Curcumin reverses impaired hippocampal neurogenesis and increases serotonin receptor 1A mRNA and brain-derived neurotrophic factor expression in chronically stressed rats

Ying Xu, Baoshan Ku, Li Cui, Xuejun Li, Philip A. Barish, Thomas C. Foster, William O. Ogle

ABSTRACT

Curcuma longa is a major constituent of Xiaoyao-san, the traditional Chinese medicine, which has been used to effectively manage stress and depression-related disorders in China. As the active component of curcuma longa, curcumin possesses many therapeutic properties; we have previously described its antidepressant activity in our earlier studies using the chronic unpredictable stress model of depression in rats. Recent studies show that stress-induced damage to hippocampal neurons may contribute to the pathophysiology of depression. The aim of this study was to investigate the effects of curcumin on hippocampal neurogenesis in chronically stressed rats. We used an unpredictable chronic stress paradigm (20 days) to determine whether chronic curcumin treatment with the effective doses for behavioral responses (5, 10 and 20 mg/kg, p.o.), could alleviate or reverse the effects of stress on adult hippocampal neurogenesis. Our results suggested that curcumin administration (10 and 20 mg/kg, p.o.) increased hippocampal neurogenesis in chronically stressed rats, similar to classic antidepressant imipramine treatment (10 mg/kg, i.p.). Our results further demonstrated that these new cells mature and become neurons, as determined by triple labeling for BrdU and neuronal- or glial-specific markers. In addition, curcumin significantly prevented the stress-induced decrease in 5-HT1A mRNA and BDNF protein levels in the hippocampal subfields, two molecules involved in hippocampal neurogenesis. These results raise the possibility that increased cell proliferation and neuronal populations may be a mechanism by which curcumin treatment overcomes the stress-induced behavioral abnormalities and hippocampal neuronal damage. Moreover, curcumin treatment, via up-regulation of 5-HT1A receptors and BDNF, may reverse or protect hippocampal neurons from further damage in response to chronic stress, which may underlie the therapeutic actions of curcumin.
1. Introduction

Depression is a highly debilitating disorder that has been estimated to affect up to 21% of the world population (Schechter et al., 2005). Chronic stress can induce depressive disorders, and animal stress models are also widely used in pre-clinical antidepressant evaluation (García, 2002). Although the exact neural substrates underlying depression are still under investigation, the hippocampus has received a great deal of attention, with much of the evidence based on studies using psychosocial stress paradigms (D’Sa and Duman, 2002; Warner-Schmidt and Duman, 2006). Chen et al. reported that chronic stress prolongs learned helplessness behavior and increases plasma corticosterone levels, which may be associated with decreased hippocampal cell proliferation (Chen et al., 2006). However, both of the negative effects of inescapable stress on escape behavior, changes in corticosterone levels and cell proliferation in the adult rats hippocampus, were reversed by fluoxetine (serotonin reuptake inhibitor, SSRI) administration for 9 days (Malberg and Duman, 2003). Moreover, the serotonin receptor 1A (5-HT₁A receptor) appears to mediate these effects: specific antagonists decreased cell proliferation in the adult dentate gyrus (Radley and Jacobs, 2002). Recent studies suggest that brain-derived neurotrophic factor (BDNF) plays an important signaling role in preventing neuronal death during development, and promotes cell survival during stressful conditions such as depression and post-traumatic stress disorders (Tsankova et al., 2006; Duman and Monteggia, 2006). 5-HT₁A receptors and BDNF therefore represent attractive targets for studying the pathophysiology of depression and the actions of antidepressant treatment.

There are four main types of classical antidepressants in clinical practice, monoamine oxidase inhibitors, tricyclic...
antidepressants, serotonin reuptake inhibitors and norepinephrine- serotonin reuptake inhibitors. However, the therapeutic benefits of these drugs are often accompanied by unwanted side effects and the precise mechanisms of action are not well understood (Malberg et al., 2000a,b). The development of more selective and ultimately safer antidepressants requires an understanding of the mechanisms involved in the development of depression. In addition, seeking effective antidepressant agents from traditional herbs may enable us to uncover novel treatments for depressive disorders and may further reveal as yet unknown mechanisms by which depressive symptoms can be alleviated.

Curcumin longa is commonly found in traditional Chinese herbal medicines, such as Xiaoyao-san and Jieyu-wan, which are used to treat the symptoms of mental stress, hypochondriac pain and mania (Xu et al., 2006). As the major constituent of curcuma longa, curcumin possesses many therapeutic properties including antioxidant, anti-inflammatory, immunodulatory and neuroprotective activities (Motterlini et al., 2000; Thiyagarajan and Sharma, 2004). Previous studies in our laboratory demonstrated that acute and chronic curcumin administration significantly decreased immobility time in behavioral despair tests, which suggests that the central monoaminergic neurotransmitter system may be involved in the anti-depressive effects of curcumin (Xu et al., 2005a,b). We also demonstrated that the behavioral alterations and elevated serum corticosterone levels in chronically stressed rats were reversed by chronic curcumin administration (Xu et al., 2006). However, to date, studies investigating the molecular mechanism of curcumin relevant to its antidepressant effects have not been conducted.

In the present study, we used an unpredictable chronic stress paradigm to determine whether chronic curcumin treatment (5, 10 and 20 mg/kg, p.o.), can alleviate or reverse the effects of stress on adult neurogenesis, which may be implicated in stress-related depression. In addition, to further investigate the possible molecular mechanisms underlying the therapeutic effects of curcumin, we evaluated the expressions of 5-HT_{1A} receptor mRNA and BDNF protein in the hippocampus of chronically stressed rats.

2. Results

2.1. The effects of curcumin on the number of BrdU-positive cells in the dentate gyrus

The effects of 21 days of curcumin administration on cell proliferation in the dentate gyrus of stressed rats are shown in Fig. 1. An analysis of the number of BrdU-positive cells (shown as brown granules) demonstrated that chronic stress significantly decreased the number of BrdU-positive cells in the subgranular zone (SGZ) and hilus of the hippocampus relative to control rats (F(5,28)=12.34, p<0.05 vs. non-stressed control, Fig. 2). Chronic administration of curcumin (at 10 and 20 mg/kg) reversed these stress-induced changes and increased the number of BrdU-labeled cells by 20–28% relative to control-treated stressed rats. Chronic treatment with the classical antidepressant imipramine (10 mg/kg, i.p.) increased BrdU-labeled cells by 48%.

![Fig. 2 – The number of BrdU-positive cells was significantly increased after 21 days of treatment with curcumin (mean± SEM, n=5–6). *p<0.05, compared with vehicle group. †p<0.05, compared with stress + vehicle group.](image)

Using the marker for mature neurons, NSE, and the astrocyte marker GFAP, the phenotype of the BrdU-positive cells was determined by triple immunofluorescent labeling (Fig. 3). Analysis of the BrdU-positive cells in the non-stressed control and curcumin-treated (20 mg/kg) stressed rats showed that 65% and 59% expressed NSE, whereas only 12% and 13% expressed GFAP, respectively, 4 weeks after the last injection of BrdU.

2.2. The effects of curcumin on hippocampal 5-HT_{1A} mRNA

In situ hybridization analysis revealed that 5-HT_{1A} mRNA levels were significantly reduced across hippocampal CA1 (F(5,28)=8.89, p<0.01 vs. non-stressed control) and DG subfields (F(5,28)=9.73, p<0.01 vs. non-stressed control) in chronically stressed rats. Administering curcumin (10 and 20 mg/kg, p.o.) or imipramine (10 mg/kg, i.p.) prior to the stress protocol prevented these changes (F(5,28)=8.89, p<0.05; F(5,28)=9.73, p<0.05 vs. vehicle-treated, stressed rats) (Fig. 4 and Table 1).

2.3. The effects of curcumin on hippocampal BDNF levels

As shown in Fig. 5 and Table 2, chronic stress decreased BDNF levels in the DG (F(5,28)=12.32, p<0.05 vs. non-stressed control), CA1 and CA3 hippocampal subfields (F(5,28)=9.84, p<0.01; F(5,28)=11.16, p<0.05 vs. non-stressed control). Chronic administration of curcumin (5, 10 or 20 mg/kg, p.o.) increased BDNF levels throughout the DG (F(5,28)=12.32, p<0.05 vs. vehicle-treated, stressed rats), CA1 (F(5,28)=9.84, p<0.05; p<0.01 vs. vehicle-treated, stressed rats) and CA3 subfields (F(5,28)=11.16, p<0.05; p<0.01 vs. vehicle-treated, stressed rats). Similar results were obtained with the 10 mg/kg imipramine administration (p<0.05; p<0.01).

3. Discussion

The results of the present study extend previous work on the modulation of the HPA axis in chronically stressed rats by curcumin treatment with two significant findings. First, our
results demonstrate that curcumin administration increased hippocampal neurogenesis in chronically stressed rats, similar to the effects of the classic antidepressant imipramine. Second, curcumin significantly prevented the stress-induced decrease in 5-HT1A mRNA and BDNF protein levels in the hippocampus, two molecules implicated in neurogenesis (Rutherford et al., 1997; Santarelli et al., 2003; Henn et al., 2004).

Chronic stress is a risk factor for the onset of major depression. Chronic unpredictable stress is often used as an animal model of depression in rodents because it induces symptoms of depression, including increased corticosterone levels and impaired learning and memory abilities, measured by shuttle box responding (Gold and Chrousos, 2002; Xu et al., 2006; Warner-Schmidt and Duman, 2006). The theoretical premise behind this method is that depression is the outcome of an eventual inability to cope with a stream of dissimilar unpleasant stimuli imposed by the environment (Ferretti et al., 1995). To simulate this effect in animals, stressors are used to induce behavioral deficits, which can subsequently be reversed by antidepressant treatments (Takamori et al., 2001; Katz and Hersh, 1981; Maier, 1984; Kennett et al., 1986). In the model used in our earlier study, rats exposed to a regime of chronic stress exhibited altered escape performance to an aversive stimulus, namely increased failures to escape from

Fig. 3 – Triple labeling confirms that BrdU-positive cells mature into neurons. Rats received BrdU injections 2 days after the last curcumin treatment and were killed 4 weeks later. A representative confocal laser-scanning image (66×) of cells triple-labeled with BrdU (green) and NSE or GFAP (red) is shown. Scale bar, 10 μm.
an electric footshock as well as disruption of the HPA axis negative feedback, characterized by an exaggerated corticosterone response. However, these abnormalities could be reversed if animals were chronically administered curcumin during the stressor period (Xu et al., 2006). In this context, it is interesting to find that neurogenesis in the adult hippocampus is sensitive to and markedly regulated by chronic stress. Curcumin (10 and 20 mg/kg, p.o., the effective doses for behavioral responses) reversed the stress-induced decrease in progenitor cell proliferation in subgranular zone, indicating that it can increase hippocampal neurogenesis in stressed rats.

Table 1 – The effects of curcumin on 5-HT\textsubscript{1A} receptor mRNA expression in stressed rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Brain region 5-HT\textsubscript{1A} receptor mRNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CA1</td>
</tr>
<tr>
<td>Control</td>
<td>100.0±2.2</td>
<td>100.0±1.9</td>
</tr>
<tr>
<td>Stress+vehicle</td>
<td>60.7±2.1**</td>
<td>77.5±0.7</td>
</tr>
<tr>
<td>Curcumin 5</td>
<td>72.9±1.9</td>
<td>73.8±1.4</td>
</tr>
<tr>
<td>10</td>
<td>80.1±1.7&quot;</td>
<td>79.1±1.2</td>
</tr>
<tr>
<td>Curcumin 20</td>
<td>85.7±0.9*</td>
<td>81.2±0.8</td>
</tr>
<tr>
<td>Imipramine 10</td>
<td>91.4±1.3**</td>
<td>85.6±1.3</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. and expressed as a percentage of the control group (n=5–6). **p<0.01, compared with control group; *p<0.05, **p<0.01, compared with stress+vehicle group.  

Preclinical studies have suggested that stress, depending on the species and the stressor (immobilization, restraint and psychosocial conflict), may alter brain structure and activity (Kim and Diamond, 2002; Rosenbrock et al., 2005). The stress-sensitive hippocampus is one region that has recently received significant attention in mood disorder research. Chronic stress and consequent elevated glucocorticoid exposure are associated with structural and functional changes in the hippocampus, including atrophy of apical dendrites of CA3 pyramidal neurons (Campbell and MacQueen, 2004; Li et al., 2004). More importantly, chronic unpredictable stress decreases neurogenesis in the adult hippocampus, which may contribute to this hippocampal atrophy (Li et al., 2006;
Neurogenesis has been documented in the adult brains of a variety of species, such as rodents and humans (Duman et al., 2001; Kempermann, 2006). In the hippocampus, progenitor cells are located in the subgranular zone where they divide and give rise to new neurons, which have physiological characteristics that are similar to existing neuron populations (Duman, 2004). A recent analysis reports that there are approximately 250,000 new neurons formed each month in the adult rodent hippocampus and about 50% of these cells differentiate and express neuronal markers (Cameron and McKay, 2001; Li et al., 2004; Kempermann, 2006). In the present study, curcumin reversed the stress-induced decrease in hippocampal neurogenesis in stressed rats. The majority (59%) of the surviving BrdU-positive cells expressed a neuronal marker (NSE) after 4 weeks. A much smaller number (13%) of cells expressed the glial marker GFAP. The remaining 28% of cells were not labeled with either cell marker and may represent either another phenotype or quiescent undifferentiated cells. These effects are consistent with previous studies examining neurogenesis following chronic treatment with classic antidepressants (imipramine and fluoxetine) in stressed rats (Santarelli et al., 2003; Malberg et al., 2000a,b). Therefore, we raise the possibility that an up-regulation of neurogenesis would oppose the action of stress or/and depression, and it may contribute to the beneficial actions of curcumin.

Table 2 – The effects of curcumin on BDNF expression in the hippocampus of stressed rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Brain region BDNF expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CA1</td>
</tr>
<tr>
<td>Control</td>
<td>100.0±5.0</td>
<td>100.0±4.0</td>
</tr>
<tr>
<td>Stress + vehicle</td>
<td>40.1±3.1***</td>
<td>43.0±4.8*</td>
</tr>
<tr>
<td>Curcumin 5</td>
<td>71.2±3.2**</td>
<td>55.7±6.1</td>
</tr>
<tr>
<td>Curcumin 10</td>
<td>62.3±3.0***</td>
<td>76.1±5.6*</td>
</tr>
<tr>
<td>Curcumin 20</td>
<td>90.5±4.7**</td>
<td>87.6±4.7**</td>
</tr>
<tr>
<td>Imipramine 10</td>
<td>96.7±3.2**</td>
<td>90.5±7.2**</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. based on the average of three sections for each brain region per animal and are expressed as a percentage of the control group (n=5–6). *p<0.05, **p<0.01, compared with the control group; *p<0.05, **p<0.01, compared with the stress + vehicle group.
Previous work has shown that alterations in 5-HT transmission are associated with changes in adult cell proliferation, since significant decreases in the number of newborn cells in the hippocampus are observed following 5-HT depletion (Brezun and Daszuta, 1999, 2000). Other studies further suggest that increases in adult neurogenesis after fluoxetine (a selective 5-HT reuptake inhibitor) administration require the activation of 5-HT₁A receptors (Santarelli et al., 2003), which is consistent with the results that 5-HT₁A receptor antagonists or knockouts decrease or lack, respectively, cell proliferation in the dentate gyrus (Radley and Jacobs, 2002; Santarelli et al., 2003). The 5-HT₁A receptor has been identified as an inhibitory somatodendritic autoreceptor in the raphe serotonergic cells and as a postsynaptic receptor in serotonergic terminal fields (Hall et al., 1985). Animal studies suggest that increases in neurotransmission at postsynaptic 5-HT₁A receptors may mediate the therapeutic effects of some antidepressant drugs (Welner et al., 1989). The highest density of 5-HT₁A receptor binding sites occurs in the hippocampus and other limbic regions (Marcinkiewicz et al., 1984). In addition to regulating HPA axis dysfunction, earlier studies in our laboratory demonstrate that curcumin treatment increased brain levels of several monoamines, including 5-HT and norepinephrine (NE) in animal behavioral despair tests (Brezun and Daszuta, 1999, 2000). Other studies further suggested that increases in neurotransmission at postsynaptic 5-HT₁A receptors may mediate the therapeutic effects of some antidepressant drugs (Welner et al., 1989). The highest density of 5-HT₁A receptor binding sites occurs in the hippocampus and other limbic regions (Marcinkiewicz et al., 1984). In addition to regulating HPA axis dysfunction, earlier studies in our laboratory demonstrate that curcumin treatment increased brain levels of several monoamines, including 5-HT and norepinephrine (NE) in animal behavioral despair tests (Xu et al., 2005a,b). From this line of reasoning, we explored whether curcumin-induced neurogenesis in stressed rats was related to the trophic effects of 5-HT and activation through the 5-HT₁A receptor. We identified a decrease in 5-HT₁A mRNA levels across hippocampal CA1 and DG subfields in chronically stressed rats. This reduction of 5-HT₁A mRNA was significantly ameliorated in rats administered 10 and 20 mg/kg curcumin. These results were paralleled by the curcumin-induced increase in cell proliferation in the dentate gyrus of stressed rats. It is possible that 5-HT, through activity at 5-HT₁A receptors, is a potent stimulator of cell proliferation and subsequent neurogenesis in the hippocampus following curcumin administration.

Among the molecular factors, only serotonin and some neurotrophic factors can increase cell proliferation and neurogenesis in the subgranular layer of the dentate gyrus (Banasr et al., 2004). Activation of 5-HT receptors coupled to cAMP production and CREB activation can induce transcription of brain derived neurotrophic factor (BDNF) gene (Mattson et al., 2004). BDNF plays a central role in brain development and plasticity by opposing neuronal damage and promoting neurogenesis and cell survival (Takahashi et al., 1998; Banasr et al., 2004). In the present study, chronic curcumin administration (5, 10 and 20 mg/kg, p.o., 21days) reversed the stress-induced decrease in BDNF levels across all hippocampal subfields in a similar time course to the up-regulation of neurogenesis, these effects were similar to those observed for the classical antidepressant imipramine (10 mg/kg, i.p.). These results suggest that BDNF expression in the hippocampus may mediate the antidepressant effects of curcumin following chronic stress.

4. Experimental procedures

4.1. Animals

Male Sprague-Dawley (SD) rats, weighing 190–200 g at the beginning of the experiment, were obtained from the Animal Center at Peking University Health Science Center. The rats were housed six per cage under standard colony conditions, with a 12-h light/12-h dark cycle and provided food and water ad libitum. They were allowed to acclimatize to the colony for at least 5 days prior to any experimentation. The experimental procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (revised 1996).

4.2. Drugs and drug administration

Curcumin and the clinically effective tricyclic antidepressant imipramine hydrochloride were purchased from Sigma Chemical (USA). Bromodeoxyuridine (BrdU) and the BrdU monoclonal antibody immunohistochemistry detection kit were purchased from Boster Biological Technology Corporation (Beijing, China). The anti-NSE antibodies, anti-glial fibrillary acidic protein (GFAP) antibodies, anti-BDNF antibodies and the respective secondary antibodies were purchased from Zhongshan Technology (Beijing, China). For oral (p.o.) administration, fresh curcumin was dissolved in peanut oil and diluted to the desired concentration on the day of experiment. For intraperitoneal (i.p.) injections, imipramine was dissolved in double-distilled water. Curcumin (5, 10 and 20 mg/kg, p.o. or imipramine (10 mg/kg, i.p.) was administered daily for 21 days. Behavioral testing commenced 60 min after the last drug treatment. In a preliminary experiment, peanut oil (p.o., 1 ml/kg) and redistilled water (i.p., 1 ml/kg) were used as control treatments and the behavioral data did not differ between the rats that received the two vehicle solutions (Xu et al., 2006). Therefore, we chose to present only the peanut oil control group data for comparison.

4.3. Chronic stress procedure and preparation of brain sections

The stressed rats were subjected to the following conditions used by Molina et al. (1990) and Murua et al. (1991) with minor modifications. Stress was administered once per day over a period of 20 d between 8:00 am to 12:00 am. The order of stressors used was as follows: shaker stress (high speed, 45 min), cold swim (10 °C, 5 min), restraint (1.5 h), tail pinch (1 min), water deprivation (24 h), foot shock for 30 min (1 mA,
1 s duration, average 1 shock/min), cold swim (10 °C, 5 min), food deprivation (24 h), restraint (2 h), shaker stress (high speed, 1 h), tail pinch (1 min), water deprivation (24 h), 24-h social isolation (the rats were placed individually in 30×15×10 cm acrylic cages in another housing room and were returned to their home cage after 24 h), overnight illumination, cold swim (8 °C, 5 min), shaker stress (high speed, 1.5 h), restraint (2.5 h), tail pinch (2 min), food deprivation (24 h) and 24-h social isolation. On day 21, rats were treated with BrdU or euthanized for assessment of hippocampal neurogenesis and gene and protein expression.

For BrdU labeling, the rats were administered BrdU (100 mg/kg, once per day for 2 d, i.p.) after chronic stress. Twenty-four hours or 28 d after the last BrdU injection, the rats were killed and perfused with cold saline for 10 min followed by 4% cold paraformaldehyde for 15 min. The brains were post-fixed overnight in cold paraformaldehyde and stored at 4 °C in 30% sucrose. Serial sections (35 μm) were cut through the entire hippocampus (~1.72 mm to ~6.72 mm from the bregma, Paxinos and Watson, 2005) on a freezing microtome, the sections were then stored at −20 °C.

To determine whether the progenitor cells could differentiate into neurons, another group of rats administered 20 mg/kg curcumin was continued to be fed normally for another 28 days without any drug treatment after the last BrdU injection. These rats were perfused and tissue was processed as described above.

4.4. Immunohistochemistry

Sections were incubated in 2 mol/L HCl for 30 min and 0.1 mol/L borate sodium (pH 8.5) for 10 min following DNA denaturation and a PBS rinse, and then incubated in a methanol solution containing 0.5% H2O2 for 30 min to eliminate endogenous peroxidases. After preincubation in normal goat serum for 60 min at 37 °C, sections were incubated in anti-mouse BrdU (1:50) or anti-rabbit BDNF (1:50) antibodies overnight at 4 °C and the respective secondary antibodies (biotinylated goat anti-mouse IgG and goat anti-rabbit IgG) for 1 h at room temperature, which was followed by amplification with a streptavidin–biotin complex (1:200, Vector Labs). The sections were then developed in 100 mmol/L acetate buffer containing 0.02% 3,3′-diaminobenzidine (DAB) (Beijing Zhongshan Biotechnology, China), 4% nickel ammonium sulfate and 0.03% H2O2 for 20 min at room temperature. BrdU-positive cells were visualized and counted under an Olympus BX50 microscope (Olympus, Tokyo, Japan).

4.5. Immunofluorescence labeling

To determine whether the BrdU-labeled cells could differentiate into neurons, the sections were triple-labeled for BrdU, neuron specific enolase (NSE, a marker for mature neurons) and glial fibrillary acidic protein (GFAP, a marker for astrocytes). Sections were pretreated in 2× SSC containing 50% formamide for 2 h at 65 °C and 2 mol/L HCl for 30 min at 37 °C, and then rinsed with 0.1mol/L borate sodium for 10 min. After preincubation in PBS containing 3% normal goat serum and 0.1% Triton-X-100 at 37 °C for 1 h, the sections were subsequently incubated in an antibody cocktail containing mouse anti-BrdU monoclonal IgG (1:50), rabbit anti-NSE monoclonal IgG (1:100) and rabbit anti-GFAP monoclonal IgG (1:200) antibodies overnight at 4 °C. The sections were rinsed with PBS and blocked with a goat anti-mouse IgG-FITC (1:50) for 1 h at 37 °C. After a PBS rinse, the sections were re-incubated with goat anti-rabbit IgG-Cy3 (1:50) for 1 h at 37 °C and were mounted with cover slips on glass slides after rinsing with distilled water. Fluorescence was detected using a Bio-Rad Radiance 2100 confocal system (Bio-Rad Laboratories, Inc, Hercules, CA, USA) with a Nikon TE300 microscope (Nikon, Tokyo, Japan). The confocal laser microscope was equipped with an argon laser emitting at 488 nm and a helium/neon laser emitting at 543 nm. The images were viewed through a 40× lens (Nikon Plan Fluo) image in a single optical plane and the different channel images were acquired sequentially (Li et al., 2006).

4.6. In situ hybridization

A 30-mer oligonucleotide probe for rat 5-HT1A receptor mRNA containing the sequence 5′-GTGAC CGTCA GCTAC CAAGT GATCA CCTCT-3′ was synthesized and purified by Boster Biotechnology Corporation (Beijing, China). In situ hybridization (ISH) was conducted following the manufacturer’s instructions (ISH kit, Boster Biotechnology Corporation, Wuhan, China) with some modifications (Xu et al., 2005). Briefly, before hybridization, sections were digested with a solution containing 2 μl/ml proteinase K for 30 min at 37 °C. After two rinses in 0.1 M PBS and fixation with 4 °C paraformaldehyde at room temperature, the sections were incubated with 20 μl of the prehybridization solution for 3 h at 42 °C and then 20 μl of the hybridization mixture (supplied by Boster Biotechnology Corporation, Beijing, China) was added to each section. After incubation in a humidified chamber for 16–18 h at 43 °C, the sections were rinsed with descending concentrations of 2×, 0.5× and 0.2× SSC and then incubated with normal goat serum for 30 min at 37 °C. Subsequently, the sections were incubated with horseradish peroxidase streptavidin-conjugated anti-digoxigenin antiserum (from the ISH kit) and washed with 0.02 mol/L PBS. Sections were then incubated with avidin biotin peroxidase complex (from the ISH kit) for 20 min at 37 °C, washed with 0.02 mol/L PBS again and incubated in DAB (Beijing Zhongshan Biotechnology Co.) in dark chambers at room temperature for 10 min. The specificity of the digoxigenin-labeled antisense oligonucleotide probe was confirmed with control experiments hybridizing with labeled sense probe or omitting the probe in hybridization solution. In either case, no hybridization signal was detectable.

4.7. Statistical analysis

All data are presented as means±SEM. A one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls (S–N–K) test was used for statistical evaluation. Results were considered statistically significant when p<0.05.

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