Expression of glycine receptor α3 subunit in neuropathic and inflammatory central pain sensitization

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Abstract

Introduction: *Increasing evidence suggests that changes in the balance of excitatory/inhibitory neurotransmission are involved in the development of the majority of chronic pain forms. In this context, impairment in glycine mediated inhibitory neurotransmission is thought to play a critical role in the disinhibition that accounts for the development and maintenance of central pain hypersensitivity.*

Aims: *The goal of this study was to evaluate the Glycine Receptor α3 subunit (α3GlyR) expression in neuropathic (Chronic Constriction Injury, CCI) and inflammatory (Zymosan A injected) animal models of chronic pain.*

Results and conclusion: *RT-qPCR analysis of spinal cord samples showed that glra3 gene expression does not change after 3 days of CCI and 4 hours of Zymosan A injection. However, we found that protein levels evaluated by Western blot increased after inflammatory pain. These data suggest that central sensitization is differentially regulated depending on the type of pain. α3GlyR protein expression plays an important role in the first step of inflammatory pain establishment.*

Keywords: *central sensitization, neuropathic, inflammatory, hypersensitivity, glycine receptors*

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Introduction

Chronic pain constitutes a major health problem, affecting millions of people worldwide. In the United States alone there are at least 116 million of adults affected by some form of chronic pain, representing an important medical and economic burden for the families of the individual affecte[1]. In Chile, it has been estimated that approximately 40% of the population (>17 years old) is affected by non-cancer related pain[2]. Despite that in Chile pain management is covered by the system for Explicit Guarantees of Health (GES), only one out three patients is satisfactorily relie-

ved of the pain with current pharmacological and pain management programs[3].

Increasing evidence suggests that changes in the excitability of peripheral and central nociceptive pathways account for the development of the majority of forms of chronic pain[4]. In this context, impairment in GABA and Glycine mediated inhibitory neurotransmission are thought to play a critical role in the disinhibition that accounts for the development and maintenance of pain hypersensitivity[5]. Glycine is the main inhibitory neurotransmitter in the spinal cord and brain stem, playing a significant role in motor respiratory control and pain sensitization[6]. Glycine receptor (GlyR) subunits are encoded by Glra and Glrb genes[7]. In the mature spinal cord, glycine activates GlyRs, which are pentamers composed mainly of α1, α2, α3 and β subunits[8]. α3GlyRs are expressed in the dorsal horn spinal cord where the pain signaling is integrated[9]. It was reported that α3GlyRs underlies inflammatory pain sensitization [10,11].

Chronic pain comprises several different types of pain, including inflammatory pain following tissue injury (for example, arthritis), cancer pain and neuropathic pain following nerve injury, spinal cord injury and brain injury (for example, stroke and trauma)[12,13,14]. The pathogenesis of neuropathic and inflammatory pain involves the alteration of neuro-immune interface[15,16,17]. Importantly, reduced inhibitory activity in the dorsal spinal cord has been reported in both persistent neuropathic [18,19,20] and inflammatory pain [21,22,23]. However, there is a lack of information related with GlyR subunit gene expression in different types of pain. Therefore, in the present study the α3GlyR subunit expression was characterized in neuropathic (Chronic Constriction Injury, CCI) and inflammatory (Zymosan A injected) animal models of pain sensitization.

Materials and Methods

Choice of animal model: The chronic constriction sciatic nerve Injury (CCI) and inflammatory hyperalgesia induced by subcutaneous Zymosan A injection on the left hindpaw in Sprague Dawley rats are well known and extensively validated models of neuropathic and inflammatory pain [24,25,26]. Rats were maintained at the animal facility of the Universidad de Chile for about 4 weeks. Accordingly, we used preferentially adult male animals. All procedures conformed to the regulations specified by the Institutional Animal Use Committee of the University of Santiago of Chile and conducted according with the ethical protocols established by the National Institutes of Health, Bethesda, USA.

quantitative PCR: qPCR from spinal tissues of CCI rats were performed at 3 days post-surgery and were compared with data obtained from Sham (rat surgery without ligation). Quantitative PCR of spinal cord tissues derived from

Zymosan-A-injected (0.06 mg in 20 μL PBS) and control rats (saline) were extracted 4 hours after hindpaw Zymosan A injection. Total RNA was isolated from the spinal cord (L3-L5 lumbar segment) by the Guanidinium thiocyanate-phenol chloroform extraction method (Trizol, Life Technologies, Carlsbad, CA, USA). Following total RNA preparation from these tissues and cDNA synthesis, qPCR of α3 subunit was performed using 0,5 ng/uL of cDNA and SYBRgreen Mastermix (Sigma) in a final reaction volume of 20 uL in Stratagene Mx3000P equipment (Agilent Technologies, Santa Clara, CA, USA). PCR amplification cycles were: 1 at 95°C/5min, 40 at 95°C/30s, 56°C/30s and 72°C/45s. All reactions were run in triplicate. The primer sequences are shown in Table 1.

The relative expression of each gene was calculated using the Pfaffle Method [27] and normalized against GAPDH expression.

PRIMERS	5´- 3´SEQUENCE	AMPLICON (base pairs size)
GAPDH-R	GGCCTCTCTCTTGCTCTCAGTA	141
GAPDH-F	TTGTGAAGCTCATTTCCTGGTA	
alpha3-F	GCCTTCTGATTGTCATTCTGTC	109
alpha3-R	CTCTGCGTGGTCATCGTAAG	

Table 1: primer sequences used in qPCR experiments.

Western blot: Total proteins were quantified using the Micro BCA protein assay (Pierce Biotechnology, Rockford, IL). SDS PAGE was performed using 60 µg of protein on Nu-PAGE gels (Invitrogen Life Technologies, Carlsbad, CA). Proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA) and then blocked overnight by incubation with 5% dry milk in Tris-buffered saline containing.
0.1% Tween 20 (TBS). After washing with TBS, the blots were incubated with anti α3GlyR subunit (1:300, rabbit; Millipore) primary antibodies. Monoclonal anti-GAPDH antibody was used as load control.

Data analysis

All data are presented as mean ± standard error of mean (SEM). Statistical analyses were performed using GraphPad Prism 6 software. One-way ANOVA analysis was performed. P-value <0.05 was considered as significant.

Results

To understand the contribution of α3GlyRs to the central sensitization process, α3GlyR subunit expression levels were analyzed in spinal cord tissues from neuropathic and inflammatory animal models of pain. In Figure A, it can be seen that the glr3 gene expression levels did not change after 3 days of CCI. Similar results were obtained after 4 hours of Zymosan A injection. However, data show that α3GlyRs protein expression increased after inflammatory pain and no changes were observed after 3 days of CCI (Figure B). These results suggest that the central pain sensitization process is differentially regulated by glr3 gene expression.

Discussion

Previous studies have described that inhibitory glycinergic neurotransmission is altered in chronic pain conditions. In order to understand the contribution of α3GlyRs to central sensitization, Harvey et al. generated a Glra3-/- Knockout mice. Behavioral approaches demonstrated that pain sensitization induced by intrathecal PGE2 and peripheral inflammation was reduced in Glra3-/- Knockout mice [22]. In agreement with these results, Reinold et al. showed that mice deficient in EP2 receptors completely lack spinal PGE2 mediated hyperalgesia [28]. Electrophysiological experiments showed that α3GlyR mediated currents were

inhibited by PGE2. These effects were abolished by intracellular loop mutation at serine 346 and PKA inhibition, demonstrating that phosphorylation of α3GlyRs consistently determines the inflammatory pain sensitization [22]. In agreement with these reports, our data show that the α3GlyRs protein increased only in the inflammatory context, suggesting the existence of fine tune mechanisms that regulate the protein expression in different types of pain conditions. For example, previous studies have demonstrated that the activation of GlyRs triggers the chloride ion influx, which results in the membrane hyperpolarization needed to control the excitability of neural circuits. Chloride homeostasis is regulated by co-transporter KCC2 [29]. One of the critical steps in the central pain sensitization that accounts for disinhibition is related with the KCC2 downregulation. It has been reported that KCC2 expression is altered in neuropathic and inflammatory pain, leading to the collapse of chloride ionic gradient and shifting the glycine receptor reversal potential to more depolarized values [30]. In terms of the molecular mechanism involved in this process, Coull et al., showed that ATP-stimulated microglia cause a depolarizing shift in anion reversal potential in spinal neurons[31]. The authors conclude that molecular mechanisms involved in these
changes are associated with the activation of TrkB receptors by BDNF because blocking these signalings with anti-TrkB antibody or siRNA anti BDNF abolished the changes in the anion reversal potential. On the other hand, Zhou et al., demonstrated that a shift in glycine current reversal potential is mediated by KCC2 calpain cleavage [32]. A recent study showed that morphine tolerance is induced by the downregulation of KCC2, impairing the chloride homeostasis in rat spinal neurons [33,34]. Our results suggest that α3GlyRs protein expression would be modulated by changes in neuronal chloride homeostasis mediated by the KCC2 transporter. Conclusions: Our results showed that the α3GlyRs protein only increased in inflammatory pain conditions, suggesting that central pain sensitization fine-tunes gene expression regulation in different types of pain contexts. This process is regulated by intracellular chloride homeostasis. Future studies to understand if there is a crosstalk between α3GlyRs and KCC2 protein expression regulation to establish central inflammatory sensitization is necessary in order to expand the therapeutic alternatives for confronting the development and establishment of chronic pain.

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