

Reduction of the number of new cells reaching olfactory bulbs impairs olfactory perception in the adult opossum

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In adult mammals cells generated in the subventricular zone (SVZ) migrate to olfactory bulbs (OB). Functional significance of this continuous neurogenesis is not clear. We injected opossums (*Monodelphis domestica*) for seven consecutive days with a 5HT_{1A} agonist (8-OH-DPAT or buspirone), or its antagonist WAY100635. One hour after each of these injections bromodeoxyuridine (BrdU), a marker of dividing cells was also injected. Two months later, when newly generated neurons settled in the OB and matured the ability of these opossums to detect hidden food by olfactory cues was tested. Afterwards, numbers of BrdU-labeled cell nuclei in their OB were counted and a phenotype of labeled cells established. In all groups investigated the majority of new cells differentiated into neurons (55–76%) and a lower proportion into astroglia (6–12%). Numbers of BrdU-labeled cells differed depending on the applied treatment: both agonists of the 5HT_{1A} receptor increased these numbers, while its antagonist decreased them. The increased number of new OB interneurons did not change the time required for finding all three food items and therefore did not improve the opossums' performance in this test of the olfactory perception. However, opossums that had the reduced number of new generated OB cells searched longer for each food item and in consequence took three times longer to find all three crickets, than did opossums from other groups. In conclusion, lower numbers of new neurons in the opossums OB correlated with their worse behavioral performance in a test based on olfactory perception.

Key words: neurogenesis, 5-HT_{1A}, buspirone, 8-OH-DPAT, WAY100635, olfactory perception, *Monodelphis*

INTRODUCTION

Olfactory perception and discrimination are important elements of animal activities such as searching for food and mates or avoiding predators. Moreover, the olfactory memory contributes importantly to the memory of places and situations that are emotionally labeled (Savic 2002, Gottfried et al. 2004). Olfactory bulbs (OB) receive information about various odors *via* the olfactory nerve and send their projections by the olfactory tracts to other structures of the olfactory system (Martinez-Marcos and Halpern 2006, Mandaïron and Linster 2009). In the OB inputs from various types of receptors (over 1000) are segregated in such way that

each OB glomerulus receives input from only one type of olfactory receptor. These receptors have relatively low specificity for various odorous compounds and each reacts to a large spectrum of compounds. But transmission of information from axons of olfactory receptors to dendrites of mitral cells is selectively modulated by the periglomerular interneurons (GABAergic and dopaminergic) that form an inhibitory network around glomeruli. Mitral cell bodies and lower dendrites are under inhibitory influences of another inhibitory network, that of GABAergic granular cells. This two-layered lateral inhibition enhances discrimination between odors and sensitivity of odor detection. Therefore, information sent by mitral cells to the olfactory cortex is to a large extent filtered, enhanced and made more specific by the activity of OB interneurons that are constantly exchanged throughout life (Zou et al. 2001, Ache and Young 2005, Zou and Buck 2005).

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Mammals (as all vertebrates) throughout life constantly add new and remove old OB interneurons (Doetsch et al. 1999, Tramontin et al. 2003). It is postulated that this turnover allows for increased plasticity of the olfactory system and its easier adaptation to changes in composition of olfactory stimuli (Nissant et al. 2009). In mammals only two brain structures (OB and the hippocampal dentate gyrus, DG) have this unique property of being constantly remodeled by neurogenesis and apoptosis. New cells that are finally becoming the OB interneurons are generated in the subventricular zone (SVZ) of the lateral ventricles from where they migrate tangentially along the rostral migratory stream (RMS) to the OB (Altman and Das 1965, Luskin 1993, Alvarez-Buylla and Garcia-Verdugo 2002, Grabiec et al. 2009). The majority of newly generated OB cells differentiate into neurons. They settle in the granule cell layer or the periglomerular layer, becoming interneurons that are using as their neurotransmitter either GABA (the majority) or dopamine (Carleton et al. 2003, Bolteus and Bordey 2004). They are incorporated into existing neural circuits (Belluzzi et al. 2003, Lledo and Lagier 2006, Gheusi and Lledo 2007). It has been postulated that newly generated OB cells allow for remodeling of the mechanism of discrimination among various odor compounds and for extraction of the significant ones from their mixture. In several studies on eutherian mammals the number of newly generated cells was correlated with their olfactory behavior (Rocheffort et al. 2002, Seiberling and Conley 2004, Alonso et al. 2006, Mandairon et al. 2006). Another hypothesis claims that newly generated neurons may allow for creation of new olfactory memories required in maternal behavior because the rate of neurogenesis in adult female mice increases during pregnancy mediated by prolactin hormones (Shingo et al. 2003). Several other factors including the level of serotonin and pattern of expression of its receptors can also modulate the rate of neurogenesis in the adult brain. Activation of 5-HT_{1A} heteroreceptors produces elevation in the rate of neurogenesis in both SVZ and DG regions, while 5-HT_{2A} and 5-HT_{2C} receptors selectively regulate cell proliferation in the DG and SVZ respectively (Gould 1999, Radley and Jacobs 2002, Banasr et al. 2004).

So far neurogenesis has been studied in only two species of adult marsupials (Harman et al. 2003, Grabiec et al. 2009). Harman and colleagues (2003) found that no new cells were incorporated into OB of

the adult fat-tailed dunnart one month after ³H thymidine injection. Our research on the adult opossum *Monodelphis domestica* showed that the process of neurogenesis continues in their SVZ throughout life and new cells are constantly added to their OB. The rate of neurogenesis in the *Monodelphis* opossum decreased with age to a lesser degree than in rodents but more than in shrews (Bartkowska et al. 2008). Injections of buspirone, a partial agonist of the serotonergic 5-HT_{1A} receptor increased the rate of neurogenesis in brains of both young and old opossums (Grabiec et al. 2009).

Here we report the results of injection of opossums with two different 5-HT_{1A} agonists or an antagonist and testing their olfactory abilities two months later.

METHODS

Animals

Twenty four adult (14–17 months old) opossums from the Nencki Institute colony were used in this experiment. Animals were housed individually in standard cages under 14/10 hours light-dark cycle, with constant temperature (25–27°C). Opossums were experimentally naive at the beginning of the study and the behavioral training was carried out during the light phase of light-dark cycle. All efforts were made to minimize the number of animals and their stress. Our experimental procedures complied with the Polish Law on Experimentation on Animals that implements the European Council Directive of 24 November 1986 (86/609/EEC) and also as with the NIH Guide for the Care and Use of Laboratory Animals. The experiments were approved and controlled by a Local Ethics Committee in Warsaw.

Pharmacological treatment of opossums

Opossums were divided into four groups each composed of 6 animals of both sexes (3 males, 3 females). The first group was injected intraperitoneally with 0.3 mg/kg 5-HT_{1A} receptor agonist 8-OH-DPAT (Sigma-Aldrich, Germany). The second group was treated with 3.0 mg/kg of the partial 5-HT_{1A} agonist, buspirone (Sigma-Aldrich, Germany). The third group of opossums was injected with 0.3 mg/kg of the 5-HT_{1A} antagonist, WAY100635 (Sigma-Aldrich, Germany), whereas the fourth group was injected i.p. with saline

to serve as control. One hour after these injections all opossums were injected with bromodeoxyuridine (BrdU, Sigma-Aldrich, Germany, 75 mg/kg), a marker of newly generated cells. All injections were performed once daily for 7 consecutive days.

Test of food detection with olfactory cues

This behavioral test was performed during the eight weeks after the last injection. Three days before the test all animals were deprived of food overnight. During the test session opossums were placed in a rat cage ($45 \times 30 \times 40$ cm) filled with a 3 cm thick layer of fresh sawdust. Three freshly killed crickets were hidden at random places under the saw dust, without any external cues (Fig. 1). Opossums were placed in the box and left there for a maximum of 15 minutes. The situation was new to them and the test was applied only once to each animal. During the test opossums were free to act as they wish and if they found a cricket they could eat it. The test ended if an opossum found and ate all three crickets before the end of time limit. All tests were videotaped. We measured and analyzed the time to find each cricket, time of eating the crickets and time to finish the whole task. As the time of eating the crickets did not differ significantly between groups, in further analyses it was subtracted from the time to location of the third cricket.

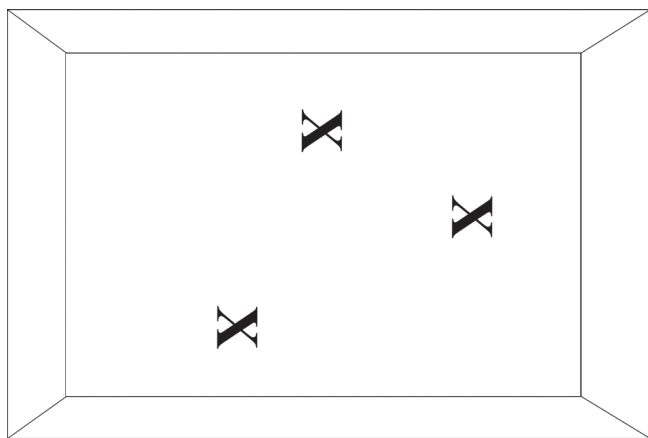


Fig. 1. Top view of the experimental box for testing olfactory perception of opossums. (X) Place where the freshly killed crickets was under 3 cm of fresh sawdust. At the beginning of testing opossums were placed in the middle of the box that they had not known before. In their home cages opossums were given crickets once or twice a month, so they knew their smell. Crickets are one of opossums' most liked foods.

Tissue processing

One day after finishing the behavioral experiments all opossums were deeply anesthetized with intraperitoneal injections of 150 mg/kg of thiopental (Biochemie) and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion brains were removed, postfixed for two weeks in the perfusion solution and then immersed in 30% sucrose for three days before sectioning into 40 μ m sections on a cryostat. All sections were collected into ten parallel series that were used for various purposes.

Immunohistochemistry

For BrdU immunohistochemistry DNA denaturation was conducted by incubation of free-floating sections for 30 min in 2N HCl at 37°C and then for 10 min in 0.1 M boric acid (pH 8.5), followed by washing with concentrated saline-sodium citrate (SSC). After further washing in the Tris-buffer saline (TBS; Tris 0.1 M, NaCl 0.150 mM, pH 7.3) sections were incubated for 30 min in 3% H_2O_2 to inactivate endogenous peroxidases. Sections were then blocked with 10% normal goat serum in TBS with 0.1% Triton X-100 (TBS-A) and TBS-A with 0.05% bovine serum albumin (TBS-B) for 60 min. Afterwards they were incubated with the rat anti-BrdU (1:1000; WAK Chemie Medical) overnight at 4°C. Then sections were rinsed in TBS-A and TBS-B followed by incubation with the secondary antibody (anti-rat Vector Laboratories, 1:200) for 45 min. The next step was incubation of sections with the extravidin complex (1:200; Sigma-Aldrich, Germany) for 40 min. BrdU-positive nuclei were visualized with diaminobenzidine (DAB kit; Vector Laboratories). Finally, after rinsing in PBS, sections were mounted on gelatinized slides, air dried, dehydrated and coverslipped with DPX (Serva, Heidelberg, Germany). Control sections taken from another series were processed in the same way, but either without the primary or secondary antibody, or both. Labeled cell nuclei were absent in all control stainings.

Double immunofluorescent stainings for BrdU and neuronal nuclei (NeuN) as a neuronal marker or BrdU and glial fibrillary acidic protein (GFAP) as an astroglial marker were performed on different series of

stions from the same brains. BrdU labeling was performed as above except that labeled nuclei were visualized with the anti-rat antibody conjugated with Alexa Fluor 488 (1:200; Molecular Probes), while presence of GFAP (rabbit, 1:500, DACO, Glostrup, Denmark) and NeuN (mouse, 1:200; Chemicon International, Temecula, CA, USA) was revealed with the anti-rabbit and anti-mouse antibodies conjugated with the Alexa Fluor 568 (1:200; Molecular Probes), respectively.

Data analysis

BrdU-labeled cells were counted in a series of every tenth section of each brain. The labeled cell nuclei were observed with the light microscope (Nikon) under 20× and 40× objectives. Only labeled nuclei with the diameter larger than about 2 μm were counted to avoid counting artifacts. To establish total numbers of the BrdU-positive cell nuclei, labeled nuclei in the SVZ and rostral migratory streams (RMS) of both hemispheres were pooled together. BrdU-labeled cells in two OBs were counted separately from the SVZ/RMS cells. Total counts for a structure were then multiplied by 10 and the product was taken as the number of labeled nuclei contained in the whole brain structure. Numbers of labeled cells given in the text are averages ±SD for each group. Statistical significance of differences between groups was evaluated with Student's *t*-test (two-tailed, two samples of unequal variance) or one-way ANOVA, using Excel Software. The level of significance of all statistical tests was set at $P < 0.05$.

Colocalization of BrdU with NeuN or GFAP was examined with the Leica TCS SP2 confocal laser scanning microscopy system. Fifty cells with BrdU-labeled nuclei from the OB of 3 opossums from each of the experimental groups were analyzed and then percentages of colocalization of BrdU with the neuronal or astrocytic markers were calculated.

Measurements of time required for location of the hidden crickets and time of eating them were performed with the ViewPoint software (ViewPoint Life Sciences, Inc, France). Data for each group were expressed as mean values ±SEM. Significance of differences between measured times was analysed with the Kruskal-Wallis test, followed by Student-Newman-Keuls *post hoc* test.

RESULTS

Influence of agonists and antagonist of the 5HT_{1A} receptor on the number and phenotype of cells incorporated into OB

Numbers of new cells (i.e. with cell nuclei containing BrdU) that were incorporated into OB were counted on immunohistochemically labeled slices. Cells generated two months earlier (at the time of BrdU injections) were found in all sections of brains containing SVZ/RMS or OB of all groups of animals. Large numbers of these cells were scattered throughout the granular and periglomerular layers of the OBs. One week long-lasting treatment of opossums with 5-HT_{1A} receptor agonists (buspirone or 8-OH-DPAT) or its antagonist (WAY100635) did not change numbers of BrdU labeled cells found two months later in the SVZ/RMS (Fig. 2A). All groups of opossums had almost the same numbers of these cells. However, numbers of BrdU-labeled cells placed in the OB were markedly higher in opossums treated with agonists of the 5-HT_{1A} receptor ($F_{2,13}=9.711$; $P < 0.001$). The average number of BrdU-labeled cells in the OB of the control group was $4\,872 \pm 589$, while in the buspirone-treated group that number was twice higher ($10\,990 \pm 2\,385$). A comparable increase (about 65%) in the rate of proliferation was also observed in the OB of opossums treated with another agonist of the 5-HT_{1A} receptor, the 8-OH-DPAT. In contrary, injections of the antagonist of 5-HT_{1A} receptor reduced numbers of BrdU-labeled OB cells to $2\,955 (\pm 868)$ i.e. by 29% in comparison to the control group ($F_{1,9}=4.871$; $P < 0.01$ Fig. 2B).

To determine the phenotype of new proliferated cells in the adult brain of opossums 50 double-labeled BrdU and NeuN cells or 50 double labeled BrdU and GFAP cells in the OB from each group were analysed by confocal microscopy (Fig. 3). In all four experimental groups a large majority of newly generated OB cells (from 55% to 76%, no significant differences among groups) showed the neuronal phenotype (Fig. 4), while 6–12% (differences n.s.) became astrocytes (Fig. 4). Phenotype of the remaining fraction of newly generated cells has not been determined.

Olfactory-guided behavior of the opossums

All opossums examined found all three crickets hidden in the saw dust by olfactory cues, dug them out and

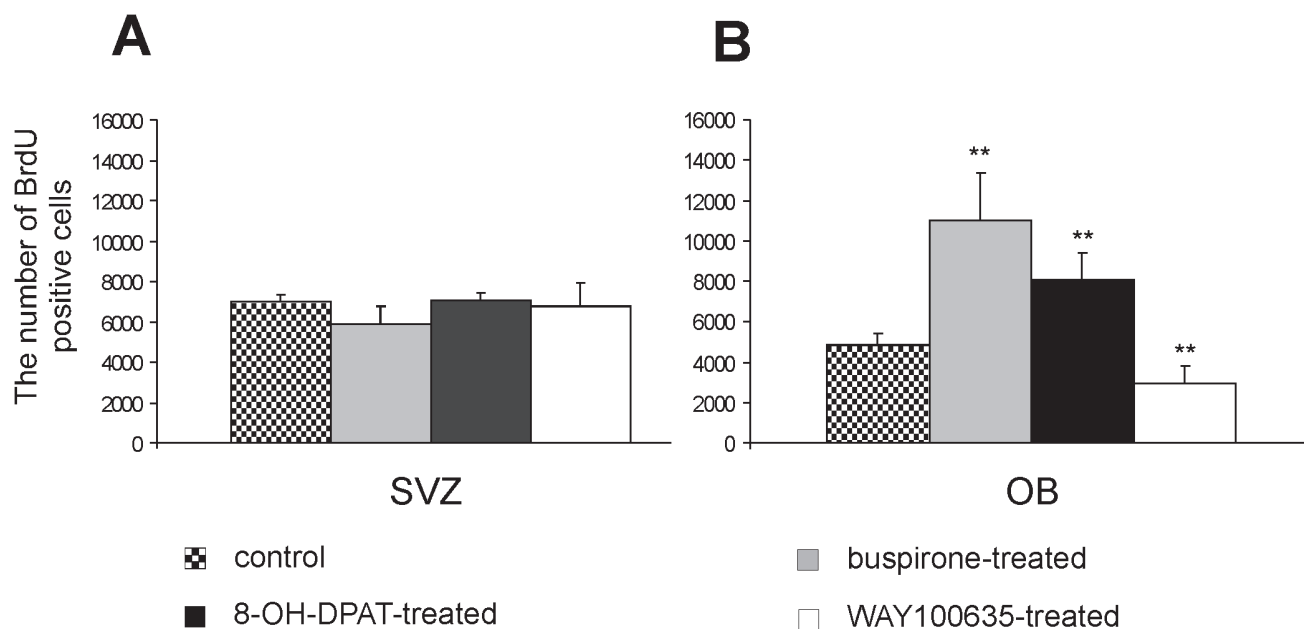


Fig. 2. Numbers (mean \pm SEM) of BrdU-positive cell nuclei in subventricular zones (SVZ) of lateral ventricles and olfactory bulbs (OB) of opossums belonging to different groups. Two months after treatment numbers of labeled nuclei in the SVZ in the groups investigated did not differ but all treatments had a significant effect on the number of BrdU labeled nuclei in the OB. Double asterisks show the significance level of $P < 0.01$.

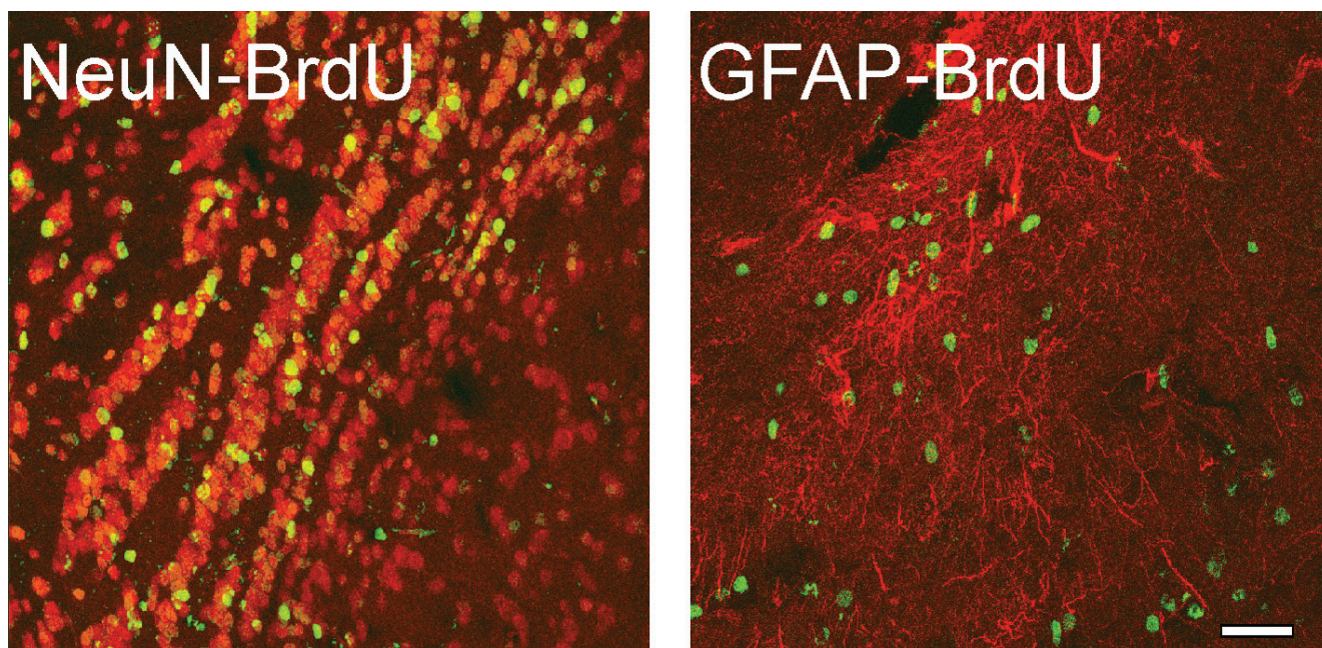


Fig. 3. Identification of phenotypes of newly generated cells in the olfactory bulb (OB). (A) Merged confocal images showing labeling for BrdU (green) and NeuN (red); (B) merged confocal images showing labeling for BrdU (green) and GFAP (red). Scale bar equals to 50 μ m.

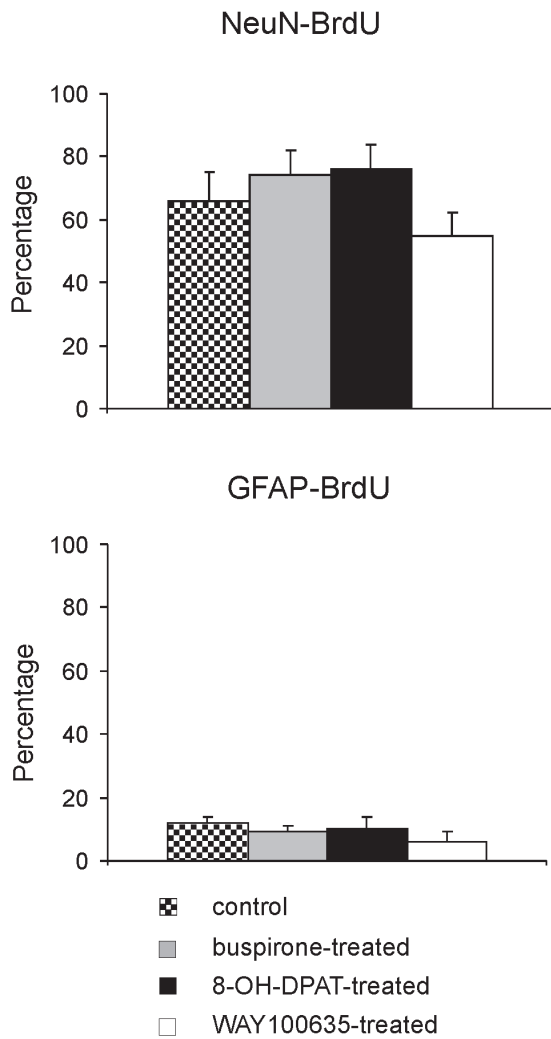


Fig. 4. Proportions of cells double-labeled for BrdU and NeuN (neurons) or BrdU and GFAP (astroglial cells) among BrdU-labeled cells in OB. Note that the two-month earlier treatment did not change proportions of neurons and glia in different groups of animals.

ate them before the end of session (15 min). There were no significant differences in the time used to finish the task between the control group and opossums treated with agonists of the 5-HT_{1A} receptor (buspirone or 8-OH-DPAT, Fig. 5). However, animals treated with the 5-HT_{1A} antagonist WAY100635 used significantly more time to locate the hidden food. Eating the first cricket took the control animals on average 64 s and the 5-HT_{1A} antagonist treated group 44 s on average (n.s.). Afterwards opossums started to look for the second cricket. This searching lasted for 22 ± 7 s in the control group and more than three times longer (77 ± 49 s) in the group treated with the 5-HT_{1A} antagonist. Eating the

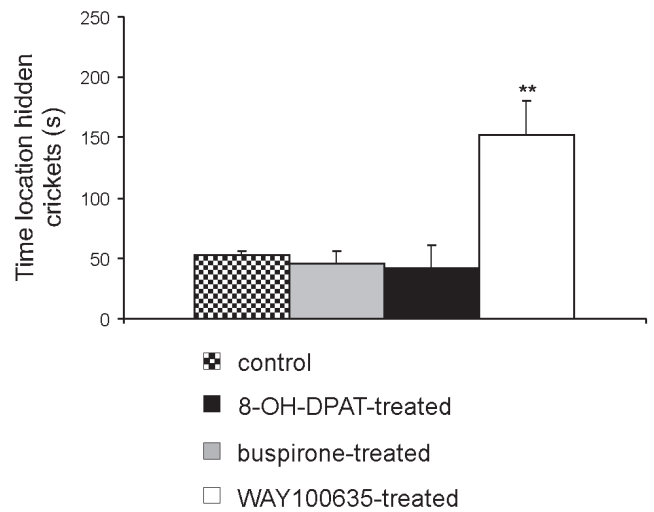


Fig. 5. Average time in seconds (mean ± SEM) required for location of all three hidden crickets by opossums from different groups. Note that injections of the agonists of 5-HT_{1A} receptor that increased the rate of neurogenesis and the numbers of new neurons in the OB did not influence that time, while injections of the antagonist of 5-HT_{1A} receptor that reduced these numbers resulted in a marked increase in the time of searching for the hidden food. Double asterisks show the significance level of $P < 0.01$.

second cricket took opossums almost the same time as the first one. Then they immediately started searching for the next morsel of food. After deduction of the time used for eating the uncovered crickets, we found that the control group of opossums was searching for all three hidden crickets on average for 63 ± 27 s, whereas opossums treated with WAY 100635 took at least three times longer to finish the task (202 ± 104 s). The difference is statistically significant ($P < 0.01$, Fig. 5).

DISCUSSION

Results of this study confirm that the Monodelphis opossum, like the majority of eutherians but in contrast to the only other investigated marsupial species (dunnart) incorporates new neurons into its OB throughout life (Harman et al. 2003). We found for the first time that treatment of opossums with agonists of the 5-HT_{1A} receptor (buspirone or 8-OH-DPAT) increases numbers of new neurons that are incorporated two months later into their OB, while administration of an antagonist of that receptor decreases these numbers. Specially we found that increased numbers of new neurons incorporated into the opossum's

OB did not improve their performance in a behavioral test requiring detection of food with use of the olfactory perception. However, reduction of numbers of newly generated mature OB interneurons resulted in threefold elongation of the time required for detection of the hidden food. Therefore, we found a correlation between decreased numbers of newly generated cells in the OB and worse olfactory perception of the opossums shown two months after the reduction of neurogenesis.

In many species of eutherians the rate of proliferation in neurogenic regions of the brain is regulated by the level of serotonin, acting *via* its 5-HT₁ and 5-HT₂ receptors (Banasr et al. 2004, Malberg and Duman 2003, Miller et al. 2008, Gardier 2009). It has been shown that chronic stress produces atrophy of hippocampal neurons due to action of glucocorticoids that are also known to reduce the rate of neurogenesis in the DG (Nichols et al. 2005, Bridges et al. 2008, Boku et al. 2009). On the other hand, an increased level of serotonin in the brain increases the rate of cell divisions in the SVZ and numbers of neurons settling in the OB. Recently we have demonstrated that buspirone, a partial agonist of 5-HT_{1A} receptors stimulates cell divisions in the DG of *Sorex* shrews that at that time (after sexual maturation) do not generate new cells in the DG (Bartkowska et al. 2008). Therefore, the mechanism of serotonergic regulation of the rate of adult neurogenesis seems to be common for marsupials and eutherians.

After divisions of the precursor cells neuroblasts migrate from the SVZ along the RMS to reach the OB, where they differentiate into granular and periglomerular interneurons, replacing older interneurons that die out there. All mammalian species investigated, including marsupials, have the same pattern of SVZ divisions, migration, differentiation of the majority of new cells into neurons, their settlement in the OB as its interneurons and their turnover (Imayoshi et al. 2009). However, mammalian species differ in the rate of those processes. Winner and others (2002) observed in the rat that four weeks after BrdU injection almost all labeled cells finished their migration from the SVZ to OB. In our previous paper we found that four weeks after the last BrdU injection only half of the newly generated cells were found in the OB of opossums, while the other half of cells were still placed in the SVZ (Grabiec et al. 2009). We postulated that the reason for this difference with the rat may be in the gener-

ally slow rate of migration of newly generated cells, which could be associated with the slow rate of brain development in the Monodelphis and marsupials in general (Saunders et al. 1989, Molnar et al. 1998, Ashwell et al. 2004, Karlen and Krubitzer 2007). In this research we found that two months after the last injection of BrdU almost as many BrdU-labeled cells were found in SVZ as in the OB. One possible explanation may be that cells still remaining in the SVZ are the progenitor cells that are permanently placed in the SVZ. Proportion of neurons in the population of new cells was very high in the Monodelphis opossum in comparison to rodents.

Lack of the effect of increased rate of neurogenesis could have been caused by the relative ease of the task of finding three hidden crickets, as all opossums finished it before the time limit. Therefore, if the foraging task would have been made more complicated, for example by hiding also some odoriferous but non-edible objects, the behavioural superiority of opossums with increased neurogenesis might have been evident. The second possible explanation is that there is a limit to the olfactory performance and the natural rate of neurogenesis is optimal. This does not seem very probable in the light of the higher rate of neurogenesis in subadults that still learn to discriminate olfactory stimuli. The third possibility is that the more numerous age cohort of the OB interneurons that had to be incorporated into the OB evoked more intense wave of apoptosis of older interneurons and therefore the surplus wave has been absorbed and did not increase numbers of the OB interneurons, changing however their age proportions. However, in that case reduction of the rate of neurogenesis should have reduced the rate of cell death and therefore also stabilized the number of interneurons and olfactory perception.

The way the test was conducted excluded a possible influence of the increased or reduced neurogenesis in the DG. Our opossums were naïve and were run in only one trial lasting a maximum of 15 minutes. They were hungry but they did not know what to expect in the empty experimental cage. Therefore the first time they picked up the smell of a cricket was accidental and they had very little opportunity to learn the task later. In these conditions they expressed their natural behavior of searching in an unknown environment. Opossums have a much stronger tendency for that activity than rats (Wesierska et al. 2003).

CONCLUSION

Temporal reduction of the rate of neurogenesis in SVS of the *Monodelphis* opossums, resulting two months later in reduction of the number of new OB neurons, lowered the effectiveness of opossums in searching for food by olfactory cues. Increased rate of neurogenesis did not improve the performance of opossums, probably because of the “ceiling effect” caused by a too simple task.

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