Effect of Paroxetine on the Neuropathic Pain: A Molecular Study

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ABSTRACT

Background: Neuropathic pain, due to peripheral nerve damage, has influenced millions of people living all over the world. It has been shown that paroxetine can relieve neuropathic pain. Recently, the role of certain proteins like BDNF, GABA receptor, and KCC2 transporter in the occurrence of neuropathic pain has been documented. In the current study, the expression of these proteins affected by paroxetine was evaluated. Methods: Male Wistar rats were allocated into two main groups of pre- and post-injury. Rats in each main group received paroxetine before nerve injury and at day seven after nerve damage till day 14, respectively. The lumbar spinal cord of animals was extracted to assess the expression of target genes and proteins. Results: In the preventive study, paroxetine decreased BDNF and increased KCC2 and GABAA gene and protein expression, while in the post-injury paradigm, it decreased BDNF and increased KCC2 genes and protein expression. In this regard, an increase in the protein expression of GABAA was observed. Conclusion: It seems that paroxetine with a change in the expression of three significant proteins involved in neuropathic pain could attenuate this type of chronic pain. DOI: 10.29252/ibj.24.5.301

Keywords: Brain-derived neurotrophic factor, Gamma-aminobutyric acid, Paroxetine

INTRODUCTION

Neuropathic pain occurs by damage to the somatosensory system, including peripheral and central neurons, through some diseases like diabetes mellitus, trauma, and cancer[1]. Although the aspects about the management of neuropathic pain have been changed, there are limited data on the pathogenesis of this pain[2,3]. In spite of the recent progress in neurosciences and pharmaceutical technology, no effective drug, with a clear mechanism, has been developed to manage the neuropathic pain[4].

Among different mechanisms involved in the neuropathic pain, the GABAergic system is the key player. GABA receptors located in pre- and postsynaptic terminals of primary afferent neurons are also found in the dorsal horn laminae I-IV[5]. Dorsal horn GABAergic interneurons play an important role in decreasing pain[6,7]. So far, the relationship between the GABAergic system and neuropathic pain has not been understood well[8]. It has been shown that GABAergic neurons transplanted to subarachnoid space attenuate hyperalgesia produced by nerve damage in rat[9]. Moreover, muscimol, as a GABAA receptor agonist decreases hyperalgesia caused by peripheral neurons injury[10]. The normal function of the GABAergic system is extremely dependent on cation-chloride cotransporters. The influx and efflux of chloride into and out of neurons are facilitated mostly by NKCC1 and KCC2, respectively[11,12]. Both NKCC1 and KCC2 expressed in spinal cord regulate intracellular chloride homeostasis. Various studies have indicated that the altered expression of these transporters can change neuropathic pain behavior[6,7,13,14]. On the other hand, the elevated concentration of intracellular chloride diminishes the inhibitory effect of GABA receptors[15].

List of Abbreviations:

BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CCI, chronic constriction injury; DRG, dorsal root ganglion; KCC2, K+-Cl- cotransporter 2; NKCC1, Na-K-2Cl cotransport 1; p38MAPK, p38 mitogen-activated protein kina; RT-PCR, reverse transcription PCR; SNL, spinal nerve ligation
Nerve injury not only increases specific microglia markers expression (Iba1) but also releases some painful mediators such as BDNF, prostaglandin E2, nitric oxide, and tumor necrosis factor-α. These factors produce hypersensitivity in CNS through rising the excitability and decreasing the inhibition of DRG neurons[16]. Most of these mediators have a significant role in the production of chronic pain[17].

Damage to nerve activates p38MAPK pathway in DRG and microglial cells[18]. A recent study has suggested that the activation of the p38MAPK pathway by ATP and purinergic P2X4 receptor in microglia results in the production and release of BDNF[19]. Investigations have also displayed that BDNF has a prominent function in neuropathic pain. Thermal hyperalgesia and mechanical allodynia are induced by intrathecal administration of BDNF[20]. Other studies have revealed that nerve injury and peripheral inflammation change gene expression and production of BDNF[21,22]. BDNF has ability to change the function of GABA receptor[23] and also alters KCC2 gene expression, thereby effluxing chloride from the neuron; these changes finally result in chloride efflux through GABA and accordingly, depolarization of neurons[24]. Based on evidence, alteration in KCC2 expression gives rise to a change in GABA behavior from inhibitory to excitatory in some subtypes of injured nerves[6]. The GABA receptor subtypes α2 and γ2 is expressed mostly in the spinal dorsal horn. Although γ2 subunit gene expression decreases after nerve injury, α2 subtype expression has no significant changes[25].

Considering P2X4 receptors role in the release of important mediators involved in neuropathic pain, inhibition of this receptor can be helpful to understand the mechanism of neuropathic pain. Up to now, no selective inhibitor of the P2X4 receptor has been presented[26]. Antidepressant drugs, specifically tricyclic antidepressants have been widely used to manage neuropathic pain. It has been demonstrated that some antidepressants and antiseizure drugs are applied to treat neuropathic pain and inhibit the P2X4 receptor[27]. Among the antidepressants, paroxetine has a significant inhibitory effect on P2X4 receptors[28,29]. In the current study, we aimed to find out any possible changes in the expression of some proteins involved in the neuropathic pain (Iba1, BDNF, KCC2, and GABAα/γ2) affected by paroxetine.

MATERIALS AND METHODS

Animals

Rats (male Wistar, 150–200 g) used in the study were housed in an environment with controlled temperature (23 ± 2 °C). Food and water were available to animals without any limitation. At least one week before surgery, all the rats were permitted to be adapted to the housing facilities.

Surgery and drug preparation

The left sciatic nerve close to trifurcation was tied loosely (4 ligatures) by chronic gut suture, and thus, a model of neuropathic pain so-called CCI was created. Except for the sham group, the left sciatic nerve was tied in both drug and control groups. After ligation, the wound was closed. All surgical procedures were under sterile condition. Ketamine (60 mg/kg) and xylazine (10 mg/kg) were administered for the induction of anaesthesia. Paroxetine hydrochloride (Sigma, USA) was dissolved in DMSO 5%.

Drug administration

Animals were placed to pre- and post-injury groups. In each group, the rats were divided into CCI vehicle-treated (control), sham, and CCI paroxetine-treated groups. CCI- and sham-operated animals received the vehicle. Paroxetine was administered (i.p.) to the drug-treated group before and after surgery. In the preemptive paradigm, 10 mg/kg of paroxetine was injected to rats one hour prior to surgery and then daily after surgery until day 14. Animals in the post-injury group received the drug the same dose as the preventive group at day seven post-injury and then daily until day 14.

Tissue collection for RT-PCR and Western blot analysis

After euthanizing by CO₂ asphyxiation, rats were decapitated immediately on day 14 post surgery. The spinal cord displaced by the normal saline from the vertebral column was frozen in dry ice. For evaluating gene and protein, the lumbar spinal cord segment was isolated from the intact frozen cord.

Gene expression study

Isothiocyanate-phenol-chloroform protocol was used for the isolation of total RNA, using RNX+ reagent (CinnaGen, Iran), according to the instructions provided by the manufacturer. Based on the manufacturer’s protocol, 2 μg of total RNA, Oligo(dT) primer (Fermentas, USA), and M-Mulv reverse transcriptase (Fermentas) was used for the synthesis of cDNA. As shown in Table 1, designing of primer sequences (CinnaGen, Iran) was performed as per sequences in the GenBank. The PCR was carried out using the synthesized cDNA, the specific primers, and Taq DNA Polymerase MasterMix. In the beginning, the PCR was run for 10 min (95 °C), then continued by amplification cycles (25 or 26), each including 1-min denaturation (95 °C), annealing (45 s, 59 °C), and extension (45 s, 72 °C) steps. PCR products were
Table 1. Primer sequences for the PCR amplification of genes of interest

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Sequence 5' to 3'</th>
<th>Primer length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iba1</td>
<td>F-iba1</td>
<td>5' ACAAGACCTCCTCGATGATC 3'</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>R-iba1</td>
<td>5' GCAACTCGAATAAGCTTCTTG 3'</td>
<td>23</td>
</tr>
<tr>
<td>BDNF</td>
<td>F-bdnf</td>
<td>5' GCTGCGCCATGAGGAAGGC 3'</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>R-bdnf</td>
<td>5' GAAACCCTACGACATGTTTG 3'</td>
<td>21</td>
</tr>
<tr>
<td>KCC2</td>
<td>F-kcc2</td>
<td>5' AGGAGGAGATGGACACCCAGCC 3'</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>R-kcc2</td>
<td>5' GCAGTAGATGGCCAGCCAGG 3'</td>
<td>20</td>
</tr>
<tr>
<td>GABAA/γ/2</td>
<td>F-gaba-a</td>
<td>5' AGATTATGCTCTAATAAAC 3'</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>R-gaba-a</td>
<td>5' CACCATTGTCTATTCAATCG 3'</td>
<td>21</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F-gapdh</td>
<td>5' GTTACCAGGCGCTTCTTCTTG 3'</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>R-gapdh</td>
<td>5' GTGGTGAGAGTGCATTGTGAC 3'</td>
<td>23</td>
</tr>
</tbody>
</table>

Ethical statement

The above-mentioned sampling and treatment protocols were approved by the Research Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (ethical code: SBMU.REC1393.327).

RESULTS

Effect of prophylactic administration of paroxetine on the gene expression

Paroxetine and control groups showed a significant difference in the Iba1 expression (p < 0.01 and p < 0.001, respectively) relative to the sham group Fig. 1). Compared to the control group, paroxetine decreased the expression of BDNF (p < 0.001). In comparison to the sham and control groups, the expression of KCC2 decreased and increased significantly in the control (p < 0.05) and paroxetine (p < 0.05) groups. However, paroxetine decreased the gene expression of GABAA/γ/2 (p < 0.05) compared to the sham group.

Effect of paroxetine on gene expression after nerve injury

As depicted in Figure 2, a significant rise was seen in the expression of Iba1 in the control (p < 0.001) and paroxetine (p < 0.01) groups compared to the sham group. The paroxetine-treated group showed a significant decline in the expression of BDNF compared to the control (p < 0.05) and sham (p < 0.01) groups. KCC2 gene expression showed a significant reduction in the control group in comparison to the sham group p < 0.01). Compared to the control, the paroxetine group showed a significant rise (p < 0.05) in KCC2 gene expression. Moreover, no significant decrease was observed in the expression of GABAA/γ/2 in the drug-treated group relative to other groups.
Fig. 1. Effect of pre-injury administration of paroxetine on the gene expression of Iba1, BDNF, KCC2, and GABA\textsubscript{A}/\gamma\textsubscript{2}. GAPDH was used as a loading control. Relative expression of mRNA: band density of genes/GAPDH band density. Data are expressed as means ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 indicate a statistically significant difference compared to the sham group, and +p < 0.05 and +++p < 0.001 show significant difference compared to the control group.

Effect of prophylactic injection of paroxetine target proteins
As it is clear from Figure 3, compared to the sham group, the expression level of the microglia marker (Iba1) rose in the control (p < 0.001) and paroxetine (p < 0.01) groups. Paroxetine could decrease the BDNF protein level significantly as compared to the sham (p < 0.01) and the control (p < 0.001) groups. Compared to the control group, KCC2 showed a significant rise in the paroxetine group (p < 0.05). On the other hand, a significant difference in GABA\textsubscript{A}/\gamma\textsubscript{2} levels (p < 0.001) was found between paroxetine and control group.

Effect of paroxetine on the expression of target proteins after nerve injury
There was a significant rise in Iba1 protein level in the control and paroxetine groups as compared to the sham group (p < 0.001; Fig. 4). On the other hand, compared to the control group, the expression of BDNF protein decreased significantly in
the paroxetine-treated group ($p < 0.01$). Paroxetine increased the KCC2 expression compared to the control group ($p < 0.05$). GABAA/γ2 protein levels showed no change in the drug-treated group in comparison with the control group.

**DISCUSSION**

In the present study, the effect of paroxetine on the expression of certain important mediators involved in neuropathic pain was investigated. Our data indicated an altered expression of $BDNF$ and $KCC2$ upon administration of paroxetine. This was also the case for $GABAA/γ2$ proteins when the drug was injected before nerve damage. Microglial cells remain in resting state under physiological conditions and have appendages morphologically. Nerve injuries and lesions activate microglial cells and change their morphology with losing the appendages as well as swelling. After nerve injury, the gene expression level of $P2X4$ receptor and the $Iba1$ protein levels increased in microglial cells\textsuperscript{[30,31]}. It has been shown that $Iba1$ gene is specifically expressed in microglial cells in the CNS\textsuperscript{[32]} but not in other cells (neurons, astrocytes, and oligodendrocytes)\textsuperscript{[33]}. Studies have evidenced the significant role of $Iba1$ in the migration and phagocytic activity of microglial cells\textsuperscript{[34]}. Moreover, the expression of this protein and mechanical allodynia elevates after SNL in the rat\textsuperscript{[35]}. As noted previously, paroxetine has the most effect on the inhibition of the $P2X4$ receptor among various antidepressants and antiepileptic drugs used in neuropathic pain. A previous study has indicated that the expression of this receptor enhances in microglia after the nerve injury, and the neuropathic pain symptoms are also observed more frequently afterward. However, there is no change in the expression of this receptor in nerve cells or astrocytes\textsuperscript{[36]}. Another report has suggested that the $P2X4$ ionotropic receptor is expressed only in microglia and its expression increases after neuropathy\textsuperscript{[37]}. Consistent with our data, some behavioral and biochemical findings have demonstrated that the activity of $P2X4$ receptors expressed in dorsal horn microglia is necessary for the induction of mechanical allodynia\textsuperscript{[38,39]}. A number of investigations have revealed that $BDNF$ neurotrophin has a critical role in neuropathic pain and its expression boosts in the spinal cord dorsal horn and DRG\textsuperscript{[40,41]}. The activation of the $P2X4$ receptor in microglia is essential for the expression and release of $BDNF$ after peripheral nerve injury, which in turn leads to the increased pain transfer in neurons\textsuperscript{[19]}. It has also been suggested that $BDNF$ indirectly facilitates the release of $GABA$ from the spinal cord interneurons\textsuperscript{[22]}, and $BDNF$ expression increases in the spinal cord (dorsal horn) 24 hours after SNL, and this elevation continues for several days\textsuperscript{[40]}. In the present study, while after CCI, the expression of the $BDNF$ gene enhanced significantly, paroxetine reduced the expression of this neurotrophin both before and after the nerve injury. Our previous surveys demonstrated that the prophylactic injection of paroxetine could diminish neuropathic pain\textsuperscript{[42]}. Considering the key role
of BDNF in neuropathic pain, this change in pain behavior is probably due to a shift in BDNF expression, which is reduced by paroxetine when administered before and after nerve damage.

KCC2 is a transporter for potassium and chloride ions and contributes to the regulation of anion gradients on both sides of the membrane. This transporter plays a very important role in regulating GABA receptor activity. The post-synaptic activity of GABA A receptors in the adult nervous system leads to the opening of the chloride channel, neuronal hyperpolarization and as a result, its inhibitory activity. The role of KCC2 has been proven in chlorine ion homeostasis in the spinal interneurons. KCC2 actively pumps the ion chloride to the outside of the neuronal cell to support conditions for the inhibitory activity of GABA receptors. Moreover, the expression of KCC2 can contribute to the neuropathic pain induced by nerve injury. In this study, paroxetine increased the expression of KCC2 as compared to the control group when used before or after nerve injury. Further researches are needed to find out whether paroxetine directly affects the expression of this protein, or secondarily, by blocking the purinergic receptor that causes this change. The GABA A receptors existing at the end of the primary afferent neurons are responsible for their synaptic inhibition. It has been proven that GABA A/2 expression decreases significantly in the DRG after the nerve injury. However, after the nerve injury, the expression of BDNF rises in DRG neurons, but the γ2 subunit of GABA A receptors decreases concurrently. As mentioned before, KCC2, as a key protein in regulating the equilibrium potential of anions, is crucial for the GABA A inhibitory activity. Reduced expression of the KCC2 protein in dorsal horn of the spinal cord neurons leads to the elimination of the inhibitory function of GABA A receptor in case of chronic neuropathy. However, considering what mentioned before, the inhibitory effect of GABA A receptor depends on two important factors: first, the expression of the receptor itself and its subunits, and the second, the post-synaptic activity of the membrane protein of KCC2. The findings of this study showed that only pre-injury injection of paroxetine resulted in the increased protein level of GABA A/2 receptors.

In conclusion, it seems that paroxetine with change in the expression of some important proteins involved in neuropathic pain (BDNF, KCC2, and GABA A) alleviates neuropathic pain. This effect was observed whether paroxetine was administered before nerve injury or when it was injected after damage to the nerve.

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Fig. 4. Effect of paroxetine on the expression of Iba1, BDNF, KCC2, and GABA A/γ2 protein levels administered after nerve injury. GAPDH was used as a loading control. Arbitrary unit: proteins optical density/GAPDH optical density. Data are expressed as means ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 indicate a statistically significant difference when compared to the sham group, and *p < 0.05, **p < 0.01 show significant difference compared to the control group.
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**CONFLICT OF INTEREST.** None declared.

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