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**Neurogenesis in the dentate gyrus of the rat hippocampus enhanced by tickling stimulation with positive emotion**

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## ABSTRACT

Hippocampal neurogenesis is influenced by many factors. In this study, we examined the effect of tactile stimulation (tickling), which induced positive emotion, on neurogenesis in the dentate gyrus (DG) of the hippocampus. Four week-old rats were tickled for 5 min/day on 5 consecutive days and received 5-bromo-2'-deoxyuridine (BrdU) administration for 4 days from the second tickling day. Then they were allowed to survive for 18 h or 3 weeks after the end of BrdU treatment. Neurogenesis in the DG was compared between the tickled and untickled rats by using immunohistochemistry with anti-BrdU antibody. The result showed that the number of BrdU- and NeuN (neural cell marker)-double positive neurons on 18 h as well as 3 weeks of the survival periods was significantly increased in the tickled group as compared with the untickled group. The expression of mRNA of brain-derived neurotrophic factor (BDNF) in the hippocampus of the tickled rats was not altered when compared with the control rats. In conclusion, tickling stimulation which induces positive emotion may affect the generation and survival of new neurons of the DG through the BDNF-independent pathway.

Key words: positive emotion, tickling, neurogenesis, hippocampus, gene expression

## 1. Introduction

New neurons are continually generated in the hippocampal dentate gyrus (DG) and the subventricular zone of the lateral ventricle of the adult mammalian brain, which are incorporated into already existing neural circuits (for reviews, see Lledo *et al.*, 2006; Balu and Lucki, 2009). In the hippocampus, the synaptic plasticity provided by new neurons is likely to mediate various aspects of learning and memory formation. It is demonstrated that various factors influence adult neurogenesis. Stress has the deleterious effects on adult hippocampal neurogenesis such as immobilization stress, footshock, subordination stress, resident intruder stress, predator odor, social isolation and others (Ibi *et al.*, 2008; Chigr *et al.*, 2009; for review, see Balu and Lucki, 2009). It decreases the survival as well as the proliferation of newly generated hippocampal neurons. On the other hand, an enriched environment increases neurogenesis in the DG of adult rodents (Kempermann *et al.*, 1997; van Praag *et al.*, 1999a; Brown *et*

*al.*, 2003; Hattori *et al.*, 2007), which is equipped with paper tubes, nesting material, a rearrangeable set of plastic tubes, a tunnel with various openings and a running wheel (Kempermann *et al.*, 1997). However, the mechanism underlying the positive effect of the exercise (van Praag *et al.*, 1999a) has not been clarified yet.

An event that leads to positive affections such as human laughter is often called eustress or good stress (Berk *et al.*, 1989; McEwen, 2000). Berk *et al.* reported that laughter changes the response of neuroendocrine and stress hormones to have a role in immunomodulation (Berk *et al.*, 1989). Laughter or sense of humor may influence specific health outcomes (Bennett and Lengacher, 2008). We have found that laughter suppresses an increase in the postprandial blood glucose level in patients with type 2 diabetes (Hayashi *et al.*, 2003). Positive affective reactions to sensory pleasure such as sweet tastes were observed in animals as well as human infants (Berridge, 2003). In this paper, we will use a term “positive stress” to describe the events for positive emotions.

It is known that rats exhibit specific ultrasonic vocalization (USVs) in response to various social interactions and stimulation. Knutson *et al.*

found that USVs with 50-kHz were drastically increased in tactile stimulated (tickled) rats (Knutson *et al.*, 1998). The tickling stimulation mimics the dorsal contact and pinning behavior of the rough- and tumble-play of rat (Panksepp and Burgdorf, 2003). The 50-kHz USV index reflecting positive emotional states has been proposed as an evolutionary antecedent to human joy (Panksepp, 2007).

We have recently found that repeated tickling stimulation alters expression of the genes related to feeding-regulation in the adolescent rat hypothalamus (Hori *et al.*, 2009). The accumulation of such experimental knowledge might prove useful in understanding the interaction between mind and gene expression (Murakami and Hayashi, 2002). The purpose of this study is to see whether tactile stimulation which induces positive emotion influences neurogenesis in the DG of the adult rat hippocampus.

## **2. Materials and Methods**

### *Animals*

Wistar rats (Japan SLC Inc., Shizuoka, Japan) were used. After weaning at postnatal day 21, rats were acclimatized by individual housing in

standard polycarbonate cages (W270×L440×H187 mm) with wood chips bedding. The cages were shielded with screens to avoid seeing each other. All rats were provided food and water *ad libitum* and maintained in constant room temperature on a 12:12 light:dark cycle (lights on 7:00 AM). Subjects weighed were divided into two groups (tickled and untickled control groups, n=6 each).

All animal experiments were carried out in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the University of Tsukuba and in accordance with the Regulation for Animal Experiments in the university and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

### *Stimulation*

For tickling stimulation, the method of Knutson *et al.* (Knutson *et al.*, 1998) was slightly modified (Hori *et al.*, 2009). After a 15-sec stimulation-free period, a 28 day-old rat was transferred into a 270×440×

187 cm opaque plastic test box covered with black soft cloth and received tickling stimulation for 15 sec. An animal was grasped on the dorsal side and tickled on the posterior neck with experimenter's fingers, rapidly overturned and vigorously tickled over the abdomen while being pushed onto the floor in a supine position, and then released. This process was repeated four times (the first tickling session). A 1-min rest period was taken, followed by the second session. The stimulus loading of total 5 min was carried out for 5 consecutive days. In the control experiment, rats were allowed without tickling stimulation in the test box for total 5 min.

### *BrdU injection*

Cell proliferation was measured by the incorporation of the thymidine analog 5-bromo-2'-deoxyuridine (BrdU). Each animal was injected intraperitoneally with BrdU (Sigma) (50 mg/kg at a concentration of 10 mg/ml in 0.9% NaCl/7 mM NaOH) daily (30 min before tickling or control sessions) for 4 consecutive days except for the first tickling day, and animals were then killed 18 h or 3 weeks after the last injection of BrdU.



### *Approach latency*

To analyze the index of positive behavior reinforcement, the approach latency to experimenter's hand was measured. After the second tickling or control session, the rat was placed at a corner of the test box and the time until the rat approached and then touched the experimenter's hand at an opposite corner was measured. The maximum 30-sec latency was recorded.

### *Ultrasonic vocalization*

To examine the index of positive emotion, 50-kHz USVs, vocalization was recorded during the tickling stimulation and resting period using a high-frequency microphone (MI-3140, Ono Sokki) on each rat, and the frequency component analysis (hanning window, frequency range: 10-100-kHz, sampling frame length: 4096 points) was performed using an ultra high-band acoustic analysis system (DS-2100, Ono Sokki). After Fourier transformation of time waveforms during vocalization recording, the peak values (dB) of the frequency components in every 15-sec period were plotted on a graph for the analysis.

### *Immunohistochemistry*

Animals were deeply anesthetized by diethyl ether and transcardially perfused with cold saline followed by 4% paraformaldehyde (PFA). Their brains were removed and post-fixed for 24 h in PFA before further processing. Serial coronal sections (70  $\mu\text{m}$  thickness) were cut for the entire hippocampus using a vibration microtome (Linear Slicer Pro7, Dosaka EM).

To assess the differentiation of the newly generated cells, every tenth section was immunostained to detect BrdU and a neuronal marker (NeuN). Free-floating sections were washed in 0.1 M phosphate-buffered saline (PBS), incubated in 2N HCl for 30 min at 37 °C and 0.1 M Tris-HCl for 10 min at room temperature and washed thoroughly with 0.01 M PBS. Following incubation with a blocking solution composed of 0.01 M PBS/0.3% Triton X-100/1% normal bovine serum albumin, they were incubated with primary antibody cocktail containing polyclonal sheep anti-BrdU antibody (1:500, BioDesign) and mouse anti-NeuN antibody (1:4000, Chemicon) in the blocking solution overnight at 4 °C. Following washing, sections were incubated in a cocktail of secondary Cy-3 conjugated anti-sheep IgG antibody (1:500, Jackson ImmunoResearch) and Alexa Fluor 488 conjugated

anti-mouse IgG antibody (1:1000, Invitrogen) for 1.5 h at 4 °C. Sections were mounted onto gelatin coated slides, cover slipped using Fluoromount (Cosmo Bio) and stored at 4 °C until analysis. Fluorescently labeled cells were observed with a confocal laser scanning microscope (LSM510META, Carl Zeiss). Confocal scans were processed with Adobe Photoshop CS4. To assess the phenotype of BrdU-labeled cells, BrdU-positive cells were carefully matched by NeuN-positive cells within the DG cell layer including the adjacent hilar margin or subgranular zone (SGZ) in the sections taken from the hippocampus. The total number of double-positive cells was scored.

#### *RNA preparation and real-time PCR analysis*

Hippocampal tissues dissected were suspended in RNAlater RNA Stabilization Reagent (Qiagen GmbH) and stored at -80 °C. Total RNA fraction was prepared from stored tissue specimens using an RNeasy Mini Kit (Qiagen GmbH) as described in the manufacturer's protocol. The cDNA was synthesized by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and real-time quantitative PCR was performed using a 7300

real-time PCR System (Applied Biosystems) according to the protocol provided by Applied Biosystems. The relative amount of the BDNF mRNA obtained by using Assay ID: Rn 02531967\_s1 (Applied Biosystems) was normalized to the level of an internal control,  $\beta$ -actin (Assay ID: Rn 00667869\_m1).

### *Statistics*

Data were expressed as mean  $\pm$  SEM. Statistically significant differences were tested by unpaired Student's *t*-test with a two-tailed *p*-value. Differences are considered significant, when the *p*-value is  $< 0.05$ .

## **3. Results**

### *Behavioral analysis*

All rats in the tickled group chirped at a frequency close to 50-kHz, an index of positive emotion, during the tickling sessions from the initial session on the first day. In contrast, no such chirp was noted in the control group throughout the experimental period. The mean number  $\pm$  SEM per rat of 50-kHz vocalization emitted for 5 days was as follows:  $48.2 \pm 4.0$  in the

tickled rats and  $0.0 \pm 0.0$  in the untickled control rats ( $p < 0.001$ ,  $n = 6$  each).

The approach latencies of the tickled and untickled groups just after tickling stimulation were  $7.1 \pm 1.9$  sec and  $24.7 \pm 1.8$  sec ( $p < 0.001$ ,  $n = 6$ ), respectively. These data demonstrated that tickling stimulation was effective to cause positive emotion to rats.

#### *Effect of tickling on neurogenesis in the DG of hippocampus*

To examine the effect of tickling on neurogenesis, animals were killed 18 h after the last injection (the 5th day) of BrdU and the number of both BrdU- and NeuN-labeled cells was counted in the SGZ of the DG. As shown in Fig. 1, the BrdU- and NeuN-double positive neurons were increased significantly by 57% in the tickled rats as compared with those in the control rats ( $1165.0 \pm 163.5$  versus  $743.3 \pm 69.5$ ,  $p < 0.05$ ). Although the number of BrdU-positive and NeuN-negative cells showed a tendency to increase in the tickled rats towards controls, no significant difference was found. The ratio of BrdU- and NeuN-double positive cells to total BrdU-positive cells was 20.3% in the tickled rats and 17.9% in the control rats, respectively.

Next, we investigated the effect on the survival of newly divided cells by

allowing animals for 3 weeks after the last injection of BrdU. As shown in Fig. 2, the tickling increased the BrdU-positive cells, irrespective of the NeuN-immunoreactivity. Thus, BrdU- and NeuN-double positive neurons were significantly increased by 40% in the tickled rats as compared with those in the control rats ( $2305.0 \pm 145.1$  versus  $1641.7 \pm 235.4$ ,  $p < 0.05$ ). In addition, the BrdU-positive and NeuN-negative cells were also significantly increased by 46% in the tickled rats as compared with controls ( $3288.3 \pm 288.3$  versus  $2251.7 \pm 316.9$ ,  $p < 0.05$ ). The ratio of BrdU- and NeuN-double positive cells to total BrdU-positive cells was 41.2% in the tickled rats and 42.2% in the control rats, respectively.

#### *Change in BDNF in the DG by tickling*

BDNF is the neurotrophin that has been well studied in the relationship between neurogenesis and stress. To see if the expression of BDNF is changed by tickling stimulation, the expression levels of BDNF mRNA after tickling were examined by real-time PCR. BDNF mRNA was not altered in the hippocampus of the tickled groups 4 h after the stimulation (101%,  $p > 0.05$ ), as shown in Fig. 3.

#### 4. Discussion

We have previously found that repeated stimulation with tickling alters the expression of feeding-regulation genes in the rat hypothalamus (Hori *et al.*, 2009), which suggests that positive stress may generally change the gene expression in various regions of the mammalian brain. In this study, we report that the tickling stimulation with positive emotion increases neurogenesis in the rat hippocampus.

In the SGZ of the DG of young adult rat, about 9,000 new cells are produced per day, and some of these progenitors mature locally into granule neurons of the DG, sending axonal projections to the molecular layer in CA3 region (Cameron and McKay, 2001). It is known that many extrinsic and intrinsic factors influence this process: composition of cell proliferation, differentiation and migration, incorporation into networks and survival (for review, see Lledo *et al.*, 2006) . Kempermann *et al.* reported that an enriched environment had a survival-promoting effect on proliferating neuronal precursor cells in the DG of mice (Kempermann *et al.*, 1997). van Praag *et al.* found that voluntary physical exercise such as running induced neuronal progenitor proliferation and then enhanced the survival (van Praag

*et al.*, 1999a). Brown *et al.* pointed out that the running effect was specific in the hippocampus, which suggested that newly formed neurons of the DG were integrated into the hippocampal functioning (Brown *et al.*, 2003). It has been reported that voluntary exercise enhances long-term potentiation as well as neurogenesis in rodents (van Praag *et al.*, 1999b; Farmer *et al.*, 2004). We showed that positive stress by tickling stimulation affected the proliferation of hippocampal neuronal progenitors (Fig. 1). In addition, the increased number of newly-formed neurons was maintained up to 3 week of survival (Fig. 2). Tickling stimulus loading has a similar effect to spontaneous physical activity. When we compared the neurogenesis at 18 h after the last BrdU injection (Fig.1) and at 3 weeks after the last injection (Fig.2), the number of BrdU- and NeuN-double positive neurons was doubled in the latter as compared with the former. This may be due to the further proliferation of BrdU-positive cells and the differentiation into neurons between 18 h and 3 weeks.

Woehr *et al.* have recently reported the relationship of adult hippocampal cell proliferation and emotional affect assessed by tickling-induced ultrasonic vocalizations (Woehr *et al.*, 2009). They



examined BrdU and proliferating-cells-nuclear-antigen immunolabeled cells after tickling for 5 days using Wistar rats of 6-8 week age. According to their results, hippocampal cell proliferation correlated positively with appetitive 50-kHz-calls, whereas negatively with aversive 22-kHz-calls. But they did not identify neurons by specific markers and examined only the effects on the proliferation but not the survival. In the present study, we used only the rats that chirped better at a frequency of 50-kHz in the tickled group in the experiments on neurogenesis. We have found the proliferation of the neuronal progenitors and the survival of these neurons in DG of rats chirping 50-kHz calls with positive emotional states.

What kinds of molecules are involved in the induction of neurogenesis accompanied by positive stress? It was reported that the expressions of *c-fos* gene during rough and tumble play (Gordon *et al.*, 2002) and playback of 50-kHz USV (Sadananda *et al.*, 2008) were differential in rat brain regions, but the hippocampus was not referred in their studies. BDNF is one of the most favorable molecules among many factors, because adult brain neurogenesis is potently stimulated by BDNF (Pencea *et al.*, 2001; Lee *et al.*, 2002; Scharfman *et al.*, 2005) and the BDNF-dependent neurogenesis is

inhibited by stress (for review, see Blau and Lucki, 2009). Voluntary exercise altered BDNF mRNA (Farmer *et al.*, 2004). In our study, BDNF mRNA in the hippocampus was not altered after tickling stimulation (Fig. 3). The gene expressions of several neurotrophic factors besides BDNF in the hippocampus between the tickled and untickled rats were compared using genome-wide gene expression analyses, but obvious changes were not observed (data not shown). Neurogenesis by tickling stimulation may be BDNF-independent. Further analysis is necessary to see how positive stress causes the change of the phenotype such as neurogenesis.

### **Acknowledgements**

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**Fig. 1** Effect of tickling for 5 days on the proliferation of newly divided cells in the DG of 4 week-old rats. BrdU (50 mg/kg) was intraperitoneally injected daily for 4 days from the second tickling day. Animals were killed 18 h after the last injection of BrdU. The total number of BrdU-positive (BrdU+) cells was counted on serial coronal sections throughout the rostro-caudal extent of hippocampus and then BrdU-positive cells were estimated to be NeuN-positive (BrdU+/NeuN+) or negative (BrdU+/NeuN-) cells. (A) The numbers of immunoreactive cells using anti-BrdU antibody or anti-NeuN antibody in the DG of control and tickled rats are shown. Values indicate mean  $\pm$  SEM (n=3 per group). \* $p$ <0.05 versus control. (B) Confocal laser scanning microscope was used to detect newly generated neurons (NeuN-positive cells, green) among the population of newborn cells (BrdU-positive cells, red). Double positive cells appeared as yellow (marked with arrowheads). A boxed area of the SGZ in DG is shown in the right.

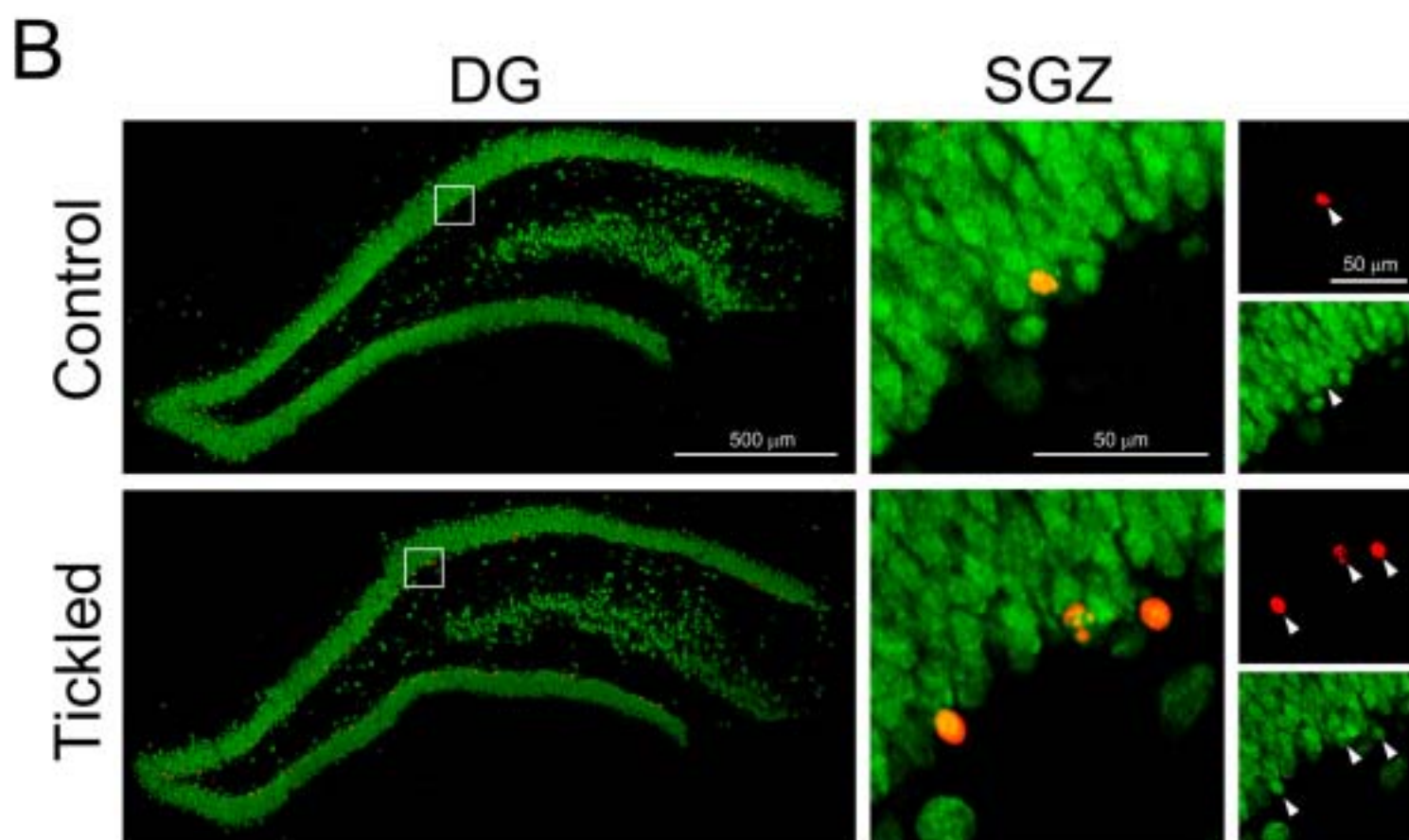
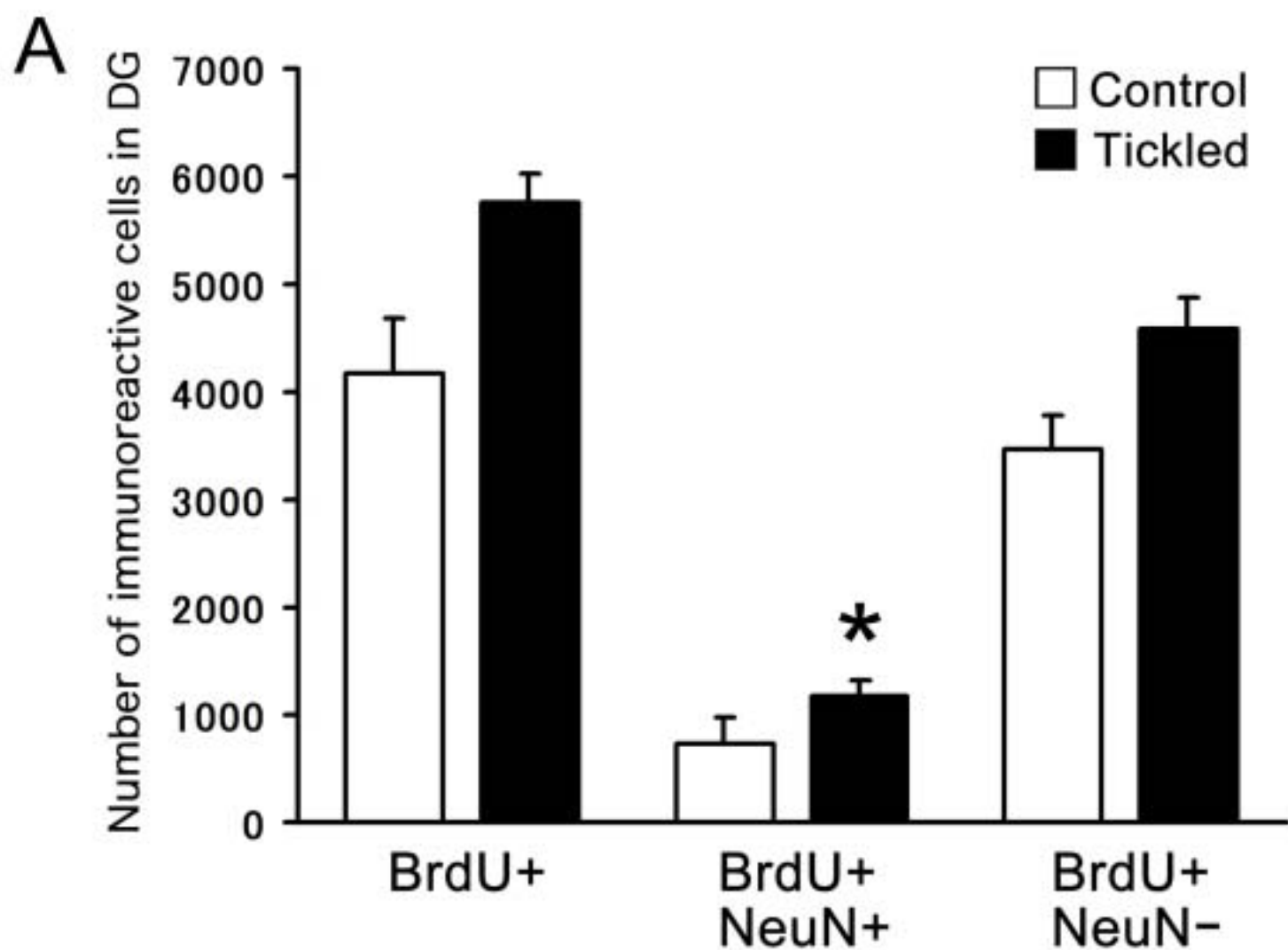
**Fig. 2** Effect of tickling for 5 days on the survival of newly divided cells in the DG of 4 week-old rats. BrdU (50 mg/kg) was intraperitoneally injected daily from the second tickling day. Animals were killed 3 weeks after the



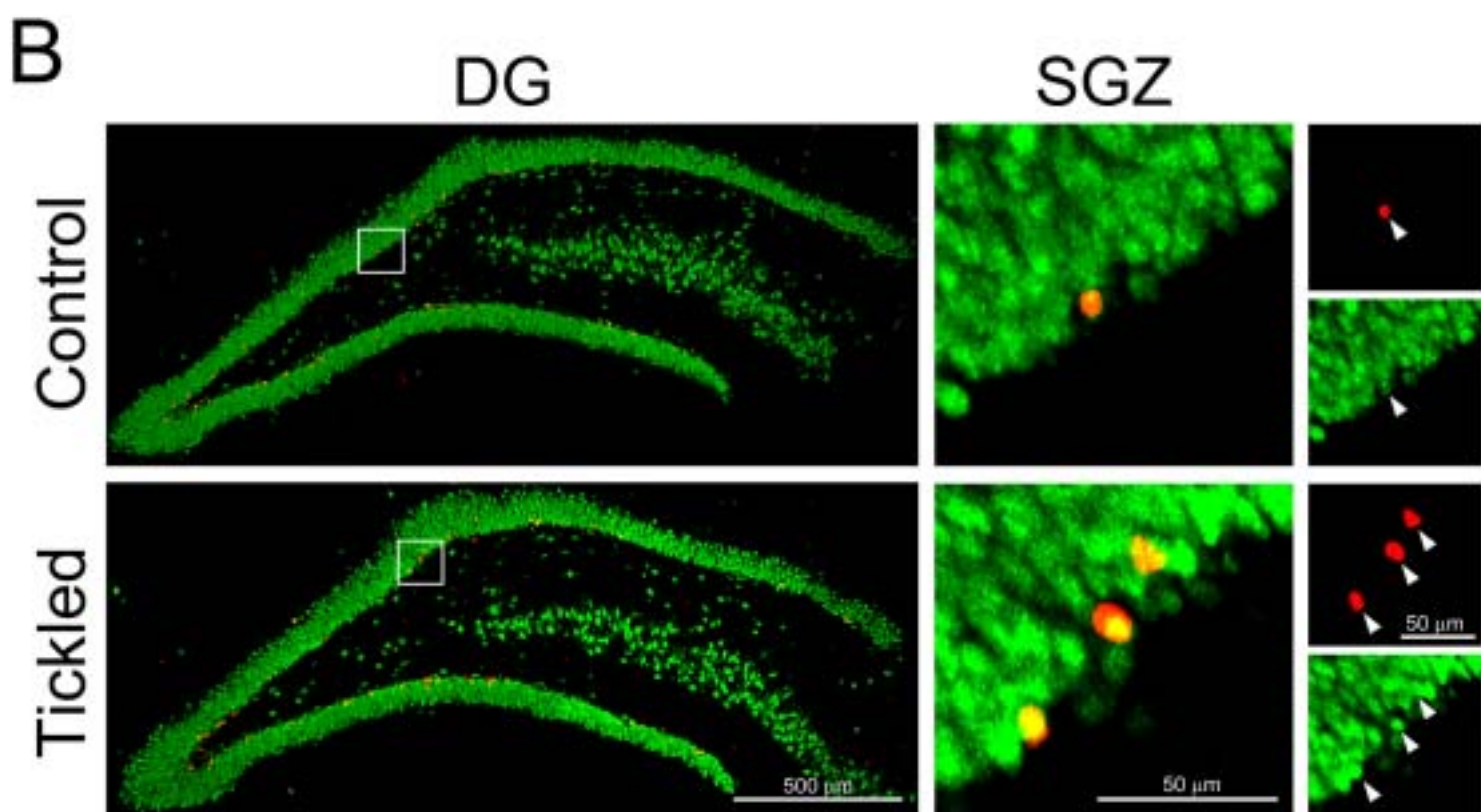
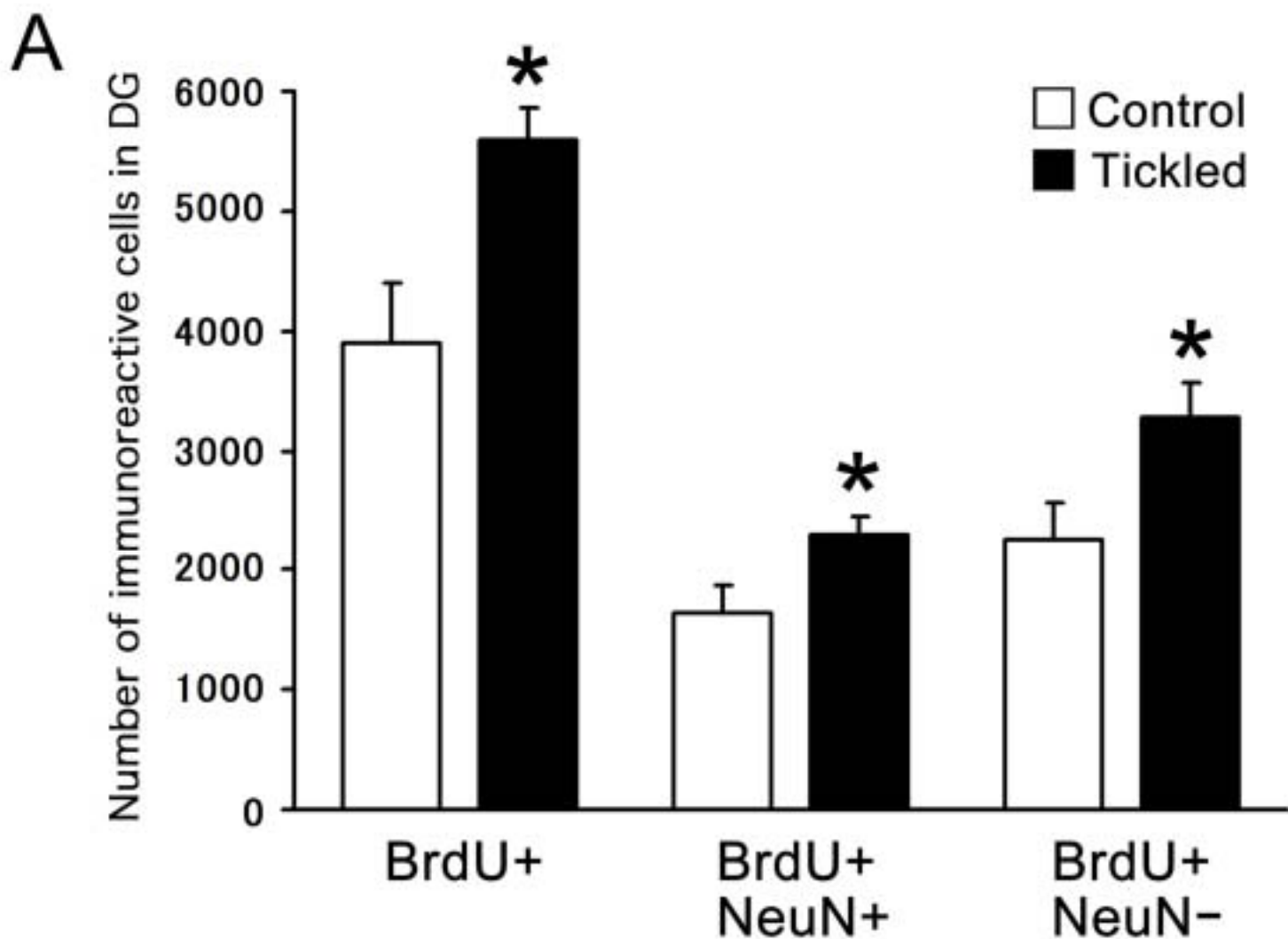
last injection of BrdU. The total number of BrdU-positive (BrdU+) cells was counted and then BrdU-positive cells were estimated to be NeuN-positive (BrdU+/NeuN+) or negative (BrdU+/NeuN-) cells. (A) The numbers of immunoreactive cells in the DG of control and tickled rats are shown. Values indicate mean  $\pm$  SEM (n=3 per group). \* $p$ <0.05 versus control. (B) Confocal laser scanning microscope was used to detect neurons (NeuN-positive cells, green) among the population of newborn cells (BrdU-positive cells, red). Double positive cells appeared as yellow (marked with arrowheads). A boxed area of the SGZ in DG is shown in the right.

**Fig. 3** Effect of tickling for 5 days in 4 week-old rats on BDNF expression in the hippocampus. Total RNA fraction of the hippocampus was prepared from the control or tickled rats 4 h after the last stimulation and then the real-time PCR analysis was carried out on BDNF mRNA. Values indicate mean  $\pm$  SEM (n=3 per group).

Yamamuro T\_Fig. 1



# Yamamuro T\_Fig. 2



Yamamuro T\_Fig. 3

