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Research report

Prolonged zymosan-induced inflammatory pain hypersensitivity in mice lacking glycine receptor alpha2

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ABSTRACT

Glycinergic synapses play a major role in shaping the activity of spinal cord neurons under normal conditions and during persistent pain. However, the role of different glycine receptor (GlyR) subtypes in pain processing has only begun to be unraveled. Here, we analysed whether the GlyR alpha2 subunit might be involved in the processing of acute or persistent pain. Real-time RT-PCR and *in situ* hybridization analyses revealed that GlyR alpha2 mRNA is enriched in the dorsal horn of the mouse spinal cord. Mice lacking GlyR alpha2 (Glra2$^{-/-}$ mice) demonstrated a normal nociceptive behavior in models of acute pain and after peripheral nerve injury. However, mechanical hyperalgesia induced by peripheral injection of zymosan was significantly prolonged in Glra2$^{-/-}$ mice as compared to wild-type littermates. We conclude that spinal GlyRs containing the alpha2 subunit exert a previously unrecognized role in the resolution of inflammatory pain.

1. Introduction

Persistent pain is accompanied by sensitization of the nociceptive system that manifests as pain in response to normally innocuous stimuli (allodynia), increased responses to noxious stimuli (hyperalgesia) or spontaneous pain. During the last decades, many peripheral and central signaling pathways have been identified that drive the sensitization during inflammatory and neuropathic pain (for review, see Refs. [1–4]). In addition, perceived pain intensity is negatively regulated by pain modulatory systems. A major mechanism of endogenous pain control is the release of inhibitory neurotransmitters in the spinal cord, both from brainstem descending pathways and local inhibitory interneurons. γ-Aminobutyric acid (GABA), the most abundant inhibitory neurotransmitter in the brain, and glycine, an inhibitory neurotransmitter acting mainly in the caudal part of the CNS, are the two fast inhibitory neurotransmitters in the mammalian spinal cord. Whereas recent studies have defined the roles of distinct GABA receptor subtypes in pain modulation [5], information about the functions of glycine receptors (GlyRs) in inhibitory pain control is still limited.

GlyRs are expressed at high levels in the spinal cord [6]. They are strychnine-sensitive pentameric chloride channels, which upon agonist binding mediate chloride influx to stabilize the resting potential of neurons [7]. To date, four ligand-binding GlyR alpha subunits (α1, α2, α3 and α4) and one GlyR beta subunit have been identified in mammals [8,9]. GlyRs containing the α3 subunit represent an essential target of PGE_2-mediated inflammatory pain sensitization in the spinal cord [10–12]. In contrast, the contribution of other GlyR subunits to pain processing is poorly investigated, although both GlyRα1 and GlyRα2 transcripts have been found in the dorsal horn of the rat spinal cord [13,14]. Here, we investigated whether GlyRs containing the α2 subunit might contribute to pain processing. We analysed the distribution of GlyRα2 in the mouse spinal cord and characterized the nociceptive behavior of mice lacking GlyRα2 (Glra2$^{-/-}$ mice) in models of acute, inflammatory and neuropathic pain.

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2. Materials and methods

2.1. Animals

Experiments were performed in 6- to 14-week-old mice lacking GlyRα2 (Glra2$^{-/-}$) and littermate wild-type (WT) control mice of either sex backcrossed onto C57BL/6N background for >10 generations [9]. C57BL/6N mice (Harlan Laboratories, Venray, The Netherlands) were additionally used for RT-PCR analyses. Animals were housed on a 12/12 h light/dark cycle with standard rodent chow and water available *ad libitum*. All experiments were approved by the local Ethics Committee for Animal Research.

2.2. Real-time RT-PCR

Lumbar (L4-L6) dorsal root ganglia (DRGs), lumbar spinal cord and brain were rapidly dissected and snap-frozen in liquid nitrogen. Total RNA was isolated under RNase-free conditions using RNA isolation kits (for DRGs: RNAqueous Micro Kit, Ambion, Austin, TX; for spinal cord and brain: RNeasy Lipid Tissue Mini Kit, Qiagen, Hilden, Germany), DNase treated for 15 min to minimize genomic DNA contamination and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA was synthesized from 200 ng RNA using random hexamers of the Verso cDNA kit (Thermo Fisher Scientific, Waltham, MA). Real-time RT-PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) using Taqman gene expression assays for GlyRα2 (cat # Mm00806742_m1) and GAPDH (cat # Mm99999915_g1) purchased from Applied Biosystems. Reactions (total volume, 10 μl) were performed in duplicate by incubating at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Water controls were included to ensure specificity. Relative expression of target gene levels was determined using the comparative $2^{-\Delta\Delta Ct}$ method, with Ct indicating the cycle number at which the signal of the PCR product crosses an arbitrary threshold set within the exponential phase of the PCR. The amount of GlyRα2 mRNA was normalized to GAPDH.

2.3. Laser microdissection

Lumbar spinal cords (L4-L6) were rapidly dissected, frozen in tissue freezing medium on dry ice and stored at −80 °C until sectioning. Serial transverse cryostat sections (16 μm) were cut and collected on PEN (polyethylene-naphthalene, RNase-free) slides (Leica Microsystems, Wetzlar, Germany). The sections were briefly stained in 1% cresyl-violet solution (Sigma–Aldrich, Munich, Germany) and dehydrated rapidly through 75–100% ethanol. A Leica DM6000B LMD system (Leica Microsystems) was used to collect the superficial dorsal horn (laminae I–III) and ventral horn (laminae VII–IX) separately of each other [15]. Total RNA was isolated from the microdissected tissue by using RNeasy Plus Micro Kit (Qiagen) according to the manufacturer's instructions. Integrity of the isolated RNA was determined using the Agilent 2100 bioanalyzer and RNA 6000 Pico LabChip Kit (Agilent Technologies, Böblingen, Germany). Electropherograms demonstrated clear 18S and 28S rRNA peaks and no significant shift of RNA fragments to shorter migration times, indicating high RNA quality (RIN > 7). Real-time RT-PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) as described above.

2.4. In situ hybridization

Total RNA from mouse lumbar spinal cord was extracted and transcribed into cDNA using the One Step RT-PCR Kit (Qiagen). The primers 5′-atgcagctggagagttttgg-3′ and 5′-ggagacctttggcagagatg-3′ were used to synthesize a fragment corresponding to nucleotides 674–1048 of GlyRα2 mRNA (accession number NM_183427.4) by PCR [13]. Agarose gel electrophoresis of the PCR product revealed a single band of the expected size. The PCR product was eluted from the agarose gel using the QIAEX II Agarose Gel Extraction Kit (Qiagen), cloned into the pCR4-TOPO plasmid vector (Invitrogen, Darmstadt, Germany), and amplified in One Shot TOP10 Chemically Competent *Escherichia coli* cells (Invitrogen). Then the plasmid-DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen), quantified with a NanoDrop spectrophotometer and sequenced for verification (LGC Genomics, Berlin, Germany). Restriction digests of the plasmid DNA were performed by incubation with *Pme*I and *Not*I restriction endonucleases. Finally, sense and antisense probes labeled with digoxigenin were transcribed *in vi*tro using T3 or T7 RNA polymerases (Roche Diagnostics, Mannheim, Germany).

Mice were intracardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS, pH 7.4) under deep anesthesia. The lumbar spinal cords were dissected, post-fixed in 4% PFA for 10 min, cryoprotected into 20% sucrose overnight and frozen in tissue freezing medium on dry ice. Cryostat sections were cut at a thickness of 16 μm and stored at −80 °C until use. Tissue sections were incubated with 3% H_2O_2 for 5 min, post-fixed in 4% PFA for 5 min, prehybridized in hybridization buffer (50% formamide, 5× SSC, 5× Denhardt's solution, 500 μg/ml herring sperm DNA, 250 μg/ml yeast tRNA in nuclease-free water) at 65 °C for 1.5–2 h and finally incubated with digoxigenin-labeled GlyRα2 antisense or the corresponding sense probes (840 ng/ml in hybridization buffer) at 65 °C overnight. After hybridization, the slides were washed in 0.2× SSC and PBS, blocked for 1–2 h in blocking buffer (0.12 M maleic acid, 0.15 M NaCl, pH 7.5; 1% Blocking Reagent; Roche Diagnostics) and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:1000; Roche Diagnostics) at 4 °C overnight. Sections were permeabilized in PBS containing 0.1% Tween and equilibrated in alkaline phosphatase-buffer (0.1 M Tris–HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5, 0.2 mM levamisole, 0.2% Tween). Fluorescent detection of digoxigenin-labeled probes using 2-hydroxy-3-naphtoic acid-2′-phenylanilide phosphate (HNPP; Roche Diagnostics) was performed at room temperature for 15 min. Sections were finally rinsed in PBS and coverslipped. Images were taken on an Axio Observer.Z1 microscope (Carl Zeiss, Oberkochen, Germany) equipped with a monochrome CCD camera (AxioCam Mrm; Carl Zeiss) using the Zeiss AxioVision 4.7.2 software. Brightness and contrast of the images were adjusted using Adobe Photoshop CS5 software.

2.5. Behavioral testing

Littermate WT and Glra2$^{-/-}$ mice were used in all behavioral tests. Animals were habituated to the experimental room and were investigated by observers blinded for the genotype and treatment of the animals.

2.5.1. Rotarod test

Motor coordination was assessed with a Rotarod Treadmill for mice (Ugo Basile, Comerio, Italy) at a constant rotating speed of 32 rpm. Animals had two training sessions before the day of the experiment. The fall-off latency was averaged from 3 to 5 tests, and the cut-off time was 120 s.

2.5.2. Hot-plate test

Mice were placed into a Plexiglas cylinder (diameter, 20 cm; height, 18 cm) on a metal surface maintained at 50 or 52 °C (Hot Plate, Ugo Basile). Cut-off times were 60 and 40 s, respectively, to prevent tissue damage. The time between placement and shaking or licking of the hindpaws or jumping off the plate was recorded. Only one test per animal was performed, since repeated measures might cause profound latency changes [16].

2.5.3. Hargreaves test

Sensitivity to noxious thermal stimulation [17] was measured using a Plantar Analgesia Meter (model 390G; IITC, Woodland Hills, CA). Animals were placed in observation chambers on top of a warmed glass surface (32 °C). The radiant heat source was aimed at the plantar surface of the mid-hindpaw of an inactive mouse. The paw withdrawal latency was calculated as the mean of three to four consecutive trials with at least 30 s in-between.

2.5.4. Dynamic-plantar test

The mechanical sensitivity of the plantar side of a hindpaw was assessed with an automated von Frey-type testing device (Dynamic Plantar Aesthesiometer, Ugo Basile) [18,19]. The stainless steel probe of the touch stimulator unit was pushed against the paw with ascending force until a strong and immediate withdrawal occurred. The force went from 0 to 5 g over a 10 s period (linear increase of 0.5 g/s) and then remained constant at 5 g for an additional 10 s (total cut-off time, 20 s). The paw withdrawal latency was calculated as the mean of four to six consecutive trials with at least 20 s in-between.

2.5.5. Formalin test

Formalin (15 μl of a 5% formaldehyde solution) was injected subcutaneously (s.c.) into the dorsal surface of one hindpaw [20,21]. The time spent licking the formalin-injected paw was recorded in 5 min intervals up to 60 min after formalin injection.

2.5.6. Inflammatory hyperalgesia

A zymosan A suspension (15 μl, 5 mg/ml in 0.1 M PBS, pH 7.4; Sigma–Aldrich) or complete Freund's adjuvant (CFA; 20 μl; containing 1 mg/ml heat-killed Mycobacterium tuberculosis in paraffin oil 85% and mannide monooleate 15%; Sigma–Aldrich) was injected into the plantar subcutaneous space of a hindpaw [22,23], and the paw withdrawal latencies upon mechanical or thermal stimulation were measured using the dynamic-plantar test and Hargreaves test, respectively.

2.5.7. Spared nerve injury model

Mice were anesthetized with isoflurane and the tibial and common peroneal branches of the sciatic nerve were ligated and sectioned distally, while the sural nerve was left intact [24]. Mechanical allodynia was determined using the dynamic-plantar test.

2.6. Statistical analysis

Statistical evaluation was done with IBM SPSS Statistics 19. The Kolmogorov–Smirnov test was used to assess normal distribution of data within groups. Normally distributed data were analysed with Student's *t*-test or repeated measures ANOVA, and are presented as the mean ± standard error of the mean (SEM). Rotarod fall-off latencies were analysed with Mann–Whitney *U*-test and are expressed as median and interquartile range. For all tests, a probability value $P < 0.05$ was considered as statistically significant.