NEURAL REGENERATION RESEARCH
February 2014, Volume 9, Issue 3
www.nrronline.org

Special function of nestin$^+$ neurons in the medial septum-diagonal band of Broca in adult rats

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doi:10.4103/1673-5374.128229
http://www.nrronline.org/

Accepted: 2013-11-25

Abstract

Nestin$^+$ neurons have been shown to express choline acetyltransferase (ChAT) in the medial septum-diagonal band of Broca in adult rats. This study explored the projection of nestin$^+$ neurons to the olfactory bulb and the time course of nestin$^+$ neurons in the medial septum-diagonal band of Broca in adult rats during injury recovery after olfactory nerve transection. This study observed that all nestin$^+$ neurons were double-labeled with ChAT in the medial septum-diagonal band of Broca. Approximately 53.6% of nestin$^+$ neurons were projected to the olfactory bulb and co-labeled with fast blue. A large number of nestin$^+$ neurons were not present in each region of the medial septum-diagonal band of Broca. Nestin$^+$ neurons in the medial septum and vertical limb of the diagonal band of Broca showed obvious compensatory function. The number of nestin$^+$ neurons decreased to a minimum later than nestin$^-$/ChAT$^+$ neurons in the medial septum-diagonal band of Broca. The results suggest that nestin$^+$ cholinergic neurons may have a closer connection to olfactory bulb neurons. Nestin$^+$ cholinergic neurons may have a stronger tolerance to injury than Nestin$^-$/ChAT$^+$ neurons. The difference between nestin$^+$ and nestin$^-$/ChAT$^+$ neurons during the recovery process requires further investigations.

Key Words: nerve regeneration; nestin; choline acetyl transferase; neurotransmitter; medial septum-diagonal band of Broca; olfactory bulb; olfactory nerve transection; the Guangdong Natural Science Foundation of China; neural regeneration

Funding: This study was supported by the Guangdong Natural Science Foundation of China, No. S2011040004372; the Fundamental Research Funds for the Central Universities, No. 11ykpy05.

Zhao YH, Guo KH, Li DP, Yuan QF, Yao ZB. Special function of nestin$^+$ neurons in the medial septum-diagonal band of Broca in adult rats. Neural Regen Res. 2014;9(3):308-317.

Introduction

Nestin is a cytoskeletal intermediate filament protein which belongs to the sixth class of intermediate filament proteins. Nestin was first discovered transiently expressed in neural progenitor cells. Upon differentiation, nestin becomes downregulated and is replaced by other specific intermediate filament proteins. Nestin is also expressed in reactive mature glial cells[1-4]. The expression of nestin in these cells was associated with their differentiation or sustenance of their active state, and may be implicated in neurogenesis, remodeling and repair of the adult central nervous system[5-9]. Although nestin always appears during particular periods of cell activation, the relationship between the expression of nestin and activation of cell specific function is not clear. Studies on the effects of nestin on neural cells could further explain the internal relationship between the structure and function of neural cells. However, the expression of nestin in these cells is transient, so it is difficult to further disclose the role of nestin on the physiological function of neural cells.

The medial septum-diagonal band of Broca is the richest region where cholinergic neurons exist in the basal forebrain[10-11]. The degeneration of cholinergic neurons in the medial septum-diagonal band of Broca is strongly associated with neurodegenerative disorder[12-13]. Memory and cognitive deficits exist in these disorders, and are usually associated with the loss of cholinergic neurons[14-15], but the amount of cholinergic loss does not correlate with the memory and cognitive deficits[16]. Further studies are needed to disclose the internal relationship between function of cholinergic neurons and cognitive and memory functions.

During studies of neurodegenerative diseases, our team was the first to find a cluster of nestin-positive neurons in the medial septum-diagonal band of Broca of normal adult rats. The nestin-positive neurons were also found by our team in the medial septum-diagonal band of Broca of adult human beings[17-19]. Using immunofluorescence and single cell-reverse transcription-PCR, these nestin-positive neurons were confirmed to express choline acetyltransferase (ChAT). Patch clamp whole cell recording also revealed that nestin-positive neurons had wide action potentials and large after hyperpolarization potentials, and that membrane properties were similar to nestin$^-$/ChAT$^+$ neurons in the medial septum-diagonal band of Broca. Nevertheless, these neurons had higher hyperpolarization-activated cation currents (*Ih*), which were different to nestin$^-$/ChAT$^+$ neurons in the medial septum-diagonal band of Broca[19-20]. Our previous studies

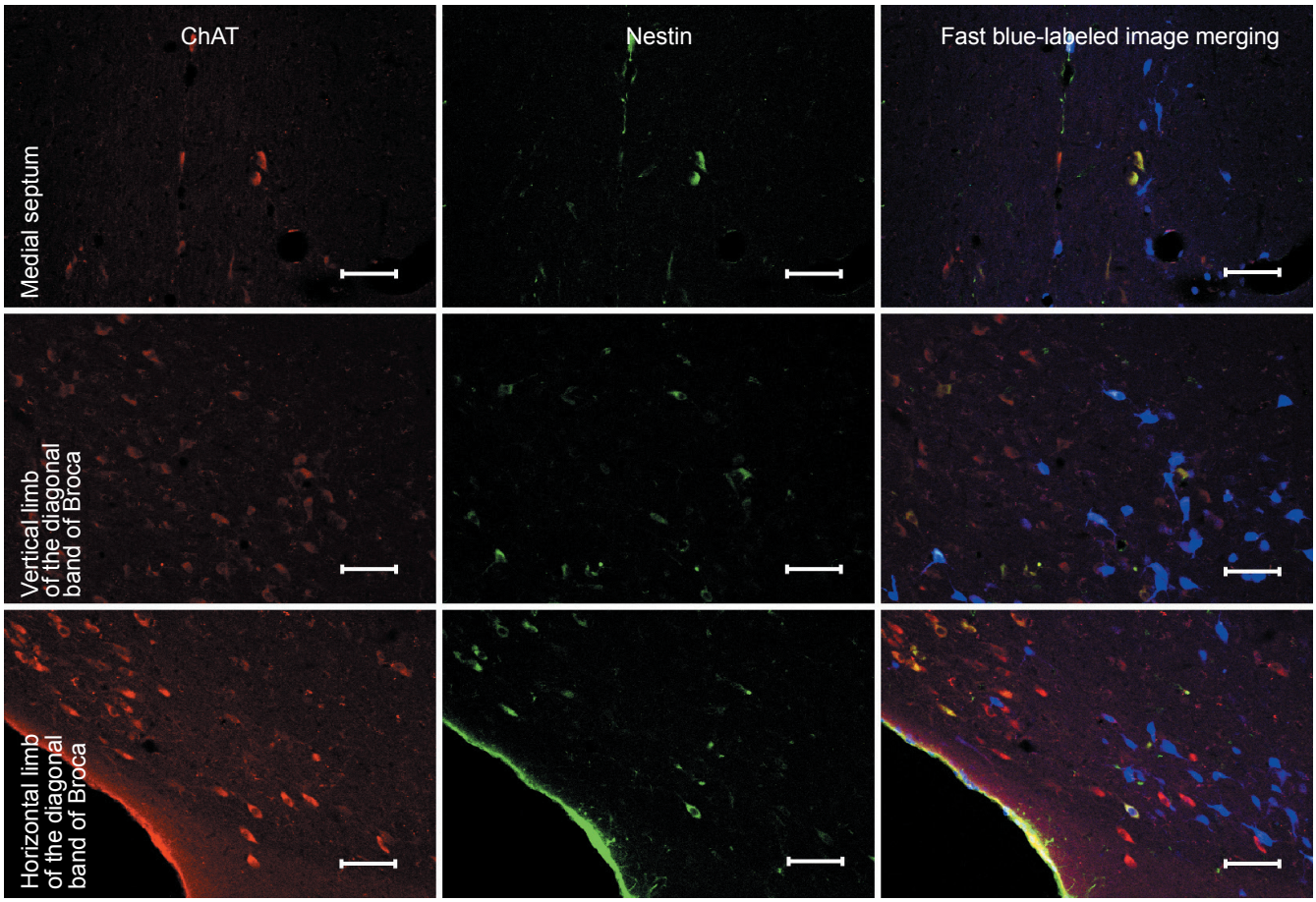

Figure 1 Retrograde labeling shows that nestin$^+$/ChAT$^+$ and nestin$^-$/ChAT$^+$ neurons projected to the olfactory bulb.
Cholinergic neurons show clear ChAT immunoreactivity (red) in the cell body. Nestin immunoreactivity (green) was clearly labeled and was also specific to the cell body. Fast blue fluorescence could be seen in the cell body and was distributed in the medial septum-diagonal band of Broca. Scale bars: 50 μm. ChAT: Choline acetyltransferase.

showed that nestin$^+$ neurons could constantly express ChAT, and may be a special type of cholinergic neuron.

Based on these findings, we propose the following questions: Do nestin$^+$ neurons play a role in neurological disorders? Do nestin$^+$ neurons have different manifestations after injury when compared to other ChAT$^+$ neurons? Do ChAT$^+$ neurons express nestin? These problems are worthy of further exploration. The present study investigated two areas. First, we explored if nestin$^+$ neurons in the medial septum-diagonal band of Broca projected to the olfactory bulb. Second, we explored the change in nestin expression in mature neurons and the differences between nestin-positive neurons and nestin-positive cholinergic neurons in response to olfactory nerve transection injury.

Results

Quantitative analysis of experimental animals

Of the 102 rats, 12 rats were randomly selected and divided into a retrograde tracing group and a control group. The projection of nestin$^+$ neurons in the adult rat medial septum-diagonal band of Broca to the olfactory bulb was analyzed. Six rats in the retrograde tracing group received a bilateral olfactory bulb injection of fast blue. The control group received an injection of PBS. An additional 60 rats received bilateral olfactory nerve transection (olfactory nerve transection group). The remaining 30 rats were designated the control group and received sham operation. The analysis was carried out at 3, 7, 14, 28 and 56 days after operation.

Projection of nestin$^+$ neurons from the medial septum diagonal band of Broca to the olfactory bulb of adult rats

At 48 hours after injection of fast blue in the basal forebrain region, blue fluorescence could be visualized throughout the entire medial septum-diagonal band of Broca region. Cholinergic neurons with red color fluorescence were round, elliptical, and large in size. ChAT$^+$ neurons could be seen in the medial septum, vertical limb of the diagonal band of Broca and horizontal limb of the diagonal band of Broca. ChAT$^+$ neurons were mainly distributed in horizontal limb of the diagonal band of Broca, and were seldom found in medial septum. Nestin$^+$ neurons, presenting with green fluorescence, were large in size, and were distributed at different places in the medial septum-diagonal band of Broca. These places also had cholinergic neurons. Moreover, all nestin$^+$ neurons co-labeled with ChAT. Nestin$^+$ neurons were mainly distributed in horizontal limb of the diagonal band of Broca and vertical limb of the diagonal band of Broca, but were seldom observed in medial septum. Fast blue-labeled

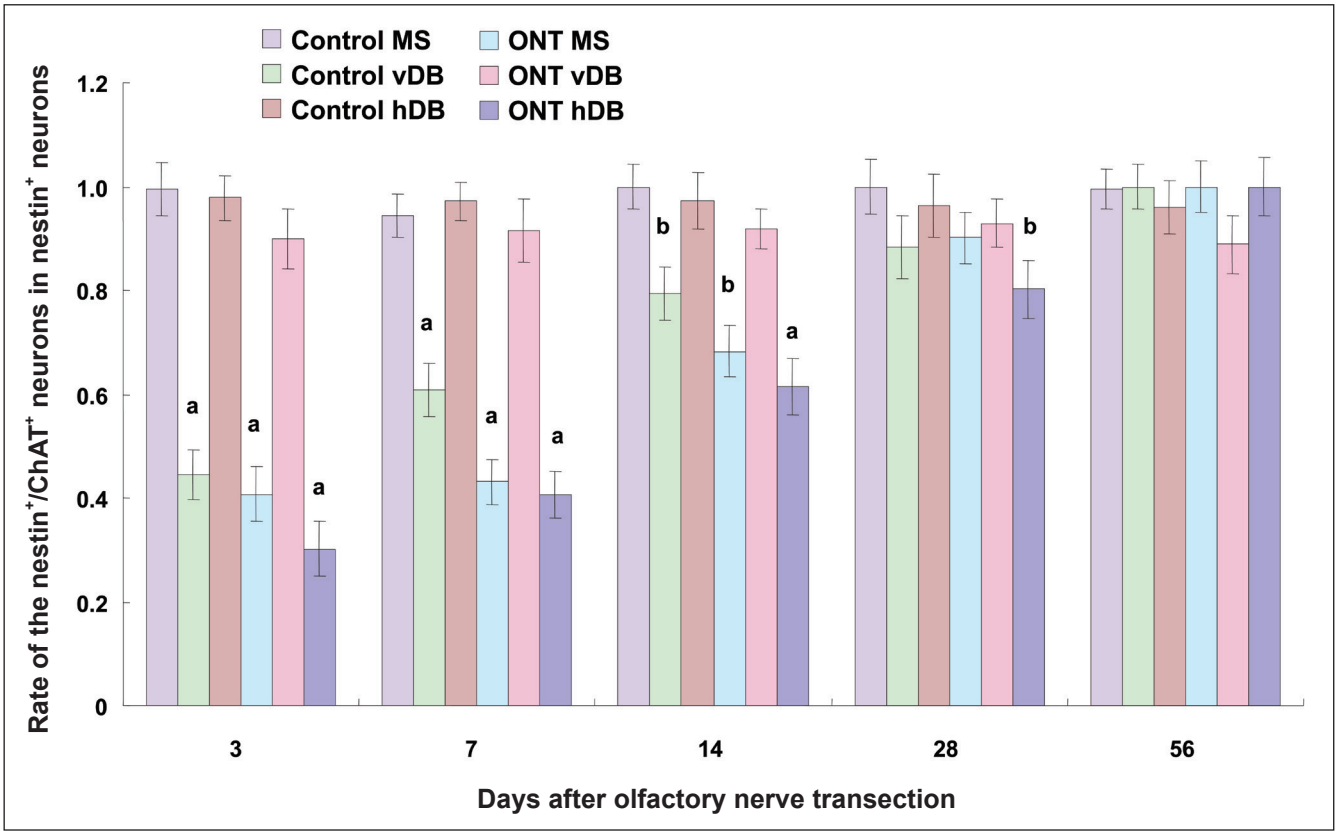

Figure 2 The number of ChAT$^+$ and nestin$^+$ neurons in the MS, vDB and hDB after olfactory nerve transection.
Data are presented as mean ± SD. Group comparisons were performed by analysis of variance with *post hoc* analysis. $^aP < 0.01$, $^bP < 0.05$, *vs.* control group. Control group contained six rats. ONT group contained 12 rats. ChAT: Choline acetyltransferase; MS: medial septum; vDB: vertical limb diagonal band; hDB: horizontal limb diagonal band; ONT: olfactory nerve transection.

cells showed the same shape as neurons or glial cells. Some ChAT$^+$ neurons were also co-labeled with fast blue. Moreover, among them, some nestin$^+$ immunoreactive neurons were tri-labeled with fast blue. Some fast blue-labeled cells were not co-labeled with ChAT or nestin neurons (Figure 1).

Approximately 22.2% of fast blue-labeled neurons in the medial septum-diagonal band of Broca area were ChAT$^+$ neurons, in which 53.6% were nestin$^+$/ChAT$^+$ neurons. The results showed that nestin$^+$ neurons in the medial septum-diagonal band of Broca could project to the olfactory bulb.

Time course of the number of ChAT$^+$ neurons and nestin$^+$ neurons in the medial septum-diagonal band of Broca

In the control group, all nestin$^+$ neurons were ChAT$^+$ in the medial septum, vertical limb of the diagonal band of Broca and horizontal limb of the diagonal band of Broca at 3, 7, 14, 28 and 56 days after sham operation of the olfactory nerve transection. No significant difference was observed in rate of nestin$^+$ neurons to ChAT$^+$ neurons in the medial septum, vertical limb of the diagonal band of Broca and horizontal limb of the diagonal band of Broca ($P = 2.03$). No significant difference was observed between the different time points in the control group ($P = 2.87$).

In the olfactory nerve transaction group, some nestin$^+$ neurons in the medial septum-diagonal band of Broca were not immunoreactive for ChAT$^+$, and the number of nestin and ChAT co-labeled cells changed at different time points after olfactory nerve transection. The number of positive cells decreased and was lowest at 3 days after olfactory nerve transection, and then gradually restored to initial levels at 56 days ($P = 4.25$; Figure 2).

Time course of nestin$^+$ neurons and nestin$^-$/ChAT$^+$ neurons in the medial septum-diagonal band of Broca

At 3, 7, 14, 28 and 56 days after olfactory nerve transection, no significant difference was observed in the number of nestin$^+$ neurons and ChAT$^+$ neurons in the medial septum, vertical limb of the diagonal band of Broca and horizontal limb of the diagonal band of Broca ($P = 3.53$). No significant difference was observed among the different time points in the sham operation group ($P = 2.87$). In the medial septum and vertical limb of the diagonal band of Broca, away from the incision, the number of nestin$^+$ neurons in the medial septum-diagonal band of Broca increased to 163.8% and 113.9% at 3 days after olfactory nerve transection, respectively, and there were significant differences when compared to the control group ($P = 0.004$; 0.043; Table 1). The number of nestin$^+$ neurons decreased rapidly to a minimum at 14 days; whereas in the horizontal limb of the diagonal band of Broca located near the incision, the number of nestin$^+$ neurons decreased after olfactory nerve transection. The number of nestin$^+$ neurons decreased to a minimum at 7 days ($P = 0.031$; Table 1). From

Table 1 Number of nestin$^+$ neurons in the MS-DBB after olfactory nerve transection

Time after olfactory nerve transection (day)	MS		vDB		hDB	
	Control	ONT	Control	ONT	Control	ONT
3	361.5±75.3	624.8±78.4[a]	768.3±71.3	798.3±253.4	1,787.4±150.5	1,165.5±151.5[a]
7	342.3±79.3	241.5±84.3[b]	794.4±142.2	616.6±183.5[b]	1,783.5±172.2	808.8±183.4[a]
14	364.4±89.8	238.2±94.2[b]	806.7±79.8	353.5±102.2[b]	1,792.0±125.3	945.6±293.4[a]
28	398.2±114.2	325.6±36.7[b]	759.6±62.5	532.2±249.3[b]	1,721.4±154.4	829.3±140.3[a]
56	343.3±94.1	140.4±66.3[b]	783.3±69.4	309.3±82.2[b]	1,696.6±112.6	681.2±69.9[a]

All values are presented as mean ± SD. Group comparisons were performed by analysis of variance with *post hoc* analysis. [a]$P < 0.01$, [b]$P < 0.05$, *vs.* control group. Control group contained six rats. ONT group contained 12 rats. MS: Medial septum; vDB: vertical limb diagonal band; hDB: horizontal limb diagonal band; MS-DBB: medial septum-diagonal band of Broca; ONT: olfactory nerve transection.

Table 2 Number of nestin$^-$/ChAT$^+$ neurons in the MS-DBB after olfactory nerve transection

Time after olfactory nerve transection (day)	MS		vDB		hDB	
	Control	ONT	Control	ONT	Control	ONT
3	2,235.2±302.7	1,890.3±206.1[b]	4,065.6±324.3	2,457.0±243.4[b]	8,681.4±586.4	4,473.0±309.5[a]
7	2,051.4±250.8	1,557.5±165.8[b]	4,018.8±311.6	2,121.0±247.5[b]	8,603.3±516.5	4,609.5±427.8[a]
14	2,467.5±257.5	2,180.5±152.5[b]	4,316.7±418.5	2,460.5±287.4[b]	8,729.4 ±686.6	4,980.5±468.6[a]
28	2,422.3±350.9	2,107.2±187.2[b]	4,279.9±422.5	3,230.5±233.6[b]	8,963.3±612.3	5,330.5±590.3[a]
56	2,218.4±244.9	1,869.2±182.3[b]	3,992.5±393.7	2,614.5±198.6[b]	9,018.4±634.86	4,963.5±569.6[a]

All values are presented as mean ± SD. Group comparisons were performed by analysis of variance with *post hoc* analysis. [a]$P < 0.01$, [b]$P < 0.05$, *vs.* control group. Control group contained six rats. ONT group contained 12 rats. MS: Medial septum; vDB: vertical limb diagonal band; hDB: horizontal limb diagonal band; MS-DBB: medial septum-diagonal band of Broca; ONT: olfactory nerve transection.

28 to 56 days, the number of nestin$^+$ neurons remained unchanged, decreased to 58.9%, 58.8% and 54.8% at 56 days in the medial septum, vertical limb of the diagonal band of Broca and horizontal limb of the diagonal band of Broca, respectively ($P = 0.027$, 0.032, 0.026). No significant difference was observed at 28 and 56 days in the medial septum, vertical limb of the diagonal band of Broca and horizontal limb of the diagonal band of Broca ($P = 3.42$; Table 2).

After olfactory nerve transection, the number of nestin$^-$/ChAT$^+$ neurons decreased. In the medial septum and vertical limb of the diagonal band of Broca, away from the incision, the number of nestin$^-$/ChAT$^+$ neurons decreased to a minimum at 7 days ($P = 3.92$). In the horizontal limb of the diagonal band of Broca located near the incision, the number of nestin$^-$/ChAT$^+$ neurons was lowest at 3 days ($P = 0.0062$; Table 2).

Double fluorescent immunocytochemistry for nestin and BrdU in the rostral migratory stream-subventricular zone after olfactory nerve transection

At 14 days after olfactory nerve transection, we examined sagittal serial sections in the basal forebrain region by double fluorescent immunocytochemistry for nestin and BrdU. In the control group, we could clearly distinguish the subventricular zone and rostral migratory stream from surrounding brain structures. Nestin immunoreactivity was clear in cell bodies and neurites, which labeled green. Cells presented with filamentous shapes, and small cell bodies. BrdU immunoreactivity was clear in the cell nuclei and labeled red. Double-labeled cells of nestin and BrdU were visible along the caudorostral course of the migratory pathway (Figure 3A). The double labeled cells were small and also presented with a filamentous and spindle shape similar to glial cells. The rostral migratory stream migrated to the center of olfactory bulb. In the olfactory nerve transection group, the subventricular zone and rostral migratory stream were clearly distinguishable from surrounding brain structures, as determined by fluorescence microscopy. The migration route of the rostral migratory stream did not change. Migrating cells gathered at the edge of the incision. The cells aggregated on the edge of the cut, which made the cell flow diameter wide. Double-labeled cells were small and also presented with elongated and spindle morphology. No double-labeled cells were found migrating in the other direction (Figure 3B).

Double fluorescent immunocytochemistry for nestin and BrdU in the medial septum-diagonal band of Broca after olfactory nerve transection

To confirm if the increased nestin$^+$ neurons in the medial septum-diagonal band of Broca came from the differentiation of immature cells, the rats were injected with BrdU after olfactory nerve transection. Using double fluorescent immunocytochemistry for nestin and BrdU, nestin$^+$ neurons in the olfactory nerve transection group showed clear nestin immunoreactivity in cell bodies. Clear BrdU immunoreactivity was visible in nuclei labeled by red fluorescence in the medial septum-diagonal band of Broca (Figure 4B). However, nestin$^+$/BrdU$^+$ neurons were not observed in cells of sagittal

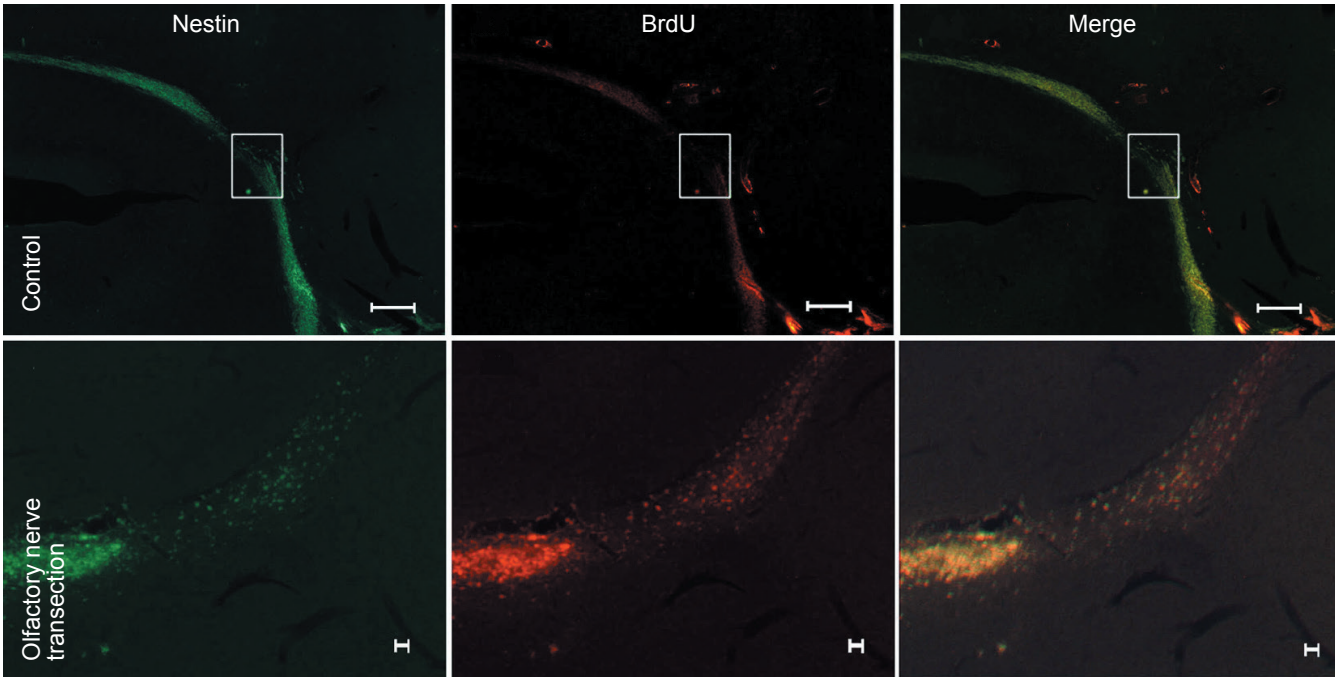

Figure 3 Double immunofluorescent labeling for nestin$^+$ and BrdU$^+$ neurons in the rostral migratory stream after 14 days of olfactory nerve transection.
Precursor cells in the rostral migratory stream show clear BrdU immunoreactivity (red) in cell nuclei. Nestin immunoreactivity (green) was clear in the cell body. Pane in the control group displays the same position of the olfactory nerve transection group. Scale bars: 500 μm in control group; 25 μm in olfactory nerve transection group. Arrows show the direction of cell migration. BrdU: 5-Bromo-2′-deoxyuridine.

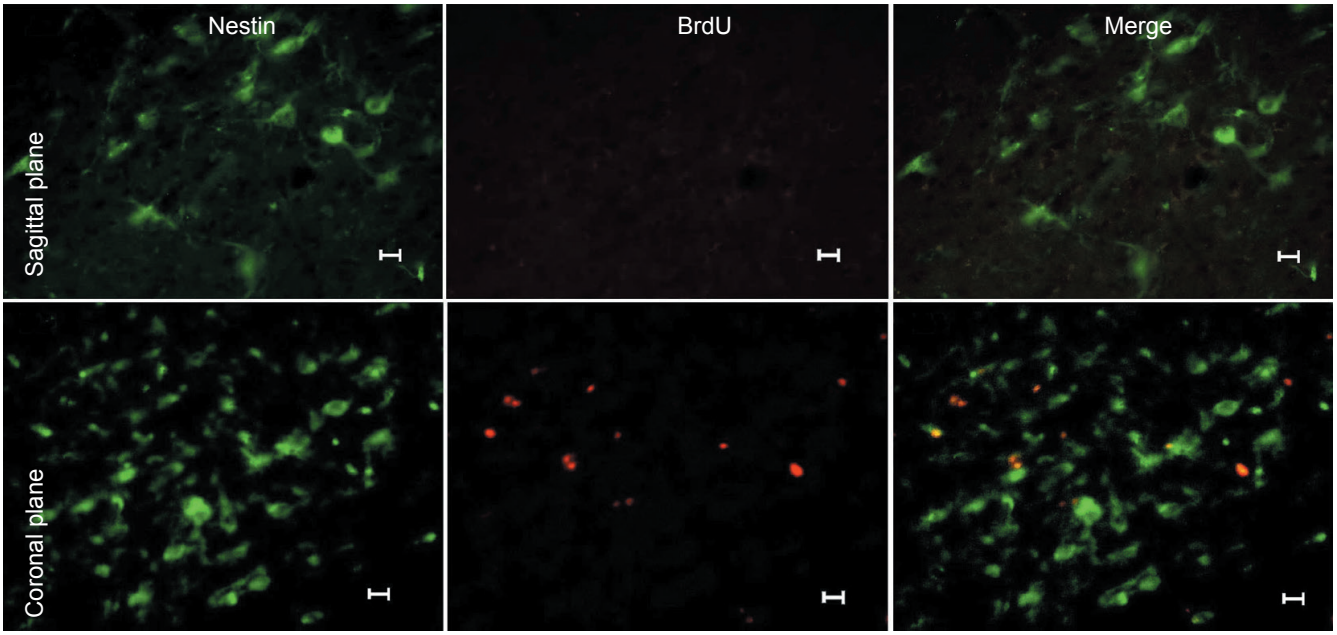

Figure 4 Double immunofluorescent labeling for ChAT$^+$ and BrdU$^+$ neurons in sagittal and coronal sections in the medial septum-diagonal band of Broca.
Precursor cells in the medial septum-diagonal band of Broca show clear BrdU immunoreactivity (red) in cell nuclei. Nestin immunoreactivity (green) was clear in cell bodies. Scale bars: 25 μm. BrdU: 5-Bromo-2′-deoxyuridine; ChAT: choline acetyltransferase.

or coronal slices.

Discussion

Previous studies on cholinergic neurons have tended to focus on the hippocampus, but the projection of hippocampal cholinergic nerve fibers are more extensive and relatively complex[21]. In this study, we analyzed the relationship of cholinergic innervations in olfactory and nestin$^+$ neurons in the medial septum-diagonal band of Broca firstly because, in the central nervous system, the cholinergic innervations

to the olfactory bulb are relatively simple. The cholinergic innervations to the olfactory bulb are predominantly from the cholinergic neurons in the horizontal limb of the diagonal band of Broca, and a few are from neurons in the medial septum and vertical limb of the diagonal band of Broca[22]. Morphological studies have shown that olfactory bulbectomy can reduce the number of ChAT immunoreactive neurons in the medial septum-diagonal band of Broca[23-25]. Second, neurodegenerative disorders involving the cholinergic system, such as Alzheimer's disease, are accompanied by olfactory dysfunctions[26-27]. Fell et al.[28] showed that some feedback signals of cholinergic neurons were influenced by the olfactory bulb: in memory processing, discharge of olfactory neurons is earlier than hippocampal neurons, and only when the activities were absolutely synchronous in both districts, was vocabulary remembered. Thus, memory dysfunction could have a close relationship with cholinergic neurons in the olfactory bulb and horizontal limb of the diagonal band of Broca.

Parallel projection of nestin$^+$/ChAT$^+$ neurons and nestin$^-$/ChAT$^+$ neurons in the medial septum-diagonal band of Broca to the olfactory bulb

In the brain, cholinergic neurons mainly aggregate in several areas. The medial septum-diagonal band of Broca is one of the areas where cholinergic neurons aggregate to in the brain and extensively project to the hippocampus, olfactory bulb and frontal cortex[10, 21-22, 26]. The medial septum-diagonal band of Broca is one of the most important inputs to olfactory neurons. The olfactory bulb receives its cholinergic projections predominantly from the horizontal limb of the diagonal band of Broca. In our experiment, retrograde labeling combined with ChAT and nestin immunofluorescence suggested that approximately 22.2% of cholinergic neurons in the medial septum-diagonal band of Broca area projected to the olfactory bulb, which was in agreement with previously reported studies[23, 29-30]. Our experiment also suggested that nestin$^+$ and nestin$^-$ cholinergic neurons projected to the olfactory bulb. Therefore, we concluded that there were two parallel septo-olfactory bulb cholinergic pathways. One pathway originates from medial septum/diagonal band of Broca nestin-positive cholinergic neurons, and the other pathway originates from nestin-negative cholinergic neurons. Moreover, after recovery from olfactory nerve transection, the amount of cholinergic neurons in the medial septum vertical limb of the diagonal band of Broca and horizontal limb of the diagonal band of Broca projecting to the olfactory bulb was different (7.9%, 28.9% and 43.0%), but the loss in the amount of nestin$^+$/ChAT$^+$ neurons in the medial septum vertical limb of the diagonal band of Broca and horizontal limb of the diagonal band of Broca projecting to the olfactory bulb was almost the same. The outcomes suggested that nestin$^+$/ChAT$^+$ cells may undertake more neurofibril connections between the olfactory bulb and the correlated projection area in the brain. Previous studies have shown that ChAT$^+$ neurons in the medial septum-diagonal band of Broca may be related to the regulation of exciting or inhibiting olfactory bulb neurons[31-37]. Nestin$^+$/ChAT$^+$ cells had different intrinsic electrophysiological properties from nestin$^-$/ChAT$^+$ cells, indicating they may have different functions in maintaining the electrical activity in projection-related brain areas. However, this area requires further investigations. Our previous studies showed that nestin$^+$ neurons are a subpopulation of cholinergic neurons in the medial septum-diagonal band of Broca complex[17-20, 38].

Source of increased nestin$^+$ neurons after olfactory nerve transection in the medial septum-diagonal band of Broca

At early time points of olfactory nerve transection, nestin$^+$ neurons increased in the medial septum and vertical limb of the diagonal band of Broca. The perplexing question was whether the injury caused by olfactory nerve transection could make nestin$^+$ cells in the subventricular zone migrate to the medial septum-diagonal band of Broca and differentiate into nestin$^+$ neurons, or do they come from newly generated neurons from elsewhere. Studies concerning olfactory bulbectomy in rats found that loss of the olfactory bulb did not change the generation and migration route of subventricular zone cells, and cells generated from the subventricular zone do not alter their migration along the rostral migratory stream into the olfactory bulb[31-34]. In this study, by administering BrdU immunofluorescence after olfactory nerve transection, we found that migration along the subventricular zone-rostral migratory stream were not changed; the cells in the subventricular zone-rostral migratory stream were glial cells, as identified in the sham operation group. Studies also demonstrated that olfactory bulb removal may not inhibit cell generation from the subventricular zone, cannot induce differentiation of nestin$^+$ cells in rostral migratory stream and did not change morphology and routine of the rostral migratory stream[39-40]. Therefore, we conclude that increased nestin$^+$ neurons in the medial septum and vertical limb of the diagonal band of Broca may not migrate from differentiated precursor cells of the rostral migratory stream.

To explore whether nestin$^+$ neuronal cells come from differentiated stem cells or from elsewhere, sagittal and coronal slices in the olfactory nerve transection group were made. No double-labeled neuronal cells (BrdU and nestin) were observed in the medial septum-diagonal band of Broca. The results showed that nestin$^+$ neurons did not originate from the differentiation of precursor cells. It has been reported that mild injury can down-regulate the expression of choline acetyl transferase in cholinergic neurons, and when the injury is repaired, the expression of choline acetyl transferase can be restored[41-43]. Vaittinen et al.[44-46] reported that skeletal muscle cells were innervated by nestin-positive cholinergic neurons. The expression of nestin increased when muscle cells were denervated. Nestin expression returned to normal levels when muscle cells were re-innervated by cholinergic nerves. The changes in nestin expression were thought to be related to cholinergic receptors in the neuron-muscular junction.

We speculated that the increased number of nestin$^+$ neurons in the medial septum and vertical limb of the diagonal band of Broca may be due to increased expression of nestin in cholinergic neurons. The increased expression of nestin in cholinergic neurons may be a response to injury. We sup-

posed that the different increases of nestin$^+$ neurons in the medial septum, vertical limb of the diagonal band of Broca and horizontal limb of the diagonal band of Broca may be due to their different projections to olfactory neurons. These cells have a direct and rich connection between the horizontal limb of the diagonal band of Broca neurons and afferent olfactory neurons. Neurons in the horizontal limb of the diagonal band of Broca were more vulnerable after transection and led to weak compensation reaction to the injury. However, the medial septum is the main cholinergic fiber input area of the hippocampus, and was far from the place of injury and only had scattered and indirect fiber links with the olfactory bulb. Thus, neurons were mildly injured and the compensatory reaction was obvious. The vertical limb of the diagonal band of Broca was the transition region between the medial septum and horizontal limb of the diagonal band of Broca, and the compensatory reaction was less in the medial septum.

Different responses of nestin$^+$ neurons and nestin$^-$/ChAT$^+$ neurons to injury and the repair process

In the medial septum and vertical limb of the diagonal band of Broca, the number of nestin$^+$ neurons reduced to a minimum at 14 days after olfactory nerve transection, whereas the number of nestin$^-$/ChAT$^+$ neurons reduced to a minimum at 7 days. In the horizontal limb of the diagonal band of Broca, the number of nestin$^+$ neurons reduced to a minimum at 7 days, whereas the number of nestin$^-$/ChAT$^+$ neurons reduced to a minimum at 3 days. That is, the response of nestin$^+$ neurons to injury was later than nestin$^-$/ChAT$^+$ neurons. These results suggest that nestin$^+$ cholinergic neurons had better tolerance to injury. The reaction of nestin$^+$ and nestin$^-$/ChAT$^+$ neurons to the injury stabilized at 28 days at the medial septum-diagonal band of Broca. These results suggest that nestin$^+$ neurons and nestin$^-$/ChAT$^+$ neurons had no obvious differences in the reparative process. Previous studies revealed that nestin$^+$ neurons had better plasticity than nestin$^-$/ChAT$^+$ neurons. Intracerebroventricular injection of colchicines led to irreversible reduction of nestin$^-$/ChAT$^+$ neurons, but only caused transient reduction of nestin$^+$ cholinergic neurons in the medial septum-diagonal band of Broca[47].

In this study, the response of nestin$^+$ neurons to injury was later than nestin$^-$/ChAT$^+$ neurons, so we suggest that nestin$^+$ cholinergic neurons may have a stronger tolerance to injury. The different reaction of nestin$^+$ and nestin$^-$/ChAT$^+$ neurons and the mechanisms underlying this protective plasticity require further investigation using ChAT$^+$ selective neurons in a moderate damage model of the medial septum-diagonal band of Broca.

Materials and Methods

Design

A controlled, observational animal experiment.

Time and setting

This study was performed in the Laboratory of the Department of Anatomy and Neurobiology, Zhongshan School of Medicine, Sun Yat-sen University, China between July 2010 and March 2012.

Materials

Male, specific-pathogen free rats, aged 2 months and weighing 200–220 g, were obtained from and bred by the Experimental Animal Center of Sun Yat-sen University in China (license No. SCXK (Yue) 2009-0011). During the experiment, the animals were housed in individual cages (30 cm × 20 cm × 18 cm) and maintained under standard laboratory conditions (12-hour light/dark cycle, 25 ± 1°C, humidity 50 ± 10%). The animals were allowed free access to a standard dry diet and tap water. All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

Methods

Analyses of the projection of nestin$^+$ and nestin$^-$/ChAT$^+$ neurons

Rats were anesthetized with sodium pentobarbital (325 mg/kg, i.p.) and placed in a stereotaxic apparatus (Huaibei Zhenghua, Anhui Province, China). The olfactory bulb injection was according to established procedures[48-49]: in brief, the skin was incised and the skull was exposed. Two 3 mm holes were drilled through the skull for placement of the injection cannula into the olfactory bulb. The stereotaxic coordinates for olfactory bulb injection were made according to the stereotaxic atlas of Paxinos and Watson[50]. The rats were administered 0.5 μL of 3% (w/v) fast blue dye (Sigma, St. Louis, MO, USA) into the olfactory bulb (anteroposterior –8 mm, lateral 1.0 mm, vertical 3.0 mm). The rats in the control group were administered 0.5 μL PBS. Forty-eight hours later, animals were sacrificed. Immunofluorescence of nestin and ChAT was performed with standard protocols. Rhodamine red was the fluorescent label for ChAT$^+$, Cy2 for nestin$^+$ neurons[51]. The localization of fast blue in nerve cell bodies of the medial septum-diagonal band of Broca was observed and analyzed.

Olfactory nerve transection

Bilateral olfactory nerve transection was performed under sodium pentobarbital (325 mg/kg, i.p.) anesthesia. The surgical procedure was conducted according to established procedures[52]. In brief, the skull above the olfactory nerve and olfactory bulb was removed to expose the olfactory tract via craniotomy. The olfactory tract near the olfactory bulb was incised to completely transect the olfactory nerve under direct vision by inserting a Teflon blade. Animals were allowed to recover for 3, 7, 14, 28 and 56 days prior to death. A total of 30 sham operated animals were subjected to the same procedure without nerve transection.

BrdU administration

To detect the migration of the rostral migratory stream, rats were given 50 mg/kg (50 mg/mL) BrdU *via* intraperitoneal injection[53-54] every 12 hours after nerve transection for 5 days. The control group received an injection of an equal volume of PBS (pH 7.4). After olfactory nerve transection,

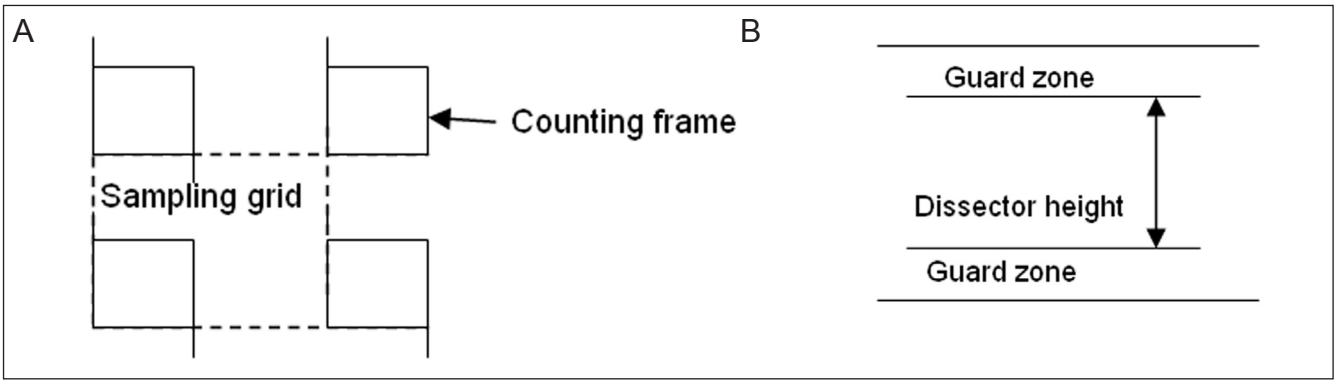

Figure 5 Area of stereological evaluation.
(A) X and Y dimensions; (B) Z dimension.

animals were allowed to recover for 28 days prior to death.

Sample collection

Rats were injected with a lethal dose of sodium pentobarbital and killed by intracardiac perfusion with 350 mL 0.9% (w/v) sodium chloride, and then fixed with 4% (w/v) paraformaldehyde in 0.1 mol/L PBS (pH 7.4). The skull was opened and the brain was stored in the same paraformaldehyde in PBS for 24 hours at 4°C, and equilibrated in 10%, 20%, 30% (w/v) sucrose PBS buffer at 4°C until saturated. Tissue was then sectioned on a freezing microtome, and all 40 μm-thick sagittal sections were collected. One 40 μm-thick coronal section was collected from every five sections. A total of six serial sections were collected per subject[51].

Detection of ChAT and nestin in the rat medial septum-diagonal band of Broca by dual immunohistochemical staining

In one of the six coronal serial sections of each rat, four representative sections of the brain containing the medial septum-diagonal band of Broca were selected using the Paxinos and Watson atlas. In serial sagittal sections, the sections of the brain containing the medial septum-diagonal band of Broca were also selected. After rinsing in PBS, the selected sagittal or coronal sections were treated with 0.3% (v/v) hydrogen peroxide (0.1 mol/L PBS) for 15 minutes, washed three times in PBS for 15 minutes, and then treated with 1% (v/v) bovine serum albumin (containing 0.5% (v/v) Triton X-100 in 0.1 mol/L PBS) for 30 minutes. The sections were then simultaneously incubated with mouse anti-rat nestin monoclonal antibody (1:800; Pharmingen, San Diego, CA, USA) and rabbit anti-rat ChAT monoclonal antibody (1:1,000; Chemicon, Temecula, CA, USA) at room temperature for 120 minutes, and then incubated overnight at 4°C (PBS was used as a negative control). After rinsing three times in PBS for 5 minutes, sections were simultaneously incubated in the corresponding Cy2 conjugated goat anti-mouse IgG (1:100; Jackson, Sufolk CB, UK) and rhodamine coupled goat anti-rabbit IgG (1:800; Jackson) at room temperature for 120 minutes, and then thoroughly rinsed with PBS, mounted and examined under a Zeiss motorized upright microscope (Carl Zeiss, Meditec, Jena, Germany)[51].

Detection of BrdU and nestin in the rat medial septum-diagonal band of Broca by dual immunohistochemical staining

One side of the brain was selected for coronal sections and the other side for sagittal serial sections. The sagittal and coronal sections containing the medial septum-diagonal band of Broca were selected as above. The sagittal serial sections containing the rostral migratory stream were also selected according to the Paxinos and Watson atlas. Floating sections were treated with 0.3% (v/v) hydrogen peroxide and 1% (v/v) bovine serum albumin as described above, and incubated overnight at 4°C with mouse anti-rat nestin monoclonal antibody (1:800; Pharmingen).

After rinsing three times in PBS, the sections were treated with 4% (w/v) paraformaldehyde for 15 minutes, and then thoroughly rinsed twice in PBS for 15 minutes. Sections were treated with 2 mol/L HCl for 30 minutes in a 60°C water bath to denature the DNA and with borate buffer (pH 8.3) for 25 minutes. Sections were then treated with 1% (v/v) bovine serum albumin and incubated with donkey anti-rat BrdU monoclonal antibodies (1:400; Jackson) overnight at 4°C, followed by rinsing twice in PBS for 15 minutes, and incubation in rhodamine conjugated goat anti-donkey IgG (1:400; Jackson) and Cy2 conjugated goat anti-mouse IgG (1:100; Jackson) at room temperature for 120 minutes. The sections were then thoroughly rinsed in PBS, mounted and examined under the Zeiss motorized upright microscope (Carl Zeiss)[51-55].

Stereological evaluation

The total number of $ChAT^+$ and $nestin^+$ cells in the medial septum-diagonal band of Broca was estimated using the optical fractionator method[56-59]. In every individual set of coronal sections of each rat, four representative sections containing the medial septum-diagonal band of Broca were selected using the Paxinos and Watson atlas. Horizontal lines through the superior border of the bilateral anterior commissural and the sulcus above the optic nerve were utilized to define the boundary of the medial septum, vertical limb of the diagonal band of Broca and horizontal limb of the diagonal band of Broca. To exclude $nestin^+$ neuroglial cells, only cells with a diameter above 15 μm were counted.

Cell counting was performed by a stereological worksta-

tion (MicroBrightField, Williston, ND, USA). The fraction of section-sampling (ssf) was 1/6, the top of the section (guard zone) in the z-dimension was set at 5 μm to the plane of the top of the counting frame (Figure 5). The mean thickness of the mounted sections was 38.8 μm. The counting frame had the size of 40 μm × 40 μm. The total number of labeled neurons in different subregions of the medial septum-diagonal band of Broca was estimated as follows:

$$N = \sum Q - 1/ssf \times 1/asf \times 1/hsf$$

Where, ssf represents the fraction of section-sampling; tsf is the sampling fraction thickness (dissector height/section thickness); asf is the sampling fraction area (counting frame/sampling), and ∑Q represents all the dissector numbers of the labeled neurons.

Statistical analysis

Data are presented as the mean ± SD. All figures were imaged using a Zeiss motorized upright microscope (Carl Zeiss) and prepared using Adobe Photoshop CS (Adobe). Statistical calculations were carried out by SPSS software version 11.0 for Windows (SPSS, Chicago, IL, USA). Multiple group comparisons were made by analysis of variance with *post hoc* analysis. In all cases, a value of $P < 0.05$ was considered statistically significant.

Author contributions: *Zhao YH designed and carried out the study, performed the statistical analysis and drafted the manuscript. Guo KH was in charge of the funding, participated in the immunohistochemistry study. Li DP participated in the design of the study and neuronal projection study. Yuan QF conceived the study, and participated in its design and coordination, and helped to draft the manuscript. Yao ZB offered the technical support.*

Conflicts of interest: *None declared.*

Peer review: *This paper addresses a very interesting issue on the connections of the diagonal band of Broca to the olfactory bulb, and most importantly on the effects that cutting afferences induce in the medial septum/diagonal band. This is a very interesting issue, since it shows that peripheral damage may induce long lasting changes in these areas, some of the changes are reverted after some time. Thus, this paper may cast light on the relationships of various areas of the brain, involved in many neurological diseases like Alzheimer's disease.*

References

[1] Bramanti V, Tomassoni D, Avitabile M, et al. Biomarkers of glial cell proliferation and differentiation in culture. Front Biosci (Schol Ed). 2010;2:558-570.
[2] Lépinoux-Chambaud C, Eyer J. Review on intermediate filaments of the nervous system and their pathological alterations. Histochem Cell Biol. 2013;140(1):13-22.
[3] Michalczyk K, Ziman M. Nestin structure and predicted function in cellular cytoskeletal organisation. Histol Histopathol. 2005;20(2):665-671.
[4] Ernst C, Christie BR. The putative neural stem cell marker, nestin, is expressed in heterogeneous cell types in the adult rat neocortex. Neuroscience. 2006;138(1):183-188.
[5] Mobarakeh ZT, Ai J, Yazdani F, et al. Human endometrial stem cells as a new source for programming to neural cells. Cell Biol Int Rep (2010). 2012;19(1):e00015.
[6] Mohseni P, Sung HK, Murphy AJ, et al. Nestin is not essential for development of the CNS but required for dispersion of acetylcholine receptor clusters at the area of neuromuscular junctions. J Neurosci. 2011;31(32):11547-11552.
[7] Bramanti V, Tomassoni D, Avitabile M, et al. Biomarkers of glial cell proliferation and differentiation in culture. Front Biosci (Schol Ed). 2010;2:558-570.
[8] Calderone A. Nestin$^+$ cells and healing the infarcted heart. Am J Physiol Heart Circ Physiol. 2012;302(1):H1-9.
[9] Zhou Y, Xu Q, Li D, et al. Early neurogenesis during caudal spinal cord regeneration in adult Gekko japonicus. J Mol Histol. 2013;44(3):291-297.
[10] Semba K. Multiple output pathways of the basal forebrain: organization, chemical heterogeneity, and roles in vigilance. Behav Brain Res. 2000;115(2):117-141.
[11] Semba K. Phylogenetic and ontogenetic aspects of the basal forebrain cholinergic neurons and their innervation of the cerebral cortex. Prog Brain Res. 2004;145:3-43.
[12] Müller ML, Bohnen NI. Cholinergic dysfunction in Parkinson's disease. Curr Neurol Neurosci Rep. 2013;13(9):377.
[13] Robinson L, Platt B, Riedel G. Involvement of the cholinergic system in conditioning and perceptual memory. Behav Brain Res. 2011;221(2):443-465.
[14] Chen KH, Reese EA, Kim HW, et al. Disturbed neurotransmitter transporter expression in Alzheimer's disease brain. J Alzheimers Dis. 2011;26(4):755-766.
[15] Schliebs R, Arendt T. The cholinergic system in aging and neuronal degeneration. Behav Brain Res. 2011;221(2):555-563.
[16] Peth-Nui T, Wattanathorn J, Muchimapura S, et al. Effects of 12-week bacopa monnieri consumption on attention, cognitive processing, working memory, and functions of both cholinergic and monoaminergic systems in healthy elderly volunteers. Evid Based Complement Alternat Med. 2012;2012:606424.
[17] Wang S, Yao Z, Wang J, et al. Evidence for a distinct group of nestin-immunoreactive neurons within the basal forebrain of adult rats. Neuroscience. 2006;142(4):1209-1219.
[18] Gu H, Wang S, Messam CA, et al. Distribution of nestin immunoreactivity in the normal adult human forebrain. Brain Res. 2002;943(2):174-180.
[19] Guo KH, Zhu JH, Yao ZB, et al. Chemical identification of nestin-immunoreactive neurons in the rat basal forebrain: a re-examination. Neurochem Int. 2010;56(5):694-702.
[20] Zhu J, Gu H, Yao Z, et al. The nestin-expressing and non-expressing neurons in rat basal forebrain display different electrophysiological properties and project to hippocampus. BMC Neurosci. 2011;12:129.
[21] Amaral DG, Kurz J. An analysis of the origins of the cholinergic and noncholinergic septal projections to the hippocampal formation of the rat. J Comp Neurol. 1985;240(1):37-59.
[22] Senut MC, Menetrey D, Lamour Y. Cholinergic and peptidergic projections from the medial septum and the nucleus of the diagonal band of Broca to dorsal hippocampus, cingulate cortex and olfactory bulb: a combined wheatgerm agglutinin-apohorseradish peroxidase-gold immunohistochemical study. Neuroscience. 1989;30(2):385-403.
[23] Bobkova NV, Nesterova IV, Nesterov VV. The state of cholinergic structures in forebrain of bulbectomized mice. Bull Exp Biol Med. 2001;131(5):427-431.
[24] Yamamoto Y, Shioda N, Han F, et al. Donepezil-induced neuroprotection of acetylcholinergic neurons in olfactory bulbectomized mice. Yakugaku Zasshi. 2010;130(5):717-721.
[25] Paolini AG, McKenzie JS. Effects of lesions in the horizontal diagonal band nucleus on olfactory habituation in the rat. Neuroscience. 1993;57(3):717-724.
[26] Mundiñano IC, Hernandez M, Dicaudo C, et al. Reduced cholinergic olfactory centrifugal inputs in patients with neurodegenerative disorders and MPTP-treated monkeys. Acta Neuropathol. 2013;126(3):411-425.
[27] Ruan Y, Zheng XY, Zhang HL, et al. Olfactory dysfunctions in neurodegenerative disorders. J Neurosci Res. 2012;90(9):1693-1700.

[28] Fell J, Ludowig E, Staresina BP, et al. Medial temporal theta/alpha power enhancement precedes successful memory encoding: evidence based on intracranial EEG. J Neurosci. 2011;31(14):5392-5397.
[29] Záborszky L, Carlsen J, Brashear HR, et al. Cholinergic and GABAergic afferents to the olfactory bulb in the rat with special emphasis on the projection neurons in the nucleus of the horizontal limb of the diagonal band. J Comp Neurol. 1986;243(4):488-509.
[30] Lucidi-Phillipi CA, Gage FH. The neurotrophic hypothesis and the cholinergic basal forebrain projection. Prog Brain Res. 1993;98:241-249.
[31] Linster C, Hasselmo ME. Neural activity in the horizontal limb of the diagonal band of broca can be modulated by electrical stimulation of the olfactory bulb and cortex in rats. Neurosci Lett. 2000;282(3):157-160.
[32] Smith RS, Araneda RC. Cholinergic modulation of neuronal excitability in the accessory olfactory bulb. J Neurophysiol. 2010;104(6):2963-2974.
[33] Salcedo E, Tran T, Ly X, et al. Activity-dependent changes in cholinergic innervation of the mouse olfactory bulb. PLoS One. 2011;6(10):e25441.
[34] Ma M, Luo M. Optogenetic activation of basal forebrain cholinergic neurons modulates neuronal excitability and sensory responses in the main olfactory bulb. J Neurosci. 2012;32(30):10105-10116.
[35] Devore S, Linster C. Noradrenergic and cholinergic modulation of olfactory bulb sensory processing. Front Behav Neurosci. 2012;6:52.
[36] Li G, Cleland TA. A two-layer biophysical model of cholinergic neuromodulation in olfactory bulb. J Neurosci. 2013;33(7):3037-3058.
[37] Chapuis J, Wilson DA. Cholinergic modulation of olfactory pattern separation. Neurosci Lett. 2013;545:50-53.
[38] Li D, Wang J, Yew DT, et al. Age-related alterations of Nestin-immunoreactive neurons in rat basal forebrain with aged memory deficit. Neurochem Int. 2008;53(6-8):270-277.
[39] Hu H, Tomasiewicz H, Magnuson T, et al. The role of polysialic acid in migration of olfactory bulb interneuron precursors in the subventricular zone. Neuron. 1996;16(4):735-743.
[40] Petreanu L, Alvarez-Buylla A. Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. J Neurosci. 2002;22(14):6106-6113.
[41] Hu X, Zhang Y, Li W, et al. Preconditioning with sevoflurane ameliorates spatial learning and memory deficit after focal cerebral ischemia-reperfusion in rats. Int J Dev Neurosci. 2013;31(5):328-333.
[42] Raslan A, Ernst P, Werle M, et al. Reduced cholinergic and glutamatergic synaptic input to regenerated motoneurons after facial nerve repair in rats: potential implications for recovery of motor function. Brain Struct Funct. in press.
[43] Zhang L, Plotkin RC, Wang G, et al. Cholinergic augmentation with donepezil enhances recovery in short-term memory and sustained attention after traumatic brain injury. Arch Phys Med Rehabil. 2004;85(7):1050-1055.
[44] Vaittinen S, Lukka R, Sahlgren C, et al. Specific and innervation-regulated expression of the intermediate filament protein nestin at neuromuscular and myotendinous junctions in skeletal muscle. Am J Pathol. 1999;154(2):591-600.
[45] Chang HM, Wei IH, Tseng CY, et al. Differential expression of calcitonin gene-related peptide (CGRP) and choline acetyltransferase (ChAT) in the axotomized motoneurons of normoxic and hypoxic rats. J Chem Neuroanat. 2004;28(4):239-251.
[46] Yang J, Dominguez B, de Winter F, et al. Nestin negatively regulates postsynaptic differentiation of the neuromuscular synapse. Nat Neurosci. 2011;14(3):324-330.
[47] Yu J, Guo KH, Li DP, et al. Tolerance of nestin$^+$ cholinergic neurons in the basal forebrain against colchicine-induced cytotoxicity. Neural Regen Res. 2011;6(24):1860-1864.
[48] Yoshikawa A, Atobe Y, Takeda A, et al. A retrograde tracing study of compensatory corticospinal projections in rats with neonatal hemidecortication. Dev Neurosci. 2011;33(6):539-547.
[49] Miyamichi K, Amat F, Moussavi F, et al. Cortical representations of olfactory input by trans-synaptic tracing. Nature. 2011;472(7342):191-196.
[50] Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. 3rd ed. New York: Academic Press. 1997.
[51] Härtig W, Reichenbach A, Voigt C, et al. Triple fluorescence labelling of neuronal, glial and vascular markers revealing pathological alterations in various animal models. J Chem Neuroanat. 2009;37(2):128-138.
[52] Christensen MD, Holbrook EH, Costanzo RM, et al. Rhinotopy is disrupted during the re-innervation of the olfactory bulb that follows transection of the olfactory nerve. Chem Senses. 2001;26(4):359-369.
[53] Wojtowicz JM, Kee N. BrdU assay for neurogenesis in rodents. Nat Protoc. 2006;1(3):1399-1405.
[54] Martončíková M, Lievajová K, Orendáčová J, et al. Odor enrichment influences neurogenesis in the rostral migratory stream of young rats. Acta Histochem. 2011;113(3):326-332.
[55] Ishikawa Y, Ida-Yonemochi H, Nakakura-Ohshima K, et al. The relationship between cell proliferation and differentiation and mapping of putative dental pulp stem/progenitor cells during mouse molar development by chasing BrdU-labeling. Cell Tissue Res. 2012;348(1):95-107.
[56] Miettinen RA, Kalesnykas G, Koivisto EH. Estimation of the total number of cholinergic neurons containing estrogen receptor-alpha in the rat basal forebrain. J Histochem Cytochem. 2002;50(7):891-902.
[57] Lyck L, Santamaria ID, Pakkenberg B, et al. An empirical analysis of the precision of estimating the numbers of neurons and glia in human neocortex using a fractionator-design with sub-sampling. J Neurosci Methods. 2009;182(2):143-156.
[58] Erbagci H, Kizilkan N, Ozbag D, et al. Post-natal growth in the rat pineal gland: a stereological study. Anat Histol Embryol. 2012;41(5):353-357.
[59] Gritti I, Henny P, Galloni F, et al. Stereological estimates of the basal forebrain cell population in the rat, including neurons containing choline acetyltransferase, glutamic acid decarboxylase or phosphate-activated glutaminase and colocalizing vesicular glutamate transporters. Neuroscience. 2006;143(4):1051-1064.

Copyedited by Diwakarla S, Gao F, Yu J, Qiu Y, Li CH, Song LP, Zhao M