*Neuroscience* 248 (2013) 180–193

# DISTRIBUTION OF TRANSMEMBRANE AMPA RECEPTOR REGULATORY PROTEIN (TARP) ISOFORMS IN THE RAT SPINAL CORD

**M. LARSSON,** [a,b]* **N. AGALAVE,** [b] **M. WATANABE** [c] **AND C. I. SVENSSON** [b]

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

[b] *Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden*

[c] *Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan*

**Abstract—The transmembrane α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor regulatory proteins (TARPs) are a family of auxiliary AMPA receptor subunits that differentially modulate trafficking and many functional properties of the receptor. To investigate which TARP isoforms may be involved in AMPA receptor-mediated spinal synaptic transmission, we have mapped the localization of five of the known TARP isoforms, namely γ-2 (also known as stargazin), γ-3, γ-4, γ-7 and γ-8, in the rat spinal cord. Immunoblotting showed expression of all isoforms in the spinal cord to varying degrees. At the light microscopic level, immunoperoxidase labeling of γ-4, γ-7 and γ-8 was found throughout spinal gray matter. In white matter, γ-4 and γ-7 immunolabeling was observed in astrocytic processes and in mature oligodendrocytes. In pepsin-treated spinal cord, γ-7 often colocalized with GluA2 immunopositive puncta in the deep dorsal horn as well as in the ventral horn, but not in the superficial dorsal horn. Postembedding immunogold labeling was further used to assess the synaptic localization of γ-2, γ-7 and γ-8 in the dorsal horn. Synaptic immunogold labeling of γ-2 was sparse throughout the dorsal horn, with some primary afferent synapses weakly labeled, whereas relatively strong γ-7 immunogold labeling was found at deep dorsal horn synapses, including at synapses formed by low-threshold mechanosensitive primary afferent terminals. Prominent immunogold labeling of γ-8 was frequently detected at synapses established by primary afferent fibers. The spinal localization patterns of TARP isoforms reported here suggest that AMPA receptors at spinal synaptic populations and in glial cells may exhibit different functional characteristics owing to differences in auxiliary subunit composition.**


**Key words: glutamate receptor, synaptic transmission, astrocyte, dorsal horn, ventral horn, electron microscopy.**

## INTRODUCTION

Glutamate is the main fast transmitter at most excitatory synapses in the spinal cord, including synapses formed by primary afferent fibers, corticospinal projections and excitatory interneurons. Members of all ionotropic glutamate receptor families are expressed in the spinal cord. Of these, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (AMPARs) mediate most of the basal excitatory transmission in spinal sensory (Larsson, 2009; Larsson and Broman, 2011) and motor pathways (Hori et al., 2002; Liu et al., 2009; Talpalar and Kiehn, 2010). Synaptic or membrane trafficking of AMPARs in the spinal dorsal horn has been implicated in several models of hyperalgesia (Galan et al., 2004; Katano et al., 2008; Larsson and Broman, 2008; Park et al., 2008, 2009; Pezet et al., 2008; Choi et al., 2010). Moreover, AMPAR activation and trafficking have been associated with excitotoxicity of motorneurons and white matter in pathological conditions such as amyotrophic lateral sclerosis and spinal cord injury (Park et al., 2004; Van Den Bosch et al., 2006; Beattie et al., 2010; Hideyama and Kwak, 2011).

AMPARs are conventionally considered heterotetrameric assemblies of the four subunits GluA1–4. The core subunit composition determines crucial characteristics of the receptor, including trafficking, $Ca^{2+}$ permeability and voltage dependence (Ashby et al., 2008; Anggono and Huganir, 2012). However, in the last decade, a number of auxiliary subunits have been identified (Jackson and Nicoll, 2011; Schwenk et al., 2012; Straub and Tomita, 2012). The most well-studied of these is the transmembrane AMPAR protein (TARP) family. The six TARP isoforms, named γ-2 (or stargazin), γ-3, γ-4, γ-5, γ-7 and γ-8 differentially influence key properties of the AMPAR. Type I TARPs (γ-2, γ-3, γ-4 and γ-8) regulate surface and synaptic trafficking of the receptor, whereas type II TARPs (γ-5 and γ-7) have little or no effect on trafficking. Moreover, all TARPs except γ-5 slow decay kinetics and increase glutamate affinity to varying degrees. For instance, receptors containing γ-2

*Correspondence to: M. Larsson, Department of Neuroscience, Karolinska Institutet, Retzius väg 8, S-171 77 Stockholm, Sweden. Tel: +46-8-524-838-89.

E-mail address: max.larsson@ki.se (M. Larsson).

*Abbreviations:* AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; AMPAR, AMPA receptor; APC, adenomatous polyposis coli; DSA, dense sinusoid axon terminal; EDTA, ethylenediaminetetraacetic acid; GFAP, glial fibrillary acidic protein; HEPES, hydroxyethyl piperazineethanesulfonic acid; LTM, low-threshold mechanosensitive primary afferent fiber/terminal; PBS, phosphate-buffered saline; PSD, postsynaptic density protein; TARP, transmembrane AMPAR regulatory protein; TBST, Tris-buffered saline with Triton X-100.

0306-4522/13 $36.00 
http://dx.doi.org/10.1016/j.neuroscience.2013.05.060

or γ-3 show more rapid kinetics than those containing γ-4 or γ-8. Thus, TARP isoforms confer an additional layer of functional diversity to AMPARs, and these proteins may be important for the modulation of AMPAR function and synaptic plasticity.

Although spinal AMPARs are now widely regarded as having a crucial role in hyperalgesia, the role of TARPs in pain signaling is essentially unknown. Knockdown of γ-2 in the spinal cord has been shown to attenuate formalin-induced nocifensive behavior in the rat (Tao et al., 2006). Moreover, *Cacng2*, the gene encoding γ-2, was recently implicated in a nerve injury-induced behavioral phenotype in mice (Nissenbaum et al., 2010). The latter study also indicated human *CACNG2* as a susceptibility factor for neuropathic pain. However, the role of the other TARP isoforms in spinal neurotransmission has not been investigated. Whereas TARPs show differential distribution between regions and cell types in the rodent brain (Tomita et al., 2003; Fukaya et al., 2006; Kato et al., 2007; Yamazaki et al., 2010), only γ-2 has been examined at the protein level in the spinal cord (Tao et al., 2006). It is distinctly possible that spinal AMPARs show considerable variability in TARP content that is reflected in their trafficking and functional properties, and that could potentially be exploited for selective modulation of AMPA receptor subpopulations in pathological conditions of the spinal cord. The objective of this study was therefore to examine the presence and distribution of TARP isoforms in the rat spinal cord.

## EXPERIMENTAL PROCEDURES

### Animals and tissue preparation

Adult male Sprague–Dawley rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and transcardially perfused with phosphate-buffered saline (PBS, 300 mOsm, ~30 s) followed by PBS containing (for light microscopy) 4% paraformaldehyde or (for electron microscopy) 4% paraformaldehyde and 0.1% glutaraldehyde (~1 L, 30 min). After perfusion, lumbar segments of the spinal cord and (in some cases) the brain were removed. Transverse sections of dorsal horn and (in one instance) parasagittal sections of cerebellar cortex were cut at a thickness of 250 μm on a Vibratome and embedded in Lowicryl as described (Larsson and Broman, 2005). Ultrathin sections were cut from embedded specimens and placed on Formvar-coated single-slot nickel grids. For light microscopy, 30- or 40-μm thick transverse spinal cord sections were sectioned on a freezing microtome or cryostat. To obtain tissue for Western blots, rats were deeply anesthetized and decapitated. Their spinal cords were ejected from the vertebral column by a pre-chilled saline-filled syringe and the lumbar part harvested. Cerebellum, forebrain, hippocampus, liver and kidney were collected by dissection. The tissues were immediately flash frozen and stored at −70 °C until analysis. All animal experiments were approved by the local Animal Care and Use Committee.

### Antibodies

For the detection of γ-2 and γ-7, we used affinity purified polyclonal antibodies raised in rabbits against synthetic peptides containing amino acids 302–318 and 260–274 of mouse γ-2 and γ-7, respectively (Yamazaki et al., 2010). A rabbit polyclonal antibody against γ-3 was raised against amino acids 294–307 of the mouse protein (254 003, Synaptic Systems, Göttingen, Germany). For the detection of γ-4, we used a polyclonal antibody raised in rabbit against a peptide derived from a sequence between amino acids 180–280 of human γ-4 (ab81107, Abcam, Cambridge, UK). For γ-8, an affinity purified polyclonal rabbit antibody raised against amino acids 362–421 of mouse γ-8 was used (TARPg8-Rb-Af1000-1, Frontier Institute, Ishikari, Japan). Thus, the antibodies against γ-2, γ-3, γ-7 and γ-8 were all directed toward epitopes within the intracellular C-terminal domain. While the exact immunogen for the γ-4 antibody was not available, most of the implicated region (amino acids 208–280) is putatively within the intracellular C-terminal domain. All antibodies against γ-2, γ-7 and γ-8 have been validated for Western blot in mouse tissue devoid of the respective antigen. Rabbit γ-2, γ-7 and γ-8 antibodies have also been validated for immunohistochemistry and postembedding immunogold labeling in tissue from knock-out mice (Fukaya et al., 2006; Yamazaki et al., 2010). Monoclonal mouse antibodies against glial fibrillary acidic protein (GFAP; MAB360, Millipore, Billerica, MA, USA), adenomatous polyposis coli (APC; clone CC-1, ab16794, Abcam) and GluA2 (MAB397, Millipore) were used for double immunofluorescence labeling.

### Western blots

For whole tissue homogenates, the tissue was homogenized in protein extraction buffer (50 mM Tris buffer, pH 8.0, containing 0.5% Triton X-100, 1% sodium dodecyl sulfate, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail (P-8340, Sigma, St. Louis, MO, USA; 1:100) by sonication. For crude synaptosome fractions, spinal cord tissue was homogenized using a Dounce homogenizer in extraction buffer (20 mM HEPES, pH 7.4, containing 0.32 M sucrose, 1 mM EDTA, 5 mM NaF, 1 mM $NaVO_3$, 1× protease inhibitor cocktail). The extracts were centrifuged at 2800 rpm for 10 min, and the supernatant collected and further centrifuged for 12,000 rpm for 10 min. The supernatant (cytosolic fraction) was removed and the pellet (crude synaptosomal fraction) sonicated in lysis buffer (50 mM Tris buffer, pH 7.5, containing 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 5 mM NaF, 1 mM $NaVO_3$, 1× protease inhibitor cocktail, 1% sodium dodecyl sulfate). Whole tissue homogenates and crude synaptosomal fractions were subjected to denaturing NuPAGE 4–12% or 10% Bis-Tris gel electrophoresis and then transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). After blocking nonspecific binding sites with 5% low-fat milk in Tris-based buffer (50 mM Tris–HCl, 6 mM NaCl) containing 0.1% Tween 20 for 1 h at room