half turn and the last 10 min, a full turn in the appropriate direction was rewarded. Training was then conducted daily for 2 days according to a continuous reinforcement schedule. Each rat was required to perform 150 complete turns (= 150 m) in these two sessions. The clockwise or anti-clockwise turning direction was randomly selected for each experimental group (three to five animals) in the first session and kept until the last session. Non-trained control animals were also deprived of water and placed in the CT apparatus for 30 min each day, but no training was performed. We have never recorded more than 10 turns per session for any control animal. Running behavior performed by trained animals was not observed in controls. The times were recorded in order to calculate the running speed. The animals with similar motor performance (speed) were grouped together for correlation studies. The direction of turning was randomly selected in each individual experiment and all the trained animals turned in that direction.

2.2. Synaptic plasma membranes (SPM) preparation

The experiments were performed 3 to 6 times using three to five animals for each experimental group. Left vs. right striata were grouped as ipsi vs. contralateral according to the turning direction (clockwise or anti-clockwise) of its respective trained group. Rats were killed by decapitation at 0 or 30 min post-training. Brains were quickly removed, left and right striata were dissected separately on ice (within 60 s) and weighed. Tissue was added to ice-cold 20 mM Tris–HCl buffer pH 7.4 (10% w/v solution), containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μM pepstatin and 10 μM leupeptin. SPM preparations were obtained according to a modification of the method described by Azcurra and De Robertis [7]. To inhibit GAP-43/B-50 dephosphorylation SPM were prepared in the presence of EGTA as described Gnegy et al. [26].

Tissue homogenization was performed at 1300 rpm by 40 strokes in a potter-Elvehjem glass homogenizer fitted with a loose teflon pestle (clearance 0.25 mm). 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 20 min (mitochondrial pellet). Following two washes, the pellets were resuspended in double distilled water pH 6.8 (osmotic shock) and centrifuged at $20\,200 \times g$ for 30 min. Then, the pellets were resuspended in homogenization buffer, loaded on a discontinuous sucrose gradient (0.8 to 1.0 M) and centrifuged at $100\,000 \times g$ max for 80 min at 4°C in a Beckman 90 Ti rotor. The material floating on the 1 M band was collected, washed in the same buffer, pelleted by centrifugation at $150\,000 \times g$ for 80 min and finally resuspended in buffer A (10 mM Tris–HCl buffer pH 7.4,

10 mM $MgCl_2$, 1 mM $CaCl_2$). Protein concentration was determined by the method of Lowry et al. [37] using bovine serum albumin as standard.

2.3. GAP-43 / B-50 phosphorylation assays

In vitro endogenous phosphorylation was performed in conditions known to activate PKC [36]. Briefly, aliquots of SPM (10–20 μg) were preincubated for 5 min at 30°C in a final volume of 25 μl in buffer 10 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$ and 1 mM $CaCl_2$. The reactions were initiated by the addition of 7.5 μM ATP and 2 μCi [γ-^{32}P]ATP (Dupont 6000 Ci mmol^{-1}) in buffer A and terminated 1 min later by the addition of 12.5 μl stopping solution (187.5 mM Tris–HCl pH 6.5, 6% SDS, 15% β-mercaptoethanol, 30% glycerol and 0.003% bromophenol blue). In some experiments, exogenous PKC, purified from bovine brain as indicated Kitano et al. [35] and according to the modifications done by Gómez et al. [27], was added to the incubation media of heat-inactivated SPM (100°C, 5 min). Phosphorylation reactions were allowed to proceed for 10 min and 10 μl of PKC (2 pmol min^{-1} μl^{-1}) were added for each 10 μg of SPM protein (Back phosphorylation).

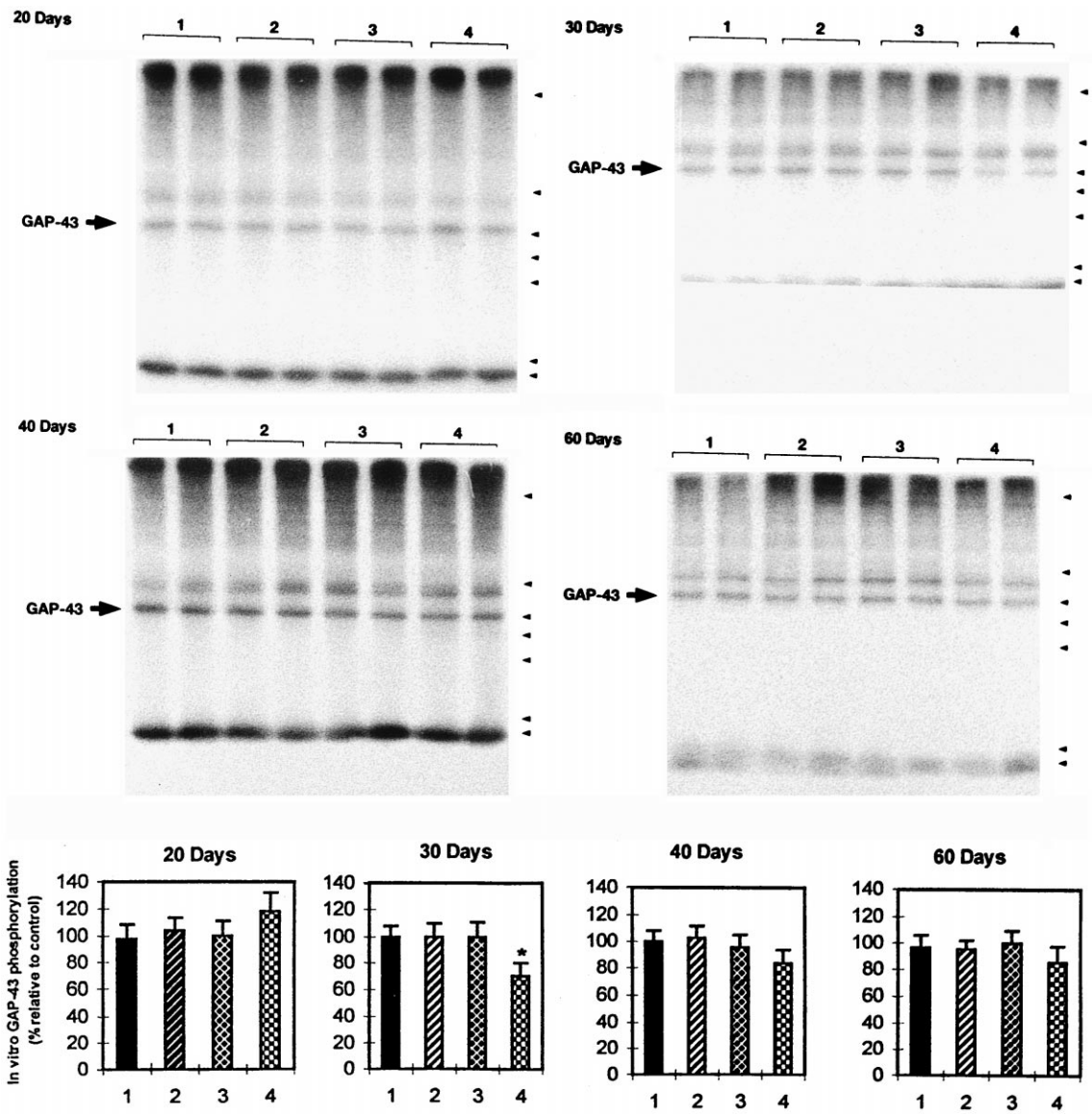

Fig. 1. Upper panel: representative autoradiograms showing the in vitro phosphorylation of striatal SPM proteins from control and trained rats of 20, 30, 40 and 60 days of age. The animals were sacrificed 30 min after CT. Lane 1, ipsilateral control; lane 2, ipsilateral trained; lane 3, contralateral control and lane 4, contralateral trained. Arrowheads indicate the position of molecular weight markers: 95.5, 55, 43, 36, 29, 18.5, and 12 kDa. Lower panel: quantification of GAP-43/B-50 in vitro phosphorylation. Data are expressed as percentages of the maximal (non-trained) control value and represent the mean ± S.D. for three to four independent experiments. Each determination was assayed in quadruplicate. $* p < 0.001$ vs. controls by two-tail Student's *t*-test for unpaired samples and vs. ipsilateral trained by two-tail Student's *t*-test for paired samples.

2.4. GAP-43 / B-50 immunoprecipitation

GAP-43/B-50 immunoprecipitation was performed according to the method described by Oestreicher et al. [42]. Samples containing 25 μg of in vitro phosphorylated membrane proteins were incubated overnight at 4°C with a polyclonal rabbit anti B-50/GAP-43 antibody (1/200 final dilution from serum 8615) in 400 μl buffer B (200 mM NaCl; 10 mM EDTA; 10 mM NaH_2PO_4; 0.5% Nonidet P-40 (v/v)). Antigen complexes were precipitated with washed pansorbin (Calbiochem), solubilized in 30 μl of stop–mix solution, and boiled for 10 min. Immunoprecipitates were analyzed by SDS-PAGE in duplicate. Incorporation of ^{32}P into GAP-43/B-50 was detected using an AGFA-Curix film and quantified using a MCID Image Analysis System (5.02v, Image Research, Ontario, Canada).

2.5. Electrophoresis

Samples containing 10 to 20 μg of SPM protein were subjected to a one-dimensional 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were subsequently stained with Coomasie blue, destained and autoradiographed by exposing the gels to an AGFA-Curix film. Finally, densitometric analysis of the X-ray film was performed using a MCID Image Analysis System (5.02v, Image Research).

2.6. Western blotting of GAP-43 / B-50

SPM proteins (20 μg) were electrophoretically transferred (12 h at 250 mA) to nitrocellulose membranes (pore size 0.45 μm) as described by Towbin et al. [55]. After blocking, the membrane was incubated for 12 h at room temperature with a specific affinity-purified anti B-50 IgGs (1/1000) obtained from serum 8920 [42]. Finally, immunoreactivity against GAP-43/B-50 was detected by using a biotinylated secondary antibody and a horseradish peroxidase-conjugated biotin–avidin complex (Vectastain Elite ABC Kit, Vector Labs) and visualized with diaminobenzidine as chromogen.

2.7. Determination of PKC activity

PKC activity was determined by an in vitro phosphorylation assay using histone III-S as substrate [13]. The reactions were performed using 10 μg of SPM proteins in 25 μl (final volume) of 50 mM Tris–HCl buffer, pH 7.4; 0.2 mM EDTA; 10 mM $MgCl_2$; 5 mM β-mercaptoethanol; 10 μM ATP; 100 μg ml^{-1} PKA inhibitor (Sigma); 0.6 μg μl^{-1} histone H III-S and 1 μCi [γ-^{32}P]ATP, during 1 min at 30°C. Phosphate incorporation was evaluated in the presence or absence of PKC activators: 0.1 μM phorbol ester TPA; 100 μg ml^{-1} phosphatidylserine (Sigma) and 1 mM $CaCl_2$. The reaction was terminated by addition of 12.5 μl stopping solution. The labeled phosphoproteins were separated on a 15% SDS-PAGE and following staining and destaining, the gels were dried and exposed to an AGFA-Curix film. The histone protein band was cut from the dried stained gel and the radioactivity incorporated counted in a liquid scintillation counter. The total PKC activity was calculated as pmol ^{32}P incorporated min^{-1} mg of $protein^{-1}$ above the basal activity, which was determined in the absence of PKC activators.

2.8. Statistical analysis

A two-way analysis of variance (ANOVA) was used to analyze the results. The ANOVA design considered control vs. trained as one factor, and ipsi vs. contralateral hemisphere as another. Differences between groups were assessed by Student's *t*-test for unpaired and paired samples. The correlation coefficient (r) was obtained studying the degree of linear association between two variables using Graph Pad Instat Software.

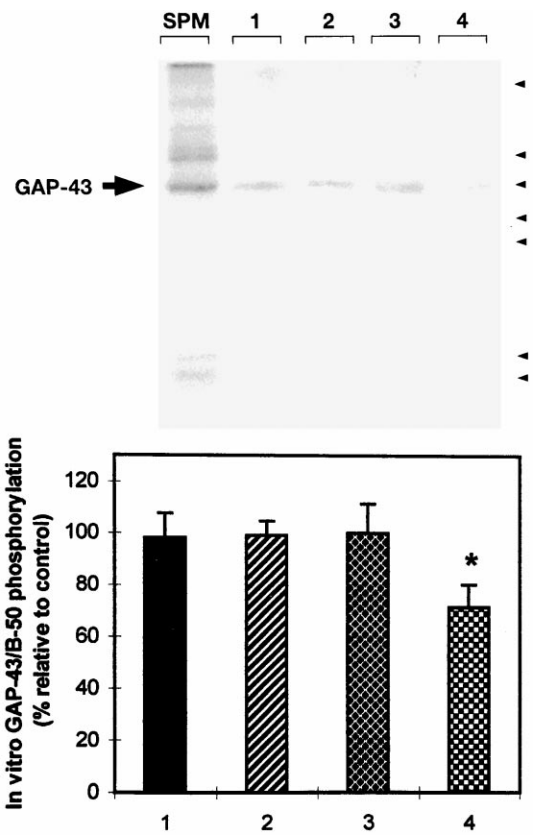

Fig. 2. Upper panel: representative autoradiogram showing a specific immunoprecipitation of GAP-43/B-50 phosphorylated from striatal SPM. Animals of 30 days of age were sacrificed 30 min post-training. Lane 1, ipsilateral control; lane 2, ipsilateral trained; lane 3, contralateral control and lane 4, contralateral trained. Arrowheads indicate the position of molecular weight markers: 95.5, 55, 43, 36, 29, 18.5, and 12 kDa. Lower panel: quantification of the GAP-43/B-50 phosphorylated band. Data are expressed as percentages of the maximal (non-trained) control value and represent the mean ± S.D. for four independent experiments assayed in duplicate. $* p < 0.05$ vs. controls by two-tail Student's *t*-test for unpaired samples and vs. ipsilateral trained by two-tail Student's *t*-test for paired samples.

3. Results

3.1. Endogenous phosphorylation of GAP-43/B-50 after CT

SPM preparations were used to assess phosphorylation of GAP-43/B-50 because this protein is a prominent phosphoband in this fraction and is readily detected under incubation conditions which are optimal for PKC activation. The identity of GAP-43/B-50 was established on the basis of electrophoretic properties on unidimensional SDS-PAGE, selective phosphorylation by PKC, and specific immunoreactivity detected on Western blots and by immunoprecipitation.

To assess whether GAP-43/B-50 endogenous phosphorylation is altered after CT during striatal development, we isolated SPM from ipsi and contralateral hemispheres, and subjected them to an in vitro phosphorylation assay under conditions known to activate PKC. As can be seen in Fig. 1, only trained animals of 30 days of age showed an average decrease of 29.5% in the level of GAP-43/B-50 endogenous phosphorylation in the contralateral striatum relative to the ipsilateral side ($p < 0.001$). Significant interhemispheric differences were not found in control animals, neither when tested left vs. right nor when striata were grouped as ipsi vs. contralateral, according to the respective trained group.

Furthermore, at this age, the CT produced a reduction of 29.3% in the level of endogenous phosphorylation of GAP-43/B-50, in comparison with control animals ($p < 0.001$). In contrast, animals trained at 20, 40 or 60 days of age did not show significant differences in the endogenous phosphorylation of GAP-43/B-50 with respect to the control animals nor between hemispheres (Fig. 1). In 30-day old trained animals, the changes were seen 30 min post-training in the striatum contralateral to the sense of turning, independently of the direction of turning chosen (clockwise or anti-clockwise).

In contrast, animals sacrificed immediately after CT activity (0 min) did not show any significant modification in the in vitro GAP-43/B-50 phosphorylation when compared with controls in both ipsilateral ($+11\% \pm 3$) and contralateral striata ($-10\% \pm 2$).

To confirm the identity of GAP-43/B-50 and the magnitude of the change obtained using endogenous phosphorylation and autoradiography of SPM phosphoproteins, specific immunoprecipitation of GAP-43/B-50 phosphorylated was performed (Fig. 2).

Densitometric analysis shows an average drop of 28.5% ($p < 0.05$) with respect to the control animals, and of 28.0% ($p < 0.05$) between ipsi vs. contralateral striata from 30-day old trained animals.

To study whether CT affects the amount of GAP-43/B-50 protein in SPM isolated from control and trained animals, we performed immunoblots using affinity-purified antibodies against GAP-43/B-50. The reduced phosphorylation observed in the present study does not seem to be due to a difference in GAP-43/B-50 protein levels, because densitometric quantitation of the Western blots did not show any difference between control and trained ani-

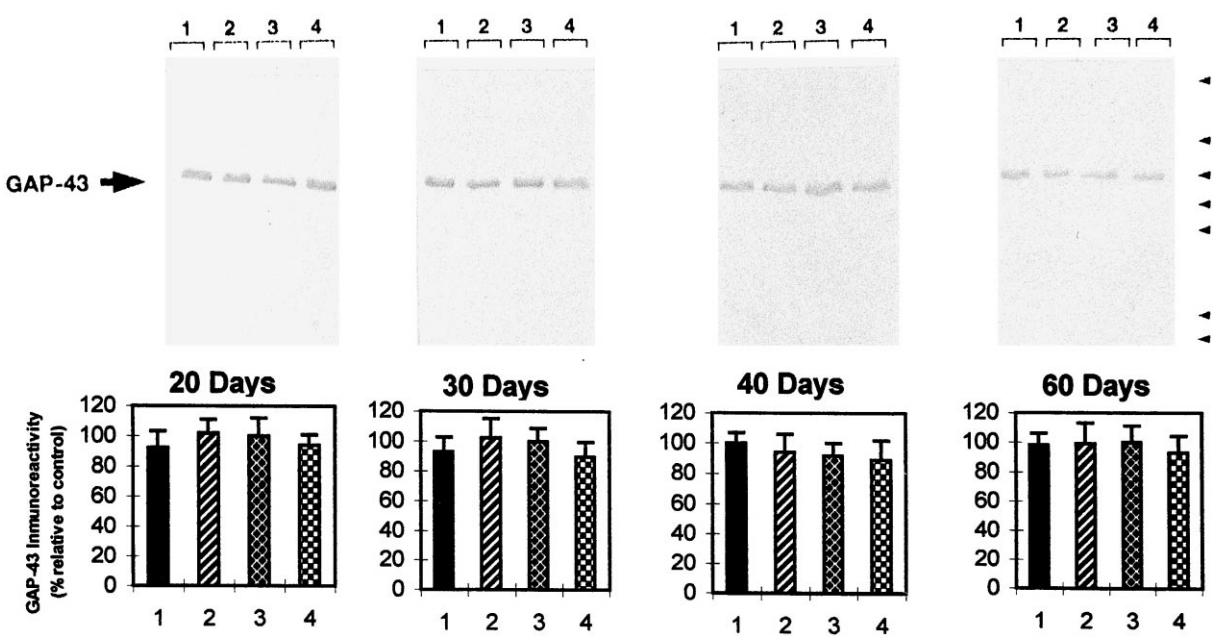

Fig. 3. Upper panel: a representative Western blot analysis of GAP-43/B-50 immunoreactivity, using striatal SPM proteins (20 μg) from control and trained animals of 20, 30, 40 and 60 days of age, isolating separately the ipsi and contralateral striata. Lane 1, ipsilateral control; lane 2, ipsilateral trained; lane 3, contralateral control; and lane 4, contralateral trained. In all cases, the animals were sacrificed 30 min post-training. Arrowheads indicate the position of molecular weight markers: 95.5, 55, 43, 36, 29, 18.5, and 12 kDa. Lower panel: quantification of GAP-43/B-50 immunoreactivity. Data are expressed as percentages of the maximal (non-trained) control value and represent the mean ± S.D. for three to four independent determinations performed in triplicate.

mals of 30 days of age (Fig. 3). No significant differences were found in the GAP-43/B-50 immunoreactivity when the animals were trained at 20, 40 or 60 days of age ($p > 0.1$) (Fig. 3).

PKC phosphorylates the Ser 41 residue of GAP-43/B-50 [12,40]. However, this protein can be also in vitro phosphorylated by casein kinase II (CKII) on Ser 192, 193 and Thr 88, 89 and 95 [6]. In order to exclude a dual participation of PKC and CKII in our preparations, we performed the phosphorylation assays in the presence of heparin (a potent inhibitor of CKII). The presence of heparin did not alter the differences observed in the in vitro GAP-43/B-50 phosphorylation (data not shown).

3.2. GAP-43/B-50 phosphorylation variation is correlated with speed

To study a possible correlation of this change with the motor performance developed by the animals during the CT, the rats were grouped according to their running speed (S.D. < 10% on each group). Plotting the percentage of drop in the in vitro GAP-43/B-50 phosphorylation (relative to control animals) as a function of motor intensity, we were able to find a significant correlation between these two variables ($r = 0.9443$; $p = 0.0046$; $n = 6$) (Fig. 4a). Similarly, a significant correlation was found plotting the speed of turning and the percentage of decrease in the endogenous GAP-43/B-50 phosphorylation relative to the ipsilateral trained striatum ($r = 0.8813$; $p = 0.0203$; $n = 6$) (Fig. 4b).

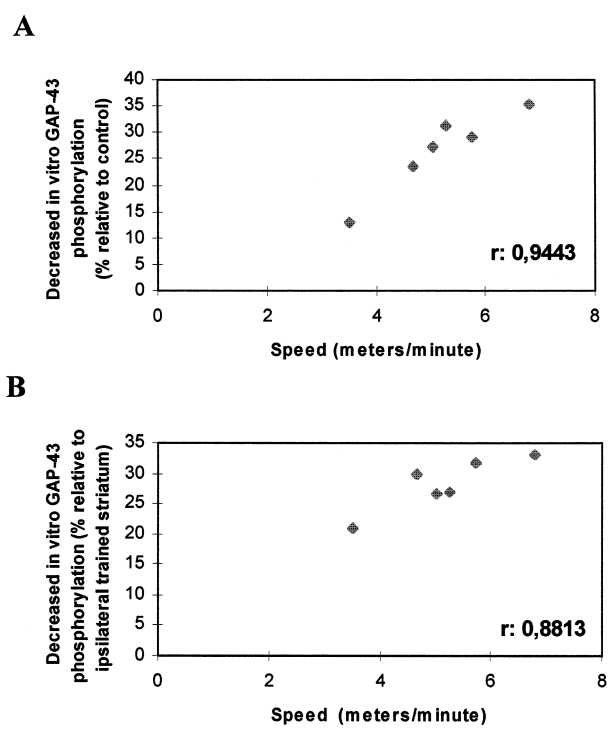

Fig. 4. Correlation between percentage of decrease of the in vitro GAP-43/B-50 phosphorylation in the contralateral striatum and motor activity (speed) accomplished during CT. (a) Percentage relative to control group. (b) Percentage relative to ipsilateral side also trained. Animals of 30 days old were sacrificed 30 min post-CT. (a) $r = 0.9443$, $p = 0.0046$, $n = 6$ and (b) $r = 0.8813$, $p = 0.0203$, $n = 6$.

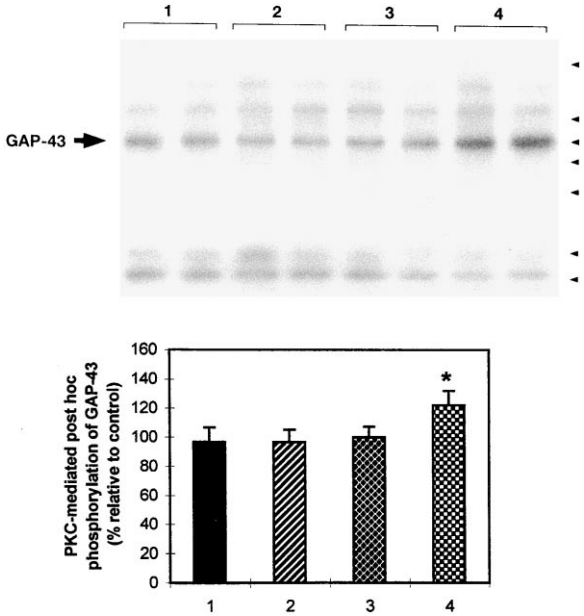

Fig. 5. Upper panel: representative autoradiogram showing a PKC-mediated posthoc phosphorylation of striatal SPM proteins from control and trained rats, 30 days old. Animals were sacrificed 30 min post-training. Heat-inactivated SPM were phosphorylated in the presence of partially purified bovine PKC and [γ-^{32}P]ATP and subjected to SDS-PAGE as described in Section 2. Lane 1, ipsilateral control; lane 2, ipsilateral trained; lane 3, contralateral control; and lane 4, contralateral trained. Arrowheads indicate the position of molecular weight markers: 95.5, 55, 43, 36, 29, 18.5, and 12 kDa. Lower panel: quantification of GAP-43/B-50 back phosphorylation. Data are expressed as percentages of the maximal control value and represent the mean ± S.D. of four independent experiments. Each determination was assayed in quadruplicate. $* p < 0.05$ vs. controls by two-tail Student's *t*-test for unpaired samples and vs. ipsilateral trained by two-tail Student's *t*-test for paired samples.

3.3. Post-hoc phosphorylation of GAP-43/B-50 in striatal SPM after CT

As GAP-43/B-50, levels were not modified among the different experimental groups, we investigated whether the endogenous phosphorylation state of GAP-43/B-50 was altered 30 min post-training. To address this question, the endogenous kinase systems present in SPM were inactivated by heating (5 min at 100°C) and protein phosphorylation present in heated SPM was carried out by adding purified bovine PKC (back phosphorylation). Fig. 5 shows an enhanced ability of GAP-43/B-50 to be phosphorylated in the contralateral striatum from trained animals. Densitometric analysis shows an increment of 21% ($p < 0.05$) with respect to control group and of 22% ($p < 0.05$) with respect to the ipsilateral striatum of the same trained animals (Fig. 5).

Table 1
Effect of CT on ratios of PKC activity in striatal SPM during development

Training age (days)	Ratios of PKC activity				PKC activity (pmol ^{32}P min^{-1} mg^{-1})	
	Cc/Ic	Ct/It	It/Ic	Ct/Cc	Ic	Cc
20	0.94 ± 0.02	1.07 ± 0.11	0.99 ± 0.04	1.13 ± 0.05	71.65 ± 8.47	67.05 ± 9.30
30	0.90 ± 0.03	0.67 ± 0.10*	1.09 ± 0.09	0.80 ± 0.07#	68.67 ± 10.7	62.40 ± 11.56
40	0.98 ± 0.03	0.90 ± 0.07	0.99 ± 0.05	0.90 ± 0.03	67.20 ± 7.95	66.30 ± 6.05
60	0.98 ± 0.07	0.89 ± 0.06	1.01 ± 0.04	0.91 ± 0.03	67.20 ± 6.56	65.70 ± 5.73

Animals were sacrificed 30 min post-CT. Values are expressed as means ± S.D. for three to four independent determinations. The PKC activity was measured in quadruplicate as described in Section 2. The different experimental groups are: Cc: contralateral control; Ic: ipsilateral control; Ct: contralateral trained and It: ipsilateral trained. This table also shows the values of PKC activity presents in striatal SPM from control rats during development. They are expressed as pmol ^{32}P incorporated min^{-1} mg^{-1} of protein above basal activity. * $p < 0.05$ vs. Cc/Ic by two-tail Student's *t*-test for unpaired samples and # $p < 0.05$ vs. It/Ic by two-tail Student's *t*-test for paired samples.

3.4. Determination of PKC activity after CT during development

To test whether the change in endogenous GAP-43/B-50 phosphorylation caused by CT could be due to a reduced PKC activity, SPM preparations of control and trained animals were used to measure total PKC activity at 30 min post-training. Particulate PKC activity was determined using specific PKC activators (PS and TPA and calcium) and an excess of histone H-III S as substrate. Table 1 shows that the ratio of PKC activity between both 30-day old trained striata is significantly different to the ratio of PKC activity detected between both control striata (It/Ct vs. Ic/Cc, [$p < 0.05$]). Moreover, the PKC activity relative to respective control group was different between both 30 days of age trained striata (It/Ic vs. Ct/Cc, ($p < 0.05$)). Similar changes were not observed in animals of 20, 40 or 60 days of age (Table 1). These results demonstrate the PKC participation in the asymmetric changes detected in the striatum of 30-day old trained animals, and suggest that this variation is mainly due to a 20% decrease in the contralateral PKC activity relative to the corresponding control hemisphere.

4. Discussion

We have studied GAP-43/B-50 phosphorylation and PKC activity in the rat striatum after CT activity during postnatal development. Results show that this circling motor behavior elicits a unilateral drop in the phosphorylation state of GAP-43/B-50, when the animals were trained at 30 days of age, indicating that this variation is not only related to the physiological stimulation but also with a specific period during development. This reduction was seen at 30 min post-training in the striatum contralateral to the sense of turning, independently of the direction of turning chosen (clockwise or anti-clockwise). Similar results were not reached when the animals were trained at 20, 40 or 60 days old.

It has been shown that PKC-dependent GAP-43/B-50 phosphorylation increases neurotransmitter release [18,26]. However, we have found a reduction in the GAP-43 phosphorylation state in the specific site for PKC (Ser 41), which may indicate a decrease of striatal neurotransmission. A point of concern is that GAP-43 modification was found 30 min post-training, but not immediately after that CT activity was performed. Delayed changes in GAP-43 phosphorylation are related with molecular events associated with plastic processes of synaptic function [24,25,47]. Thus, it is likely that GAP-43 alteration is playing a modulatory role in the plastic events triggered after completion of CT. In adult striatum, a role for GAP-43 phosphorylation in priming or modulation of neurotransmitter release in rat striatum after amphetamine (AMPH) treatment has been proposed by Gnegy et al. [26].

Since the level of GAP-43/B-50 immunoreactivity in SPM was similar between trained and non-trained control animals of 30 days of age, the decrease in the phosphorylation might be the consequence of a reduced amount of membrane bound protein kinase, a reduced concentration of endogenous activators (i.e., diacylglycerols, phosphatidylserine or arachidonic acid) or an increased phosphatase activity. To determine if the drop in the endogenous phosphorylation of GAP-43 was related to a reduced PKC activity, we measured the activity of this kinase in control and trained animals at different postnatal ages. These results also demonstrate an asymmetric alteration in the particulate PKC activity. Thus, the present findings suggest that the decreased GAP-43/B-50 phosphorylation could be related to a reduced contralateral PKC activity in SPM isolated from 30-day old trained animals. Our results are in agreement with other neurochemical and anatomical studies indicating that the experience may modify different developing neuronal systems through the mediation of PKC phosphorylation events [21]. In this context, it has been reported that PKC activity can be regulated by sensory deprivation in the olfactory and visual system. Therefore, different sensory and motor systems may use common molecular mechanisms to process information. Specifically in rat striatum, it has been shown that activation of DA D1 and D2 receptors exert a direct inhibitory effect on particulate PKC activity [22,23]. These reports support a

role for DA receptor-mediated modulation of PKC phosphorylation. Furthermore, there is evidence that the circling motor behavior and the turning behavior induce speed-dependent contralateral activation of the nigrostriatal dopaminergic pathway [56,57]. The reported data involve changes in DA, DOPAC (3,4-dihydroxyphenylacetic acid) and in the presynaptic turnover rate of this neurotransmitter. However, there is an unresolved question with respect to the reported asymmetries in the striatal dopaminergic system, since other reports have failed to confirm these findings [19,39,48,49,54]. Since the CT represents an asymmetric motor behavior, the contralateral reduction in GAP-43 phosphorylation might be a consequence of the asymmetric motor stimulation, putatively associated with DA-mediated effects.

Recently, we have demonstrated that the motor activity developed by the animals in the CT test triggers a long-term reduction in the levels of striatal muscarinic and dopaminergic D2 receptors, only when the animals were trained at 30 days of age [32,33]. Interestingly, these changes in GAP-43/B-50 phosphorylation were only detected in the same sensitive period.

Due to the role of GAP-43/B-50 in activity-dependent plastic mechanisms, the above mentioned modifications in GAP-43 phosphorylation could be related to the long-lasting alterations of neuroreceptors in rat striatum during this postnatal critical period. However, future experiments are required to determine whether the variation in GAP-43/B-50 is involved in the induction of the permanent changes of striatal neuroreceptors. The decrease of GAP-43 phosphorylation does not seem to be related with a differential motor activity (speed) of the animals of 30 days of age when compared with other age groups. At the end of the 2 days of training the speed values for each age group tested were as follow: 20 days old = 5.34 ± 0.93 m min^{-1}, 30 days old = 5.50 ± 0.82 m min^{-1}, 40 days old = 5.88 ± 0.63 m min^{-1}, and 60 days old = 5.06 ± 0.91 m min^{-1}). However, at 30 days of age, we detected a significant correlation between the percent reduction in the endogenous GAP-43 phosphorylation and the speed developed by the animals (Fig. 5a and b). Recently, using a spontaneous motor activity paradigm devoid of associative learning components, we showed that the physical exercise reduces the phosphorylation of GAP-43/B-50 in striatal synaptic membranes isolated from rats of 30 days old. Taken together, our experiments suggest that a combination of physiological motor stimulus and a genetically fixed period of development are necessary in the striatum for activity dependent-neurochemical changes to occur.

Since the CT is a conditioned motor behavior, associative learning activities could be involved in the neurochemical changes described here. Thus, the associative errors (AE) were recorded (AE = number of changes to the rewarded turning direction), but we did not find a significant correlation with the GAP-43/B-50 phosphorylation (data not shown). Moreover, only the animals of 20 days of age showed a significant increase in the associative errors (second training session) in relation to other ages (20 days old = $11.30 \pm 2.38*$, 30 days old = 5.40 ± 1.87, 40 days old = 4.33 ± 1.36, and 60 days old = 3.83 ± 1.88; $p < 0.001$ by Student–Newman–Keuls test for unpaired samples). Thus, it seems unlikely that this variation in GAP-43/B-50 phosphorylation is related to associative learning activity (at least evaluated through associative errors).

Pharmacological experiments with apomorphine (APO) support a late state of rat striatal neurochemical maturation around 28 days of age [30,50]. APO, a mixed D1/D2 agonist, has been shown to produce dose-dependent effects on locomotor activity in adult rats [53]. Several studies have documented that the behavioral sedation produced at low doses of APO was not observed until 28 days of age and suggest that in rat striatum the DA autoreceptor does not modulate DA release until this age [30,50]. However, the use of APO-induced behavioral depression as an index of autoreceptor activation is probably not appropriate, as has been suggested by others [4,38,53].

The development of appropriate neuronal circuits involves activity-dependent processes [28,51]. Forms of synaptic plasticity such as LTP and LTD have been suggested as cellular mechanisms for these activity-dependent processes. For example, maximal susceptibility to LTP and LTD coincides with critical periods for cortical plasticity [16,20,34].

Interestingly, coactivation of DA D1 and D2 receptors is involved both in motor control and generation of striatal LTD [9]. Recently, Choi and Lovinger [11] demonstrated that striatal LTD maintenance appears to involve a decrease in the probability of neurotransmitter release from presynaptic terminals and suggest that the combination of the dopaminergic activation and an up-state transition in response to cortical input may set the stage for striatal LTD in vivo. As discussed above, a reduction in GAP-43 phosphorylation may be indicating a decrease in the probability of neurotransmitter release and reflect a process of synaptic depression like LTD or a mechanistically related process.

In conclusion, the present findings demonstrate the participation of PKC and its presynaptic substrate GAP-43/B-50 in response to motor stimulation, during an experience-dependent sensitivity period, in rat striatum. In this same period, motor activity triggers long-lasting neuroreceptor alterations. Whether these findings are related to the present results remains to be determined.

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