The effect of serotonin on GABA synthesis in cultured rat spinal dorsal horn neurons

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Abstract

The spinal dorsal horn (SDH) is the first step in the integration of primary nociceptive information, which is controlled by the descending serotonin (5-HT) system as well as the principal inhibitory neurotransmitter γ-aminobutyric acid (GABA). However, the influence exerted by 5-HT on GABA synthesis remains poorly understood. The major pathway for GABA synthesis is the enzymatic decarboxylation of glutamate by glutamic acid decarboxylase (GAD) 65 and 67. In the present research, western blotting results show a time- and dose-dependent enhancement of GAD65 and GAD67 expression induced by 5-HT treatment and a concentration of 100 nM 5-HT applied for 3 days is shown to be the optimal condition for maximal expression of GAD67 and a significant expression of GAD65. Under the stimulation of such 5-HT application the phosphorylation of Akt and p42/p44 mitogen-activated protein (MAP) kinase is activated and specifically blocked by inhibitors of phosphatidylinositol 3-kinase (PI3-K) (LY294002) or the p42/p44 MAP kinase (PD98059 and U0126) pathways. Moreover, LY294002, or PD98059, or U0126 partially inhibit 5-HT-stimulated increases in GAD67 or GAD65 expression. Further, 5-HT application has no effect on the number of GAD65/GAD67-immunopositive neuronal cells; but it can induce an increase in the total area, process length and number of primary neurites of GAD65/67-immunoreactive neurons and terminals. The results of this study provide an in vitro model of the regulation of 5-HT on synthesis of GABA in the SDH that is putatively thought to occur in vivo as a result of excitatory neural activity.

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Descending serotonin (5-hydroxytryptamine; 5-HT) pathways are considered to play a major role in spinal antinociception (Cervero et al., 1995; Fields and Basbaum, 1994; Willis and Coggeshall, 1991). The descending 5-HT-immunoreactive terminals, which mainly originate from the rostral ventromedial medulla (RVM), including the nucleus raphe magnus (NRM) and its surrounding reticular formation, are found in all laminae of the spinal dorsal horn (SDH), with the densest concentration in the superficial laminae (laminae I and II) (Jones and Light, 1990; Kwiat and Basbaum, 1992; Potrebic et al., 1994). In addition to 5-HT, γ-aminobutyric acid (GABA) has been widely studied in relation to spinal analgesia (Barber et al., 1978; Carlton et al., 1992; Levy, 1977; Roberts, 1976, 1984). In the SDH, GABA-like immunoreactive neurons and terminals and GABA_A receptors are intensely located in the superficial laminae (Antal et al., 1996; Bohlhalter et al., 1996; Hiura et al., 1991; Mitchell et al., 1996). The fact that the distribution of GABAergic neuron is similar to that of 5-HT-containing terminals, indicates possible links and interactions between the two neuronal systems in the SDH. Indeed, it has been reported that 5-HT released from descending terminals originates from supraspinal structures that have been shown to evoke GABA release from interneurons in the spinal cord, resulting in antinociception (Alhaider et al., 1991; Yoshimura and Furue, 2006). Additionally, some authors have demonstrated that 5-HT potentiates the inhibitory effects of GABA and glycine on neurons of the superficial laminae in the rat SDH (Kang et al., 1998; Wang et al., 1999). However, the effect of 5-HT on GABA synthesis remains to be elucidated.

Two isoforms of the GABA-synthesizing enzyme, glutamic acid decarboxylase (GAD) 65 and 67, have been identified (Erlander et al., 1991; reviewed in Erlander and Tobin, 1991). Eaton et al. (1998) have reported that intrathecally administrated transplants of bioengineered serotonergic nerve cells could reverse the decreased number of GABA-like immunoreactive and GAD-like
immunoreactive cells in the SDH after chronic constriction injury (CCI). Moreover, our previous studies (Wang et al., 2003) have demonstrated an increase in the time course of the expression of GAD mRNA after administration of 5-HT. These results suggest a possible role of 5-HT in the regulation of GABA synthesis.

Recently, two signaling pathways, phosphatidylinositol 3-kinase (P3K) and p42/44 MAP kinase, have been suggested to be involved in pain signal processing at the spinal cord level (Hu and Gereau, 2003; Ji et al., 1999; Ma et al., 2006; Sun et al., 2006a,b, 2007; Wei et al., 2006).

Therefore, to gain further insights into the interaction between the serotonergic descending system and the spinal GABA systems in antinociception, the present study examined the effects of 5-HT, with or without the inhibitors of P3-K (LY294002) or p42/p44 MAP kinase (PD98059 and U0126) pathways, on the expression of GAD65 and GAD67 proteins, on the number and on the morphology of GAD65/67-like immunoreactive cells, in primary cultured SDH neurons.

1. Materials and methods

1.1. Antibodies

Rabbit anti-GAD 65/67 antibody and rabbit anti-β-actin antibody were purchased from Sigma Chemical Company (St. Louis, MO, USA) (Shimizu et al., 2005). Monoclonal antibodies against anti-p44/42 MAP Kinase (MAPK) (L34F12), anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-Akt (SGC) and anti-phospho-Akt (Ser473) antibody detects endogenous levels of total p44/42 MAPK (Erk1/Erk2) protein. Mouse phospho-p44/42 MAPK (Thr202/Tyr204) antibody detects endogenous levels of p44 and p42 MAPK (Erk1 and Erk2) dually phosphorylated at threonine 202 and tyrosine 204. Mouse Akt (SGC) antibody detects endogenous levels of total Akt, Akt2 and Akt3 proteins. Mouse phospho-Akt (Ser473) antibody detects endogenous levels of Akt only when phosphorylated at serine 473.

1.2. Primary culture of SDH neurons

All animal experiments have been conducted in accord with the Policies on the Use of Animals and Humans in Neuroscience Research, revised and approved by the Committee on Animal Care and Use for Research and Education at The Fourth Military Medical University (Xi’an, Shanxi Province, P.R. China). All efforts were made to minimize the number of animals used and their suffering.

The procedures for the primary cultures of SDH neurons have been described previously (Wang et al., 2003). Briefly, 3- to 4-day-old Wistar rats (provided by the Animal Centre of the Fourth Military Medical University, Xi’an, Shanxi Province, P.R. China) were deeply anesthetized with diethyl ether and a laminectomy was performed. The dorsal third of the spinal cord was carefully removed with a razor blade. The tissue pieces were digested for 30 min at 37 °C in oxygenated divalent free Eagle’s balanced salt solution (EBSS, Gibco-BRL, Renfrewshire, Scotland, UK) containing 20 U/ml papain. The enzymatic digestion was stopped by adding 3 ml EBSS containing 1 mg/ml bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA), 10 mg/ml trypsin inhibitor (Sigma), and 0.01% DNase (Sigma), and the tissue was mechanically dissociated with a 1 ml plastic pipette. The homogenate was deposited on top of 4 ml of an EBSS solution similar to that described above except that the concentration of bovine serum albumin was increased to 10 mg/ml. After centrifugation (5 min at 500 rpm), the supernatant was removed and replaced by Eagle’s minimal essential medium (MEM; Gibco-BRL) containing 5% fetal calf serum (Gibco-BRL), 5% heat-inactivated horse serum (Gibco-BRL), 10 mg/ml transferrin (Sigma), 5 mg/ml insulin (Sigma), 100 nM putrescine (Sigma), and 20 mg/ml progesterone (Sigma). Transferin, insulin, putrasecine, and progesterone were added to promote neuronal cell survival. After trituration with a fire-polished Pasteur pipette, the neuronal cells with a density of 50,000 cells/ml were plated onto 24-well plates or 100 mm culture dishes, which had been precoated with 0.1 mg/ml poly-o-lysine (Sigma) for morphological staining or Western blot analysis, respectively. The cultures were incubated in medium, nutrient mixture F-12 Ham (N-6760, Sigma), pH 7.4, supplemented with 10% dialyzed fetal bovine serum (dialyzed in membranes with 1000 Da molecular weight cutoffs against a 100-fold greater volume of 150 mM NaCl to remove endogenous 5-HT), 1% penicillin-streptomycin, and 400 mg/ml gentamicin at 37 °C in a 5% CO2 incubator. Cytosine-b-arabinofuranoside (1 mM, Sigma) was added to the medium to inhibit glial cell growth.

Primary cultures of the SDH neurons were used because a previous study (Wang et al., 2003) has demonstrated that these cultures show a high level of GAD mRNA expression, which can be increased by the addition of 5-HT at a 100 nM concentration.

1.3. Application of drugs

The experiments on the effect of 5-HT alone on GAD65/67 protein expression are divided into two parts: first to study the temporal effects of 5-HT at a constant final concentration for different periods of application, second to observe the effect of different concentrations of 5-HT over the same application period. The procedures in detail were as follows. First, the medium was removed after 4 days in vitro (DIV) and replaced by N2-supplemented medium to deprive the cells of serum for 24 h. Then 5-HT at a final concentration of 100 nM (Sigma) was added. The 5-HT was dissolved in N2-supplemented medium with BSA (6.6 mg/ml). Cultures containing 5-HT were incubated for different times (1, 2, 3, 4, 5 or 6 days) and examined for GAD65/67 content. Cells used as baseline controls were treated with phosphate-buffered saline (PBS, pH 7.2), 10 μg/ml of the culture medium in each series of experiments. There were several reasons for using the concentration of 100 nM of 5-HT to induce the GAD expression: one is that the effect of 5-HT on the expression of prodynorphin, the pain-related gene, is concentration-dependent between 0.1 and 100 nM (Jose et al., 1993), another is that the expression of GAD mRNA is significantly induced by applications of 100 nM 5-HT (Wang et al., 2003). Further, 5-HT at different concentrations (including 0.1, 1, 10 and 100 nM, and 1 mM) was added and cultures containing 5-HT were incubated for 3 days, based on the observations in the first step which is described in Section 2. Baseline controls were the same as those for the first step. In other sets of experiments, the cultures were divided into three treatment conditions: 5-HT-only, inhibitor-only, and inhibitor-plus-5-HT. Pre-treatment and treatment procedures are shown in Fig. 1. The design was implemented so that all cells were of the same age when they were harvested for subsequent Western blotting, or fixed for further immunocytochemical staining. As shown in Fig. 1, the medium was removed and replaced as described in the preceding paragraph. In the 5-HT-only group, 5-HT was added at a final concentration of 100 nM and maintained at that concentration for 1–3 days. In the inhibitor-plus-5-HT group, the cultures containing 100 nM 5-HT were incubated for different periods, with or without the presence of phosphatidylinositol 3-kinase (P3K) and p42/p44 MAPK pathway inhibitors. In the inhibitor-only group, the cultures were treated with the P3K and p42/p44 MAPK pathway inhibitors without application of 5-HT. The inhibitor of the PI3K signaling pathway, LY294002, was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). The inhibitors of p42/p44 MAPK pathways, PD98059 and U0126, were purchased from Calbiochem (San Diego, CA). Two concentrations were selected for each inhibitor, for LY294002, 25 or 50 μM; for PD98059, 25 or 50 μM, and for U0126, 5 or 10 μM. All inhibitors were initially dissolved in dimethyl sulfoxide (DMSO), prepared in semi-darkness and freshly prepared just before each use. Cells used as baseline controls were treated with the same vehicle as that used to dissolve the inhibitors (DMSO). At no time did the DMSO concentration exceed 0.5% of the total volume in the tissue culture well.

1.4. Western blotting analysis

At different examination time points, cultured SDH neurons were rinsed rapidly in ice-cold PBS, and lysed with lysis buffer (50 mM Tris–HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 10 mM EGTA and 150 mM NaCl) plus protease inhibitors (2 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM orthovanadate). The cell lysates were collected and immediately added to ice. The samples were removed (i) into NaOH for protein determination and (ii) into gel sample buffer, containing sodium dodecyl sulfate and β-mercaptoethanol, for electrophoresis (Patel et al., 2006). Protein concentrations of the homogenate were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL).

SDV

| 1 – 4 DIV | SDIV |
| 5-HT-only group |
| inhibitors-only group |
| inhibitors-only group |
| 5-HT plus inhibitor group |
| 5-HT added |
| inhibitor added without 5-HT |
| S2 applied N2 medium |
| 5-HT and inhibitor added at the same time |

Fig. 1. Pre-treatment and treatment procedures.
The electrophoresis samples were heated to 100 °C for 5 min and then loaded onto 10% SDS polyacrylamide gels for examination of the expression of GAD65/67 or 8% SDS gels for that of phosphorylated forms of p42/p44 and Akt, in the Mini-protein system (Bio-Rad). After that, the proteins were electroblotted onto a polyvinylidene difluoride (PVDF, Immobilon-P, Millipore, Billerica, MA) membrane. The membranes were placed in a blocking solution, which contained Tris-buffered saline with 0.02% Tween (TBS-T) and 5% non-fat dry milk, for 1 h, and incubated overnight under gentle agitation with primary antibodies with specific dilutions, as follows: GAD 65/67 (1:5000), β-actin (1:5000), Phospho-Akt (1:2000), p44/p42 MAPK (1:500), and Phospho-p44/42 MAPK (1:1000). Bound primary antibodies were detected with the anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000). Between each step the immunoblots were rinsed with TBS-T. All reactions were detected by the enhanced chemiluminescence (ECL) detection method (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The densities of protein bands were analyzed by using Labworks Software (Ultra-Violet Products, UK). The densities of approximately 65 and 67 kDa GAD65/67, 46 kDa β-Actin, 60 kDa total or phosphorylated Akt, 42 and 44 kDa total or phosphorylated p44/p42 MAPK were normalized with the background subtraction.

1.5. Immunocytochemistry

At the end of the culture period, cultures were washed twice in 0.1 M PBS (pH 7.4) and fixed in 4% paraformaldehyde in 0.1 M PBS for 20 min at room temperature. After 4 washes (10 min each) in PBS, cultures were preincubated for 60 min in PBS containing 0.1% Triton X-100 (Sigma) and 30% normal horse serum (Gibco-BRL). After that, if washing was timed, the cultures were incubated with the antibody against GAD 65/67 (1:1000) overnight at 4 °C with primary antibodies diluted in PBS containing 0.05% Triton X-100 and 2.5% horse serum. After 3 washes (5 min each) in PBS, cultures were incubated for 2 h with biotinylated secondary antibodies (anti-mouse, anti-rabbit, or anti-goat antibodies; Vector Laboratories, Burlingame, CA) diluted 1:200 in PBS containing 0.05% Triton-X100 and 2.5% horse serum. The cultures were then washed three times in PBS and exposed to the secondary antibody: Alexa Fluor 488 (A488)-conjugated goat anti-rabbit antibody (1:1000, Invitrogen Corporation, Carlsbad, CA), for 2 h at room temperature. Cultures were then mounted in PBS containing 50% glycerol and examined under a Leitz epifluorescence microscope.

1.6. Detection of cell death

Neuronal injury was assessed by a short exposure of cells to the vital dye—trypan blue (TB, at concentration 0.2% for 1 min), which stains dead neurons in blue, and then manually counting live and dead (trypan positive) cells. The percent of dead cells was determined from the ratio of the number of TB positive cells to the total number of TB positive cells plus live phase-bright cells. The validity of this method was earlier demonstrated (Patterson, 1979; Palliam et al., 1998).

Dying neurons at 8DIV were detected 3 days after treatment with 100 nM 5-HT and/or without the presence of inhibitors (50 μM LY294002 or 50 μM PD98059). Cells were fixed with 4% paraformaldehyde for 1 h at room temperature, followed by three rinses in PBS. Neurons were incubated with TB (1:100; Sigma) for 5 min and then rinsed twice with PBS.

1.7. Quantitative analyses

The total numbers of GFP65/67-immunopositive neurons and of dying cells stained by TB were counted in 20 fields at 200× magnification. Results are given as number of cells/cm² of the magnified fields or as a percentage of the value for control cultures. Four to six wells were analyzed per condition from three different experiments. Morphological parameters were assessed using a PC-image analysis system from Foster Findlay on a computer attached to an Olympus microscope. GAD65/67-immunopositive neurons were selected randomly from control and treated cultures. Five to eight cultures per group and five to eight cells per culture for GAD65/67-immunopositive neurons were analyzed from two to three independent experiments. For quantitative morphometry only clearly visible primary neurites that could be undoubtedly assigned to single neurons were included for measurements. The following parameters were recorded for all neurons: cell soma area (μm²), number of primary neurites emerging from the cell body (n) and the neurite length of the longest process of an individual neuron (μm) were identified by means of a radius measure (Ducray et al., 2006).

1.8. Statistical analysis

For western blotting, the optical density of the bands of GAD65 and GAD67 was divided by that of its respective β-actin band to yield the corrected band intensity. For the optical density of the bands of phospho-p44/42 MAPK (p42 and p44), and phospho-Akt (pAkt), the ratio of bands of the experimental culture was expressed as means ± S.E.M. and analyzed by ANOVA followed by the Scheffe’ post hoc test. P < 0.05 was considered as significant.

2. Results

2.1. 5-HT treatment enhances GAD65 and GAD67 expression in a time- and dose-dependent manner

Experiments were performed firstly to determine whether 5-HT could alter the expression of GAD65 or GAD67 proteins in cultured SDH neurons. The cells were treated by 5-HT at 5 DIV with a fixed concentration (100 nM), but different incubation times (including six time points: 1, 2, 3, 4, 5 or 6 days) were used. In the control group, PBS was added and incubated for the same durations. As can be seen in Fig. 2A, 5-HT (100 nM) stimulation enhanced the expression of both GAD65 and GAD67 proteins in a time-dependent manner. The expression of GAD65 protein was increased from about 2.5-fold at 2 days of 5-HT exposure, to 3.5-fold at 3 days and maintained this maximum level (about 3.7-fold) at 4 days, decreasing to the control level at 5 days. Similarly, the elevated expression of GAD67 was observed to increase at 2 days (about 2.3-fold), reach the maximum level at 3 days (about 4.0-fold), decrease but remain above the control at 4 days (about 3.0-fold) and 5 days (about 2.0-fold), and return to the baseline level at 6 days. In addition, when 5-HT (100 nM) was added to the incubation medium for 4 and 10 days, the levels of GAD65 and GAD67 blotting bands were not significantly different from those measured after 6 days of the 5-HT treatment (P > 0.05, data not shown).

We next examined the effects of 5-HT application with different concentrations in order to determine the range of 5-HT doses that regulate the expression of GAD65 and GAD67. We included five concentrations: 0.1, 1, 10, 100 nM or 1 mM, but with a fixed incubation time. Based on the results described in the last paragraph we selected 3 days for incubation with 5-HT to get the maximal expression of GAD65 and almost the highest level of GAD65. PBS treatment for 3 days was used as a control. As shown in Fig. 2B, the changes of GAD65 and GAD67 expression induced by 5-HT treatment showed similar tendencies: the protein levels of the two proteins were increased in a dose-dependent manner; and the lowest levels were observed in the absence of 5-HT, whereas the highest levels were seen at 100 nM 5-HT.

Overall, 5-HT treatment enhanced GAD65 and GAD67 expression in a time- and dose-dependent manner. Moreover, a concentration of 100 nM 5-HT applied for 3 days was shown to be the optimal condition for maximal expression of GAD67 and significant expression of GAD65 (Fig. 2).

2.2. 5-HT induces the activation of PI3-K and p42/p44 MAP kinase pathways

In order to test whether 5-HT was able to activate the PI3-K and p42/p44 MAP kinase pathways in SDH cultures, cells were stimulated with 5-HT (100 nM) at SDIV, and the phosphorylation of Akt and p42/p44 was examined by Western blot at different time points (including 1, 2, 3, 4, 5 or 6 days). Our results show that Akt (Fig. 3A) and p42/p44 (Fig. 3B) were phosphorylated after 5-HT treatment with a similar time course. The Akt results showed an enhancement of phosphorylation from 1 to 3 days after 5-HT treatment (P < 0.05, Fig. 3A). At 6 days, the phosphor-Akt expression returned to control levels (P > 0.05, Fig. 3A). The p42/p444 results showed an enhancement of phosphorylation from...
1 to 4 days after 5-HT treatment ($P < 0.05$, Fig. 3B), whereas at 5 days the levels were already decreased ($P < 0.05$, Fig. 3B). However, the expression of phosphor-p42/p44 was still above the control levels even at 6 days ($P < 0.05$, Fig. 3B). In order to determine the efficacy of the signaling pathways in blocking the 5-HT effects, cultures were incubated with 100 nM 5-HT in the absence or presence of selective inhibitors of PI3-K (LY294002) or of p42/p44 MAP kinase (PD98059 or U0126) pathways. 5-HT application alone to cultures significantly activated phosphorylation of Akt ($P < 0.05$, Fig. 4A), which was reduced by the treatment with 25 or 50 $\mu$M LY294002 ($P < 0.05$, Fig. 4A). Furthermore, the inhibition of the p42/p44 MAP kinase pathway (50 $\mu$M PD98059 or 10 $\mu$M U0126) did not modify 5-HT-induced Akt phosphorylation ($P > 0.05$, Fig. 4B). In contrast, the inhibition of the PI3-K pathway (50 $\mu$M LY294002) did not modify the 5-HT-induced p42/p44 phosphorylation ($P > 0.05$, Fig. 4B), indicating that there was not an interaction between these two pathways.

![Figure 2](image2.png)  
**Fig. 2.** 5-HT enhanced GAD65/67 expressions in vitro in a time- and dose-dependent manner. (A and B) showed the quantification of the blots with results expressed as percentages of control, whereas autoradiograms were obtained from representative experiments. Bars showed GAD65 and GAD67 protein levels. (A) Time course exposure to 5-HT in cultured SDH neurons was shown. 5-HT (100 nM) was added to the cultures and GAD65/67 protein levels were examined by Western blot at different time points. (B) Dose-dependent effect of 5-HT on GAD65/67 protein expressions in cells was shown. CTL, control. Results are expressed as the mean ± S.E.M. *$P < 0.05$ compared to control values. ANOVA followed by the Scheffe’ post hoc test. Experiments were conducted three times, with each sample measured in duplicate.

![Figure 3](image3.png)  
**Fig. 3.** 5-HT induced the activation of PI3-K and p42/p44 MAP kinase pathways. 5-HT (100 nM) was added to the cultures and phosphorylation of Akt and p42/p44 was examined by Western blot at different time points. Figures showed the quantification of the blots with results expressed as percentages of control, whereas autoradiograms were obtained from representative experiments. (A) Bars showed phospho-Akt protein levels. (B) Bars showed phospho-p42/p44 protein levels. Experiments were conducted three times, with each sample measured in duplicate. CTL, control. Results are expressed as the mean ± S.E.M. *$P < 0.05$ compared to control values. ANOVA followed by the Scheffe’ post hoc test. Experiments were conducted three times, with each sample measured in duplicate.
compared to control values. ANOVA followed by the Scheffe' post hoc test. Experiments 10
HT + PD50); U0126 5
HT + LY50). CTL, control. Results are expressed as the mean
required for the action of 5-HT on the expression of GAD65 and
inhibitors was examined. The concentration of each inhibitor that
independent both the PI3K and the p42/p44 MAP kinase pathway.
ly294002, or PD98059, or U0126 alone, either in low or in high concentrations, has
significantly enhanced the expression of GAD65 and GAD67 proteins in all treated groups, compared to the control
groups (P < 0.05, Fig. 5A–C), and this was consistent with the
results above. In addition, application of LY294002, or PD98059, or U0126 alone, either in low or in high concentrations, has
significant effect on the expressions of GAD65, compared to the control
groups (P > 0.05, Fig. 5A and B). Similar results were also
seen for the GAD67 protein levels (P > 0.05, Fig. 5A and B). These
data suggest that in the present in vitro environment the action of
these inhibitors is specific, which provides an important basis for
the further evaluation of the role of the two signaling pathways in
the regulation process. We found that two doses of the PI3K
inhibitor LY294002 blocked the potentiation of 5-HT on GAD65
(P < 0.05), and also on GAD67 expression (P < 0.05). As shown in
Fig. 5A, LY294002 in the higher concentration (50 μM) did not
have a greater suppression effect (71.7%) on the GAD67 expression
increase of the 5-HT treatment, than the effect (44.3%) of the
lower concentration. Similarly, the potentiation of 5-HT on GAD65
was significantly blocked by LY294002 (P < 0.05, Fig. 5A), with
a smaller effect at the lower concentration (33.3%) and greater effect
46.4%) at the higher concentration. As shown in Fig. 5B, the two
inhibitors of the p42/p44 MAP kinase pathway, PD98059 and
U0126, exerted different effects on the potentiation of 5-HT on
GAD65 and GAD67 expression. PD98059 in the higher concentra-
tion (50 μM) significantly inhibited the 5-HT-potentiation of
GAD65 by 74.1% (P < 0.05, Fig. 5B) and had a less suppressive effect
on GAD67 expression by 36.4% (P < 0.05, Fig. 5B). However, it was
noted that PD98059 in a lower concentration of 25 μM did not
affect the 5-HT-potentiated GAD65 or GAD67 expression (P > 0.05,
Fig. 5B). Similarly, U0126 at the lower concentration (5 μM) did
not suppress 5-HT-potentiation of GAD65 production (P > 0.05,
Fig. 5B) or of the GAD67 enhancement induced by 5-HT (P > 0.05,
Fig. 5B). Moreover, U0126 with a higher concentration (10 μM)
significantly blocked 5-HT-potentiated GAD67 expression by
23.1% (P < 0.05, Fig. 5B), and produced a greater suppression of
5-HT-potentiated GAD65 expression by 63.1% (P < 0.05, Fig. 5B).

As a further step in our analysis, the treatment of the cultures
with the combination of the PI3K and p42/p44 MAP kinase pathway
inhibitors was examined. The concentration of each inhibitor that
has the greatest effect on the protein block was selected, specifically
as follows: LY294002 (50 μM), PD98059 (50 μM), and U0126
(10 μM). As shown in Fig. 5C, 5-HT induced significant increases in
GAD65 and GAD67 protein expression (P < 0.05). Both combina-
tions of LY294002 and PD98059, and LY294002 and U0126 showed
greater inhibitory effects on GAD65 expression induced by 5-HT
(100 nM), by 74.0 and 71.4%, respectively (P < 0.05, Fig. 5C).
Similarly, 5-HT-stimulated GAD67 increases were prevented
partially by the combinations of LY294002 with PD98059, or
LY294002 with U0126, by 55.7 and 50.8%, respectively (P < 0.05,
Fig. 5C). Meanwhile, the combination of PD98059 with U0126
partially blocked the expression of GAD65 and GAD67 elicited by
5-HT, by 68.8 and 37.7%, respectively (P < 0.05, Fig. 5C).

Therefore, in cultured SDH neurons 5-HT is able to activate
independently both the PI3K and the p42/p44 MAP kinase pathway.

2.3. Inhibitors of the PI3K and p42/p44 MAP kinase pathway suppress
5-HT-stimulated increases in GAD65 and GAD67 expression

To further determine whether a possible signaling pathway is
required for the action of 5-HT on the expression of GAD65 and
GAD67 proteins in cultured SDH neurons, the effects of the PI3K
and p42/p44 MAP kinase pathway inhibitors were examined.

Fig. 4. Inhibitors of PI3-K and p42/p44 MAP kinase pathways inhibited 5-HT-
stimulated Akt and p42/p44 phosphorylations. Figures showed the quantification of
the blots with results expressed as percentages of control. Autoradiograms were
obtained from representative experiments. (A) Bars showed phospho-Akt protein
levels as assessed by Western blot analysis. 5-HT (100 nM) induced an increase in
phospho-Akt, which was selectively blocked by the addition of LY294002 25 μM (5-
HT + LY25) or 50 μM (5-HT + LY50); PD98059 50 μM (5-HT + PD50) or U0126
10 μM (5-HT + U10). (B) Bars showed phospho-p42/p44 protein levels as assessed
by Western blot analysis. 5-HT (100 nM) induced the phosphorylation of p42/p44, which was
selectively blocked by the addition of PD98059 25 μM (5-HT + PD25) or 50 μM (5-
HT + PD50); U0126 5 μM (5-HT + U5) or 10 μM (5-HT + U10); LY294002 50 μM (5-
HT + LY50); CTL, control. Results are expressed as the mean ± S.E.M. *P < 0.05
compared to control values. ANOVA followed by the Scheffe’ post hoc test. Experiments
were conducted three times, with each sample measured in duplicate.

2.4. 5-HT application has no effect on the number of GAD65/GAD67-
immunopositive cells

Because 5-HT supplements increased the expression of both
GAD65 and GAD67 proteins in the SDH cultures, we investigated
whether or not the 5-HT (100 nM) application could alter the
number of GAD65/67-immunopositive cells. Firstly the detection
by TB staining showed that 5-HT treatment did not affect the total
number of neuronal cells in the cultures with 100.0 ± 6.6 and
100.7 ± 4.9% for control and 5-HT treatment, respectively (mean ± S.E.M.; n = 20 from two independent experiments). In control conditions, the total number of cells was similar at different DIV examined (5DIV: 34,056 ± 1770 cells/cm²; 10DIV: 37,190 ± 1044 cells/cm²) and did not change when 5-HT was added to the cultures (5DIV: 25,826 ± 2809 cells/cm²; 10DIV: 28,631 ± 495 cells/cm²).

Secondly, the immunocytochemical staining with the antibody against GAD65/67 showed that the number of GAD65/67-positive neurons was similar at different DIV examined (5DIV: 18,056 ± 1240 cells/cm²; 6DIV: 19,590 ± 1071 cells/cm²; 7DIV: 18,560 ± 1460 cells/cm²; 8DIV: 18,490 ± 1300 cells/cm²; 9DIV: 18,010 ± 1340 cells/cm²; 10DIV: 17,090 ± 1001 cells/cm²), in the culture without 5-HT application. When 5-HT (100 nM) was added to the cultured neurons at 5DIV and maintained for 1, 2, 3, 4, 5 or 6 days, the number of GAD65/67-positive neurons increased to 10% at 7DIV, 15.4% at 8DIV, 10% at 9DIV and 7.1% at 10DIV. However, these values showed no statistically significant differences, compared with those obtained in the control group at the corresponding time points (P > 0.05, Fig. 6A).

Since both the PI3-K and the p42/p44 MAP kinase pathways were activated by 5-HT, we further examined the effect of the two...
The involvement of the PI3K and p42/p44 MAP kinase pathways in neurite number of GAD65/67-positive neurons (5-HT) induced an increase in the total area, process length and primary neurite number (P < 0.05, Fig. 7). PD98059 had a similar effect on GAD65/67-positive neurons (P < 0.05, Fig. 7). The treatment with LY294002 (25 μM) or PD98059 (50 μM) alone induced a decrease in all the morphological parameters analyzed but these never reached more than a 15% reduction (data not shown).

3. Discussion

The SDH is important in the transduction and modulation of various pain signals (Cervero et al., 1995; Fields and Basbaum, 1994; Willis and Coggshall, 1991). In this area, 5-HT and GABA interact with each other's receptors and show overlapping biological activities. The modulatory role of descending 5-HT inhibition in GABA transmission, which is involved in the spinal processing of nociception, has been well studied (Morgan, 1996; Lee et al., 1995; Otsuka and Yanagisawa, 1990; Stamford, 1995), but the effect of 5-HT on GABA synthesis is not clear. This point is important in relation to the intrathecal administration of 5-HT agonists or the simultaneous activation of descending serotonergic systems and potentiation of GABAergic neurotransmission, which may be useful alternatives to addictive opioid analgesics. Our study demonstrates that the chronic application of 5-HT affects the expression of two isoforms of the enzyme for GABA synthesis, GAD65 and GAD67, as well as the morphological properties of GAD65/67-positive cells in vitro. We have shown that: (1) the application of 5-HT to primary cultured SDH neurons induces a dose- and time-dependent increase in both GAD65 and GAD67 proteins; (2) both the PI3-K and the p42/p44 MAP kinase pathways are involved in the effect of 5-HT on the up-regulation of GAD65 and GAD67 proteins; (3) 5-HT treatment increases the total area, process length and primary neurite number of GAD65/67-positive neurons, without affecting the number of these cells, and (4) this effect is blocked when specific inhibitors of the PI3-K (LY294002) or of the p42/p44 MAP kinase (PD98059) pathways were added to the cultures.

3.1. Effect of 5-HT on the levels of GAD65 and GAD67 proteins

Previous studies have demonstrated that 5-HT can exert an influence over both translation and transcription in sensory neurons (Barzilai et al., 1989; Eskin et al., 1984; Garcia-Gil et al., 1995; Hu et al., 2004; Udo et al., 2005). The time course and sequence of changes induced in specific proteins suggests the possibility that 5-HT can lead to the activation of a gene cascade whereby regulatory genes, act to modify the expression of one or more rounds of late effector genes. Previous studies have used primary cultured spinal cord neurons of rats to mimic how the stimulation by pain application acts in vivo on the prodynorphin-expressing neurons of the SDH. These studies have exposed the cultured neurons to different concentrations of a variety of neurotransmitters, including 5-HT, shown to be involved in pain transmission or its control at the spinal cord level (Jose et al., 1993). Reverse transcription-polymerase chain reaction analysis revealed an increase in the time course of the expression of mRNAs encoding c-fos, preprodynorphin (ppDyn), preproenkephalin (ppEnk) and GAD after administration of 5-HT (100 nM) (Wang et al., 2003). The results of the current study are consistent with the idea that the expressions of GAD proteins in the SDH can be regulated by the descending serotonergic effectors.

However, our present work has not detected the changes of either the basal or the evoked release of GABA from the cultured neurons.
SDH neurons. In addition, it has been indicated that GAD67 is responsible for basal GABA production; in contrast, GAD65 is transiently activated in response to the demand for extra GABA in neurotransmission (Fenalti et al., 2007). These data reveal the molecular complexity for regulation of GABA homeostasis. Therefore, it is not evident that the release of GABA at sensory synaptic terminals would also be potentiated, although the levels of GAD65 and GAD67 are increased in the presence of 5-HT.

The present results of a dose-dependent upregulation of GAD65 and GAD67 proteins induced by 5-HT application relates to the pattern shown by intrathecal 5-HT injection in terms of the dose-dependent antinociception demonstrable in acute pain tests in rats (Yaksh and Wilson, 1979). The mechanisms underlying this phenomenon are not known. We have not undertaken pharmacological investigations of the agonists or antagonists of the different 5-HT receptor subtypes, but, our in vitro observations

### Table 1 Quantitative analyses of differentiation by chronic 5-HT treatment on GAD65/67-immunopositive spinal dorsal horn neurons

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total area (µm²) n = 40</th>
<th>Process length (µm) n = 40</th>
<th>Primary neurites (µm) n = 40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.3 ± 2.6 [70.7 - 153.9]</td>
<td>78.5 ± 2.6 [50.5 - 142.5]</td>
<td>3.2 ± 0.1 [2.0 - 8.0]</td>
</tr>
<tr>
<td>5-HT</td>
<td>120.0 ± 1.7* [76.6 - 186.3]</td>
<td>90.4 ± 3.1* [77 - 108.9]</td>
<td>3.9 ± 0.1* [2.0 - 8.0]</td>
</tr>
<tr>
<td>5-HT + LY</td>
<td>68.5 ± 2.4# [30.5 - 160.7]</td>
<td>59.3 ± 2.9# [28.5 - 160.4]</td>
<td>2.9 ± 0.1# [2.0 - 6.0]</td>
</tr>
<tr>
<td>5-HT + PD</td>
<td>70.2 ± 1.5# [33.8 - 119.0]</td>
<td>69.0 ± 2.9# [30.5 - 142.4]</td>
<td>3.1 ± 0.1# [2.0 - 6.0]</td>
</tr>
</tbody>
</table>

Fig. 7. 5-HT promotes GAD65/67-positive neurons differentiation through PI3-K and ERK MAP kinase pathways. GAD65/67 immunocytochemistry was performed at 8DIV. Photomicrographs show GAD65/67-positive neurons from the SDH cultures treated with (A) vehicle, (B) 5-HT (100 nM), (C) 5-HT + LY294002 25 µM or (D) 5-HT + PD98059 50 µM. Scale bar = 32 µm. (E) Quantitative analysis of 5-HT effects on GAD65/67-positive cells morphology within the SDH cultures and involvement of PI3-K and ERK MAP kinase pathways. For each parameter and condition examined, 40 neurons were analyzed in three different experiments. Results are expressed as the mean ± S.E.M. *P < 0.05 compared to control values, #P < 0.05 compared with 5-HT values. ANOVA followed by the Scheffe’ post hoc test.
show that the mode of action may be attributed to the varied distribution of multiple 5-HT receptor subtypes in the SDH. Of the 5-HT receptor families, the major class found in the SDH is the 5-HT1 family (Zemlan and Schwab, 1991; Murphy and Zemlan, 1992). High densities of 5-HT3 receptors are found in the substantia gelatinosa at all levels of the spinal cord (Hamon et al., 1989; Fonseca et al., 2001). In addition, other 5-HT receptor subtypes cannot be excluded from a role in the modulation of the expression of two GAD proteins because they may also play a role in dorsal horn excitability, including 5-HT2 (Fonseca et al., 2001; Garraway and Hochman, 2001) and 5-HT7 (Garraway and Hochman, 2001; Meuser et al., 2002). In order to elucidate the exact 5-HT receptor subtypes in relation to this modulation process in the SDH, further studies are necessary.

3.2. Effect of 5-HT on the GAD65/67-immunoreactive cells

5-HT promotes the emergence of the GABA phenotype as demonstrated by the graft of embryonic raphe cells in the injured spinal cord (Dumoulin et al., 2000) and by immortalized serotonergic precursors (Eaton et al., 1998). Because 5-HT supplements increased the expression of both GAD65 and GAD67 proteins in the SDH cultures, we expected that 5-HT application could alter the number of GAD65/67-positive cells. However, our results have demonstrated a different role of the 5-HT on the GABA phenotype. At present, it is not clear how the number of GAD65/67-positive cells is modulated at the spinal cord level. Our observations show that 5-HT induces an increase in the total area, process length and primary neurite numbers of GAD65/67-positive neurons. It is well known that GAD65 and GAD67 are the products of two independently regulated genes that show differences in structure, cellular distribution, and dependence upon the cofactor, pyridoxal 5-phosphate (Kaufman et al., 1991; Esclapez et al., 1994). Immunohistochemistry has shown that GAD67 is primarily localized to neuronal cell bodies whereas GAD65 is seen more prominently in synaptosomes (Erlander et al., 1991; reviewed in Erlander and Tabin, 1991). In addition, Asada et al. (1996, 1997) have generated mice carrying a targeted disruption of either the GAD67 or GAD65 gene to show that GAD67 has a more pronounced effect on brain GABA synthesis than GAD65. Hence, the present study, demonstrating the effects of 5-HT on GAD65/67-positive neuronal cells at spinal levels, may result from the up-regulation of the GAD65 protein, rather than the GAD67 protein. Of the possible signaling molecules that link 5-HT to GAD65 and GAD67, phosphatidylinositol 3-kinase (PI3K) is a lipid kinase that phosphorilates the D3 position of phosphatidylinositol lipids to produce Pi(3,4,5)P3, acting as a membrane-embedded second messenger (Whitman et al., 1988; Toker and Cantley, 1997). Serine/threonine protein kinase B/Akt (PKB/Akt) is embedded as a second messenger (Whitman et al., 1988; Toker and Cantley, 1997). Serine/threonine protein kinase B/Akt (PKB/Akt) is embedded

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