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TRANSCRIPT EXPRESSION OF VESICULAR GLUTAMATE TRANSPORTERS IN LUMBAR DORSAL ROOT GANGLIA AND THE SPINAL CORD OF MICE – EFFECTS OF PERIPHERAL AXOTOMY OR HINDPAW INFLAMMATION

M. MALET,[a,b1] C. A. VIEYTES,[a1] K. H. LUNDGREN,[c] R. P. SEAL,[d] E. TOMASELLA,[a,b] K. B. SEROOGY,[c] T. HÖKFELT,[e] G. F. GEBHART[d] AND P. R. BRUMOVSKY[a,b,d*]

[a] *Faculty of Biomedical Sciences, Austral University, Buenos Aires, Argentina*

[b] *CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Buenos Aires, Argentina*

[c] *Department of Neurology, University of Cincinnati, Cincinnati, OH, USA*

[d] *Pittsburgh Center for Pain Research, Department of Neurobiology, University of Pittsburgh, Pittsburgh, PA, USA*

[e] *Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden*

Abstract—Using specific riboprobes, we characterized the expression of vesicular glutamate transporter (VGLUT)$_1$–VGLUT$_3$ transcripts in lumbar 4–5 (L4–5) dorsal root ganglions (DRGs) and the thoracolumbar to lumbosacral spinal cord in male BALB/c mice after a 1- or 3-day hindpaw inflammation, or a 7-day sciatic nerve axotomy. Sham animals were also included. In sham and contralateral L4–5 DRGs of injured mice, VGLUT$_1$-, VGLUT$_2$- and VGLUT$_3$ mRNAs were expressed in ~45%, ~69% or ~17% of neuron profiles (NPs), respectively. VGLUT$_1$ was expressed in large and medium-sized NPs, VGLUT$_2$ in NPs of all sizes, and VGLUT$_3$ in small and medium-sized NPs. In the spinal cord, VGLUT$_1$ was restricted to a number of NPs at thoracolumbar and lumbar segments, in what appears to be the dorsal nucleus of Clarke, and in mid laminae III–IV. In contrast, VGLUT$_2$ was present in numerous NPs at all analyzed spinal segments, except the lateral aspects of the ventral horns, especially at the lumbar enlargement, where it was virtually absent. VGLUT$_3$ was detected in a discrete number of NPs in laminae III–IV of the dorsal horn. Axotomy resulted in a moderate decrease in the number of DRG NPs expressing VGLUT$_3$, whereas VGLUT$_1$ and VGLUT$_2$ were unaffected. Likewise, the percentage of NPs expressing VGLUT transcripts remained unaltered after hindpaw inflammation, both in DRGs and the spinal cord. Altogether, these results confirm previous descriptions on VGLUTs expression in adult mice DRGs, with the exception of VGLUT$_1$, whose protein expression was detected in a lower percentage of mouse DRG NPs. A detailed account on the location of neurons expressing VGLUTs transcripts in the adult mouse spinal cord is also presented. Finally, the lack of change in the number of neurons expressing VGLUT$_1$ and VGLUT$_2$ transcripts after axotomy, as compared to data on protein expression, suggests translational rather than transcriptional regulation of VGLUTs after injury.

Key words: VGLUTs, DRGs, hybridization, axotomy, hindpaw inflammation, spinal cord.

INTRODUCTION

Glutamate has an essential role in sensory neuron signaling, and it also participates in both acute and chronic pain mechanisms (Chiosa and Gane, 1956; Curtis et al., 1960; Karim et al., 2001; Minami et al., 2001; Sung et al., 2003; Binns et al., 2005; Liaw et al., 2005; Tao et al., 2005; Moechars et al., 2006). Presence of glutamate in dorsal root ganglion (DRG) neurons and their projections was first identified by Rustioni and Weinberg (1989). Recently, vesicular glutamate transporters (VGLUTs), the proteins responsible for the uptake of glutamate into synaptic vesicles, have been identified and characterized in numerous neuron types in both the central (CNS) and peripheral nervous systems (PNS) (see Kaneko and Fujiyama, 2002; Fremeau et al., 2004; Seal and Edwards, 2006; Takamori, 2006). Because their expression is directly related to the glutamatergic phenotype of neurons, VGLUTs have emerged as the definitive markers to histochemically identify neurons that use glutamate as a neurotransmitter (see Brumovsky et al., 2011a and references therein). Three subtypes have been described thus far, VGLUT$_1$, (Ni et al., 1994; Bellocchio et al., 2000; Takamori et al., 2000), VGLUT$_2$ (Aihara et al., 2000; Bai et al., 2001; Fremeau et al., 2001; Hayashi et al., 2001; Herzog et al., 2001; Sakata-Haga et al., 2001; Takamori et al., 2001; Varoqui et al., 2002) and VGLUT$_3$ (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002).

*Correspondence to: P. R. Brumovsky, Faculty of Biomedical Sciences, Austral University, Av. Juan D. Perón 1500, Pilar B1629AHJ, Buenos Aires, Argentina. Tel: +54-0230-448-2699.
E-mail addresses: pablo_brumovsky@yahoo.com, brumovskypr@upmc.edu (P. R. Brumovsky).
[1] These authors contributed equally to this work..

Abbreviations: ANOVA, analysis of variance; Axo, axotomy; CFA, Complete Freund's Adjuvant; DRG, dorsal root ganglion; EGFP, enhanced green fluorescent protein; IR, immunoreactive; L, lumbar; Li, like-immunoreactivity; LS, lumbosacral; NPs, neuron profiles; TL, thoracolumbar; VGLUT, vesicular glutamate transporter.

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Rodent DRG neurons are divided between those that innervate visceral and non-visceral tissues (see Robinson and Gebhart, 2008 and references therein). Initial studies focused on the immunohistochemical expression of $VGLUT_1$ and $VGLUT_2$ in rat DRG neurons projecting to non-visceral tissues (lumbar (L) 4 and 5), showing that $VGLUT_1$ was primarily expressed in large non-peptidergic neurons whereas $VGLUT_2$ was expressed in neurons of all sizes, including peptidergic ones (Tong et al., 2001; Oliveira et al., 2003; Hwang et al., 2004). These observations found a correlate in mouse (Brumovsky et al., 2007; Scherrer et al., 2010) and guinea pig (Morris et al., 2005) DRG neurons, as well as those targeting visceral organs such as the colorectum (Brumovsky et al., 2011a) or the urinary bladder (Brumovsky et al., 2012). The identification of $VGLUT_3$ in DRGs has been more difficult, mainly because none of the commercially available antibodies against $VGLUT_3$ efficiently labels these neurons or their projections. However, the recent generation of transgenic mice where reporter proteins such as the enhanced green fluorescent protein (EGFP) are under control of the $VGLUT_3$ promoter ($VGLUT_3$-EGFP), demonstrated the presence of $VGLUT_3$ in subpopulations of sciatic nerve- (Seal et al., 2009; Lou et al., 2013) and urinary bladder-projecting (Brumovsky et al., 2012) DRG neurons.

VGLUTs are also immunohistochemically detected in the neuropil (but not in cell bodies) of the rodent spinal cord and exhibit different patterns of distribution, including dorsal vs. ventral or lateral horns (Varoqui et al., 2002; Li et al., 2003a; Olave and Maxwell, 2003; Oliveira et al., 2003; Todd et al., 2003; Alvarez et al., 2004; Hwang et al., 2004; Landry et al., 2004; Morris et al., 2005; Persson et al., 2006; Llewellyn-Smith et al., 2007; Seal et al., 2009). Some of the immunoreactivity to VGLUTs in the spinal cord represents transporter molecules synthesized in DRG neurons and then axonally transported through the central branches of dorsal roots to the spinal cord. This was first suggested by Varoqui et al. (2002), and later demonstrated by Todd et al. (2003), showing that $VGLUT_1$ and $VGLUT_2$ proteins are present in transganglionically labeled primary afferent terminals in the dorsal horn of the spinal cord of rat. In support, dorsal rhizotomy, a procedure normally utilized to unveil the contribution of molecules produced by DRG neurons and actively transported to the spinal cord, results in a dramatic (although not complete) decrease in $VGLUT_1$-like-immunoreactivity (Li) in nerve fibers in the ventral and to some extent also in the dorsal horn of rats (Li et al., 2003a; Oliveira et al., 2003; Alvarez et al., 2004) and mice (Brumovsky et al., 2007). A decrease, although restricted to the superficial laminae of the dorsal horn, has been described for $VGLUT_3$ as well as for $VGLUT_3$-EGFP in transgenic mice (Seal et al., 2009). In contrast, and with one exception in the rat showing an ipsilateral decrease (Li et al., 2003a), dorsal rhizotomy fails to alter the immunoreactivity of $VGLUT_2$, both in rat (Oliveira et al., 2003; Alvarez et al., 2004) and mouse (Brumovsky et al., 2007). These observations suggest presence of more than one source of VGLUT proteins detected in the spinal cord. In fact, brainstem- (Oliveira et al., 2003; Du et al., 2012) and cortical-derived (Fremeau et al., 2001; Persson et al., 2006; Du et al., 2012) descending fibers express $VGLUT_1$ and/or $VGLUT_2$.

Analyses of VGLUT transcripts in DRGs and spinal cord also have received attention. Thus, the use of oligo- (Oliveira et al., 2003) and riboprobes (Landry et al., 2004) in the adult rat revealed that $VGLUT_1$ (Oliveira et al., 2003; Landry et al., 2004) and $VGLUT_2$ (Landry et al., 2004) transcripts are expressed by a considerable number of neurons in L4–5 DRGs. $VGLUT_3$ mRNA was also identified in rat DRGs by means of reverse transcription polymerase chain reaction (RT-PCR) (Gras et al., 2002). More recently, the presence of $VGLUT_3$ transcripts has been reported (but not quantified) in neonatal mouse DRG neurons (Lou et al., 2013). In the spinal cord, neuronal expression for all VGLUTs has been described in adult rats (Oliveira et al., 2003; Landry et al., 2004; Llewellyn-Smith et al., 2007), neonatal rats (Kullander et al., 2003), as well as in neonatal mice (Lou et al., 2013). However, the pattern of distribution for each VGLUT differs among studies (Kullander et al., 2003; Oliveira et al., 2003; Landry et al., 2004; Llewellyn-Smith et al., 2007; Lou et al., 2013).

To date, neither a detailed description of the transcript expression of VGLUTs in mouse DRG and spinal cord neurons is available, nor knowledge of possible changes in expression after peripheral nerve injury or inflammation. Thus, in the present study, we have characterized the expression of the three VGLUTs in lumbar DRG neurons and the thoracolumbar, lumbar and lumbosacral spinal cord of BALB/c mice by means of *in situ* hybridization. We used selective riboprobes that have been validated in their specificity to identify VGLUTs in peripheral neurons (Brumovsky et al., 2011a). Animals with a 1- or 3-day hindpaw inflammation or a 7-day axotomy of the sciatic nerve were included in the analysis. Portions of these data have been reported in abstract from (Vieytes et al., 2013).

EXPERIMENTAL PROCEDURES

Animals

The experiments were performed on 51 male BALB/c mice (b.wt. 20–30 g). The animals were maintained under standard conditions on a 12-h day/night cycle (light on at 7 AM), with water and food *ad libitum*. All experiments were performed following the Society for Neuroscience and the International Association for the Study of Pain guidelines for the use of animals in research, and approved by the institutional ethics committees (University of Pittsburgh and Austral University).

Axotomy (Axo)

Under aseptic conditions, mice were anesthetized with Isoflurane (Hospira Inc., Lake Forest, IL, USA), and a complete transection of the right sciatic nerve was performed. Briefly, the right sciatic nerve was exposed