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Partial serotonergic denervation decreases progenitor cell proliferation in the adult rat hippocampus, but has no effect on rat behavior in the forced swimming test

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Abstract

It has been hypothesized that impaired serotonin (5-HT) neurotransmission contributes to the pathophysiology of depression. Recently, it was shown in adult animals that antidepressants which influence 5-HT levels increase hippocampal progenitor cell proliferation, suggesting an important role of the 5-HT system in the regulation of adult neurogenesis. In this study, we investigated the effects of partial 5-HT denervation of the adult rat forebrain caused by a single *para*-chloroamphetamine (PCA) administration (10 mg/kg, s.c.) on hippocampal progenitor cell proliferation measured by Ki-67 immunohistochemistry over a 3-week time period. Moreover, behavior of rats was analyzed by using the forced swimming test which serves as a so-called animal model of depression. One week after PCA administration, a significant decrease of Ki-67 immunopositive cells and cell clusters (−25% and −53%, respectively) was found which was recovered over the 3-week period. The decrease in progenitor cell proliferation in the dentate gyrus per animal was positively correlated with the 5-HT denervation. Behavioral analysis of rats revealed no significant differences between vehicle and PCA-treated animals at any of the examined time points. These findings indicate a regulative role of 5-HT for hippocampal progenitor cell proliferation and neurogenesis, but its relation to animal models of depression remains to be elucidated.

Keywords: Serotonin; Progenitor cell; Neurogenesis; Hippocampus; Ki-67; Depression; Forced swimming test

1. Introduction

The monaminergic theory of the pathophysiology of depression has put much emphasis on the deficiency of serotonergic function in the brain, and the use of selective serotonin (5-HT) reuptake inhibitors, increasing 5-HT concentrations in the synapse, has proved to be an effective treatment for depression (Middlemiss et al., 2002). Recent data showed that, after chronic administration, antidepressants also exhibit increasing effects on adult cell proliferation in the rat hippocampus (Malberg et al., 2000; Lee et al., 2001; Santarelli et al., 2003). The mammalian hippocampus is one of the brain regions which retains the ability to form new neurons during adulthood, and the process of neurogenesis takes place in the subgranular zone (SGZ) within the dentate gyrus (Taupin and Gage, 2002). Progenitor cells dividing within the SGZ migrate into the granule cell layer (GCL) and differentiate into neurons exhibiting the morphology and features of mature granule cells (van Praag et al., 2002). The rat hippocampus receives dense serotonergic innervation from the dorsal raphe via the medial forebrain bundle, and, among other things, the fact that antidepressants could increase neurogenesis led to the suggestion that the serotonergic system plays an important role in stimulating neurogenesis in the adult hippocampus. Indeed, cell proliferation in the adult rat hippocampus could be increased by activation of 5-HT1A receptors (Santarelli

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et al., 2003; Banasr et al., 2004) and decreased by a blockade of 5-HT1A receptors or depletion of 5-HT in the forebrain by specific neurotoxic lesions (Brezun and Daszuta, 2000; Radley and Jacobs, 2002). These findings also indicate that dysregulation of hippocampal neurogenesis might be a pathological feature of depression and increased neurogenesis might contribute to the actions of antidepressants (Jacobs et al., 2000; Duman et al., 2001; Kempermann, 2002).

Despite the contributions that so-called animal models of depression have made to the understanding of the mechanisms of antidepressant action, they have remained imperfect representations of pathophysiology. Several animal tests for compounds with antidepressant activity are used (Cryan et al., 2002), and the forced swimming test is one of the most commonly employed, originally described and validated by Porsolt et al. (1977). Its relation to depression has been reviewed extensively (Willner, 1984; Borsini, 1995), and it became a screening test for (putative) antidepressant agents. However, other than studies investigating the impact of chronic stress and/or partial 5-HT depletion on rat behavior (Page et al., 1999; Harro et al., 2001), little information is available about the effect of hippocampal 5-HT denervation on possible changes in rat behavior in the forced swimming test in conjunction with adult hippocampal progenitor cell proliferation and neurogenesis.

Thus, the aim of this study was to examine, over a 3-week time period, the consequences of partial 5-HT denervation in the rat forebrain by *para*-chloroamphetamine (PCA) on rat behavior in the forced swimming test, and subsequently, on hippocampal progenitor cell proliferation. Single systemically administered PCA causes a rapid and selective degeneration of most of the 5-HT axons in the rat forebrain (Mamounas et al., 2000) through a mechanism proposed to involve toxic free-radical formation within nerve terminals (Colado et al., 1997). The extent of hippocampal 5-HT denervation was measured by immunohistochemical staining of 5HT transporter (SERT) which serves as an appropriate marker of 5-HT axons (Sur et al., 1996). Hippocampal progenitor cell proliferation was measured by Ki-67 immunohistochemistry described to be a suitable tool for determination of such cells (Kee et al., 2002).

2. Materials and methods

2.1. Materials and antibodies

Superfrost®-slides were supplied by Carl Roth AG (Karlsruhe, Germany), bovine serum albumin (BSA) of a grade suitable for immunohistochemistry came from Dianova (Hamburg, Germany), D,L-*para*-chloroamphetamine hydrochloride was supplied by Sigma (Taufkirchen, Germany), mowiol® came from Calbiochem-Novabiochem (Schwalbach, Germany) and the cryomedium Tissue-Tek® was from Sakura (Zoeterwoude, NL). Ketamin (Ketanest®) was purchased from Albrecht (Aulendorf, Germany) and Xylazin (Rompun®) from Bayer AG (Leverkusen, Germany). All other chemicals were purchased in highest quality from the usual suppliers.

Monoclonal anti-Ki-67 (clone B56) antibodies were supplied by BD Biosciences Pharmingen (Heidelberg, Germany) and monoclonal anti-SERT antibodies were supplied by Chemicon Int. (Hofheim, Germany). Secondary goat anti-mouse (GAM)-IgG conjugated with rhodamine red-X™ came from Dianova (Hamburg, Germany). For immunohistochemistry, primary and secondary antibodies were diluted in phosphate buffered saline (PBS) containing 0.1% (w/v) Triton X-100, 1% (w/v) BSA and 0.1% (w/v) sodium azide (NaN_3).

2.2. Animals and experimental design

Procedures involving animals and their care were conducted in conformity with institutional and European Union guidelines (EEC Council Directive 86/609), and were approved by the Ethical Committee of the Regional Council of Upper Swabia (Tübingen, Germany).

Adult male Sprague–Dawley rats (Charles River, Sulzfeld, Germany), weighing 200–250 g, were housed in groups of 4 with controlled temperature (22±1 °C) and a 12-h light–dark cycle (lights on at 6 a.m.). Standard rodent feed and tap water were available ad libitum. After arrival of animals, rats were left undisturbed for a minimum of 7 days in order to adjust to new environment until the beginning of experiment. Following this acclimatization period, rats were habituated to experimental handling for 3 days.

Rats were injected subcutaneously with PCA hydrochloride dissolved in saline on the same day (10 mg/kg free base, single injection 1 ml/kg); control rats received a single injection with saline. The entire study was performed according to a time course design. It consisted of 3 group pairs of control and PCA-treated rats (7–8 per group) which were subjected to the forced swimming test on days 6, 13, and 20 respectively, after PCA injection. Immediately following their last forced swimming period, rats were killed for brain analysis on days 7, 14, and 21 respectively, after PCA injection.

2.3. Forced swimming test

The test was performed between 2:30 and 4:30 p.m., and the method utilized was as described (Porsolt et al., 1977). The animals were individually forced to swim for 15 min in a cylinder (height: 40 cm; diameter: 18 cm) containing 18 cm of water at 25 °C (pre-test session). They were then removed and allowed to dry for 20 min in a cage placed below an infrared lamp. Twenty-four hours after the pre-test session, they were again placed in the cylinder for 5 min (test session), and their behavior videotaped. At the end of the test session, animals were killed for brain analysis.

The videotapes showing rat behavior during the 5-min test session were analyzed by an observer blind to the treatment, and duration of the following behavior was recorded: immobility=floating and making only those movements necessary to keep nose above water; swimming=when an animal exhibits active motion, i.e., moving around the tank including diving; climbing=when rats energetically move their forepaws in and out of the water, usually against the walls.

2.4. Preparation of brain tissue and immunofluorescence staining

Rats were anesthetized by intraperitoneal administration of a mixture of Ketamin (70 mg/kg) and Xylazin (6 mg/kg). After 10 min of transcardial perfusion with Ringer solution brains were immediately removed and cut sagittaly into two halves. The hemibrains were frozen in sagittal position in Tissue Tek® cryomedium on cork supports by immersion in isopentane precooled in liquid nitrogen and stored at −70 °C until use.

Frozen, TissueTek®-embedded hemibrains were sliced parasagittally in 12 μm thin sections (−18 °C chamber temperature; −15 °C object holder temperature) and removed from the knife by attachment to Superfrost® slides. After 1 h drying at room temperature (RT) and subsequent fixation in acetone/methanol (1:1, v/v) for 15 min at −20 °C, brain slices were dried at RT and then stored at −20 °C until use. After rehydration of frozen slices in PBS 3×5 min at RT, unspecific protein binding sites were blocked with PBS containing 3% (w/v) BSA, 0.1% (w/v) NaN_3 for 1 h at RT. Then, brain sections were directly incubated with anti-Ki-67 antibodies (1:200) or anti-SERT antibodies (1:1000) overnight at 4 °C. After 3×5 min washing with PBS, the slices were incubated with secondary antibodies (1:600) for 2 h at RT to visualize the binding sites of the primary antibodies. Then, brain slices were rinsed for 15 min in triple changes of PBS, subsequently washed with water and embedded in 0.2 mM Tris/HCl (pH 8.5) containing 12% (w/v) mowiol and 1.5% 1,4-diazoabicyclo[2.2.2]octane as anti-fading agent. Fluorescent signals were analyzed using a fluorescence microscope (Axioplan 2 Imaging, Carl Zeiss Göttingen, Göttingen, Germany) with a CCD camera (AxioCam s/w, Carl Zeiss Göttingen) and AxioVision 3.0 software to capture and manage the digitized images. Negative controls were performed by omitting the primary antibodies and did not show any signals.

For quantification of the proteins of interest in the hippocampus, consecutive slices from 0.9 mm to 1.2 mm lateral (according to Paxinos and Watson, 1998) were made, and 3 sections per animal with 24 μm space in between were used for immunohistochemistry. After digitizing the images, immunopositive SERT-fiber (=5-HT axon) density as well as Ki-67 immunopositive cells and cell clusters were automatically calculated in the region of interest (expressed in relation to mm^2) using Halcon imaging software (MVTech Software, München, Germany). The region of interest consisted of the SGZ and the hilus of the dentate gyrus (also see Figs. 1 and 2). A cell cluster was defined as an irregularly shaped object greater than or equal to 1.5 the average size of a single Ki-67-labelled cell as described previously for BrdU-labelled cell clusters (Bai et al., 2003). The mean of the 3 values derived from 3 slices per animal was used for further statistical analysis regarding group comparisons. Filtering criteria were applied to exclude small Ki-67-labelled particles (<1 μm^2) and linear immunostained (endothelial-like) objects as described previously (Parent et al., 1997).

2.5. Statistical analysis

Values were normally distributed and variances were homogenous. Therefore, quantitative data are presented as mean±S.E.M. Mean and S.E.M. were calculated from 7 to 8 animals per group for immunohistochemical analyses and the forced swimming test. Comparisons between control and stressed group as well as monitoring at different time points after PCA injection were all performed by two-way ANOVA followed by Tukey's test. Correlations shown are Pearson correlation coefficients. $P<0.05$ was considered significant. All statistical procedures were carried out using SigmaStat version 2.03.

3. Results

3.1. Forced swimming test in rats

Behavioral analysis of rats in the 5-min test session revealed no significant differences between vehicle and PCA-treated rats in any of the recorded parameters (Table 1). Looking at the time course after PCA injection, none of the behavioral parameters were significantly changed over the 3-week period. In addition, behavioral analysis of rats in the 15-min pre-test session revealed no significant differences between vehicle and PCA-treated rats in any of the recorded parameters and time points (not shown).

3.2. Analysis of serotonergic axon density in the hippocampus after PCA injection

By using SERT immunofluorescence staining, 5-HT axons are normally found in relatively high densities in the hilus of the dentate gyrus of the rat hippocampus which were severely decreased after a single subcutaneous administration (10 mg/kg) of PCA (Fig. 1A, B). Furthermore, a severe loss of 5-HT axons was also found throughout the forebrain including the neocortex after PCA administration (not shown). As shown in Fig. 1C, using quantitative analysis, a single PCA administration caused an immense reduction of 5-HT axon density in the region of interest

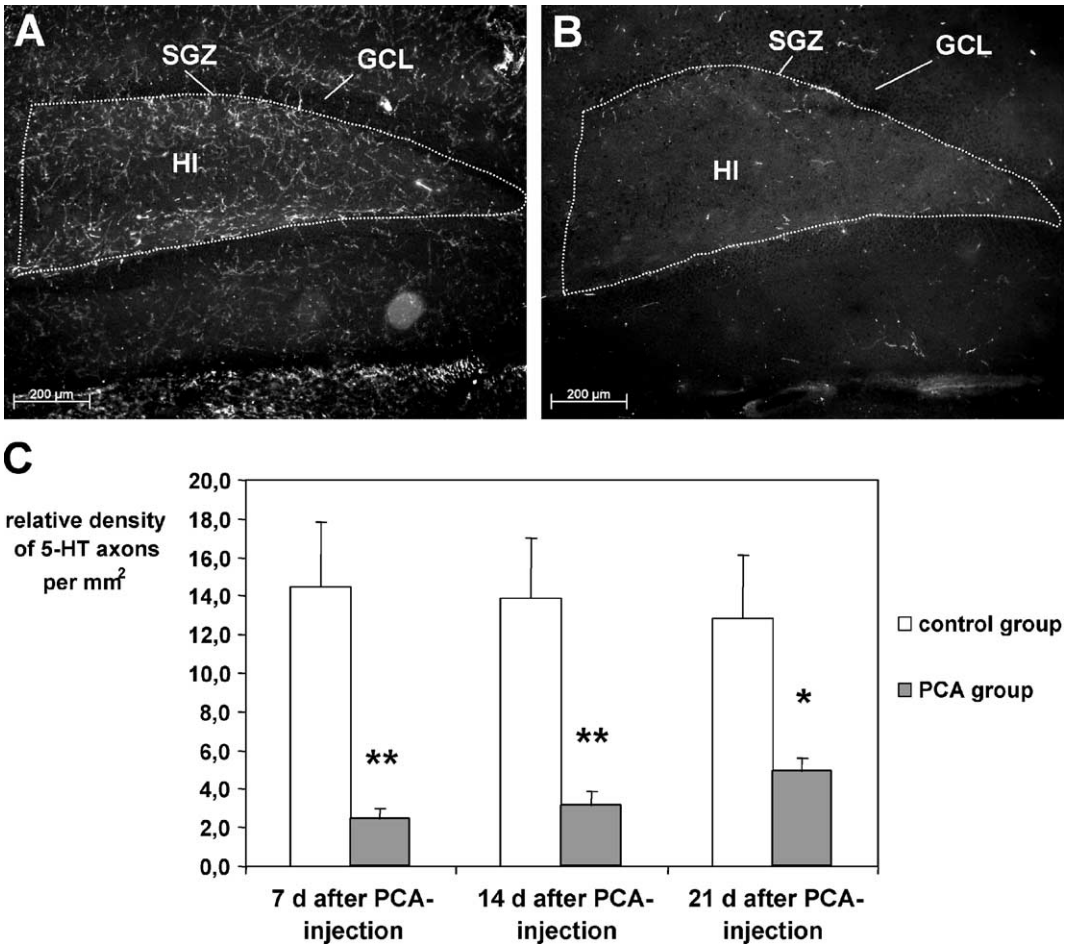

Fig. 1. Immunofluorescence staining of serotonin transporter (5HTT) as marker for 5HT-axons in a representative tissue section of the rat dentate gyrus of a control (A) and *para*-chloroamphetamine (PCA)-treated animal (B) 7 days after single PCA injection (10 mg/kg, s.c.). The area within the dotted curve represents the region of interest for quantification of 5HT-axons. (C) Quantification of 5HT axons in the region of interest 7, 14, and 21 days after single PCA injection. Data are presented as mean±S.E.M. of the relative density of 5-HT axons per mm^2, 7–8 animals per group. *P<0.05, **P<0.01 compared to respective control, two-way ANOVA followed by Tukey's test. SGZ—subgranular zone; GCL—granule cell layer; HI—hilus.

which consists of the granule cell layer and the hilus. Statistical analysis using two-way ANOVA revealed a significant effect of PCA teatment [$F(1,40)=27.67$, $P<0.001$]. Using post hoc analysis (Tukey's test), a significant reduction of 5-HT axon density was found 7 days after PCA injection (−83%, $P<0.01$) which is still significant after 14 days (−78%, $P<0.01$) and 21 days (−62%, $P<0.05$). Although not significant, 5-HT axon regeneration seemed to proceed gradually over the ensuing 3 weeks after a single PCA administration.

3.3. Immunofluorescence analysis of hippocampal Ki-67 expression after PCA injection

Ki-67 immunopositive cells reside in the so-called subgranular zone on the border between the granule cell layer and the hilus (Fig. 2). Some cells were also present in the hilus, the crest of the dentate gyrus, and throughout Ammon's horn (not shown) which was previously shown for the adult mouse hippocampus (Rietze et al., 2000). Consistent with previous reports using the BrdU labelling

Table 1
Effects of a single *para*-chloroamphetamine (PCA) dose (10 mg/kg, s.c.) on the time spent immobile, swimming, or climbing in the rat forced swimming test recorded at the 5-min test session

Parameter	7 days		14 days		21 days	
	Vehicle	PCA	Vehicle	PCA	Vehicle	PCA
Immobile [s]	243.9±9.4	240.6±7.9	242.9±12.8	215.5±16.5	243.5±11.7	236.1±19.5
Swimming [s]	27.8±5.6	30.6±7.0	35.3±11.4	41.6±9.5	37.4±10.5	18.4±8.6
Climbing [s]	26.3±8.4	25.4±4.1	22.0±5.5	39.5±11.7	18.3±4.9	44.8±18.6

The forced swimming test session was performed 7, 14, and 21 days after PCA injection using different group pairs (see Materials and Methods). Data are presented as mean±S.E.M., 7–8 animals per group.

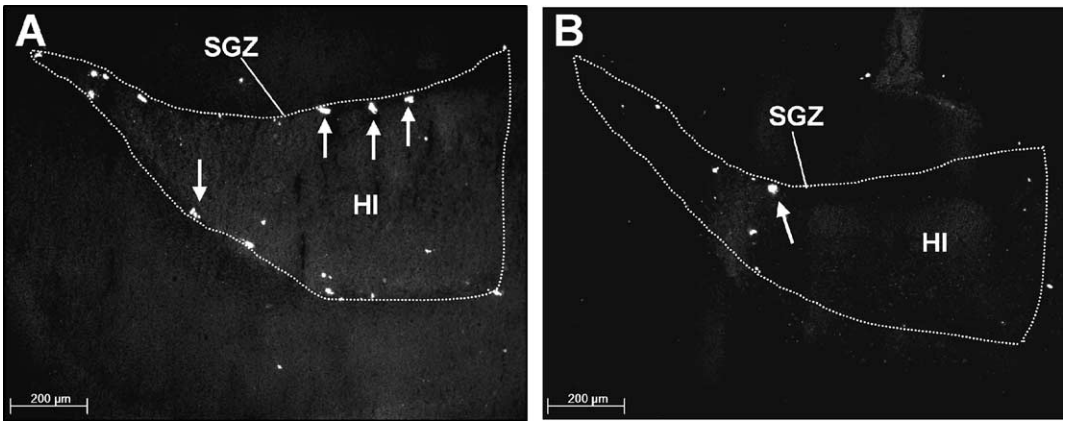

Fig. 2. Immunofluorescence staining of Ki-67 as proliferative marker of adult neurogenesis in a representative tissue section of the rat dentate gyrus of a control (A) and *para*-chloroamphetamine (PCA)-treated animal (B) 7 days after single PCA injection (10 mg/kg, s.c.). The area within the dotted curve represents the region of interest for quantification of Ki-67 immunopositive cells and cell clusters. Arrows show Ki-67 immunopositive cell clusters indicating newly formed cells. SGZ—subgranular zone; HI—hilus.

method (Parent et al., 1997; Bai et al., 2003), a small number of Ki-67 immunopositive cells in the SGZ were arranged in clusters indicating mitotically active progenitor cells which had just formed daughter cells (Fig. 2, arrows).

As shown in Fig. 3, using quantitative analysis, a single PCA administration (10 mg/kg, s.c.) caused a strong reduction of hippocampal progenitor cell proliferation as measured by counting Ki-67 immunopositive cells and cell clusters in the region of interest (granule cell layer and the hilus). Statistical analysis using two-way ANOVA revealed a significant effect of PCA teatment for both Ki-67 cell and cell clusters [$F(1,46)=7.56$, $P<0.01$; $F(1,46)=24.13$, $P<0.001$, respectively], and a significant interaction of treatment x days for Ki-67 cell clusters [$F(2,46)=3.27$, $P<0.05$]. Using post hoc analysis (Tukey's test), a significant decrease of Ki-67 cells (−25%, $P<0.05$) and Ki-67 cell clusters (−50%, $P<0.001$) was found 7 days after single administration of PCA. Regarding Ki-67 positive cell clusters, a significant suppression was still present 14 days after PCA injection (−43%, $P<0.001$). Post hoc analysis also revealed that there was significant recovery with regard to supression of Ki-67 cell clusters over the 3-week period after PCA administration ($P<0.05$).

Using the Pearson correlation, the number of Ki-67 immunopositive cells and cell clusters in the dentate gyrus per animal was significantly and positively correlated with the 5-HT axon density as calculated for the 7th-day time point ($P<0.05$, $r=0.653$, $r=0.580$, respectively) and tended to be significant for the number of Ki-67 cell clusters even 14 days after PCA injection ($P<0.07$, $r=0.543$). For the other time points, there were no significant correlations.

4. Discussion

In this study, we were able to show that partial 5-HT depletion in the adult rat brain by a single injection of PCA significantly reduces the proliferation of hippocampal progenitor cells as measured by counting Ki-67 immunopositive cells and cell clusters in the SGZ and hilus of the hippocampal dentate gyrus subfield. This result is in line with a recently published study showing decreased hippocampal cell proliferation after *para*-chlorophenylalanine treatment by using the BrdU labelling method (Banasr et al., 2004). In general, Ki-67 immunohistochemistry has been shown to be a suitable alternative to the BrdU labelling method (Kee et al., 2002; Rosenbrock et al., 2003; Heine et al., 2004) which requires a systemic injection of BrdU 1 day before killing the animal which may cause a disturbing effect on animal behavior in the forced swimming test. Ki-67 positive cells show mitotically active, single progenitor cells that, when clustered, give rise to immature daughter cells. These newborn cells are found in clusters because they have yet to migrate out into the granule cell layer. Thus, determination of Ki-67 cell clusters can be considered an index of proliferative activity of the SGZ as previously described (Bai et al., 2003). Since several studies demonstrated that the majority of these proliferating cells in the SGZ differentiate into neurons rather than glial cells (Malberg et al., 2000; Santarelli et al., 2003; Banasr et al., 2004), the reduced number of hippocampal Ki-67 positive cells and cell clusters also indicates a decreased hippocampal neurogenesis after partial 5-HT depletion. Furthermore, the extent of the reduction of Ki-67 cells and cell clusters in the dentate gyrus of the hippocampus correlates with the loss of SERT immunoreactivity which serves as a marker of 5-HT denervation or 5-HT depletion. In addition, over the examined 3-week period examined following a single PCA administration, there is a significant recovery in the reduction of progenitor cells, which seems to be paralleled by a recovery of hippocampal 5-HT axons corroborating recently published data (Brezun and Daszuta, 2000; Mamounas et al., 2000). Thus, our findings clearly indicate that 5-HT – in addition to other functions – contributes to the regulation of progenitor cell proliferation in the dentate gyrus. A possible mechanism underlying the

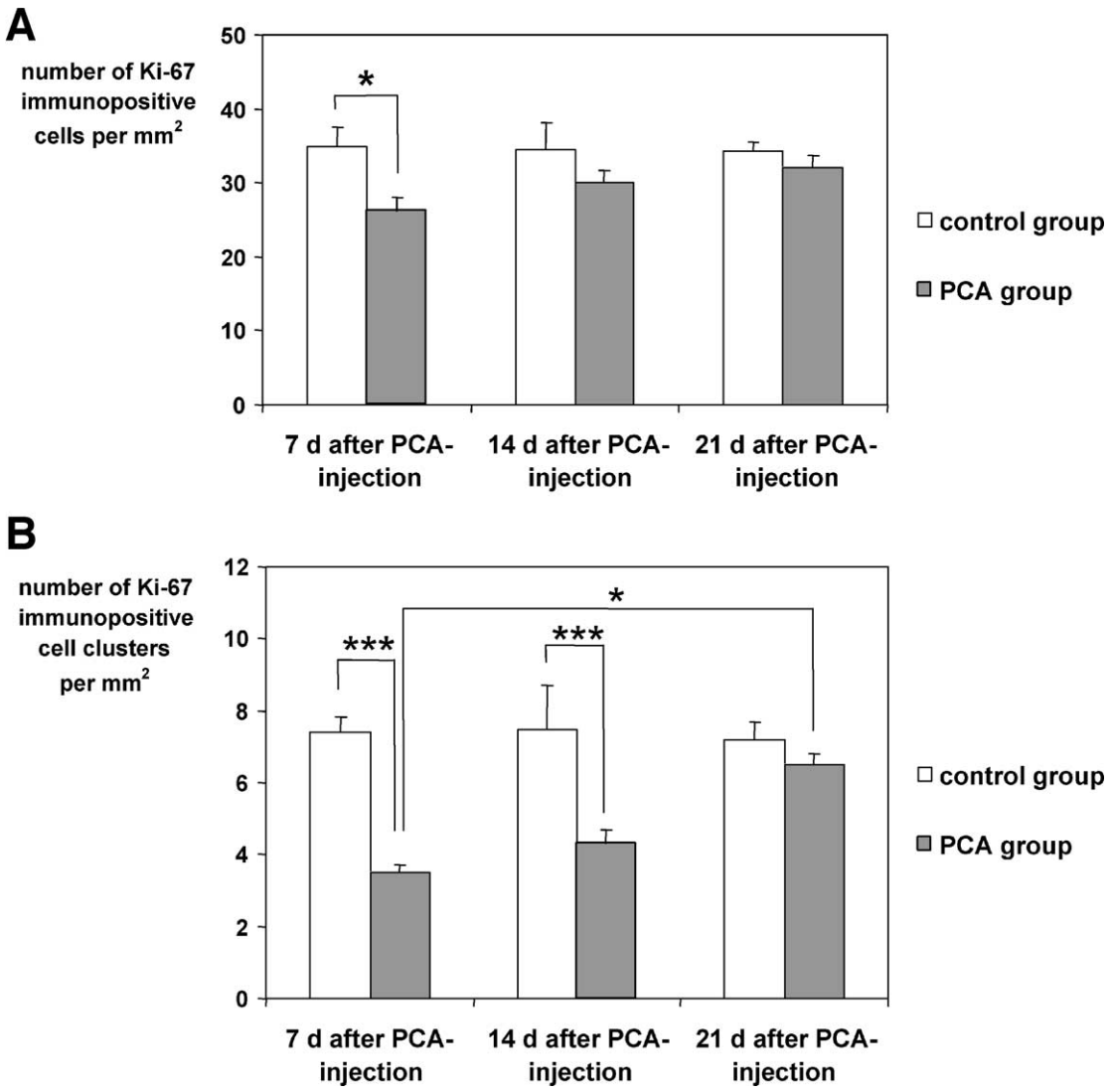

Fig. 3. Quantification of Ki-67 immunopositive cells (A) and cell clusters (B) in the region of interest described in Fig. 2 of control and *para*-chloroamphetamine (PCA)-treated rats 7, 14, and 21 days after a single PCA administration (10 mg/kg, s.c.). Data are presented as mean±S.E.M. of the number of cell or cell clusters per mm^2, 7–8 animals per group. *P<0.05, ***P<0.001, two-way ANOVA followed by Tukey's test.

influence of 5-HT on cell proliferation might be the activation of postsynaptic 5-HT receptors such as 5-HT1A receptors (Gould, 1999; Santarelli et al., 2003). Indeed, pharmacological studies showed that 5-HT1A receptor agonists, but not 5-HT1B or 2A or 2C receptor (ant)agonists increase hippocampal cell proliferation and neurogenesis (Banasr et al., 2004). All these data also point out that antidepressants affecting 5-HT transmission exert their effects in patients not only via normalization of 5-HT levels, but possibly also via normalization of impaired hippocampal neurogenesis, which was outlined as neurogenic hypothesis of the pathophysiology of depression (Jacobs et al., 2000; Duman et al., 2001; Kempermann, 2002). Clinical findings in depressed patients are in line with this hypothesis, showing that the days of unmedicated depressive episodes were significantly and inversely correlated with total hippocampal volume (Sheline et al., 2003). This could be interpreted as a result of decreased hippocampal neurogenesis, synaptic remodeling, or altered cell packing density. Nevertheless, this clinical study indicates at least a neuroprotective effect of antidepressants during treatment of depression.

In order to evaluate possible behavioral alterations of rats after partial 5-HT denervation of the forebrain, we subjected the animals to the forced swimming test before determining hippocampal progenitor cell proliferation. This test was chosen because it is the most widely used and also a fast test for detecting antidepressant activity of agents and for validating new targets regarding depression (Cryan et al., 2002). However, although it could be shown that partial 5-HT depletion causes progenitor cell suppression in the hippocampus, no behavioral changes of rats could be detected between the PCA- and saline-treated animals at any time point regardless of whether or not pre-test or test session analysis had been carried out. Interestingly, a decrease in immobility time was previously found after

chronic variable stress and/or partial 5-HT depletion only in the pre-test session, but the behavior of animals in the test session was not altered (Harro et al., 2001). Normally, the test session of the forced swimming test is used for behavioral analysis when searching for compounds with potential antidepressant activity. Even the role of the 5-HT system in the forced swimming test was questioned, since 5-HT reuptake inhibitors showed unreliable efficacy in this test (Borsini, 1995; Lucki, 1997; Cryan et al., 2002). Another possibility in explaining the lack of behavioral changes after PCA treatment may be the compensatory mechanisms of progenitor cell survival in response to initial changes in proliferation. In addition, the partial 5-HT lesion caused by PCA might not be severe enough to evoke behavioral changes, or in general, the baseline immobility of rats is too high to detect a further increase which would indicate depressive-like behavior. However, the latter idea seems to be unlikely, since increased immobility in the forced swimming test can be observed in principle (Schramm et al., 2001; Bonilla-Jaime et al., 2003). Taken together, our results point out that the forced swimming test – despite its predictivity in finding antidepressants – is not an adequate test for investigating the impact of hippocampal progenitor cell proliferation on the pathophysiology of depression. Its impact on so-called animal models of depression is in fact questionable, because, as in the forced swimming test, animal behavior in the learned helplessness model was not correlated with alterations of hippocampal cell proliferation (Vollmayr et al., 2003) whereas in the chronic mild stress model a correlation could be found (Alonso et al., 2004).

In summary, this study has provided further evidence that partial 5-HT denervation of the adult rat forebrain by a single PCA administration causes a suppression of hippocampal progenitor cell proliferation which starts to recover over the examined 3-week time period examined. However, 5-HT depletion together with decreased hippocampal progenitor cells did not produce behavioral alterations of rats in the forced swimming test. Therefore, the impact of adult neurogenesis on experimental animal models of depression remains to be elucidated.

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