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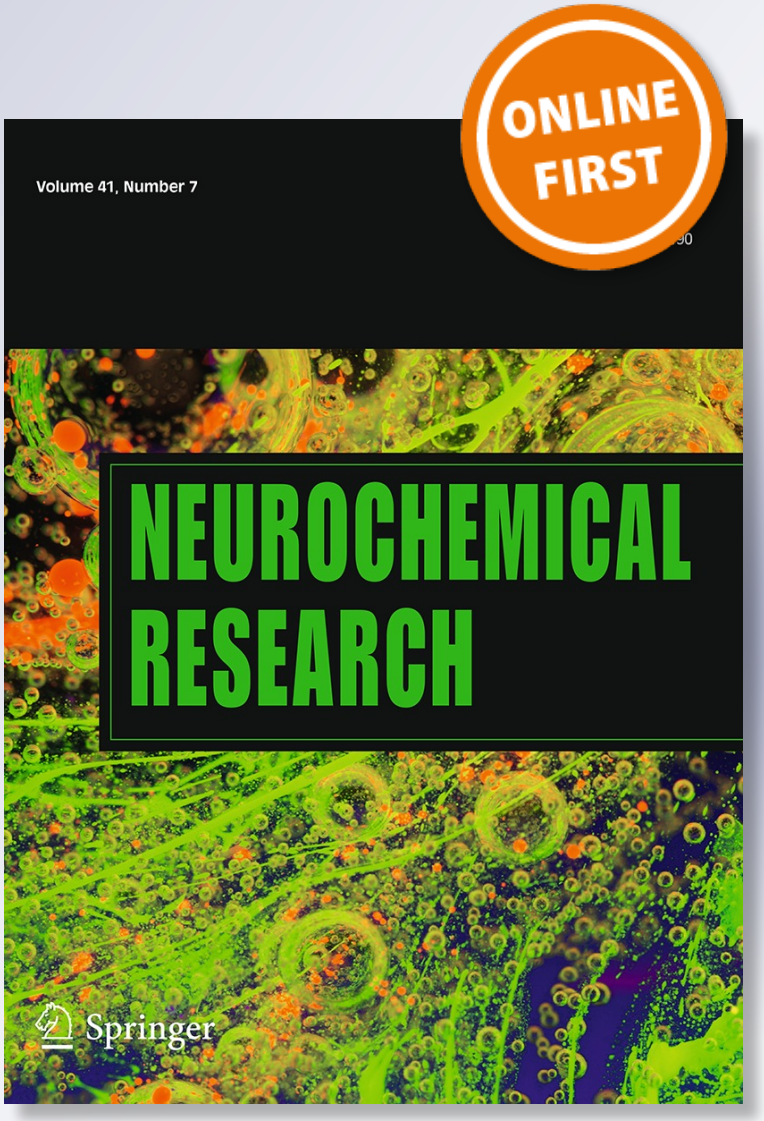
Neurochemical Research

ISSN 0364-3190

Neurochem Res
DOI 10.1007/s11064-016-1983-0



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Neurochem Res
DOI 10.1007/s11064-016-1983-0

ORIGINAL PAPER

Effects of GnRH on Neurite Outgrowth, Neurofilament and Spinophilin Proteins Expression in Cultured Spinal Cord Neurons of Rat Embryos

J. Luis Quintanar[1] · Denisse Calderón-Vallejo[1] · Irma Hernández-Jasso[1]

Received: 2 March 2016 / Revised: 13 June 2016 / Accepted: 15 June 2016

Abstract It has been previously described the presence of GnRH receptor in spinal cord neurons of rat embryos and adult rats. However, the functional role of these receptors has not been studied. In this work, the effect of GnRH on neurite outgrowth and cytoskeletal protein expression in cultured spinal cord neurons of rat embryos was analyzed. Specifically, neurofilaments of 68 and 200 kDa by immunoblot assays and spinophilin mRNA expression by RT-PCR. Results show that GnRH stimulates neurite outgrowth in addition to an increase in neurofilaments and spinophilin expression. These findings suggest that GnRH may play a role as neuromodulator in neuronal plasticity and that could be considered as a potential factor for neuronal regeneration in spinal cord injuries.

Keywords Spinal cord neurons · Gonadotropin-releasing hormone · GnRH-R · NFs · Neurotrophic effect

Introduction

Gonadotropin releasing hormone (GnRH) was initially described as an hypothalamic decapeptide involved in the neuroendocrine reproductive axis, but it also has other extrapituitary roles including neurotrophic effects. These effects are mediated by activation of the GnRH receptor (GnRH-R). Presence of GnRH-R has been reported in extrapituitary tissues, particularly in different areas of the nervous system such as rat hippocampus [1], cerebral cortical neurons of embryos and adult rats [2], mouse cerebellum [3], mouse and sheep brain [4], and sheep [5] and rat spinal cord [6]. Furthermore, exposure of FNC-B4 cells to GnRH changes their polyhedral to spindle-shape morphology accompanied by an increase in axonal growth and actin cytoskeletal remodeling to a motile phenotype [7]. GnRH administration modifies the density of dendritic spines in hippocampus [8] and cultured cerebral neurons of rat embryos. Additionally, GnRH increases both growth and number of neurites, as well as expression of neurofilaments (NFs) [9].

NFs are intermediate filaments of the neuronal cytoskeleton that are classified in three groups according to their molecular weight: NF-68, NF-160, and NF-200 kDa. Besides with other axonal components such as microtubules, they maintain and regulate neuronal cytoskeletal plasticity through neurite outgrowth, axonal caliber and axonal transport [10, 11].

On the other hand, spinophilin is an actin-associated scaffold protein that is enriched in dendritic spines [12], which is involved in regulating the morphology, function and formation of these spines [13–15]. Spinophilin has been used as a reliable spinogenesis marker [16].

The aim of the present study was to investigate the effect of GnRH on neurite outgrowth in cultured spinal cord neurons of rat embryos as well as cytoskeletal NF-68 and NF-200 kDa and spinophilin expression.

Material and Methods

Adult female Wistar rats (200–250 g body weight) were maintained in a temperature- and light controlled room and food (purina chow) and water ad libitum. Animals were

✉ J. Luis Quintanar
jlquinta@correo.uaa.mx

[1] Laboratory of Neurophysiology, Department of Physiology and Pharmacology, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Av. Universidad 940, Col. Ciudad Universitaria, Aguascalientes, AGS C.P. 20131, Mexico

Published online: 23 June 2016

euthanized by an overdose of sodium pentobarbital (50 mg/kg i.p.) and used as donors of embryos. Rats were treated according to the Institutional Normative on animal welfare (Universidad Autónoma de Aguascalientes).

Spinal Cord Neurons Culture

Dissociated cell cultures of whole spinal cord were prepared [17]. Briefly, 15-day-old embryos were removed from anesthetized rats and their spinal cords were placed in ice-cold (4 °C) supplemented medium: Minimum Essential Medium Eagle, MEM (Gibco, USA) with 10 % fetal calf serum and 30 mM glucose. Spinal cord was disaggregated mechanically by gentle trituration employing a Pasteur pipette as well enzymatically (0.25 % trypsin, Gibco, USA). Dissociated cells for cytochemistry and RT-PCR mRNA were plated at different densities into sterile 30 mm (polyornithin-coated) plastic culture dish (Nunc, USA). Plating medium was Eagle MEM containing 10 % heat-inactivated fetal calf serum, 10 % horse serum, 30 mM glucose, penicillin–streptomycin (10 mg/ml) and fungizone (2.5 mg/ml). Cultures were maintained at 37 °C under an atmosphere of 95 % O_2 and 5 % CO_2 for 24 h prior to analysis.

Morphological Analysis

Spinal cord neurons were stained with hematoxilin-eosin and neurite length was measured as an indicator of neuronal outgrowth. Analysis of neurite length (total length of all neurites from a neuron) was performed according to Nathan et al. [18]. Briefly, ten randomized fields of each dish were quantified and ten neurons (40× magnification) per field were considered. Cells with at least one neurite longer than a cell body were counted as positive.

Concentration–Response of GnRH on Neurite Length Outgrowth

To obtain a concentration–response curve, cultured neurons (5×10^5 cells/dish per triplicate) were incubated at different concentrations of GnRH (Sigma Chemicals, St. Louis, MO, USA) (0.1, 1, 10, 100 and 1000 nM) for 24 h. Control cultures were treated with vehicle solution.

Time-Course of GnRH on Neurite Length Outgrowth

Neurite length outgrowth was assessed incubating neurons (5×10^5 cells/dish per triplicate) with or without GnRH (10 nM) at different times (4, 24 and 72 h). After each incubation, neurite length outgrowth was measured as described above.

NFs Study

Isolation of NFs

Cytoskeletal fraction was obtained as described previously [19]. Neurons were disrupted using homogenization buffer containing 1 % Triton X-100, 1 mM EGTA (ethylene glycol tetra-acetic acid), 20 mM Tris–HCl and 1 mM phenylmethylsulfonyl fluoride, at pH 7.4. Cells were scraped from the plate and incubated in this solution at 4 °C for 10 min. Triton-insoluble cytoskeletal proteins were isolated by low speed sedimentation (17,000 g for 10 min). Total protein was measured by Bradford method [20].

Western Blot Analysis of NFs and β-Actin Proteins

Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (7.5 % SDS–PAGE) was carried out using mini-protean system (Bio-Rad, Hercules, CA, USA) and running on 30 mg of proteins of each sample. Equal amounts of protein were compared in each experiment. After electrophoresis, gels were electrotransferred to polyvinylidene difluoride membranes (Sigma, St. Louis, MO, USA). Membranes were blocked in a solution consisting of 3 % bovine serum albumin in TBS (0.5 M NaCl, 20 mM Tris–HCl pH 7.5) for 1 h at room temperature and then incubated at 4 °C overnight with the monoclonal anti-NF 68 kDa or rabbit polyclonal anti-NF 200 kDa or monoclonal anti-β-actin (Sigma, St. Louis, MO, USA), diluted 1:1000 in blocking buffer. After several washes the membranes were incubated for 2 h with alkaline phosphatase-conjugated secondary antibody (diluted 1:20,000), and after repeat washes, alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma, St. Louis, MO, USA). Quantification of protein bands was carried out by densitometry, using a Kodak Digital Science imaging system (Eastman Kodak Company, Rochester, NY, USA). Values were expressed as relative densitometric units of Media of Intensity (MI) per 30 mg of protein. This analysis was carried out using culture dishes (1×10^6 cells/dish/per triplicate) incubated with GnRH during 24 h and three culture dishes untreated as a control.

Spinophilin Expression

RNA Isolation and RT-PCR Analysis of Spinophilin

Total RNA was isolated from neuron cultures by cell disruption with TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcriptase (RT) reaction was performed with a High Capacity cDNA RT kit (Applied Biosystems: Cat. 4,368,813) using 3 µg of RNA with 0.8 µl of 25× dNTPs (100 mM), 2 µl

10× random primers, 2 µl 10× RT buffer and 1 U of Multiscribe RT in a final volume reaction of 20 µl completed with Nuclease-Free water using a thermocycler (Techne Genius: FGEN02TP) with the following incubation conditions: 25 °C/10 min, 37 °C/120 min, 85 °C/5 min. Polymerase chain reaction (PCR) was performed in 25 µl of final volume with 0.25 µl of cDNA, 10X PCR Buffer Minus Mg, 10 mM dNTPs mix, 50 mM $MgCl_2$, 10 µM of each spinophilin and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primer and 0.125 U of Taq polymerase. A 139 bp DNA fragment coding for rat spinophilin was amplified with forward primer 5′-TCA ACT TCT CCG TGT GCC TC-3′ and reverse primer 5′-TAA GCT GAC CTC CCT CCC TC-3′, oligonucleotide designed from the sequence of gene (GenBank, accession number NM_012664.2). As an internal control for amplification, a fragment of 207 bp rat GAPDH was amplified from the same cDNA, forward primer 5′-AGA CAG CCG CAT CTT CTT GT-3′ and reverse primer 5′-CTT GCC GTG GGT AGA GTC AT-3′ designed from the sequence of gene (GenBank, accession number NM_017008.4). Optimal PCR conditions were: 3 min at 94 °C, 30 cycles of 45 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C and a final extension of 10 min at 72 °C. PCR products were analyzed in ethidium bromide-stain agarose gels (2%). Intensity of the amplified bands was analyzed using QuantityOne® software. Band intensities were normalized to GAPDH signal (SPI/GAPDH rate).

To study the effect of GnRH on spinophilin mRNA expression, neurons (1.5×10^6 cells/dish per triplicate) were incubated with or without GnRH for 4, 24 and 72 h at different concentrations (0.1, 1, 10, 100, 1000 nM/twice/day). GAPDH was used as a marker for constitutive expression. Results were analyzed by one-way ANOVA with Dunnett's post-test.

Results

Cultured spinal cord neurons of 15 days rat embryos showed that exposure to GnRH induced changes in morphological characteristics such as neurite length (Fig. 1). In control cultures, neurite length was shorter than those incubated with GnRH.

Concentration and Time–Response of GnRH on Neurite Length

To assess this effect, neurons were incubated with different concentrations of GnRH at different times and neurite length was analyzed. Is noteworthy that shows that GnRH concentrations from 1 to 1000 nM induced a significant increase in neurite length (Fig. 1). However, response to lower GnRH concentration (0.1 nM) did not differ from that of control conditions (Fig. 1).

Effect of GnRH on NFs and β-Actin Protein

To determine whether GnRH modifies NFs expression, spinal cord neurons were incubated with GnRH for 24 h and protein content was analyzed by western blot. A significant increase in the level expression of both NF-68 (18.3%) and NF-200 kDa (10.5.6%) was observed in neurons incubated with GnRH compared with the control (Fig. 2). In this experiment, specific effect of GnRH on β-actin protein (42 kDa) expression was also analyzed. Results showed similar expression in both conditions (with and without GnRH treatment) (Fig. 2).

Concentration and Time–Response of GnRH on Spinophilin Expression

Spinophilin mRNA in spinal cord neurons of rat embryos was examined by semiquantitative RT-PCR. The study revealed that neurons incubated with GnRH (1000 nM) for 4 h induced a significant increase in the expression of spinophilin, while lower concentrations had no significant effect (Fig. 3a). Similar results were obtained when neurons were incubated for 24 h, showing a significant difference from the control, only with the highest concentration (Fig. 3b). However, incubations with GnRH at 10, 100 and 1000 nM for 72 h, neurons showed a significant increase in spinophilin mRNA expression compared to control neurons without GnRH (Fig. 3c).

Constitutive expression of GAPDH was considered to normalize the expression values of spinophilin (Fig. 3a, b and c).

Discussion

In addition to its role in the neuroendocrine reproductive axis, GnRH may have other extra-pituitary roles in the central nervous system. This fact is strongly suggested by studies showing the presence of GnRH-R in different areas of the nervous system [2–5, 15, 21]. It has been reported previously, the presence of GnRH-R in spinal cord neurons of embryos and adult rats [2]; however, there is no information available the receptor activation, as well as its effect on cultured spinal cord neurons. In the present work, we have found that GnRH has neurotrophic effects on neurites length in cultured spinal cord neurons. Similar results were obtained in neurons of cerebral cortex of rat embryos incubated with GnRH [9]. An increase in neurites length was found progressive with a higher concentration of GnRH at 4, 24 and 72 h. This growth was higher with concentration of 1000 nM at 72 h. Results show that effect on neurite outgrowth was induced directly by GnRH and that it is possible due to receptor activation. Neurons incubated at 4 h

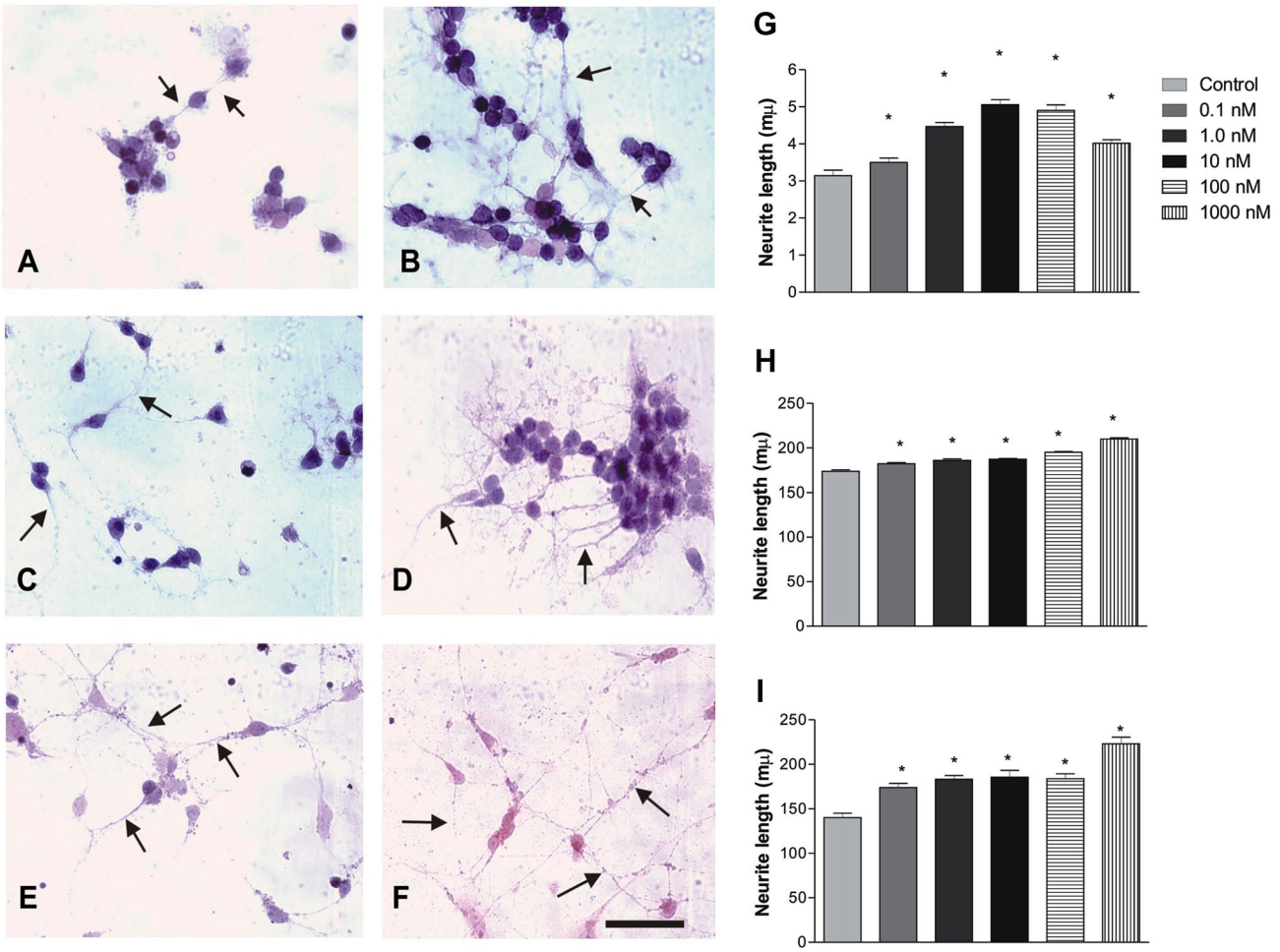

Fig. 1 Representative cytochemical staining with H-E of spinal cord neurons in culture of 15 days rat embryos. At different incubation times (4, 24 and 72 h) in control cultures (**a, c, e** respectively), neurons were treated with vehicle (saline solution); whereas in **b, d, f**, cultures were incubated with GnRH (100 nM for 4, 24 and 72 h respectively). Note that in **a, c**, and **e**, neurite length is lower than in **b, d** and **f**, respectively (*arrows*). Magnification was ×40. GnRH dose–response in *right panel*. Neurons were incubated with GnRH (0.1, 1.0, 10, 100 and 1000 nM) at different times (4, 24 and 72 h; **g, h**, and **i** respectively). Control cultures were incubated with saline solution. Ten neurons of ten randomized fields of each dish per triplicate were quantified. Data are presented as mean ± SEM. *$p < 0.01$ compared to control

increased neurite length in response to GnRH, as occur in cortical cerebral neurons at the same conditions. However, at concentration of 1 nM of GnRH, cortical cerebral neurons did not increase neurite outgrowth [9]. This difference could be due to the presence of a greater number of GnRH receptors responding to GnRH stimulation or its lower sensitivity to this neurohormone in spinal cord neurons. This action of GnRH resembles that observed in cultured spiral ganglion neurons, where neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor induced an increase in neurite length [22]. Likewise, neurotrophic effect of GnRH may also include the regulation in expression of structural proteins similar to BDNF which has been reported that can upregulate protein synthesis in dendrites of hippocampal neurons within hours [23].

NFs are referred to as type IV intermediate filament proteins that are exclusively expressed in neurons in central as well in peripheral nervous systems. Together with other axonal components such as microtubules-associated protein, maintain and regulate neuronal cytoskeletal plasticity, thus affecting axonal transport, axonal caliber, and neurite outgrowth [10, 24]. In the present experiment, GnRH did not change the expression of β-actin levels, whereas NFs expression was increased. These results indicate a specific effect of GnRH on NFs, which is similar to that found in spinal cord of injured animals treated with GnRH [25]. In this study, we observed a significant increase in the level expression of both NF-68 and NF-200 kDa and this fact is according with an increase in outgrowth length of neurites. These results were similar to those observed in cultured cerebral cortical neurons of rat embryos [9].

Spinophilin is a protein that plays an important role in synaptic transmission and neuronal plasticity [13]. Previously, it has been demonstrated that spinophilin is a dendritic spine

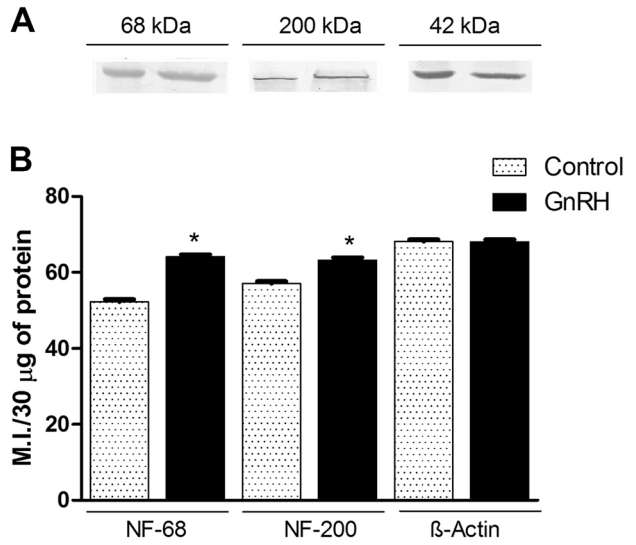

Fig. 2 Western blot analysis of NF-68, NF-200 kDa and β-actin of spinal cord neurons in culture of 15 days rat embryos. Cultured neurons (1×10^6 cells/dish) were incubated without treatment (control) or GnRH (100 nM/twice/day) for 24 h. **a** Representative immunoblot and (**b**) densitometric analysis of NF-68, NF-200 kDa, and β-actin. Values were expressed as media of intensity (MI) per 30 µg of protein. One hundred micrograms of protein/lane were processed for western blot analysis. *$p < 0.05$ (GnRH vs Control)

marker [15, 16]. We have found that neurons incubated with GnRH have a greater number of neuritic contacts, which is consistent with increasing expression of spinophilin. It is possible that GnRH through activation of its receptor is capable of inducing an increase in protein synthesis, specifically of spinophilin, considering that GAPDH constitutive expression remains constant. According to our results, high concentrations of GnRH (1000 nM) are needed to produce a greater expression of spinophilin as well as longer incubation time (72 h).

It is possible that these effects could be related to receptor unsaturation and to neuronal culture maturity.

Furthermore, in relation to GnRH-R activation and spinophilin expression, Schang et al. [26] have also observed an increase of this protein in response to a synthetic GnRH agonist (Triptorelin) in hippocampal neurons of mice during postnatal development. Likewise, Calderón-Vallejo et al. [27] found that in animals with spinal cord injury treated with GnRH, spinophilin expression was significantly increased in spinal cord.

Together, our findings indicate that GnRH has neurotrophic effects on neurite outgrowth in cultured neurons of rat spinal cord embryos. These effects are associated with an increase in NFs of 68 and 200 kDa and spinophilin expression. These results suggest that GnRH may play a role as neuromodulator of neuronal plasticity. It is also possible that GnRH could be considered as a potential factor for neuronal regeneration in spinal cord injuries.

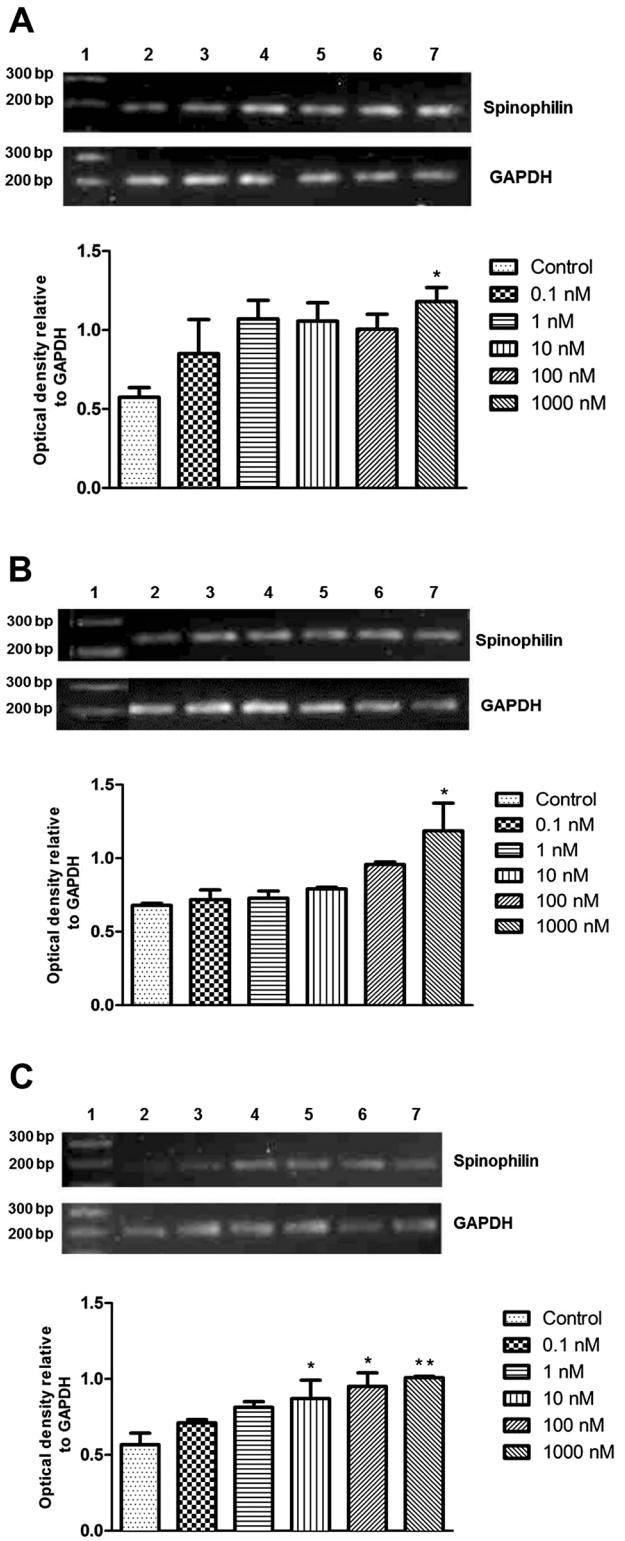

Fig. 3 GnRH effect on spinophilin mRNA expression. Neurons were incubated with or without GnRH for 4 h (**a**), 24 h (**b**) and 72 h (**c**) at different concentrations (0.1, 1, 10, 100, 1000 nM/twice/day). RT-PCR products of spinophilin and GAPDH mRNAs were analyzed on ethidium bromide-stained agarose gels. In lane 1: DNA size marker is given in base pairs (bp); and lanes 2, 3, 4, 5, 6 and 7, products of mRNAs at different concentrations of GnRH. 1.5×10^6 cells/dish/triplicate were used. Data are presented as mean ± SEM. *$p < 0.05$

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