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Spinal glycine receptor alpha 3 cells communicate sensations of chemical itch in hairy skin

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Author contributions

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Conflict of interest

The authors declare no conflict of interest.

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Abstract

Glycinergic neurons regulate nociceptive and pruriceptive signaling in the spinal cord, and the identity and role of the glycine-regulated neurons are not fully known. Herein, we have characterized spinal glycine receptor alpha 3 (*Glra3*) subunit-expressing neurons in *Glra3*-Cre female and male mice. *Glra3*-Cre(+) neurons express *Glra3*, are located mainly in laminae III–VI, and respond to glycine. Chemogenetic activation of spinal *Glra3*-Cre(+) neurons induced biting/licking, stomping, and guarding behaviors, indicative of both a nociceptive and pruriceptive role for this population. Chemogenetic inhibition did not affect mechanical or thermal responses, but reduced behaviors evoked by compound 48/80 and chloroquine, revealing a pruriceptive role for these neurons. Spinal cells activated by compound 48/80 or chloroquine express *Glra3*, further supporting the phenotype. Retrograde tracing revealed that spinal *Glra3*-Cre(+) neurons receive input from afferents associated with pain and itch, and dorsal root stimulation validated the monosynaptic input. In conclusion, these results show that spinal *Glra3*(+) neurons contribute to acute communication of compound 48/80- and chloroquine-induced itch in hairy skin.

Significance Statement

Spinal glycinergic neurons regulate itch (pruriception), suggesting that components of the glycinergic system have great potential as drug targets to treat pruritus. Nonetheless, thus far, the pruriceptive roles of any of the glycine receptor (GLR) subunits have not been evaluated. Here, we successfully linked the *Glra3*-Cre populations to a pro-pruriceptive role in itch, indicating that GLRA3-expressing neurons may be a potential novel target for itch treatment. The spontaneous stomping and guarding behaviors observed from activating the *Glra3*-Cre populations are indicative of a role in sensory hypersensitivity and hence, raises questions regarding the hypersensitivity involvement of these populations for future investigations.

Introduction

Spinal somatosensory circuits transmitting the sensation of pain and itch from the body are regulated locally by inhibitory inputs, including glycinergic transmission (Beyer et al., 1985; Yamamoto and Yaksh, 1993; Takazawa et al., 2017; Freitag et al., 2019). For instance, ablation of glycine transporter 2 (GLYT2) neurons results in mechanical, heat, and cold hyperalgesia, and behaviors associated with persistent itch, e.g. extensive localized biting (Foster et al., 2015). Conversely, selective activation of GLYT2 neurons *in vivo* reduces the sensitivity to mechanical-, heat-, and cold-induced pain, and the behavioral responses against chloroquine and histamine, suggesting that the glycinergic system is essential for controlling pain and itch transmission (Foster et al., 2015). In addition, the glycinergic system is activated by nociceptive counter stimuli, which decrease itch transmission in the spinal cord (Akiyama et al., 2011).

Glycinergic receptors (GLRs) are ligand-gated ion channels, which induce inward hyperpolarizing chloride currents upon binding of glycine (Lynch, 2004; Zeilhofer, 2005; Lein et al., 2007; Dutertre et al., 2012). In mice, the glycine receptor alpha 3 (*Glra3*) gene is expressed in both excitatory and inhibitory spinal dorsal horn neuronal clusters (Häring et al., 2018; Zeisel et al., 2018; Ceder et al., 2023) and GLRA3 immunoreactivity is detected in the dorsal (Harvey et al., 2004; Wang et al., 2018; Werynska et al., 2021) and ventral horns of the spinal cord (Harvey et al., 2004; Wang et al., 2018). The other *Glr* genes; *Glra1*, *Glra2*, *Glra4* and *Glrb*, are also expressed in the dorsal horn of the spinal cord (Groemer et al., 2022). In addition to the expression in the spinal cord, *Glra3* is detected in the amygdala, hypothalamus, nucleus accumbens, tegmentum, and brainstem, but not in the dorsal root ganglia (DRG) (Lein et al., 2007; Usoskin et al., 2015; McCracken et al., 2017; Häring et al., 2018; Tudeau et al., 2020; San Martin et al., 2021; Groemer et al., 2022). Expression analyses have shown that spinal cord injury decreases levels of GLRA3 in the dorsal spinal cord,

whereas zymosan A-induced inflammation increases GLRA3 levels (Berrocal et al., 2014; Mariqueo, 2020). Additionally, in an endometriosis mouse model, *Glra3* expression was found to be upregulated in the insula (Li et al., 2018), emphasizing this receptor subunit's role in pain and its potential as a novel pain treatment.

Thus far, studies have focused on examining the nociceptive role of the GLRA3 subunit. Herein, we investigated the molecular and electrophysiological characteristics, along with the sensory role of spinal *Glra3*-expressing cells in pruriceptive, mechanical, and thermal transmission, using a transgenic *Glra3*-Cre mouse line and *fos* measurements. Moreover, we established neuronal inputs to the population, using replication deficient rabies tracing and dorsal root stimulations.

Materials and methods

Key Resources table

Viral Vectors and Serotypes					
	Vector	Source	Company	Lot and titer	Injection
AAV8.hSyn-DIO-hM3D(Gq)-mCherry	pAAV-hSyn-DIO-hM3D(Gq)-mCherry	The vector was a gift from Bryan Roth (Krashes et al., 2011) (Addgene viral prep #44361-AAV8; http://n2t.net/addgene:44361; RRID:Addgene_44361)	Addgene, USA	first lot#: v27924 with titer: 2.2x 10^{13}GC/ml; second lot # v78582 with titer: 2.1x 10^{13}GC/ml	One site
AAV8.hSyn-DIO-hM4D(Gi)-mCherry	pAAV-hSyn-hM4D(Gi)-mCherry	The vector was a gift from Bryan Roth (Krashes et al., 2011) (Addgene viral prep #44362-AAV8;	Addgene, USA	v86749 with titer: 1.8 x 10^{13} GC/ml	Two sites (unilateral)

		http://n2t.net/addgene:44362; RRID:Addgene_44362)			
AAV8.hSyn-DIO-mCherry	pAAV-hSyn-DIO-mCherry	The vector was a gift from Bryan Roth (Addgene viral prep # 50459-AAV8; http://n2t.net/addgene:50459 ; RRID:Addgene_50459)	Addgene, USA	v61605 with titer: 2.2x 10^{13}GC/ml	One or two sites (unilateral)
AAVDJ.EF1a-DIO-HTB	pAAV.EF1a-DIO-HTB	The vector was a gift from Edward Callaway (Addgene plasmid # 44187; http://n2t.net/addgene:44187; RRID:Addgene_44187). The vector was packaged into AAVDJ by Salk institute GT3 (Gene Transfer, Targeting, and Therapeutics) core facility (provided by John Naughton) with funding from NIH-NCI CCSG:	Salk institute, USA	lot date: 20/12-2018 with titer: 1.09x 10^{12}VG/ml	Two sites (bilateral)

		P30 014195, an NINDS R24 Core Grant and funding from NEI			
AAV8.Syn-flex-TVA.E66T-P2A-oG-WPRE3	pAAV.Syn-flex-TVA.E66T-P2A-oG-WPRE3	Charité, Germany, with technical assistance from Salk investigator John Naughton	Charité, Germany	BA-229a with titer: 3.98x 10^{12}VG/ml	One site
BRVenvA-1o Rabies Virus, pseudotyped EnvA, mCherry	pSADB19dG-mCherry	Charité, Germany, the material was originally provided by Edward Callaway and distributed through Addgene (plasmid #32630, #32631, #32632, #32633, #32634) (Osakada et al., 2011)	Charité, Germany	unknown, with titer: 1.00x 10^{8} particles/ml	One site
Antibodies					
Antibody name	Host animal	Dilution	Company	Catalogue number	Lot
NEUN	Mouse	1:500–1:1,000	Millipore, USA	MAB377	
PKCγ	Rabbit polyclonal	1:500	Santa Cruz Biotechnology, USA	sc-211	
IB4		1:500–1:1,000	Invitrogen, USA	I32450	

PAX2	Rabbit polyclonal	1:500	Covance/BioLegend, USA	Poly19010	
GFP	Chicken	1:1,000	Aves Lab, USA	GFP-1020	
NF200	Rabbit	1:1,000	Sigma-Aldrich, USA	N4142	
TRKA	Rabbit monoclonal	1:1,000	Abcam, USA	ab8871	
CGRP	Rabbit polyclonal	1:1,000	Peninsula Laboratories, USA	T-4239.0050	
TH	Rabbit	1:1,000	Millipore, USA	AB152	
SST	Rabbit polyclonal	1:500	Invitrogen, USA	XJ371918	PA5-85759
Anti-rabbit 488	Goat	1:500	Jackson ImmunoResearch, USA	111-095-144	
Anti-rabbit 647	Donkey	1:200 (SST assay); 1:500	Invitrogen, USA	A31573	
Anti-mouse 488	Donkey	1:200	Abcam, USA	ab150105	
Anti-mouse 647	Donkey	1:200	Invitrogen, USA	A31571	
Anti-chicken 488	Donkey	1:500	Invitrogen, USA	SA1-72000	
RNAscope probes					

Probe name		Dilution	Company	Catalogue number	Channel
fos		1:50	Advanced Cell Diagnostics, USA	31692	C1
Glra3		1:50	Advanced Cell Diagnostics, USA	490591	C2
mCherry		1:50	Advanced Cell Diagnostics, USA	431201	C2
Nppb		1:50	Advanced Cell Diagnostics, USA	425021	C1
Mrgpra3		1:50	Advanced Cell Diagnostics, USA	548161	C3
Mrgprd		1:50	Advanced Cell Diagnostics, USA	417921	C1
Trpm8		1:50	Advanced Cell Diagnostics, USA	420451	C3
Trpv1		1:50	Advanced Cell Diagnostics, USA	313331	C1
Vglut2		1:50	Advanced Cell Diagnostics, USA	319171	C3
Viaat		1:50	Advanced Cell Diagnostics, USA	319191	C3

Animals

Procedures related to the mice used in this study were approved by the local animal research ethical committee (Uppsala djurförsöksetiska nämnd) and followed the Swedish Animal Welfare Act (Svensk författningssamling (SFS) 2018:1192), The Swedish Animal Welfare Ordinance (SFS 2019:66), and the Regulations and General Advice for Laboratory Animals (SJVFS 2019:9, Saknr L 150), permit numbers: 5.8.18-01428/2023, 5.2.18-17971/2019, 5.8.18-11551/2019, 5.8.18-19421/2019, 5.8.18-01217/2019, 5.8.18-01503/2023 and 5.8.18-03266/2023. The constitutive knock-in *Glra3*-Cre mouse line was generated by Cyagen, with the homology arms having been amplified from a bacterial artificial chromosome (BAC), for which the *Glra3* gene is located on chromosome 8 (GenBank: NM_080438.2, Ensembl: ENSMUS00000038257). *Glra3*-Cre(+) mice were crossed with C57BL/6J mice (Taconic, Denmark) and *tdTomato* reporter mice (Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$, Allen Brain Institute). The *Glra3*-Cre allele was kept hemizygous and both female and male mice were included in the analyses, unless otherwise stated. The mice were housed with littermates in approximately 501cm^2 cages (maximum 5 mice per cage) in room temperature ranging between 20–24°C and humidity of 45–65% on a 12-hour light:dark cycle with lights on at 6 am. All mice were provided food (Diet Pellets, Scanbur, Sweden) and tap water *ad libitum*.

Genotyping by polymerase chain reaction

Tissue biopsies from ear marking, taken at the age of 3–4 weeks, were incubated in 50µL of buffer, consisting of 25mM NaOH and 200µM ethylenediaminetetraacetic acid (EDTA), in a shaking block (BIOER Mixing Block MB-102, China, 300 speed) at 96°C for 25min, whereafter the samples were neutralized with 50µL of Tris-HCl (40mM), pH 8.0. The following primers were used to identify *Cre* and *tdTomato*, respectively; *Cre* 5'-acgagtgatgaggttcgcaaga-3' (forward,

mutant allele), 5’-accgacgatgaagcatgtttag-3’ (reverse, mutant allele), *tdTomato* 5’-ctgttcctgtacggcatgg-3’ (forward, mutant allele), 5’-ggcattaaagcagcgtatcc-3’ (reverse, mutant allele), 5’-aagggagctgcagtggagta-3’ (forward, wild type allele), 5’-ccgaaaatctgtgggaagtc-3’ (reverse, wild-type allele).

Spinal Cord Viral Injections

The viral injections into the spinal cord were performed for the chemogenetic sensory tests, monosynaptic retrograde tracing, and the electrophysiological recordings of adult *Glra3*-Cre(+) neurons. The injections were performed as previously described (Freitag et al., 2019); in brief, *Glra3*-Cre mice (>6 weeks old) were initially anesthetized in a 4% isoflurane (FORANE, Baxter, USA) box. When fully anesthetized, the mice were moved to a stereotaxic frame with a breathing mask, where the isoflurane concentration was kept at 1.5–2% throughout the entire procedure. To prevent eye damage, Oftagel was applied (Santen Oy, Tammerfors, Finland) and the body temperature was monitored and maintained at 35–37°C using a heating pad (FHC, Bowdoin, Maine, USA). Adjacent to the incision sites, the mice were administered subcutaneously with bupivacaine (Marcain®, 2mg/kg, AstraZenica AB, Cambridge, England). For post-surgery analgesia, the mice were administered subcutaneously with karprofen (Norocarp vet, 5mg/kg, N-vet, Uppsala, Sweden or Rimadyl® Bovis vet, 4mg/kg, Zoetis Finland Oy, Finland). Within 24h post-surgery, the mice were again administered 5mg/kg karprofen for post-surgery analgesia. The dorsal skin was shaved and cleaned with sterile saline (B Braun medical AB, Stockholm, Sweden) and chlorhexidine (Fresenius Kabi, Bad Humburg, Germany) before a 1cm skin incision was made to expose the T13 and L1 vertebrae. Sterile saline was continuously applied to keep the tissue moist. The connective tissue was gently separated along these vertebrae and a clamp was inserted

ventral of the L1 transverse process for stabilization of the spine. When stabilized, the posterior longitudinal ligament and ligament flavum connecting T13 and L1 were cut to expose the spinal cord. Thereafter, 500nl of the respective viral vector (*AAV8.hSyn-DIO-hM3D(Gq)-mCherry* (Krashes et al., 2011), *AAV8.hSyn-DIO-mCherry* or *AAVDJ.EF1a-DIO-HTB*) (please see Key Resources Table for detailed information) was injected into the L5/L6 spinal dorsal horn (as caudal as possible from zeroed midline, ML: 0.4mm, DV: 0.4mm, with needle eye directed rostrally), using a 10µl Nanofil Hamilton syringe (World Precision Instruments, Sarasota, Florida, USA) with a 34g beveled needle (World Precision Instruments), monitored by a micro syringe pump controller (World Precision Instruments) at 50nl/min. For injections of *AAV8.hSyn-DIO-hM4D(Gi)-mCherry* (Krashes et al., 2011), the virus was injected at two sites into the right dorsal horn (RC: 0/-0.5mm, ML: 0.3mm, DV: 0.6mm), with the eye of the needle pointing lateral. To prevent leakage and withdrawal of virus, the needle was left in the injection site for 5min. When the injection was completed, the spine was detached from the clamp and the skin and connective tissue were sutured and cleaned with sterile saline before the mice were removed from the breathing mask and administered subcutaneously with Buprenorfin (Vetergesic® Vet, Orion Pharma, Finland, 0.05mg/kg). The mice were subsequently placed on a heating pad in their cages to wake up. The mice were subjected to behavioral experiments or sacrificed for tissue analyses after a minimum of 2–4 weeks to allow sufficient expression of viral genes.

Adult *Glra3*-Cre mice (7+7, 7 females, 7 males, 6–26 weeks old) for the Randall-Selitto test were injected with *AAV8.hSyn-DIO-hM4D(Gi)-mCherry* (Krashes et al., 2011) or *AAV8.hSyn-DIO-mCherry* between L1/L2 to target the sacral 2 (S2) segment, affecting the tail (Bennett et al., 1999). The virus was injected into the dorsal horn at two sites with the eye of the needle directing lateral

(RC: 0/-0.5mm, ML: 0.25mm, DV: 0.45mm). The mice were assessed to the Randall-Selitto test 2–3 weeks after injection.

The injections for monosynaptic retrograde tracing of adult *Glra3*-Cre mice (*Glra3*-Cre(+): 5 females, 5 males, 7–15 weeks old; *Glra3*-Cre(-): 3 females, 3 males, 7–17 weeks old) were conducted in the same manner as described above for the *AAV8.hSyn-DIO-hM3D(Gq)-mCherry* experiments. The mice were initially injected with helper virus (herein abbreviated as AAV8.Syn-flex-TVA-oG-GFP). To allow sufficient expression of the helper vector genes required for rabies virus host cell entry, the mice were injected with pseudotyped rabies virus *BRVenvA-1o Rabies Virus, pseudotyped EnvA, mCherry* (please see Key Resources Table for detailed information) 7–8 days after the helper virus injection. One week after the final injection, the mice were sacrificed.

Immunohistochemistry Tissue Preparation of Developmental and Adult Virally labelled Glra3-Cre(+) mice

Adult *Glra3*-Cre;*tdTomato* mice (4 females, 7–23 weeks old) and virally spinal cord injected *Glra3*-Cre.mCherry mice (2 females, 2 males, 17–25 weeks old, from the behavioral experiments) were anesthetized in isoflurane (FORANE, Baxter, USA). All mice were subsequently injected intraperitoneally with 0.6ml (1:1) Ketamin (Ketalar, 10mg/ml, Pfizer, Sweden) and Medetomidine (Domitor, 1mg/ml, Orion Pharma, Sweden). When fully anaesthetized, the mice were perfused through the left ventricle with 1x PBS, followed by 4% formaldehyde (FA) (Histolab, Sweden). The spinal columns were isolated and placed in 1x PBS, followed by dissection of the tissue area of interest (spinal cord and dorsal root ganglia (DRG)). The tissues were placed in 4% FA (Histolab, Sweden) at 4°C overnight. The spinal cords and DRG were dehydrated for 24h in 15% sucrose and then for 24h in 30% sucrose for cryoprotection. The tissues were thereafter embedded

in optimal cutting temperature (OCT) medium (Bio-Optica, Milan, Italy) and snap-frozen on dry ice in -80°C isopentane (Sigma-Aldrich, USA), at which temperature the tissues were stored until sectioning. The spinal cords and DRG were sectioned into 16–18µm sections using a cryostat (Leica cryocut 1800, Leica, Germany) and the sections were collected on glass slides (Superfrost® Plus, Thermo Scientific, USA) as a series of six slides/series. The completed slides were stored at -80°C until further immunohistochemical analyses were performed.

In the tracing experiment, following brain dissections, the brains were fixated in 4% FA (Histolab, Sweden) at 4°C overnight and thereafter stored in 1x PBS at 4°C until vibratome sectioning. Upon sectioning, the brains were superficially and unilaterally cut with a razor blade to keep track of orientation, and subsequently mounted in 4% agarose. The brains were sliced into 70µm sections (Leica VT1000S, Leica, Germany), which were collected into wells as series of five wells/series with five sections/well. All sections were examined for traced mCherry(+) cells using a fluorescent stereomicroscope (Leica MZ16F, Leica, Germany). For documentation, the brain sections of one well/series (every fifth brain section) were mounted and embedded in Anti-Fade Fluorescence Mounting Medium (Abcam, UK) on glass slides and covered with glass cover slides (Menzel-Gläser, Germany) for imaging.

Immunohistochemistry

All slides to be used for immunohistochemistry (*Glra3*-Cre;*tdTomato*: 4 females, 7–23 weeks old; *Glra3*-Cre.mCherry: 4 mice; 2 females, 2 males, 17–25 weeks old; traced *Glra3*-Cre(+): 4 females, 2 males, 9–17 weeks old) were placed at room temperature (RT) for 30min to thaw and dry before initializing the protocols. The assays were either PBS- (NEUN, PKCγ, IB4, NF200, TRKA, CGRP,

TH) or TBS- (PAX2, SST) based. In all assays, the sections were washed with 1x PBS/TBS for 4x 10min before and after the primary antibody incubation. Prior to the primary antibody incubation, the sections were blocked with either supermix (0.25% gelatin and 0.5% TritonX-100 in 1x PBS/TBS, [NEUN, NF200, TRKA, CGRP, TH]) or blocking solution (5% donkey or goat serum in 1x PBS/TBS, [PKCγ, IB4, PAX2, SST]) for 1h at RT. In the same solutions (supermix: [PKCγ, IB4, PAX2, SST]; blocking solution: [NEUN, NF200, TRKA, CGRP, TH]), the primary and secondary antibodies were incubated, in which the primary antibodies were incubated for 48h at 4°C and the secondary antibodies with 200nM/ml DAPI for 2h at RT. As the final step, the sections were washed 4x 10min in 1x PBST/TBST (0.1% Tween20 in 1x PBS/TBS). After completion of protocols, the slides were embedded in Anti-Fade Fluorescence Mounting Medium (Abcam, UK) and covered with glass slides (Menzel-Gläser, Germany). The slides were left at 4°C to dry and were stored at this temperature until imaging. For antibody specifics, see Key Resources Table.

In Situ Hybridization Tissue Preparation

Adult *Glra3*-Cre(+) mice microinjected with AAVDJ.EF1a-DIO-HTB (3 females, 2 males, 7–11 weeks) and mice included in the retrograde rabies tracing (1 female and 2 males, 14 weeks) in the L5/L6 spinal dorsal horn, were subjected to similar procedures as described previously (Freitag et al., 2021). The HTB protein is a histone-tagged GFP and this virus was used since the fluorescence could be detected after RNAscope protocol. In brief; 14 days post viral injection, the mice were anesthetized in isoflurane (FORANE, Baxter, USA), followed by intraperitoneal injection of 0.6ml (1:1) Ketamine (Ketalar, 10mg/ml, Pfizer, Sweden) and Medetomidine (Domitor, 1mg/ml, Orion Pharma, Sweden). To minimize the risk of contamination and altered gene expression, the mice

were perfused in autoclaved ice-cold 1x PBS. In the same solution, the spinal columns were quickly dissected and the spinal area containing the viral fluorescence was isolated. The tissues were immediately embedded in optimal cutting temperature (OCT) medium (Bio-Optica, Milan, Italy) and snap-frozen on dry ice in -80°C isopentane (Sigma-Aldrich, USA), at which temperature the tissues were stored until sectioning. The tissues were cryosectioned (Leica Cryocut 1800, Leica, Germany) into 12–14 µm sections and were collected onto Superfrost Plus (Thermo Scientific, USA) glass slides as series consisting of six slides with 7–8 sections/slide for the *Glra3*-Cre(+) AAVDJ.EF1a-DIO-HTB injected mice and eight slides with 3 sections/slide for the sensory stimulated C57BL/6J mice. To prevent mRNA degradation and contamination, the completed series were stored at -21°C until sectioning was finished. The slides were stored at -80°C until the RNAscope Fluorescent Multiplex kit (Advanced Cell Diagnostics (ACD), USA, cat #: 320850) protocol commenced.

Fluorescent In Situ Hybridization

The fluorescent *in situ* hybridization was performed using the RNAscope Fluorescent Multiplex kit (ACD, USA, cat# 320850) in accordance with ACD guidelines for fresh frozen tissues, with minor modifications (Wang et al., 2012) on sections from *Glra3*-Cre.HTB and sensory stimulated C57BL/6J mice. In brief, as performed previously (Freitag et al., 2021), the slides to be used were taken from -80°C and immediately fixated in 4% FA (Histolab, Sweden) for 15min at RT before being washed in autoclaved 1x PBS for 2min. The tissues were thereafter dehydrated in a step-wise increase of EtOH concentration; 3min in 50%, 3min in 70% and 2x 5min in 100% (Merck KGaA, Germany). The slides were placed at RT for 5min to dry, whereafter a hydrophobic barrier was made around the chosen sections (3 sections/mouse), using an ImmeEdge pen (Vector

Laboratories, USA). The sections were incubated in Protease IV for 30–40min at RT, followed by 3x 5min washing in autoclaved 1x PBS. The sections were incubated in target probes (for specifics see Key Resources Table) 1:50 in probe diluent (ACD, USA, cat# 300041) for 2h at 40°C in a hybridization oven (HybEZ^{TM} II Oven, ACD, USA). The following amplification steps were performed at 40°C in the hybridization oven and the sections were washed 2x 2min in RT washing buffer between each amplification step: AMP 1-FL for 30min, AMP 2-FL for 15min, AMP 3-FL for 30min and AMP 4-FL for 15min. The coloring step using AMP 4-FL was performed to enable the combination with the viral fluorescence. Lastly, the slides were washed 2x 2min in washing buffer before 30sec incubations in DAPI and mounting in Anti-Fade Fluorescence Mounting Medium (Abcam, UK). The slides were covered with glass slides (Menzel-Gläser, Germany) and were left at 4°C to dry. The slides were stored at this temperature until imaging.

Image Acquisition and Quantification

Images of immunohistochemistry treated sections were acquired using a wide field Olympus BX61WI fluorescence microscope (Olympus, Japan) with a 10x objective, for which the brightness and contrast were optimized for each channel during image acquisition and quantification. The RNAscope treated sections were acquired with wide field 20x magnification with the Olympus BX61WI fluorescence microscope (Olympus, Japan) or an Axio Imager.Z2 (ZEISS, Germany), where each channel was set to be automatically optimized for each image, but had to be further optimized during image analysis. Here, the optimal intensity and contrast was set for one image (reference image) and the settings of the other images were set to match the reference image. The images were manually quantified using the Fiji (ImageJ 1.52f) Cell Counter plugin. *Immunohistochemistry spinal cord*: All *Glra3*-Cre.*tdTomato* (2 females, n sections/mouse: 3) or

287 *Glra3*-Cre.mCherry (2 females and 2 males, n sections/mouse: 3–5) mice with DAPI overlap were
288 quantified depending on layer location (IB4: outer lamina II; PKCγ: inner lamina II) and marker
289 protein (NEUN) co-expression. *Immunohistochemistry retrograde rabies tracing, spinal cord*:
290 *Glra3*-Cre(+) mice: A DAPI cell with overlap of helper virus GFP and rabies virus mCherry was
291 considered a starter cell, and a DAPI cell with only mCherry overlap was considered a presynaptic
292 traced cell. The co-expression of starter and traced cells was quantified for NEUN and PAX2 (5
293 females, 5 males, n section/mouse/assay: 2–11); *Glra3*-Cre(-) mice: The overlap of helper virus
294 GFP and rabies virus mCherry with DAPI overlap was quantified (3 females, 3 males, every sixth
295 section analyzed). *Immunohistochemistry retrograde rabies tracing, DRG:* The overlap of traced
296 mCherry DAPI+ cells with NF200. TRKA, CGRP, IB4, TH and SST was quantified (NF200: 2
297 females, 2 males, n sections/mice: 2–5; TRKA: 2 females, 2 males, n section/mice: 1–8; CGRP: 1
298 female, 2 males, n sections/mice: 2–5; IB4: 3 females. 1 male, n sections/mice: 2–5; TH: 4 females,
299 2 males, n sections/mice: 1–7; SST: 2 females, 1 male, n sections/mice: 3–5). *Brain scanning for*
300 *mCherry(+) traced cells in Glra3-Cre(+) and Glra3-Cre(-) mice*: Whole brain section images were
301 acquired in the mCherry (500ms) and widefield black & white (15% light source intensity, 5ms)
302 channels of every fifth section, using tiles (ZEISS, Germany) to scan for mCherry(+) traced cells
303 (*Glra3*-Cre(+): 5 females, 5 males; *Glra3*-Cre(-): 3 females, 3 males). *RNAscope, Glra3-Cre.HTB*:
304 All *Glra3*-Cre.HTB cells with DAPI overlap were considered cells and one read of the targeted
305 probe could be visualized as one dot. A *Glra3*-Cre.HTB cell was considered to be expressing the
306 targeted gene (*Glra3, Vglut2* or *Viaat*) if the overlapping #dots≥3 (3 females and
307 2 males, n sections/mouse: 2–4). One section from the *Glra3/Viaat* assay was excluded
308 due to weak signal from both probes. *RNAscope, retrograde rabies tracing DRG*: DAPI cell was
309 considered expressing the targeted gene (*mCherry, Nppb, Mrgpra3, Mrgprd, Trpv1, Trpm8*) if

the #dots≥3 (*Nppb*: 1 female and 1 male, n sections/mice:4–6; *Mrgprd* and *Mrgpra3*: 1 female and 1 male, n sections/mice:4–6, *Trpv1* and *Trpm8*: 2 females and 1 male, n sections/mice:2–6). *RNAscope, fos expression in Glra3 expressing cells following sensory stimulation*: The experimenter was blinded to the treatment received by the mouse and the *Vglut2*/*Viaat* probes, so no randomization was needed in the quantification. A DAPI cell was considered to express the targeted gene (*Glra3*, *fos* and *Vglut2* or *Viaat*) if the #dots≥3 and #dots≥5 for *fos* (3 mice/stimulus, n sections/mouse: 3). One section from the scratch analysis was excluded due to poor tissue quality. To obtain a high resolution, two images of each dorsal horn were acquired and later merged together using Adobe Photoshop 22.3 to a composited representative image of the dorsal horn. The result is presented as percentage±SEM.

Electrophysiology

For patch-clamp recordings, spinal cord transverse slices were made from *Glra3*-Cre;*tdTomato* mice (13 females, 11 males, 4–35 weeks old) according to a previously described protocol (Freitag et al., 2019). For root stimulations, the spinal cord was cut at a 60^{o} angle and the slice thickness was increased to 400µm in order to get transverse slices with attached dorsal roots. After incubation, the slice was transferred to a recording chamber, where *Glra3*-Cre;*tdTomato* neurons were visualized via a fluorescent LED light source (CoolLED system, UK) on a Prime BSI Express scientific sCMOS camera (Teledyne Photometrics, USA) through 60x or 10x water-immersion objectives (LUMPlan FI, 0.90 numerical aperture (NA), Olympus, Japan). Borosilicate glass capillaries (GC150F-10 Harvard Apparatus, USA) were used to pull patch electrodes (6-10MΩ) with a flaming/brown micropipette puller (P-1000, Shutter Instrument, USA). The internal solution

of patch pipettes (in mM): 130 K-gluconate, 40 HEPES, 1.02 $MgCl_2$, 2.17 MgATP, 0.34 NaGTP, with pH adjusted to 7.2 using 1M KOH. Liquid junction potential was corrected before each recording. Whole-cell patch-clamp signals were amplified with a MultiClamp 700B amplifier (Molecular Devices, USA), digitalized at 20kHz with Digidata 1440A (Molecular Devices, USA), low pass filtered at 10kHz, and acquired in WinWCP software (Dr. J. Dempster, University of Strathclyde, Glasgow, UK).

When the whole cell configuration was achieved, action potentials were induced, in the current clamp mode via current steps from 0–150pA with increments of 10pA (pulse duration 500ms), to monitor the viability and the firing pattern of the patched neuron. The rheobase was determined by using 1pA increment current steps (pulse duration 500ms). The neuron was then held at -60mV in the voltage clamp mode. When a stable baseline was achieved in a continuous voltage clamp recording, 300µM glycine was applied through the perfusion system to the recording chamber to verify the expression of GLRs on the patched neuron. The hyperpolarization was then blocked by 10µM strychnine to further confirm that the response was due to the expression of GLRs.

In root stimulation experiments, the dorsal root was identified using the 10x objective and sucked into a suction pipette. The stimulating electric pules were applied via the suction pipette from an A365 Stimulus Isolator (World Precision Instruments, USA). Stimulation pulses with a duration of 0.2ms were used for activation of the dorsal root, while in some cases 0.5ms pulse durations were used to activate the C-fiber. The transduction velocities of different afferent fibers were used to determine monosynaptic inputs (Pan et al., 2019). Further confirmation followed by none failure responses with consistent onset latencies, in which patched cells responded to a minimum of 10 consecutive root stimulations at 1Hz and the latency variation was less than 1ms (Pinto et al., 2008; Pan et al., 2019). Data analyses were done by Clampfit 10.3 (Molecular Devices, USA), Mini

Analysis (Synaptosoft, USA), and GraphPad Prism (GraphPad Software, USA). No neurons were excluded in the post analysis.

Cell filling

Neurobiotin™ Tracer (Vector Laboratories, USA) was added into the intracellular solution (4mg/ml) and diffused into the target *Glra3*-Cre;*tdTomato* cells during patch-clamp recording. The diffusion of Neurobiotin™ was further assisted by injecting depolarizing current pulses (0.2–0.5nA, duration 150ms) into the cell at 2Hz for 10–15min. After the filling, the patch pipette was carefully detached from the cell and removed from the recording chamber. The excessive Neurobiotin™ in the tissue was removed by perfusing the slice for at least 15 more min after the removal of the pipette. The slice was then transported into an Eppendorf tube and fixed in 4% FA (Histolab, Sweden) overnight at 4 °C. Fixed slices were washed with 1x PBS (Fisher BioReagents, USA) 4x10min before the staining. Slices were stained for PKCγ using the same procedure described in previous immunohistochemistry section. Additionally, streptavidin Alexa Flour 488 conjugate (Invitrogen, USA) was added to the primary antibody staining solution with 1:1000 dilution ratio for Neurobiotin™ staining. The mounted slice was imaged using a ZEISS LSM700 confocal microscope (ZEISS, Germany) with 10x and 20x objectives. The morphology of a filled neuron was reconstructed using the Simple Neurite Tracer plug-in in the NIH ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Basal behavioral observation after chemogenetic activation or inhibition of Glra3-Cre(+) neurons

376 *Glra3*-Cre(+) mice (*Glra3*-Cre.hM3Dq and *Glra3*-Cre.mCherry: 7+8 mice, 7 females and 8 males;
377 *Glra3*-Cre.hM4Di and *Glra3*-Cre.mCherry: 8+8 mice, 11 females, 5 males) unilaterally injected in
378 L5 with AAV8.hsyn-DIO-hM3D(Gq)-mCherry, AAV8.hsyn-DIO-hM4D(Gi)-mCherry or
379 AAV8.hsyn-DIO-mCherry were acclimatized to a plastic cylinder arena (diameter: 19cm, height:
380 29cm, surface area: 283cm^2) with a mirror to obtain a 360° view for 20min. The mice were injected
381 intraperitoneally with 0.1mg/kg of freshly prepared Clozapine N-oxide (CNO) (AK Scientific,
382 USA, 0.02mg/ml dissolved in 0.02% DMSO in sterile saline). The basal behavior of the mice
383 following CNO administration was recorded for 30min (for *Glra3*-Cre.hM3Dq recordings) or
384 60min (for *Glra3*-Cre.hM4Di recordings). The duration and frequency of targeted behaviors were
385 analyzed for the total recording time. The same experimenter scored all the behavior recordings
386 and was blinded for the viral vectors used during the experiments. The licking/biting of the
387 ipsilateral paw were scored as one behavior, for which the episodes were scored when contact
388 between the paw and face could be clearly visualized. The guarding and stomping behaviors were
389 also scored. Guarding was defined as the time the mouse spent sitting still with its paw in the air.
390 Stomping was interpreted as a mouse rapidly lifting and lowering the hind paw while being either
391 still or in movement. No mice were excluded from the analysis

392

393 *Injections of saline or pruritogens*

394 Two days prior to the stimulus recording, the right calves of the mice were shaved and cleaned
395 with sterile saline. Adult *Glra3*-Cre(+) mice injected with AAV8.hsyn-DIO-hM4D(Gi)-mCherry
396 or control AAV8.hsyn-DIO-mCherry were injected with 0.1mg/kg freshly prepared CNO (AK
397 Scientific, USA, 0.02mg/ml dissolved in 0.02% DMSO in sterile saline) and thereafter returned to

their respective home cages. After 30min, the mice were placed in a plastic cylinder arena with a mirror to obtain a 360° view for 10min to acclimatize to the set up. The mice were subsequently injected subcutaneously in the dorsolateral calf with either 10µl of saline (8+8 mice; 9 females, 7 males), 20µg compound 48/80 (Sigma-Aldrich, USA, cat# c2313, dissolved in sterile saline; 8+8 mice; 8 females, 8 males), or 10mM chloroquine phosphate (Sigma-Aldrich, USA, cat# PHR1258, dissolved in sterile saline, 8+8 mice; 9 females, 7 males). The mice were returned to the plastic cylinder area and recorded for 30min. Licking of the calf is indicative of pain, while biting demonstrates itch (LaMotte et al., 2011). However, since we had difficulties separating these behaviors while scoring, the total duration and frequency of licking/biting towards the injected calf was scored as one behavior. These episodes were scored when contact between the calf and face could be clearly visualized. No mice were excluded from the analysis

Randall-Selitto test

Two days prior to the experiment, a plastic cylinder (Model 84, IITC Life Science, USA) was placed in each home cage to acclimatize the mice to the setup. Adult *Glra3*-Cre(+) mice (7+7 mice; 7 females, 7 males) injected with either AAV8.hsyn-DIO-hM4D(Gi)-mCherry or AAV8.hsyn-DIO-mCherry between L1/L2 were intraperitoneally administered 0.1mg/kg freshly prepared CNO (AK Scientific, USA, 0.02mg/ml dissolved in 0.02% DMSO in sterile saline) and thereafter returned to their respective home cages. Ten min later, the mice were allowed to enter the plastic cylinder and were placed in the Randall-Selitto setup (Analgesy-meter, UGO Basile, Italy) for approximately 30min. When 40min had passed since the CNO injection, the mechanical threshold (g), at which pressure the mouse retracted its tail, was measured twice per mouse at different

locations on the tail with at least 5min between the measurements. One female and one male injected with AAV8.hsyn-DIO-hM4D(Gi)-mCherry were excluded from the analysis due to lack of mCherry expression in the post hoc verification step.

Hargreaves test

Adult *Glra3*-Cre(+) mice (8+8 mice; 11 females, 5 males) injected with AAV8.hsyn-DIO-hM4D(Gi)-mCherry or control AAV8.hsyn-DIO-mCherry were initially acclimatized for 60min in the Hargreaves set up (transparent acrylic glass chambers on glass floor). Baseline thermal sensitivity was measured by directing the Hargreaves heat source (IITC Life Science, Woodland Hills, CA, USA), guided by a light pointer, to the plantar surface of the right hind paw, for which the time from turning on the thermal source until the mouse withdrew/flinched its paw was noted. The cut-off time was set to 20sec to avoid tissue damage and the withdrawal time was measured twice with at least 5min intervals in between each measurement. After completed measurements, the mice were injected intraperitoneally with 0.1mg/kg freshly prepared CNO (AK Scientific, USA, 0.02mg/ml dissolved in 0.02% DMSO in sterile saline) and placed back into the Hargreaves setup. Forty min after the CNO administration, the withdrawal time measurement was repeated. No mice were excluded from the analysis

Acetone drop test

Adult *Glra3*-Cre(+) mice (8+8 mice; 8 females, 8 males) injected with either AAV8.hsyn-DIO-hM4D(Gi)-mCherry or AAV8.hsyn-DIO-mCherry were allowed 60min acclimatization to the

gridded surface. Forty minutes before the first measurement, the mice were injected intraperitoneally with 0.1mg/kg freshly prepared CNO (AK Scientific, USA, 0.02mg/ml dissolved in 0.02% DMSO in sterile saline), and returned to the setup. The mice were subjected to a drop of acetone solution (9:1 acetone in water, Labscan, Dublin, Ireland) on the plantar surface of the right hind paw, where the total duration of sensory aversive behaviors, including lifting, flinching, and licking/biting of the paw, was recorded. The stimulation was performed twice with at least 5min intervals in between each application of the acetone solution. No mice were excluded from the analysis

Sensory stimulation for fos detection

Pruritic stimulation of urethane anesthetized mice: To detect activation of *Glra3* expressing cells following sensory stimulation, adult C57BL/6J mice (10–14 weeks old, 3 mice/stimulus, 15 mice in total) were initially anesthetized with 2g/kg urethane (Sigma-Aldrich, USA, cat# U2500, 125mg/ml in sterile saline) through intraperitoneal injection to minimize neuronal activity caused by prurito- and nocifensive behavior. To prevent eye-damage and dehydration, Oftagel (Santen Oy, Finland) was applied to eyes and the mouse was injected subcutaneously with 0.5ml saline. To maintain body temperature, a glove filled with body temperature water, that was continuously replaced to sustain temperature, was placed next to the mouse. When the mouse had been fully anesthetized for 10min, the mouse was subjected to the stimulus. For pruritic stimulations, the mice were injected subcutaneously into the right dorsolateral calf either with 10µl saline (1 female and 2 males), or a pruritic substance: 20µg compound 48/80 (Sigma-Aldrich, USA, cat# c2313, dissolved in sterile saline, 1 female and 2 males), or 20mM chloroquine (Sigma-Aldrich, USA, cat# PHR1258, dissolved in sterile saline, 1 female and 2 males). *Noxious mechanical stimulation*

of urethane anesthetized mice: the mouse was either subjected to pinching (1 female and 2 males) or scratching (2 females and 1 male) of the skin on the right dorsolateral calf. The pinching was performed 5x for 5sec using tweezers, with 5sec resting periods in between each pinching episode. The scratching was conducted for 30sec with 2Hz and approximately 300mN (30.6g), using an artificial mouse claw in scratch position. Forty min after application of the stimulus, the mouse was injected intraperitoneally with 0.05ml Ketamine (Ketalar, 10mg/ml, Pfizer, Sweden) and 0.05ml Medetomidine (Domitor, 1mg/ml, Orion Pharma, Sweden), followed by perfusion and tissue preparation for RNAscope, as described above. *Hargreaves stimulation for fos detection in awake mice*: adult C57BL/6J mice (2 females and 1 male, 11–17 weeks old) were subjected to the same Hargreaves protocol as described above for baseline measurements. After completed stimulation, 40min was allowed to pass until the mouse was injected intraperitoneally with 0.7–0.8ml Ketamine (Ketalar, 10mg/ml, Pfizer, Sweden) and Medetomidine (Domitor, 1mg/ml, Orion Pharma, Sweden) (1:1), followed by perfusion and tissue preparation for RNAscope, as described above. Same mice but separate sections have been used in a manuscript under revision. No mice were excluded from the analysis.

Experimental Design and Statistical Analyses

All behavioral testing was performed a minimum of 2–4 weeks after viral injection to allow sufficient expression of viral vector genes. *Glra3*-Cre.hM3Dq mice were only included in one basal behavioral analysis/mouse, except for 2 (*Glra3*-Cre.hM3Dq) + 3 (*Glra3*-Cre.mCherry) mice that also were subjected to an initial analysis (one CNO injection, data not shown) a few weeks prior to establish a proper CNO concentration. *Glra3*-Cre.hM4Di mice were included in maximum four

behavioral tests (including basal recording) with a minimum of one week between the tests. The basal recording was conducted first and the following tests were not conducted in a specific order. *Glra3*-Cre.mCherry mice were included in maximum four behavioral tests (including basal recording) with a minimum of one week between the tests. The tests were not conducted in a specific order. The mice were returned to their home cages after each completed behavioral test. No mice were excluded from the behavioral analyses presented. No randomization was used. Mice were arbitrary assigned to different treatments (e.g., injections of viral vectors) based on sex. All the behavior experiments were conducted by the same female experimenter, who was blinded to viral vectors (control vs chemogenetic). In the acetone and Randall-Selitto tests, an additional female experimenter was conducting the experiment (also blinded to the viral vector injected), so no randomization was needed or possible. Reporter expression was validated and documented in all mice after chemogenetic behavioral testing to ensure presence of DREADD (designer receptors exclusively activated by designer drugs) or control vector at the correct spinal segments. The experimental groups were matched to the best extent in terms of sex and littermates. In the sensory stimulation tests to examine *fos*, the mice were arbitrary assigned to the different stimuli, but we ensured that both sexes were used in the testing.

The number of mice per behavioral and *in situ* experiment was not based on any statistical calculations prior to the experiments. Sample sizes are in line with similar studies in the field (Bourane et al., 2015; Foster et al., 2015; Häring et al., 2018). All data were analyzed in GraphPad Prism (version 9 or 10). The normal distribution of the mean data per mouse was analyzed using the Shapiro-Wilk normality test (α=.05). To compare mean values, either a two-tailed student t-test or Mann-Whitney u test was performed. In the basal hM3Dq experiment, for which the mean value of the control mCherry groups was zero for stomping and guarding behaviors, a Chi-square test

was performed to compare the mean values between these groups. In order to compare the mean values between multiple parameters (viral vector and pre/post CNO injection) in the Hargreaves test and to compare the differences in the number of the targeted cells following saline, compound 48/80, and chloroquine injections, a one-way ANOVA with Šídák's multiple comparisons test was used. The results are presented as mean±SEM.

Results

Glra3-Cre;tdTomato neurons are predominantly located in laminae III–IV and the adult Glra3-Cre population consists of a major excitatory and a minor inhibitory population

First, immunohistochemistry was used to examine the anatomical location and molecular characteristics of the spinal *Glra3*-Cre population using the *tdTomato* reporter line (Figure 1A–C). Immunostaining for the neuronal marker NEUN (Figure 1A) showed that almost all tdTomato(+) cells were neurons (98.7±0.2%, 1637/1659). The neurons were most frequently found in the dorsal horn (dorsal horn: laminae I–VI, 89.1±2.3%, 1528/1713; ventral horn: laminae VII–X, 10.9±2.3%, 185/1713), especially in laminae III–IV (44.2±2.1%, 753/1713) and laminae V–VI (23.4±1.6%, 405/1713). Smaller tdTomato(+) populations were found in lamina I (4.8±0.6%, 78/1713), the outer lamina II defined by IB4 staining (Todd, 2017) (4.7±0.7%, 80/1713), and the inner lamina II defined by PKCγ (Polgár et al., 1999; Peirs et al., 2014) (12.1±1.6%, 212/1713), in which 52.4±5.2% (117/212) of the cells were tdTomato(+)PKCγ(+) (Figure 1B–B'). Collectively, the *Glra3*-Cre;*tdTomato* neurons were located throughout the spinal cord (Figure 1C) and were most commonly found in laminae III–IV.

Single-cell RNA sequencing (scRNAseq) has identified *Glra3* in both excitatory SCGLU10 and Glut9, and in inhibitory Gaba8–9 spinal dorsal horn neuronal clusters among others (Häring et al., 2018; Zeisel et al., 2018). To further examine the molecular characteristics of the *Glra3*-Cre population and to address adult *Glra3*-Cre expression, fluorescent *in situ* hybridization using the RNAscope approach (Wang et al., 2012) was performed. Said method targeted *Glra3*, the excitatory marker *Vglut2* (*Vesicular glutamate transporter 2, Slc17a6*), and the inhibitory marker *Viaat* (*Vesicular inhibitory amino acid transporter, Slc32a1*) in adult AAVDJ.Ef1a-DIO-HTB

labelled *Glra3*-Cre(+) neurons (Figure 1D–H). HTB is a histone-tagged protein that was used due to its ability to be detected following the RNAscope protocol. *Glra3* was expressed by 74.8±1.8% (436/571) of the *Glra3*-Cre.HTB(+) cells in the analysis also targeting *Vglut2* and by 71.7±4.3% (235/342) of the *Glra3*-Cre.HTB(+) cells in the analysis also targeting *Viaat*. These findings indicated that the mouse line and Cre-dependent virus mark the *Glra3*(+) population (Figure 1E–H). In the *Vglut2*-examining assay (Figure 1E–E'), *Vglut2*(+) and *Glra3*(+)*Vglut2*(+) were found in 74.5±2.8% (437/571) and 70.6±2.8% (318/517) of the *Glra3*-Cre.HTB(+) population, respectively (Figure 1F). Meanwhile, *Viaat*(+) was found in 34.4±3.3% (116/342) and *Glra3*(+)*Viaat*(+) in 28.0±3.75% (89/342) of the *Glra3*-Cre.HTB(+) neurons (Figure 1G–H). These results suggest that the adult spinal *Glra3*-Cre population consists of *Glra3*-expressing neurons found in a major *Vglut2*(+) excitatory population and a smaller *Viaat*(+) inhibitory population.

Glra3-Cre;tdTomato neurons respond to glycine and the populations display a heterogeneous firing pattern

Patch-clamp recordings were used to examine electrophysiological properties of *Glra3*-Cre(+) neurons. The recorded *Glra3*-Cre;*tdTomato* neurons had an average resting membrane potential of -59.9±1.2mV, input resistance of 879±70.1MΩ, and membrane capacitance of 55.1±4.3pF (Table 1). All recorded neurons fired action potentials (APs) upon electrical stimulation of 500ms duration and increments of 10pA (Figure 2A). Moreover, the APs comprised five different firing patterns (Figure 2B–B'), with 52% of APs were tonic (36/69), 17% phasic (12/69), 7% single (5/69), 13% delayed (9/69), and 10% irregular (7/69) (Figure 2B–B', Table 1). These firing patterns resemble previously identified categories of mouse dorsal horn neurons in terms of AP patterns (Hu and

Gereau, 2003, 2011; Heinke et al., 2004). The tdTomato(+) neurons had an average rheobase of 22.4±2.8pA, an AP threshold of -30.9±1.1mV, and a peak AP of 21.7±1.8mV. Inter-group comparison showed that only neurons with delayed AP patterns had lower resting membrane potentials. No differences were observed in all other measured electrophysiological properties among neurons in the five AP pattern categories (Table 1). Collectively, the *Glra3*-Cre populations constitute five categories of neurons according to their firing patterns, with homogenous intrinsic membrane properties. The presence of functional glycine receptors on the recorded neurons was determined by applying glycine to the recording chamber in a voltage clamp mode, where cells were held at -60mV. All glycine applied *Glra3*-Cre;*tdTomato* neurons showed hyperpolarizing currents (an average of -34.8±5.7pA), and the glycine-induced current was completely blocked by the glycine receptor antagonist strychnine (Figure 2C).

Two studies have described that GLRA3 is present in the superficial laminae of the dorsal horn (Harvey et al., 2004; Werynska et al., 2021), while a third study demonstrated that GLRA3 immunoreactivity is also present in the ventral horn (Wang et al., 2018). The latter study is more consistent with our observations as the *Glra3*-Cre(+) populations were localized in both the dorsal and ventral laminae (Figure 1A–C), which is also consistent with mRNA expression of *Glra3* (Ceder et al., 2023). To investigate the dendritic localization of *Glra3*-Cre(+) neurons, we performed cell fillings (Figure 2D–E). Neurobiotin was used to fill the neurons and the morphology was revealed by staining the filled neuron with Alexa Fluor™ 488 streptavidin conjugate (Figure 2D). Dendritic morphologies and locations are showed in Figure 2E. The dendritic tree of each filled neurons appeared to be local and without long projecting dendrites. All neurons showed vertical alignment, where the dendritic arbors projected predominantly in a dorsal-ventral direction.

583 *Adult Glra3-Cre(+) neurons are mainly located in laminae III–IV and selective chemogenetic*
584 *activation induces spontaneous behaviors indicative of a role in nociception and pruriception*

585 After the analysis of *Glra3*-Cre;*tdTomato* neurons, we further investigated the neuronal profile and
586 anatomical location of adult *Glra3*-Cre(+) cells. Theoretically, the *tdTomato* reporter line marks
587 both developmental and adult *Glra3*-Cre-expressing cells. Therefore, to label the adult population
588 exclusively, reporter virus AAV8.hSyn-DIO-mCherry was unilaterally microinjected into the
589 lumbar 5/lumbar 6 (L5/L6) spinal segments (abbreviated *Glra3*-Cre.mCherry). First, the specificity
590 of the reporter, and the DREADD viral vectors used for the subsequent sensory behavioral
591 analyses, were investigated by examining mCherry expression in *Glra3*-Cre(-) control mice. No
592 fluorescent cells were detected (Figure 3), thus the virally induced gene expression in subsequent
593 analyses was Cre-dependent. The histological analysis (Figure 4A–C) was conducted in the same
594 manner as in the *Glra3*-Cre;*tdTomato* analysis, showing that 89.2±3.9% (1807/2099) of the
595 mCherry(+) cells co-expressed NEUN (Figure 4A, C). In consistency with the tdTomato analysis,
596 the mCherry(+) population was predominantly located in the dorsal horn (86.4±3.2%, 1279/1524),
597 with a minor subpopulation in the ventral horn (15.0±4.5%, 245/1524) (Figure 4B–C). In the dorsal
598 horn, the mCherry(+) cells were mainly restricted to laminae III–IV (40.1±4.2%, 590/1524),
599 followed by lamina I (14.2±2.7%, 179/1524), laminae V–VI (11.6±2.0%, 222/1524), the PKCγ(+)
600 inner lamina II (11.1±1.2%, 166/1524), in which 21.3±5.4% (41/166) of the mCherry(+) neurons
601 were PKCγ(+), and the IB4(+) outer lamina II (9.4±1.4%, 122/1524, Figure 4B–C).

602 Activation of spinal GLYT2 neurons decreases pain and itch behaviors (Foster et al., 2015), and
603 the anatomical location of the *Glra3*-Cre(+) neurons showed herein indicates a sensory role of
604 *Glra3*(+) neurons. To investigate this, *Glra3*-Cre(+) mice were unilaterally injected into L5/L6
605 with AAV8.hSyn-DIO-hM3D(Gq)-mCherry (abbreviated *Glra3*-Cre.hM3Dq) and the behavioral

phenotype was compared with *Glra3*-Cre.mCherry mice (control) (Figure 3D–F). The mice were administered CNO to selectively activate the *Glra3*-Cre populations. After CNO administration, *Glra3*-Cre.hM3Dq mice displayed a higher duration and frequency of licking/biting of the ipsilateral hind paw compared with control mice (Figure 4D). In mice, licking of the hind paw is associated with pain, while biting is a sign of itch (LaMotte et al., 2011). Therefore, our phenotype indicated both a nociceptive and a pruriceptive role for the *Glra3*-Cre populations. Additionally, activation of the *Glra3*-Cre.hM3Dq population resulted in stomping (Casarrubea et al., 2019) and guarding (Wang and Wang, 2003; Mogil and Crager, 2004), which were not observed in control mice (Figure 4E–F). These behaviors further indicated nociceptive/pruriceptive-related roles of these populations. Collectively, activation of the lumbar spinal *Glra3*-Cre populations results in nocifensive and pruritofensive behaviors.

Chemogenetic silencing of the Glra3-Cre populations decreases chloroquine- and compound 48/80-induced itch

Since selective chemogenetic activation of *Glra3*-Cre(+) neurons induced spontaneous behaviors indicative of a role in pain/itch transmission, we sought to decipher the involvement of this population in different sensory modalities. For this purpose, adult *Glra3*-Cre(+) mice were unilaterally injected with AAV8-hSyn-DIO-hM4D(Gi)-mCherry in L5/L6 (abbreviated *Glra3*-Cre.hM4Di) to enable selective silencing while sensory behaviors were monitored. The results were compared with control virus-injected *Glra3*-Cre.mCherry mice (Figure 5A). First, the basal behavioral phenotype was investigated following CNO administration. Selective silencing of *Glra3*-Cre(+) neurons did not affect spontaneous licking/biting behaviors in duration nor frequency

during the 0–30- and 30–60-min intervals after CNO administration (Figure 5B). Stomping and guarding behaviors were not observed when silencing the *Glra3*-Cre populations (data not shown).

In consistency with the Bourane et al. (2015) study (Bourane et al., 2015), the mice were subjected to sensory testing 40min after CNO administration. The pruriceptive role of the spinal lumbar *Glra3*-Cre population was examined in hairy skin, for which mice were administered either control saline, compound 48/80, or chloroquine solution (chemical itch) subcutaneously into the calf (Figure 5C–E). Compound 48/80 activates sensory neurons both directly via MRGPRA1 (Schemann et al., 2012; Azimi et al., 2016, 2017) and indirectly as a mast cell degranulator by binding MRGPRB2 (Azimi et al., 2016), resulting in the release of pro-inflammatory molecules and pruritogens, including histamine and serotonin (Gupta and Harvima, 2018). Chloroquine activates primary afferents expressing MRGPRA3 (Liu et al., 2009). Saline evoked no differences in the duration or frequency of licking/biting of the injected area (Figure 5C), showing that the *Glra3*-Cre populations do not convey sensory information associated with the injection itself. When administering compound 48/80, both the duration and frequency of licking/biting of the affected area were decreased following *Glra3*-Cre(+) silencing (Figure 5D). For chloroquine administration, the same results were observed as with compound 48/80 injection (Figure 5E).

The role of the *Glra3*-Cre populations in noxious mechanical transmission was examined using the Randall-Selitto test. To target the tail dermatome, AAV8-hSyn-DIO-hM4D(Gi)-mCherry or the control virus was injected in the sacral 2 (S2) segment (Bennett et al., 1999). The mechanical threshold for *Glra3*-Cre.hM4Di mice did not differ compared with *Glra3*-Cre.mCherry mice (Figure 5F). To investigate if the *Glra3*-Cre populations are involved in thermal transmission, Hargreaves and acetone drop tests were performed (Figure 5G–H). Withdrawal response times, within groups, induced by heat stimulation of the ipsilateral hind paw were not affected when

comparing pre- and post- CNO administration in *Glra3*-Cre.mCherry or *Glra3*-Cre.hM4Di mice (Figure 5G). When further comparing the withdrawal response times between the *Glra3*-Cre.mCherry and *Glra3*-Cre.hM4Di mice following CNO administration, no differences were observed (Figure 5G). Application of a drop of acetone solution to the plantar surface of the ipsilateral hind paw did not alter sensory responses, including flinching, withdrawal, or licking/biting of the paw (Figure 5H). In conclusion, the *Glra3*-Cre populations have a pro-pruritic role in compound 48/80- and chloroquine-evoked itch, while not involved in acute noxious mechanical or thermal transmission.

Spinal neurons activated by compound 48/80 or chloroquine co-express Glra3

Based on the behavioral observations, we wanted to molecularly verify the proposed sensory role of spinal *Glra3*(+) neurons and subsequently relate it to the *Vglut2*(+) and *Viaat*(+) spinal *Glra3*-Cre subpopulations. To do so, sensory stimulations in anesthetized and awake C57BL/6J mice were performed, followed by RNAscope (Wang et al., 2012) analyses of *fos* (Sheng and Greenberg, 1990), *Glra3*, and *Vglut2* or *Viaat* in the L4/L6 dorsal spinal cord. The mice were subjected to one of six possible stimuli: a subcutaneous injection of saline, compound 48/80, or chloroquine subcutaneously in the right dorsolateral calf, a noxious mechanical stimulus (pinch or scratching) of the right dorsolateral calf, or thermal (Hargreaves) stimulation of the right hind paw (Figure 6A–L', for separate channels see Figure 7 and Figure 8). Scratching of the calf is not a natural behavior of mice, however, this stimulation was conducted in this area to enable comparison with the other stimuli. To prevent transcriptional influence from pain- and itch-responsive behaviors, all stimulations were performed under urethane anesthesia, except for the Hargreaves test that was performed on awake freely moving mice. All stimuli, except saline, were found to have a higher

number of *fos*(+) cells on the ipsilateral side compared with the contralateral side: saline contralateral 47±5 (847) and ipsilateral 48±2 (862); compound 48/80 contralateral 43±3 (779) and ipsilateral 75±5 (1347); chloroquine contralateral 24±5 (426) and ipsilateral 41±3 (745); artificial scratching contralateral 36±3 (606) and ipsilateral 52±4 (889); pinch contralateral 36±3 (573) and ipsilateral 52±12 (834); and noxious heat (Hargreaves) contralateral 21±3 (378) and ipsilateral 27±2 (485) (Figure 6M). *Fos*(+)*Glra3*(+)-expressing cells were found to be greater in number on the ipsilateral dorsal horn than the contralateral dorsal horn for both compound 48/80 and chloroquine (Figure 6N). The average number of *fos*(+)*Glra3*(+) cells in the ipsilateral dorsal horn after injection with compound 48/80 was higher than the average number of *fos*(+)*Glra3*(+) cells after saline injection, which was not observed for chloroquine administration (Figure 6N). Of *fos*(+) cells, more than half of compound 48/80- and chloroquine-activated cells expressed *Glra3* (compound 48/80=59% (795/1347); chloroquine=67% (502/745); saline=52% (449/862); scratch=50% (442/889); pinch=49% (218/443); Hargreaves=47% (230/486)). No difference in the number of *fos*(+)*Glra3*(+) expressing cells could be detected for scratch, pinch, or Hargreaves (comparing the ipsi- and contralateral sides) (Figure 6O). Altogether, these findings verify that *Glra3*(+) neurons are involved in the communication of compound 48/80- and chloroquine-induced itch, and that these neurons are not involved in acute mechanical or thermal transmission. Since *Glra3* is found in both excitatory and inhibitory neuronal populations (Häring et al., 2018; Zeisel et al., 2018), we further investigated the sensory-modality activation of these *fos*(+)*Glra3*(+) subpopulations after injections with saline, compound 48/80, or chloroquine, focusing on the ipsilateral dorsal horn (Figure 9A–H’’’’). All three stimuli led to the expression of *fos* in both *Glra3*(+)*Vglut2*(+) and *Glra3*(+)*Viaat*(+) populations (Figure 9B). Taken together, the transcriptional analysis shows that *Glra3* is expressed in compound 48/80- and chloroquine-activated neurons, suggesting a role in transmission of these two sensory stimuli. Moreover, the

sensory-modality activated *fos*(+)*Glra3*(+) cells can be found in subpopulations expressing the excitatory marker *Vglut2*(+) or the inhibitory marker *Viaat*(+).

Lumbar Glra3-Cre(+) neurons receive monosynaptic input from excitatory and inhibitory local spinal neurons

After identifying a pro-pruritic role for *Glra3*-Cre(+) neurons via behavioral experiments and co-expression of *Glra3* in compound 48/80- and chloroquine-activated *fos*(+) cells, we investigated the connectivity of lumbar *Glra3*-Cre(+) neurons. Retrograde viral tracing and dorsal root stimulation were used to deduce the mono- and poly-synaptic neurons targeting the *Glra3*-Cre populations. To enable analysis of the monosynaptic connectivity, a two-step viral injection procedure was performed. First, the helper virus AAV8.Syn-flex-TVA-oG-GFP was injected, enabling *Glra3*-Cre(+) host cell entry and subsequent retrograde monosynaptic propagation of the secondly injected EnvA pseudotyped mCherry rabies virus. In the spinal cord of control *Glra3*-Cre(-) mice, no helper GFP(+)mCherry(-) nor starter GFP(+)mCherry(+) cells were detected. Two mCherry(+) cells were found in the cervical division (1 cell in the ipsilateral dorsal horn and 1 mCherry(+) cell in the contralateral ventral horn (one in each mouse)) (Figure 10A–B). In the brain, no traced mCherry(+) cells were detected in control mice. In the lumbar DRG, 51 mCherry(+) cells (43 ipsilateral, 8 contralateral) were found in two mice (43 ipsilateral and 6 contralateral in one mouse and 2 contralateral in a second mouse), verifying the Cre-dependent robustness and reliability of this tracing system.

In the spinal cords of *Glra3*-Cre(+) mice, 94 starter GFP(+)mCherry(+) cells were localized in the lumbar enlargement (Figure 10C’’**,** D). Furthermore, 526 traced mCherry(+) cells were found in

720 the ipsilateral lumbar enlargement, and in four out of five mice, 16 traced cells were found in the
721 contralateral lumbar spinal cord. None of these mice had any starter GFP(+)mCherry(+) cells
722 located on the contralateral side (Figure 10C–D). Thus, it is possible that the *Glra3*-Cre populations
723 receive some input from the contralateral side in addition to abundant ipsilateral input. Also, one
724 mCherry(+) cell was detected in the ipsilateral dorsal horn of the cervical division, while none were
725 detected in either thoracic nor sacral divisions (Figure 10C–C’’’), indicating that the *Glra3*-Cre
726 populations receive mainly local spinal input.

727 To molecularly examine the starter and traced cells, co-localizations with NEUN and the inhibitory
728 marker PAX2 (Larsson, 2017) were investigated. Starter cells overlapped 44% (17/39), whereas
729 79% (191/241) of the traced cells co-localized with NEUN (Figure 11A–B). 30.9% (17/55) of the
730 starter cells and 35.4% (101/285) of the traced cells overlapped with PAX2 (Figure 11C–D), which,
731 in consistency with the RNAscope findings, further indicates that the *Glra3*-Cre population
732 comprises an inhibitory subpopulation. In the dorsal-ventral axis, the mCherry(+) cells were mainly
733 located in the dorsal horn (laminae I–VI, Figure 11E–F), suggesting that the spinal input to the
734 *Glra3*-Cre populations predominately constitutes of sensory-related transmission. A smaller
735 subpopulation of mCherry(+) cells was observed in the ventral horn (laminae VII–IX) and lamina
736 X (Figure 11E–F), with the former suggesting that the *Glra3*-Cre populations potentially receive
737 input from motor-related spinal neurons.

738

739 *Lumbar Glra3-Cre(+) neurons receive monosynaptic input from several brain areas*

740 In the brain, a total of 89 traced mCherry(+) cells were detected in seven out of ten *Glra3*-Cre(+)
741 mice. One mouse had a traced cell in the ipsilateral and two mice had traced cells in the contralateral

motor cortices (M1, M2, n cell=9). In a third mouse, traced cells were located in the ipsilateral somatosensory cortex, barrel field (S1BF, n cells=2) area (Figure 12A). Three mice had mCherry(+) cells in the contralateral p1 reticular formation (p1Rt, n cells=7) (Figure 12B) and in the red nucleus magnocellular part/red nucleus parvicellular part (RPC/RMC, n cells=16 cells) (Figure 12C). In addition, traced cells were detected in the ipsilateral and contralateral pontine reticular nucleus, either in the oral (PnO, n cells=10) or caudal part (PnC, n cells=7) (Figure 12D), and bilaterally in the gigantocellular vestibular nucleus (Gi, n cells=8) (Figure 12E). This demonstrates that the lumbar *Glra3*-Cre populations receive monosynaptic input from several brain areas. For details regarding brain area localization of the traced mCherry(+) cells in the individual mice, see (Table 2).

The spinal Glra3-Cre populations receive monosynaptic information from multiple subgroups of primary afferents

Mono- and pre-synaptic traced mCherry(+) cells were detected in lumbar DRG of *Glra3*-Cre(+) mice, indicating that these spinal populations receive peripheral monosynaptic input. The traced cells were mainly found ipsilateral, but a few mCherry(+) cells were also detected in contralateral lumbar DRG in two out of six mice (n cells=20). In *Glra3*-Cre(+) mice, traced cells were found in one ipsilateral thoracic DRG in two separate mice (n cells=47). In one of these mice, and in a third mouse, mCherry(+) cells (n cells=20) were found in one contralateral thoracic DRG. As mentioned above, mCherry(+) cells were observed in the contralateral DRG in two *Glra3*-Cre(-) mice, implying that the contralateral mCherry(+) cells found in the *Glra3*-Cre(+) mice may be false positives. To identify the *Glra3*-Cre(+) contacting primary afferents, overlap with the markers NF200, TRKA, CGRP, IB4, TH, *Mrgprd*, *Mrgpra3*, SST, *Nppb*, *Trpv1* and *Trpm8* (Averill et al.,

765 1995; Patapoutian et al., 2003; Li et al., 2011; Usoskin et al., 2015; Albisetti et al., 2017; Kupari
766 and Ernfors, 2023) was examined (Figure 13A–N, for separate channels see Figure 14). Of the
767 ipsilateral lumbar DRG mCherry(+) cells, 28.4% (591/2079) belonged to the neurofilament heavy
768 myelinated NF200(+) group (Figure 13A, L), which is present in Aδ- and Aα/β-fibers (Basbaum
769 et al., 2009; Meltzer et al., 2021). CGRP is a pro-pruritic and -noxious neuropeptide (McCoy et al.,
770 2012; Rogoz et al., 2014), which is highly co-expressed with noxious receptor TRKA (Woolf et
771 al., 1994; Averill et al., 1995; McCoy et al., 2012; Barker et al., 2020), and both genes have little
772 overlap with IB4-binding fibers (Averill et al., 1995; McCoy et al., 2012; Usoskin et al., 2015).
773 34.9% (687/1988) of mCherry(+) cells overlapped with the TRKA(+) population (Figure 14B, L)
774 and 20.3% (213/1049) with CGRP(+) (Figure 13C, L). Furthermore, 26.6% (391/1472) of the
775 mCherry(+) cells overlapped with small unmyelinated non-peptidergic neuronal binding marker
776 IB4 (Figure 14D, L), and 9.2% (179/1951) with TH (Figure 13E, L), which is expressed in low-
777 threshold mechanosensory C-fibers (Li et al., 2011). In contrast, 79.5% (591/743) of the NF200(+),
778 57.1% (687/1203) of TRKA(+), 58.0% (213/367) of CGRP(+), 57.9% (391/675) of IB4-binding,
779 and 29.9% (179/598) of TH(+) neurons were mCherry(+) (Figure 13M). Not all primary sensory
780 afferents are equally susceptible to retrograde tracing by rabies virus (Albisetti et al., 2017),
781 however, the overlap of the traced mCherry(+) neurons with all markers indicates that the spinal
782 *Glra3*-Cre populations receive monosynaptic peripheral information from several fiber subtypes.

783 To obtain a more detailed view of the peripheral input to lumbar *Glra3*-Cre(+) neurons, markers
784 for receptor and neurotransmitter DRG subtypes were targeted (Kupari and Ernfors, 2023) (Figure
785 13F–M). Three pruriceptive molecular clusters have previously been identified, namely NP1, NP2,
786 and NP3. *Mrgprd* is expressed in the NP1 cluster, *Mrgpra3* in the NP2, and SST/*Nppb* in the NP3
787 cluster (Usoskin et al., 2015), where *Sst* has little co-localization with IB4 (Usoskin et al., 2015;

Stantcheva et al., 2016). *Mrgprd* was detected in 37.5% (147/392) (Figure 13F, L), *Mrgpra3* in 17.6% (99/392) (Figure 14G, L), SST in 21.4% (265/1239) (Figure 13H, L), and *Nppb* in 20.3% (72/354) (Figure 13I, L) of the traced mCherry(+) cells. Strikingly, mCherry was detected in a large portion of the pruriceptive sub-clusters: 46.5% (147/316) of the *Mrgprd*(+), 69.7% (69/99) of *Mrgpra3*(+), 66.9% (265/394) of SST(+), and 40.9% (72/176) of the *Nppb*(+) neurons (Figure 14M), supporting the behavioral finding of the *Glra3*-Cre populations facilitating itch-related transmission. Lastly, the expressions of the temperature-sensitive channels *Trpv1* and *Trpm8* were investigated. *Trpv1* is activated by capsaicin and noxious temperatures (≥42°C), while *Trpm8* is activated by menthol and cooling temperatures (<26–28°C) (Patapoutian et al., 2003). Herein, 13.5% (53/392) and 2.3% (9/392) of the *mCherry*(+) cells expressed *Trpv1* or *Trpm8*, respectively (Figure 13J–L). In contrast, 13.2% (53/403) of the *Trpv1*(+) and 14.3% (9/63) of the *Trpm8*(+) populations expressed *mCherry* (Figure 13M). The low overlap of *Trpv1* in traced neurons is consistent with both the low co-expression of *Glra3* in Hargreaves-induced *fos*(+) cells and the lack of phenotype of DREADD-mediated inactivation of *Glra3*-Cre(+) neurons in the same test.

To further investigate and validate that *Glra3*-Cre(+) neurons receive peripheral monosynaptic input, patch-clamp recordings were conducted on *Glra3*-Cre(+) (reporter tdTomato(+) and viral mCherry(+)) neurons in combination with dorsal root stimulation (Figure 13N). The data revealed that the *Glra3*-Cre populations receive monosynaptic inputs from all afferent fiber subtypes. Half of the recorded neurons (16/32) received monosynaptic inputs from at least two afferent fibers, among which almost one-third (5/16) formed monosynaptic connections with all three fiber subtypes. Furthermore, the majority of synaptic inputs was delivered via Aα/β fibers (41%) or C-fibers (40%), while only 19% was transmitted by Aδ fibers (Figure 13O). Collectively, these results confirmed that *Glra3*-Cre(+) neurons receive monosynaptic information from multiple afferent

811 fiber subtypes, including myelinated and itch-associated neurons, suggesting that the *Glra3*-Cre
812 populations form complex monosynaptic connections with primary afferents.

813

Discussion

Herein, we report that the *Glra3*-Cre line labels excitatory and inhibitory primarily dorsal neuronal populations in the spinal cord that express *Glra3*. These populations respond to glycine and are heterogeneous in terms of AP firing patterns and homogenous in intrinsic membrane properties. Behavioral and expressional analyses revealed that spinal *Glra3*-Cre populations have a pro-pruritic role in compound 48/80- and chloroquine-evoked itch, and no role in the mechanical or thermal responses tested. Analyses using mono-synaptic retrograde tracing and dorsal root stimulations demonstrated that lumbar *Glra3*-Cre populations receive monosynaptic excitatory and inhibitory input from neurons within the lumbar division, several brain areas related to sensory and motor functions, and afferents belonging to the NF200(+), TRKA(+), IB4-binding, and TH(+) subpopulations. Furthermore, CGRP(+) and pruritic markers *Mrgprd*(+), *Mrgpra3*(+), SST(+), and *Nppb*(+) afferent populations were found to synapse on spinal *Glra3*-Cre(+) neurons. The multitude of sensory-modality input to this population was confirmed with dorsal root stimulations. Taken together, the data show that the spinal *Glra3*-Cre populations communicate compound 48/80 and chloroquine-evoked itch.

The glycinergic system is a fast-response inhibitory system important for modulating motor and sensory reflex activity, muscle tone, and respiratory rhythms (Manzke et al., 2010; Cioffi, 2018). The glycinergic system serves a protective role in pain and itch, where activation of glycinergic neurons leads to attenuated pain and itch responses, and ablation causes nociceptive and pruriceptive hypersensitivity (Foster et al., 2015). Blocking spinal glycine receptors decreases the nociceptive counter-stimulation effect on persistent itch-mediated spontaneous activity in the spinal cord (Akiyama et al., 2011), implementing the importance of the glycinergic system in

sensory regulation. Our chemogenetic activation experiments indicate that the adult spinal *Glra3*-Cre populations have an acute sensory role as its activation evoked spontaneous sensory behaviors, such as licking/biting, stomping, and guarding of the affected dermatome, whereas silencing decreased compound 48/80- and chloroquine-induced itch, indicative of a pro-pruritic role.

In the retrograde rabies tracing experiment, we investigated overlap of traced neurons with primary afferent subtype markers to deduce the sensory-modality input to the spinal *Glra3*-Cre populations. Almost half of the NP1-*Mrgprd*(+) (β-alanine receptor (Liu et al., 2012)) and NP3-*Nppb*(+)/*Sst*(+) (Usoskin et al., 2015; Stantcheva et al., 2016) primary afferents synapse on *Glra3*-Cre(+) neurons. Activation of SST(+) primary afferents evokes pruritofensive behaviors and deletion of *Sst* attenuates itch evoked by pruritogens, such as compound 48/80 and chloroquine (Huang et al., 2018). Furthermore, SST(+) primary afferent ablation decreases histamine, chloroquine, IL-31- and serotonin-evoked scratching (Stantcheva et al., 2016). Herein, we found that almost 70% of SST(+) primary afferents and chloroquine receptor *Mrgpra3*(+) primary afferents, found in the NP2 cluster (Liu et al., 2009; Usoskin et al., 2015), synapse on *Glra3*-Cre(+) neurons. TRKA is the receptor of NGFβ, a neurotrophic protein important for hyperalgesia (Woolf et al., 1994; Barker et al., 2020) and CGRP is a neuropeptide with pro-pruritic and -noxious functions (McCoy et al., 2012; Rogoz et al., 2014). *Ntrk1* (gene encoding TRKA) and *Calca* (gene encoding CGRP) are highly co-expressed in non-peptidergic pruriceptive NP2 neurons and in nociceptive peptidergic PEP1–2 neurons. Our retrograde monosynaptic tracing showed that *Glra3*-Cre(+) neurons receive monosynaptic input from TRKA(+) and CGRP(+) primary afferents. The dorsal root stimulation further confirmed that these populations receive monosynaptic input from C-fibers, collectively implying that spinal *Glra3*-Cre(+) neurons are central for communicating itch.

Transcriptional validation of the behavioral involvement of spinal *Glra3*-Cre(+) neurons in different sensory modalities confirmed that *Glra3* is largely expressed in compound 48/80-evoked *fos*(+) neurons compared with saline-induced *fos*(+) cells. Compared with the contralateral side, chloroquine-activated *fos*(+) cells expressed *Glra3* but this effect could not be separated from the influence of the injection itself. However, the chloroquine-activated cells constitute a smaller population than the saline-activated group ($p<.0001$), which may explain this result. Previous studies have found that itch-inducing compounds activate cells in the superficial dorsal horn (Doi-Saika et al., 1997; Nojima et al., 2003; Yao et al., 1992; Jinks and Carstens, 2000; Nakano et al., 2008; Gatto et al., 2021; Han et al., 2012; Akiyama et al., 2013), which is similar to our findings.

Consistent with the absence of thermal response alterations following *Glra3* deletion/mutation observed by the cited studies (Harvey et al., 2009; Werynska et al., 2021), chemogenetic silencing of *Glra3*-Cre neurons did not alter the withdrawal response in the Hargreaves test. Subsequent histological analysis showed that Hargreaves-activated *fos*(+) cells did not overlap with *Glra3* compared with the contralateral side in naïve mice. Moreover, silencing did not affect the response in the acetone drop test, further dismissing involvement of the spinal *Glra3*-Cre(+) neurons in acute thermal transmission. The retrograde rabies tracing revealed that the *Glra3*-Cre populations receive sparse monosynaptic input from *Trpm8*(+) primary afferents, while 13.5% of the traced DRG neurons overlapped with *Trpv1*. TRPV1(+) primary afferents are key mediators in itch-transmission (Mishra et al., 2011; Rogoz et al., 2014) and TRPV1-deficient mice show reduced responses to histamine (Imamachi et al., 2009). The *TrpV1(+)* primary afferent input to the lumbar *Glra3*-Cre populations may therefore be related to itch rather than thermal sensation. Silencing of GLYT2 neurons do however regulate both mechanical and thermal transmission (Foster et al., 2015) and activation of GLYT2 neurons has an anti-hyperalgesic effect on neuropathic-induced

mechanical allodynia (Foster et al., 2015). Meanwhile, deletion/mutation of *Glra3* does not affect the withdrawal response to mechanical and thermal stimulation following nerve injury (Harvey et al., 2009b; Werynska et al., 2021). Chemogenetic silencing of the *Glra3*-Cre populations did not affect the acute mechanical sensitivity in the Randall-Selitto test and scratch- or pinch-activated *fos*(+) cells did not express *Glra3* in higher occurrence compared with the contralateral side in naïve mice. Conclusively, our analyses indicate that the *Glra3*-Cre(+) neurons may not be the post-synaptic target of the GLYT2 population in regulation of noxious mechanical and thermal transmission. However, since GLRA3 has been connected to inflammatory-induced hypersensitivity (Harvey et al., 2009; Werynska et al., 2021), future investigations targeting the role of *Glra3*-Cre(+) neurons in inflammatory, neuropathic, thermal and mechanical allodynia are of interest.

Besides input from itch-related primary afferents, the monosynaptic tracing experiments and dorsal root stimulations revealed that the *Glra3*-Cre populations receive input from Aα/β fibers as partial overlap with NF200(+). As NF200 can be detected in Aβ low-threshold mechanoreceptors (LTMRs), Aβ high-threshold mechanoreceptors (HTMRs), and Aδ-fibers (Djouhri and Lawson, 2004; Nagi et al., 2019; Meltzer et al., 2021), thus, input from these neuronal sub-populations cannot be excluded. Furthermore, the overlap of mCherry(+) cells with TH(+) neurons, which convey low-threshold mechanical information and are possibly associated with pleasant touch (Li et al., 2011), proposes that the lumbar *Glra3*-Cre populations receive several categories of sensory input. In addition, traced cells were found in the ventral horn (laminae VII-IV), indicating that the *Glra3*-Cre populations may receive input from spinal motor-related neurons. Additionally, starter, lineage and virally labelled *Glra3*-Cre(+) cells were observed in the ventral horn. In line with these observations, we recently showed that *Glra3* is detected in the dorsal and ventral horns of the

lumbar division (Ceder et al., 2023). Thus, it remains unclear whether sensory-mediating *Glra3*-Cre(+) neurons receive motor input or if the ventrally located *Glra3*-Cre population have motor functions.

Traced cells were also detected in the brain, suggesting that the lumbar *Glra3*-Cre(+) neurons receive distant descending input. These brain areas included the contralateral motor cortices, ipsilateral primary somatosensory cortex, barrel area, contralateral p1 reticular formation, magnocellular and parvicellular parts of the red nucleus (RMC and RPC), ipsilateral oral and caudal part of the pontine reticular nucleus, and bilateral gigantocellular vestibular nucleus. Previous unilateral retrograde tracing from the cervical 1 and 2 segments in mouse shows a similar tracing pattern as observed in our tracing experiment (Liang et al., 2011). The RMC and reticular formations are related to analgesic functions (Prado et al., 1984; Martins and Tavares, 2017; Basile et al., 2021), and the RMC, RPC, and pontine reticular nucleus to motor functions (Morales et al., 1987; Kennedy, 1990; Basile et al., 2021). Moreover, a study in mice linked monosynaptic signalling from the motor and sensory cortices to distinct spinal dorsal and ventral interneuron populations, and further to different motoric functions. Here, scant monosynaptic inputs from the motor cortex to dorsal horn neurons and from the sensory cortex to ventral neurons were observed (Ueno et al., 2018), indicating that the ventrally located *Glra3*-Cre(+) neurons probably receive monosynaptic input from the motor cortex. Collectively, we showed that the spinal *Glra3*-Cre populations receive monosynaptic descending input from brain areas involved in sensory and/or motor functions.

Conclusions

927 Spinal GLYT2 neurons regulate itch (Foster et al., 2015), suggesting that the glycinergic system
928 has potential as drug targets for itch. Nonetheless, thus far, the pruriceptive roles of the glycine
929 receptor subunits have not been evaluated. Here, we successfully linked the *Glra3*-Cre populations
930 to a pro-pruriceptive role in itch, indicating that GLRA3 may be a potential novel target for itch
931 treatment. The spontaneous guarding behaviors observed from activating the *Glra3*-Cre
932 populations are indicative of a role in sensory hypersensitivity (Wang and Wang, 2003; Mogil and
933 Crager, 2004; Casarrubea et al., 2019) and raises questions regarding the hypersensitivity
934 involvement of these populations for future investigations.

935

936 *Methodological considerations*

937 From the monosynaptic retrograde viral tracing, the lumbar *Glra3*-Cre populations were found to
938 receive both inhibitory PAX2(+) and presumably excitatory, PAX2(-) input, from the lumbar
939 segments, where the majority of the traced mCherry(+) cells were PAX2(-). However, the NEUN
940 overlap analysis revealed that 44% of starter cells, 79% of traced mCherry(+) cells, and 89% of
941 virally marked *Glra3*-Cre.mCherry were NEUN(+), which can be compared with the 98%
942 NEUN(+) overlap in the *Glra3*-Cre;*tdTomato* cells. The decrease in overlap may indicate that the
943 viral injections affect expressional patterns in the infected cells, and therefore, the PAX2(+) overlap
944 in the starter and traced cells may be underestimated.

References

Akiyama T, Iodi Carstens M, Carstens E (2011) Transmitters and pathways mediating inhibition of spinal itch-signaling neurons by scratching and other counterstimuli. PLoS ONE 6:e22665.

Akiyama T, Tominaga M, Davoodi A, Nagamine M, Blansit K, Horwitz A, Carstens MI, Carstens E (2013) Roles for substance P and gastrin-releasing peptide as neurotransmitters released by primary afferent pruriceptors. J Neurophysiol 109:742–748.

Albisetti GW, Ghanem A, Foster E, Conzelmann K-K, Zeilhofer HU, Wildner H (2017) Identification of two classes of somatosensory neurons that display resistance to retrograde infection by rabies virus. J Neurosci 37:10358–10371.

Anderson CR, Ashwell KW, Collewijn H, Conta A, Harvey A, Heise C, Hodgetts S, Holstege G, Kayalioglu G, Keast JR, McHanwell S, McLachlan EM, Paxinos G, Plant G, Scremin O, Sidhu A, Stelzner D, Watson C (2009) The spinal cord: A christopher and dana reeve foundation text and atlas. In: The Spinal Cord, pp v. Elsevier. Available at: https://linkinghub.elsevier.com/retrieve/pii/B9780123742476500018 [Accessed February 23, 2021].

Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV (1995) Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. Eur J Neurosci 7:1484–1494.

Azimi E, Reddy VB, Pereira PJS, Talbot S, Woolf CJ, Lerner EA (2017) Substance P activates Mas-related G protein-coupled receptors to induce itch. J Allergy Clin Immunol 140:447-453.e3.

Azimi E, Reddy VB, Shade K-TC, Anthony RM, Talbot S, Pereira PJS, Lerner EA (2016) Dual action of neurokinin-1 antagonists on Mas-related GPCRs. JCI Insight 1:e89362.

Barker PA, Mantyh P, Arendt-Nielsen L, Viktrup L, Tive L (2020) Nerve growth factor signaling and its contribution to pain. J Pain Res 13:1223–1241.

Basbaum AI, Bautista DM, Scherrer G, Julius D (2009) Cellular and molecular mechanisms of pain. Cell 139:267–284.

Basile GA, Quartu M, Bertino S, Serra MP, Boi M, Bramanti A, Anastasi GP, Milardi D, Cacciola A (2021) Red nucleus structure and function: from anatomy to clinical neurosciences. Brain Struct Funct 226:69–91.

Bennett DJ, Gorassini M, Fouad K, Sanelli L, Han Y, Cheng J (1999) Spasticity in rats with sacral spinal cord injury. J Neurotrauma 16:69–84.

Berrocal YA, Almeida VW, Puentes R, Knott EP, Hechtman JF, Garland M, Pearse DD (2014) Loss of central inhibition: implications for behavioral hypersensitivity after contusive spinal cord injury in rats. Pain Res Treat 2014:178278.

Beyer C, Roberts LA, Komisaruk BR (1985) Hyperalgesia induced by altered glycinergic activity at the spinal cord. Life Sciences 37:875–882.

Bourane S, Duan B, Koch SC, Dalet A, Britz O, Garcia-Campmany L, Kim E, Cheng L, Ghosh A, Ma Q, Goulding M (2015) Gate control of mechanical itch by a subpopulation of spinal cord interneurons. Science 350:550–554.

Casarrubea, Aiello, Santangelo, Di Giovanni, Crescimanno (2019) Different Representation Procedures Originated from Multivariate Temporal Pattern Analysis of the Behavioral Response to Pain in Wistar Rats Tested in a Hot-Plate under Morphine. Brain Sci 9:233.

Ceder MM, Weman HM, Johansson E, Henriksson K, Magnusson KA, Roman E, Lagerström MC (2023) The glycine receptor alpha 3 subunit mRNA expression shows sex-dependent differences in the adult mouse brain. BMC Neuroscience 24:32.

Cioffi CL (2018) Modulation of Glycine-Mediated Spinal Neurotransmission for the Treatment of Chronic Pain. J Med Chem 61:2652–2679.

Djouhri L, Lawson SN (2004) Abeta-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. Brain Res Brain Res Rev 46:131–145.

Doi-Saika M, Tokunaga A, Senba E (1997) Intradermal 5-HT induces Fos expression in rat dorsal horn neurons not via 5-HT3 but via 5-HT2A receptors. Neurosci Res 29:143–149.

Dutertre S, Becker C-M, Betz H (2012) Inhibitory glycine receptors: an update. J Biol Chem 287:40216–40223.

Foster E, Wildner H, Tudeau L, Haueter S, Ralvenius WT, Jegen M, Johannssen H, Hösli L, Haenraets K, Ghanem A, Conzelmann K-K, Bösl M, Zeilhofer HU (2015) Targeted ablation, silencing, and activation establish glycinergic dorsal horn neurons as key components of a spinal gate for pain and itch. Neuron 85:1289–1304.

Freitag FB, Ahemaiti A, Jakobsson JET, Weman HM, Lagerström MC (2019) Spinal gastrin releasing peptide receptor expressing interneurons are controlled by local phasic and tonic inhibition. Sci Rep 9:16573.

Freitag FB, Ahemaiti A, Weman HM, Ambroz K, Lagerström MC (2021) Targeting barrel field spiny stellate cells using a vesicular monoaminergic transporter 2-Cre mouse line. Sci Rep 11:3239.

Gatto G, Bourane S, Ren X, Di Costanzo S, Fenton PK, Halder P, Seal RP, Goulding MD (2021) A functional topographic map for spinal sensorimotor reflexes. Neuron 109:91-104.e5.

Groemer TW, Triller A, Zeilhofer HU, Becker K, Eulenburg V, Becker CM (2022) Nociception in the glycine receptor deficient mutant mouse spastic. Front Mol Neurosci 15:832490.

Gupta K, Harvima IT (2018) Mast cell-neural interactions contribute to pain and itch. Immunol Rev 282:168–187.

Han N, Zu JY, Chai J (2012) Spinal bombesin-recognized neurones mediate more nonhistaminergic than histaminergic sensation of itch in mice. Clin Exp Dermatol 37:290–295.

Häring M, Zeisel A, Hochgerner H, Rinwa P, Jakobsson JET, Lönnerberg P, La Manno G, Sharma N, Borgius L, Kiehn O, Lagerström MC, Linnarsson S, Ernfors P (2018) Neuronal atlas of the dorsal horn defines its architecture and links sensory input to transcriptional cell types. Nat Neurosci 21:869–880.

Harvey RJ, Depner UB, Wässle H, Ahmadi S, Heindl C, Reinold H, Smart TG, Harvey K, Schütz B, Abo-Salem OM, Zimmer A, Poisbeau P, Welzl H, Wolfer DP, Betz H, Zeilhofer HU, Müller U (2004) GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. Science 304:884–887.

Harvey VL, Caley A, Müller UC, Harvey RJ, Dickenson AH (2009) A Selective Role for alpha3 Subunit Glycine Receptors in Inflammatory Pain. Front Mol Neurosci 2:14.

Heinke B, Ruscheweyh R, Forsthuber L, Wunderbaldinger G, Sandkühler J (2004) Physiological, neurochemical and morphological properties of a subgroup of GABAergic spinal lamina II neurones identified by expression of green fluorescent protein in mice. J Physiol 560:249–266.

Hu H-J, Gereau RW (2003) ERK Integrates PKA and PKC Signaling in Superficial Dorsal Horn Neurons. II. Modulation of Neuronal Excitability. Journal of Neurophysiology 90:1680–1688.

Hu H-J, Gereau RW (2011) Metabotropic glutamate receptor 5 regulates excitability and Kv4.2-containing K+ channels primarily in excitatory neurons of the spinal dorsal horn. J Neurophysiol 105:3010–3021.

Huang J, Polgár E, Solinski HJ, Mishra SK, Tseng P-Y, Iwagaki N, Boyle KA, Dickie AC, Kriegbaum MC, Wildner H, Zeilhofer HU, Watanabe M, Riddell JS, Todd AJ, Hoon MA (2018) Circuit dissection of the role of somatostatin in itch and pain. Nat Neurosci 21:707–716.

Imamachi N, Park GH, Lee H, Anderson DJ, Simon MI, Basbaum AI, Han S-K (2009) TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. Proc Natl Acad Sci U S A 106:11330–11335.

Jinks SL, Carstens E (2000) Superficial dorsal horn neurons identified by intracutaneous histamine: chemonociceptive responses and modulation by morphine. J Neurophysiol 84:616–627.

Kennedy PR (1990) Corticospinal, rubrospinal and rubro-olivary projections: a unifying hypothesis. Trends Neurosci 13:474–479.

Krashes MJ, Koda S, Ye C, Rogan SC, Adams AC, Cusher DS, Maratos-Flier E, Roth BL, Lowell BB (2011) Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. J Clin Invest 121:1424–1428.

Kupari J, Ernfors P (2023) Molecular taxonomy of nociceptors and pruriceptors. Pain 164:1245–1257.

LaMotte RH, Shimada SG, Sikand P (2011) Mouse models of acute, chemical itch and pain in humans. Exp Dermatol 20:778–782.

Larsson M (2017) Pax2 is persistently expressed by GABAergic neurons throughout the adult rat dorsal horn. Neurosci Lett 638:96–101.

1053 Lein ES et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. Nature 445:168–
1054 176.

1055 Li L, Rutlin M, Abraira VE, Cassidy C, Kus L, Gong S, Jankowski MP, Luo W, Heintz N, Koerber HR,
1056 Woodbury CJ, Ginty DD (2011) The functional organization of cutaneous low-threshold
1057 mechanosensory neurons. Cell 147:1615–1627.

1058 Li T, Mamillapalli R, Ding S, Chang H, Liu Z-W, Gao X-B, Taylor HS (2018) Endometriosis alters brain
1059 electrophysiology, gene expression and increases pain sensitization, anxiety, and depression in
1060 female mice. Biol Reprod 99:349–359.

1061 Liang H, Paxinos G, Watson C (2011) Projections from the brain to the spinal cord in the mouse. Brain
1062 Struct Funct 215:159–186.

1063 Liu Q, Sikand P, Ma C, Tang Z, Han L, Li Z, Sun S, LaMotte RH, Dong X (2012) Mechanisms of itch evoked
1064 by β-alanine. J Neurosci 32:14532–14537.

1065 Liu Q, Tang Z, Surdenikova L, Kim S, Patel KN, Kim A, Ru F, Guan Y, Weng H-J, Geng Y, Undem BJ, Kollarik
1066 M, Chen Z-F, Anderson DJ, Dong X (2009) Sensory neuron-specific GPCR Mrgprs are itch
1067 receptors mediating chloroquine-induced pruritus. Cell 139:1353–1365.

1068 Lynch JW (2004) Molecular structure and function of the glycine receptor chloride channel. Physiol Rev
1069 84:1051–1095.

1070 Manzke T, Niebert M, Koch UR, Caley A, Vogelgesang S, Hülsmann S, Ponimaskin E, Müller U, Smart TG,
1071 Harvey RJ, Richter DW (2010) Serotonin receptor 1A-modulated phosphorylation of glycine
1072 receptor α3 controls breathing in mice. J Clin Invest 120:4118–4128.

1073 Mariqueo T (2020) The Expression of Glycine Receptor α3 Subunit is Differentially Regulated in Different
1074 Types of Pain. J Neurol Neurobiol Available at:
1075 https://scholar.archive.org/work/pxkxm6bt35cdzon6ktqnppfily/access/wayback/http://sciforsch
1076 enonline.org/journals/neurology/article-data/JNNB161/JNNB161.pdf [Accessed March 23,
1077 2022].

1078 Martins I, Tavares I (2017) Reticular formation and pain: the past and the future. Front Neuroanat 11:51.

1079 McCoy ES, Taylor-Blake B, Zylka MJ (2012) CGRPα-expressing sensory neurons respond to stimuli that
1080 evoke sensations of pain and itch. PLoS ONE 7:e36355.

1081 McCracken LM, Lowes DC, Salling MC, Carreau-Vollmer C, Odean NN, Blednov YA, Betz H, Harris RA,
1082 Harrison NL (2017) Glycine receptor α3 and α2 subunits mediate tonic and exogenous agonist-
1083 induced currents in forebrain. Proc Natl Acad Sci USA 114:E7179–E7186.

1084 Meltzer S, Santiago C, Sharma N, Ginty DD (2021) The cellular and molecular basis of somatosensory
1085 neuron development. Neuron 109:3736–3757.

1086 Mishra SK, Tisel SM, Orestes P, Bhangoo SK, Hoon MA (2011) TRPV1-lineage neurons are required for
1087 thermal sensation. EMBO J 30:582–593.

1088 Mogil JS, Crager SE (2004) What should we be measuring in behavioral studies of chronic pain in
1089 animals? Pain 112:12–15.

1090 Morales F, Engelhardt J, Soja P (1987) Motoneuron properties during motor inhibition produced by
1091 microinjection of carbachol into the pontine reticular formation of the decerebrate cat. Journal
1092 of … Available at: https://journals.physiology.org/doi/abs/10.1152/jn.1987.57.4.1118 [Accessed
1093 April 27, 2023].

1094 Nagi SS, Marshall AG, Makdani A, Jarocka E, Liljencrantz J, Ridderström M, Shaikh S, O'Neill F, Saade D,
1095 Donkervoort S, Foley AR, Minde J, Trulsson M, Cole J, Bönnemann CG, Chesler AT, Bushnell MC,
1096 McGlone F, Olausson H (2019) An ultrafast system for signaling mechanical pain in human skin.
1097 Sci Adv 5:eaaw1297.

1098 Nakano T, Andoh T, Lee J-B, Kuraishi Y (2008) Different dorsal horn neurons responding to histamine and
1099 allergic itch stimuli. Neuroreport 19:723–726.

1100 Nojima H, Simons CT, Cuellar JM, Carstens MI, Moore JA, Carstens E (2003) Opioid modulation of
1101 scratching and spinal c-fos expression evoked by intradermal serotonin. J Neurosci 23:10784–
1102 10790.

1103 Osakada F, Mori T, Cetin AH, Marshel JH, Virgen B, Callaway EM (2011) New rabies virus variants for
1104 monitoring and manipulating activity and gene expression in defined neural circuits. Neuron
1105 71:617–631.

1106 Pan H, Fatima M, Li A, Lee H, Cai W, Horwitz L, Hor CC, Zaher N, Cin M, Slade H, Huang T, Xu XZS, Duan B
1107 (2019) Identification of a spinal circuit for mechanical and persistent spontaneous itch. Neuron
1108 103:1135-1149.e6.

1109 Patapoutian A, Peier AM, Story GM, Viswanath V (2003) ThermoTRP channels and beyond: mechanisms
1110 of temperature sensation. Nat Rev Neurosci 4:529–539.

1111 Paxinos G, Franklin K (n.d.) The Mouse Brain in Stereotaxic Coordinates Academic Press, San Diego.

1112 Peirs C, Patil S, Bouali-Benazzouz R, Artola A, Landry M, Dallel R (2014) Protein kinase C gamma
1113 interneurons in the rat medullary dorsal horn: distribution and synaptic inputs to these neurons,
1114 and subcellular localization of the enzyme. J Comp Neurol 522:393–413.

1115 Pinto V, Szûcs P, Derkach VA, Safronov BV (2008) Monosynaptic convergence of C- and Adelta-afferent
1116 fibres from different segmental dorsal roots on to single substantia gelatinosa neurones in the
1117 rat spinal cord. J Physiol (Lond) 586:4165–4177.

1118 Polgár E, Fowler JH, McGill MM, Todd AJ (1999) The types of neuron which contain protein kinase C
1119 gamma in rat spinal cord. Brain Res 833:71–80.

1120 Prado WA, Raghubir R, Roberts MHT (1984) Long duration antinociception induced by red nucleus
1121 stimulation in the rat. Pain 18:S329.

1122 Rogoz K, Andersen HH, Lagerström MC, Kullander K (2014) Multimodal use of calcitonin gene-related
1123 peptide and substance P in itch and acute pain uncovered by the elimination of vesicular

glutamate transporter 2 from transient receptor potential cation channel subfamily V member 1 neurons. J Neurosci 34:14055–14068.

San Martin LS, Armijo-Weingart L, Araya A, Yévenes GE, Harvey RJ, Aguayo LG (2021) Contribution of glyr α3 subunits to the sensitivity and effect of ethanol in the nucleus accumbens. Front Mol Neurosci 14:756607.

Schemann M, Kugler EM, Buhner S, Eastwood C, Donovan J, Jiang W, Grundy D (2012) The mast cell degranulator compound 48/80 directly activates neurons. PLoS ONE 7:e52104.

Sheng M, Greenberg ME (1990) The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron 4:477–485.

Stantcheva KK, Iovino L, Dhandapani R, Martinez C, Castaldi L, Nocchi L, Perlas E, Portulano C, Pesaresi M, Shirlekar KS, de Castro Reis F, Paparountas T, Bilbao D, Heppenstall PA (2016) A subpopulation of itch-sensing neurons marked by Ret and somatostatin expression. EMBO Rep 17:585–600.

Takazawa T, Choudhury P, Tong C-K, Conway CM, Scherrer G, Flood PD, Mukai J, MacDermott AB (2017) Inhibition Mediated by Glycinergic and GABAergic Receptors on Excitatory Neurons in Mouse Superficial Dorsal Horn Is Location-Specific but Modified by Inflammation. J Neurosci 37:2336–2348.

Todd AJ (2017) Identifying functional populations among the interneurons in laminae I-III of the spinal dorsal horn. Mol Pain 13:1744806917693003.

Tudeau L, Acuña MA, Albisetti GW, Neumann E, Ralvenius WT, Scheurer L, Poe M, Cook JM, Johannssen HC, Zeilhofer HU (2020) Mice lacking spinal α2GABAA receptors: Altered GABAergic neurotransmission, diminished GABAergic antihyperalgesia, and potential compensatory mechanisms preventing a hyperalgesic phenotype. Brain Res 1741:146889.

Ueno M, Nakamura Y, Li J, Gu Z, Niehaus J, Maezawa M, Crone SA, Goulding M, Baccei ML, Yoshida Y (2018) Corticospinal Circuits from the Sensory and Motor Cortices Differentially Regulate Skilled Movements through Distinct Spinal Interneurons. Cell Rep 23:1286-1300.e7.

Usoskin D, Furlan A, Islam S, Abdo H, Lönnerberg P, Lou D, Hjerling-Leffler J, Haeggström J, Kharchenko O, Kharchenko PV, Linnarsson S, Ernfors P (2015) Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. Nat Neurosci 18:145–153.

Wang F, Flanagan J, Su N, Wang L-C, Bui S, Nielson A, Wu X, Vo H-T, Ma X-J, Luo Y (2012) RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn 14:22–29.

Wang H-C, Cheng K-I, Chen P-R, Tseng K-Y, Kwan A-L, Chang L-L (2018) Glycine receptors expression in rat spinal cord and dorsal root ganglion in prostaglandin E2 intrathecal injection models. BMC Neuroscience 19:72.

Wang LX, Wang ZJ (2003) Animal and cellular models of chronic pain. Adv Drug Deliv Rev 55:949–965.

Watson C, Paxinos G (2009) Chapter 16—Atlas of the Mouse Spinal Cord. The Spinal Cord: A ….

1160 Werynska K, Gingras J, Benke D, Scheurer L, Neumann E, Zeilhofer HU (2021) A Glra3 phospho-deficient
1161 mouse mutant establishes the critical role of PKA-dependent phosphorylation and inhibition of
1162 glycine receptors in spinal inflammatory hyperalgesia. Pain Available at:
1163 https://journals.lww.com/10.1097/j.pain.0000000000002236 [Accessed March 1, 2021].

1164 Woolf CJ, Safieh-Garabedian B, Ma QP, Crilly P, Winter J (1994) Nerve growth factor contributes to the
1165 generation of inflammatory sensory hypersensitivity. Neuroscience 62:327–331.

1166 Yamamoto T, Yaksh TL (1993) Effects of intrathecal strychnine and bicuculline on nerve compression-
1167 induced thermal hyperalgesia and selective antagonism by MK-801. PAIN 54:79.

1168 Yao GL, Tohyama M, Senba E (1992) Histamine-caused itch induces Fos-like immunoreactivity in dorsal
1169 horn neurons: effect of morphine pretreatment. Brain Res 599:333–337.

1170 Zeilhofer HU (2005) The glycinergic control of spinal pain processing. Cell Mol Life Sci 62:2027–2035.

1171 Zeisel A et al. (2018) Molecular architecture of the mouse nervous system. Cell 174:999-1014.e22.

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Legends

Figure 1. *Glra3*-Cre;*tdTomato* neurons are predominantly located in laminae III–IV and the adult *Glra3*-Cre population consists of a major excitatory and a minor inhibitory population. **(A)** Overlap of spinal lumbar *Glra3*-Cre;*tdTomato* cells (magenta) and neuronal marker NEUN (yellow). Yellow dotted circles display examples of tdTomato(+)NEUN(+) cells. **(B–B')** Location of tdTomato(+) cells (magenta) in IB4(+) outer lamina II (white), PKCγ(+) inner lamina II (yellow), laminae III–IV, V–VI, and ventral laminae defined from The Spinal Cord atlas (Anderson et al., 2009). Yellow dotted circles represent examples of tdTomato(+)PKCγ(+) cells. **(C)** Scatter bar plot of the occurrence of tdTomato(+)NEUN(+) and tdTomato(+) cells in different spinal areas (2 females, n images: NEUN: 10; 2 females, n images: PKCγ/IB4: 13). **(D)** Schematic illustration of AAVDJ.Ef1a-DIO-HTB lumbar 5/lumbar 6 (L5/L6) microinjection into *Glra3*-Cre(+) mice. **(E–E')** Overlap of *Glra3* (magenta) and *Vglut2* (white) in adult *Glra3*-Cre.HTB neurons (cyan). Yellow dotted circles indicate *Glra3*(+)*Vglut2*(+), magenta dotted circles denote *Glra3*(+)*Vglut2*(-), and white circles represent *Glra3*(-)*Vglut2*(+) in *Glra3*-Cre.HTB(+) cells. **(F)** Scatter bar plot of percentages of *Glra3*-Cre.HTB(+) neurons expressing analyzed genes when targeting *Vglut2* (2 males, n sections: 6, images: 12) **(G–G')** *Glra3*-Cre.HTB neurons' expression of *Glra3* (magenta) and *Viaat* (white). Yellow dotted circles indicate *Glra3*(+)*Viaat*(-), white dotted circles represent *Glra3*(-)*Vglut2*(+), and blue dotted circles show *Glra3*(-)*Vglut2*(-) in *Glra3*-Cre.HTB(+) cells. **(H)** Scatter bar plot displaying percentages of *Glra3*-Cre.HTB(+) neurons expressing analyzed genes when targeting *Viaat* (3 females, n sections: 7, images: 15). Scale bar: (A, B', E, G): 100µm, (B): 150µm, (enlargement in A, E', G'): 50µm, (enlargement in B): 75µm. The observational dots in

1195 the scatter bar plots (C, F, H) represent a unilateral part of the spinal cord and the different dot
1196 colors signify different mice. Results are presented as mean±SEM.

1197

1198 **Figure 2. *Glra3*-Cre;*tdTomato* neurons respond to glycine and the populations display a**
1199 **heterogeneous firing pattern. (A)** Patch-clamp recordings of spinal *Glra3*-Cre;*tdTomato* neurons
1200 (magenta). A schematic patch pipette is indicated with a white arrowhead. Scale bar: 50µm. The
1201 image on the right represents a recording of action potential firing (above) upon stimulation with a
1202 depolarizing current for a duration of 500ms (below). **(B)** Pie chart of the distribution of different
1203 action potential firing patterns (8 females, 7 males, n cells: 69). **(B')** Representative recording of
1204 each firing pattern. The scale bar applies to all five traces. **(C)** Representative recordings of the
1205 hyperpolarizing current induced by glycine (300µM, n cells: 13, above) and blockage by strychnine
1206 (10µM, n cells: 6, below). The scale bar applies to both traces. **(D)** A Neurobiotin™ filled neuron
1207 in a mouse spinal cord slice. Magenta(+) cells are *Glra3*-Cre;*tdTomato* neurons, PKCγ staining is
1208 presented in white, blue is DAPI staining (scale bar: 50µm). The zoomed in image shows the
1209 Neurobiotin™ filled neuron stained with Alexa Fluor™ 488 streptavidin conjugate (in green, scale
1210 bar: 20µm). **(E)** Morphological and locational reconstruction of all Neurobiotin™ filled neurons
1211 (n cells: 13, scale bar: 100µm). Laminae are defined from The Spinal Cord atlas (Anderson et al.,
1212 2009).

1213

1214 **Figure 3. mCherry and chemogenetic viral vector fluorescent genes are not expressed in**
1215 ***Glra3*-Cre(-) wildtype injected mice.** Low mCherry fluorescence detection, but no positive cells,
1216 could be visualized in close proximity to the L5/L6 injection site of AAV8.hSyn-DIO-mCherry

(control virus, 2 females and 1 male) **(A)**, AAV8.hSyn-DIO-hM3D(Gq)-mCherry (1 female and 2 males) **(B),** or AAV8.hSyn-DIO-hM4D(Gi)-mCherry (2 females and 1 male) **(C)** injected mice, demonstrating the Cre-dependent specificity of the viral vectors. Scale bars: 150µm.

Figure 4. Adult *Glra3*-Cre(+) neurons are mainly located in laminae III–IV and selective chemogenetic activation induces spontaneous behaviors indicative of a role in nociception and pruriception

(A) Expression of NEUN (yellow) in *Glra3*-Cre.mCherry (magenta) lumbar spinal cord. Yellow dotted circles represent mCherry(+)NEUN(+) overlap and magenta dotted circles specify mCherry(+)NEUN(-) cells. **(B–B')** mCherry(+) co-localization with outer lamina II marker IB4 (white), inner lamina II marker PKCγ (yellow), and its expression in laminae III–IV, V–VI, and ventral laminae defined from The Spinal Cord atlas (Anderson et al., 2009). The white dotted circles show mCherry(+) expression in the IB4 band and magenta dotted circles indicate mCherry(+)PKCγ(-) cells in the PKCγ band. mCherry(+)PKCγ(+) cells are not shown in this image. **(C)** Scatter bar plot of percentages of mCherry(+)NEUN(+) and laminae layer localization of mCherry(+) neurons (2 females, 2 males, n sections: NEUN: 31; PKCγ/IB4: 16). Scale bars: (A, B): 100µm, (zoomed images): 50µm. The observational dots in the scatter bar plots represent a unilateral part of the spinal cord, different dot colors signify different mice. Results are presented as mean±SEM. **(D–F)** Spontaneous behaviors, including licking/biting, stomping, and guarding of the corresponding dermatome (hind paw/leg), were observed in *Glra3*-Cre.hM3Dq mice (7+8 mice; 7 females, 8 males) after 0.1mg/kg intraperitoneal administration of CNO. **(D)** Chemogenetic activation of the *Glra3*-Cre populations increased total duration and frequency of licking/biting of the ipsilateral hind paw compared with *Glra3*-Cre.mCherry mice in the 30min time window post-

CNO injection (duration p=.0006, frequency p=.0002). **(E)** Spontaneous stomping behavior was observed in *Glra3*-Cre.hM3Dq mice following CNO injection, which was not seen in *Glra3*-Cre.mCherry mice (duration and frequency, p<.0001). **(F)** *Glra3*-Cre.hM3Dq mice displayed guarding behaviors not observed in control mice. Both guarding duration and frequency were affected by *Glra3*-Cre populations activation (p<.0001). Results are presented as mean±SEM. Mann-Whitney u test was performed in **D** and Chi-square test in **E–F** to compare the group means. **p<.001, ***p<.0001.

Figure 5. Chemogenetic silencing of *Glra3*-Cre(+) neurons decreases histaminergic and chloroquine-induced itch.

(A) Dendritic and cytosolic expression of mCherry (magenta) after AAV8.hSyn-DIO-mCherry microinjection (upper). Dendritic and cytosolic localization of hM4D(Gi)-mCherry (yellow) after microinjection of AAV8.hSyn-DIO-hM4D(Gi)-mCherry (lower). **(B)** Intraperitoneal administration of CNO (0.1mg/kg) did not induce spontaneous licking/biting of the affected dermatome in the 0–30min or 30–60min intervals post-injection (duration: 0–30min: p=.7463, 30–60min: p=.4589; frequency: 0–30min: p=.4109, 30–60min: p=.3945, 8 mice/group; 11 females, 5 males). **(C)** Neither licking duration nor frequency were affected by saline administration (10µl, duration p=.7923; frequency p=.9405, 8 mice/group; 9 females, 7 males). **(D)** Silencing *Glra3*-Cre(+) neurons attenuated the licking/biting duration and frequency following compound 48/80 (20µg, 10µl) injection (duration p=.0037; frequency p=.0028, 8 mice/group; 8 females, 8 males). **(E)** *Glra3*-Cre.hM4Di mice displayed lower licking/biting duration and frequency following chloroquine (10mM, 10µl) injection (duration p=.0117; frequency p=.0084, 8 mice/group; 9 females, 7 males). **(F)** Silencing of sacral *Glra3*-Cre(+) neurons did not affect the mechanical

threshold in the tail (p=.5110, 7 mice/group, 7 females, 7 males). **(G–H)** *Glra3*-Cre(+) neurons are not involved in thermal transmission. **(G)** Thermal stimulation (Hargreaves) of the ipsilateral hind paw did neither affect the withdrawal latency in post-CNO administrated *Glra3*-Cre.hM4Di mice compared with *Glra3*-Cre.mCherry mice, nor the response pre- and post-administration of CNO in *Glra3*-Cre.hM4Di mice (*Glra3*-Cre.mCherry pre vs post CNO: p=.9981; *Glra3*-Cre.hM4Di pre vs post CNO: p=.9659; *Glra3*-Cre.mCherry vs *Glra3*-Cre.hM4Di post CNO: p=.5326, 8 mice/group; 11 females, 5 males). **(H)** Application of acetone solution (9:1 in water) did not affect the aversive response when silencing *Glra3*-Cre(+) neurons (p=.1145, 8 mice/group; 8 females, 8 males). Scale bars (A): 100µm. All results are presented as mean±SEM. To compare the mean values, Mann-Whitney u test was performed in C (saline duration) and **E**, unpaired two-tailed Student t-test was performed in **C** (saline frequency), **D**, **F** and **H**. In **G**, and a one-way ANOVA with Šídák's multiple comparisons test was used. *p<.05, **p<.01.

Figure 6: Spinal neurons activated by compound 48/80 or chloroquine co-express *Glra3*.

C57BL/6J mice were subjected to different sensory stimuli, whereafter the *Glra3* (magenta) and *fos* (cyan) co-expressional patterns were investigated (DAPI (dark blue)). **(A–B')** Saline (10µl) (1 female, 2 males), **(C–D')** compound 48/80 (20⌈g, 10µl; 1 female, 2 males), **(E–F')** chloroquine (20mM, 10µl; 1 female, 2 males), **(G–H')** scratch (30sec, approximately 300mN (30.6g); 2 females, 1 male) and **(I–J')** pinch (5 times for 5sec; 1 female, 2 males) in urethane anesthetized mice (2g/kg). **(K–L')** Hargreaves (stimulated 3 times; 2 females, 1 male) in awake and freely moving mice. **(A–L)** Representative images of the contralateral and ipsilateral (stimulated side) dorsal horns for each stimulus, with close ups. *Fos*(+)*Glra3*(+) cells are depicted by a dotted magenta circle. Scale bars: (A–L): 100µm, (zoomed in A–B, E–J): 20µm, (zoomed in C–D, K–L):

50µm. To obtain high resolution, two images of each dorsal horn were acquired and later merged together, to a composited representative image of the dorsal horn, using Adobe Photoshop 22.3. **(A'–L')** Schematic illustrations of the *fos*(+) and *fos*(+)*Glra3*(+)cells, where each cell is illustrated by a circle; *fos*(+) in cyan and *fos*(+)*Glra3*(+) in magenta (n sections; saline:16, compound 48/80: 17, chloroquine: 17, artificial scratching: 16, pinch: 17 and Hargreaves: 18). **(M)** Scatter bar plot of the average number of *fos*(+) cells per dorsal horn for each stimulus on the contralateral (white bar) and ipsilateral (grey bar) side. **(N–O)** Scatter bar plot of the average number of *fos*(+)*Glra3*(+) cells per dorsal horn for each stimulus on the contralateral (white bar) and ipsilateral (grey bar) side. Results are presented as mean±SEM. Individual mice are marked with magenta, yellow, and cyan in **M–O** to display the spread between sections and mice. Paired two-tailed Student t-tests were performed in **M–O**, and a one-way ANOVA with Šídák's multiple comparisons test to check for differences between saline, compound 48/80, and chloroquine injections. **M.** Contralateral vs. ipsilateral: saline p=.8372; compound 48/80 p<.0001; chloroquine p<.0001; artificial scratching p<.0001; pinch p=.0014; and noxious heat (Hargreaves) p=.0218. **N.** The number of *fos*(+)*Glra3*(+) cells was higher on the ipsilateral side when injecting compound 48/80 or chloroquine compared with the contralateral side (saline p=.5194, compound 48/80 p<.0001 and chloroquine p<.0001). Compared with saline injections, only compound 48/80 injection resulted in a higher number of *fos*(+)*Glra3*(+) cells (p<.0001). **O.** No differences in the number of *fos*(+)*Glra3*(+) neurons were detected for scratch (p=.6817), pinch (p=.0617), or Hargreaves (p=.1092). For separate channels, see Figure 7. For overlap with *fos*, *Glra3* and *Vglut2* or *Viaat* for the following stimuli: scratching, pinch and Hargreaves, see Figure 8.

Figure 7. Expression of *Glra3* and *fos* in the contralateral L5/L6 dorsal horn following calf or paw stimulation in anesthetized or awake freely moving mice. (A–F) The expression of *Glra3* (magenta) in the contralateral L5/L6 dorsal horn following sensory stimulation. **(A'–F')** Expression of *fos* (cyan) in the contralateral L5/L6 dorsal horn after sensory stimulation. **A–A'** Saline (10µl, 1 female, 2 males) injection in urethane (2g/kg) anesthetized mice. **B–B'** Compound 48/80 (20µg, 10µl, 1 female, 2 males) injection in urethane anesthetized mice. **C–C'** Chloroquine (20mM, 10µl, 1 female, 2 males) injection in urethane anesthetized mice. **D–D'** Scratching (30sec with 2Hz and approximately 300mN (30.6g), 2 females, 1 male) in urethane anesthetized mice. **E–E'** Pinching (5 times for 5sec, 1 female, 2 males) in urethane anesthetized mice. **F–F'** Hargreaves (stimulated 3 times, 2 females and 1 male) in freely moving awake mice. Scale bars: 50µm. To obtain high resolution, two images of each dorsal horn were acquired and later merged together to a composited representative image of the dorsal horn using Adobe Photoshop 22.3.

Figure 8. The expression of *Glra3*, sensory modality-induced *fos* cells, and *Vglut2* or *Viaat* in L5/L6 after dorsolateral calf or paw mechanical or heat stimulation in anesthetized or awake freely moving mice. (A–E''') Expressional view (A–F) of *Glra3* (', magenta), *fos* ('', cyan), and *Vglut2* or *Viaat* (''', white) in the L5/L6 ipsilateral dorsal horn after sensory stimulation of the dorsolateral calf in urethane (2g/kg) anesthetized mice (Scratch: 30sec with 2Hz and approximately 300mN (30.6g), 2 females, 1 male; Pinch: 5 times for 5sec; 1 female, 2 males). **(a–e''')** Zoomed in view of the respective marker genes after stimulation. **(F–F''')** Expressional view (F) of *Glra3* (F', magenta), *fos* (F'', cyan), and *Vglut2* or *Viaat* (F''', white) in the L5/L6 ipsilateral dorsal horn after noxious heat stimulation of the hind paw (stimulated 3 times with 20sec cut-off time; 2 females, 1 male) in awake freely moving mice. **(f–f''')** Zoomed in view of the respective marker genes after

noxious heat stimulation. Magenta dotted circles show *fos*(+)*Glra3*(+), light pink indicates *fos*(+)*Viaat*(+), and dark magenta shows *fos*(+)*Glra3*(+)*Vglut2*(+). Scale bars: (A–F): 50µm, (a–f’’’): 20µm. To obtain high resolution, two images of each dorsal horn were acquired and later merged together to a composited representative image of the dorsal horn using Adobe Photoshop 22.3.

Figure 9. Compound 48/80- and chloroquine-induced *fos* cells expressing *Glra3* are both excitatory and inhibitory. Expression of *fos*, *Glra3*, and *Vglut2* or *Viaat*, and the ipsilateral L5/L6 dorsal horn spatial location of cells expressing these genes (3 mice of mixed sex/stimulation, n sections/mice: 2–4), following control saline (10µl, n sections: 9), compound 48/80 (20µg, 10µl, n sections: *Vglut2*: 8; *Viaat*: 9), or chloroquine (20mM, 10µl, n sections: *Vglut2*: 9; *Viaat*: 9) calf injections in urethane (2g/kg) anesthetized mice. (**A**) Schematic illustration of calf injections. (**B**) The co-expression of *Vglut2* or *Viaat* in saline, compound 48/80- and chloroquine-activated *fos*(+)*Glra3*(+) cells. Both excitatory *Glra3*(+)*Vglut2*(+) and inhibitory *Glra3*(+)*Viaat*(+) neurons are activated following saline, compound 48/80, and chloroquine administration. Saline-activated *fos*(+)*Glra3*(+) population showed 40.9±3.6% (94/231) co-expression with *Vglut2* and 56.1±4.4% (124/218) with *Viaat*; compound 48/80 displayed 53.2±4.9% (208/373) and 45.7±3.2% (196/422) co-expression with *Vglut2* and *Viaat*, respectively. Chloroquine-activated *fos*(+)*Glra3*(+) neurons co-expressed both *Vglut2* and *Viaat* in similar proportions: 40.8±4.6% (98/238) and 40.9±3.5% (107/264), respectively. The graph presents data as mean±SEM. (**C–H’’’’**) Each panel shows the overview of the expression of the targeted genes with nucleus marker DAPI (dark blue) first, followed by *Glra3* (’, magenta), sensory stimulation-induced *fos* (’’, cyan), and *Vglut2* or *Viaat* (’’’, white). Magenta dotted circle: *fos*(+)*Glra3*(+) cells; white dotted circle:

fos(+)*Glra3*(+)*Vglut2*(+) or *fos*(+)*Glra3*(+)*Viaat*(+) cells. The schematic images in (’’’’) show the spatial localization of the sensory-induced *fos*(+) cells (cyan), *fos*(+)*Glra3*(+) (magenta), and *fos*(+)*Glra3*(+)*Vglut2*(+) (C’’’’, E’’’’, G’’’’; purple) or *fos*(+)*Glra3*(+)*Viaat*(+) (D’’’’, F’’’’, H’’’’; light pink) cells. **(C’’’’, D’’’’)** Saline injection resulted in a widespread *fos*(+) cell pattern in the dorsal horn, and overlapping cells with *Glra3*(+) could be found in the whole dorsal horn. Moreover, the saline-activated *fos*(+)*Glra3*(+)*Vglut2*(+) neurons were found to be located more to the lateral part of the dorsal horn, while the saline-activated *fos*(+)*Glra3*(+)*Viaat*(+) cells were more spread over the dorsal horn, with some clustering in the medial part of the dorsal horn. (**E’’’’**, **F’’’’**) Compound 48/80-activated *fos*(+) cells were clustered in the superficial layer of the dorsolateral horn, where *Glra3*(+)*Vglut2*(+) cells (E’’’’) and *Glra3*(+)*Viaat*(+) cells (F’’’’) were found in the same area. **(G’’’’, H’’’’)** Chloroquine-activated *fos*(+) cells clustered in similar patterns as observed for compound 48/80, but the chloroquine *fos*(+) cells were fewer in number compared with compound 48/80. *Fos*(+)*Glra3*(+)*Vglut2*(+) cells (G’’’’) were found more dorsolateral, similar to *fos*(+)*Glra3*(+)*Viaat*(+) cells (H’’’’), which were found mostly dorsolateral but with a higher degree of scattering. Scale bars: (C, D, E, F, G, H): 100µm; (c, d, e, f, g, h): 50µm. To obtain high resolution, two images of each dorsal horn were acquired and later merged together to a composited representative image of the dorsal horn using Adobe Photoshop 22.3.

Figure 10. Starter and traced cells were mainly found in the lumbar division in *Glra3*-Cre(+) mice and only a few cells were detected in *Glra3*-Cre(-) mice. (A–A’’’). No starter GFP(+)mCherry(+) or traced mCherry(+) cells were detected in the thoracic (A’), lumbar (A’’) or sacral (A’’’) divisions in *Glra3*-Cre(-) mice. Two mCherry(+) cells were found in the cervical (A) division in two separate mice, where 1 mCherry(+) cell was found in the contralateral ventral horn

and 1 cell in the ipsilateral dorsal horn (3 females, 3 males, every sixth section analyzed). These cells are not depicted in the image. **(B)** No starter GFP(+)mCherry(+) or traced mCherry(+) cells were observed at the injection site of *Glra3*-Cre(-) mice. **(C)** Starter GFP(+)mCherry(+) and traced mCherry(+) cells were found in the lumbar division (C'') and 1 mCherry(+) cell was observed in the distant cervical (C), whereas no traced cells were located in the thoracic (C') or sacral (C''') divisions in *Glra3*-Cre(+) mice (5 females, 5 males, every sixth section analyzed). **(D)** Starter GFP(+)mCherry(+) and traced mCherry(+) cells were detected at the injection site of *Glra3*-Cre(+) mice. The blue dotted circles represents GFP(+)mCherry(-) cells and the yellow dotted circles show GFP(+)mCherry(+) starter cells. Traced mCherry(+) is not displayed in images. GFP is displayed as cyan and mCherry as magenta (DAPI as yellow). Scale bars: (A–A''', C–C'''): 300µm; (B, D): 150µm. For high resolution, images were acquired in 10x and were thereafter merged for representation in Adobe Photoshop 22.3.

Figure 11. Lumbar *Glra3*-Cre(+) neurons receive monosynaptic input from excitatory and inhibitory local spinal neurons. (A) Co-expression of NEUN (yellow) in *Glra3*-Cre starter GFP(+)mCherry(+) and traced mCherry(+) cells. **(B)** Pie charts of NEUN overlap in ipsilateral starter *Glra3*-Cre(+) (top chart) and traced cells (bottom chart) (3 females, 2 males, n images: 29). **(C)** Co-localization of PAX2 (yellow) in *Glra3*-Cre starter GFP(+)mCherry(+) and traced mCherry(+) cells. GFP is displayed as cyan and mCherry as magenta. **(D)** Pie charts of co-expression of PAX2 in the starter *Glra3*-Cre(+) (top chart) and traced cells (bottom chart) (3 females, 2 males, n images: 31). Scale bars: (A, C): 150µm; (zoomed images): 75µm. **(E)** Schematic illustration of the spatial localization of NEUN(+) and NEUN(-) *Glra3*-Cre starter GFP(+)mCherry(+) and traced mCherry(+) cells in the ipsi- and contralateral spinal lumbar

division. **(F)** Schematic illustration of *Glra3*-Cre starter GFP(+)mCherry(+) and traced mCherry(+) cell localizations and overlap with PAX2 in the ipsi- and contralateral spinal lumbar division. The marker(+) starter cells are shown as cyan dots and the marker(-) starter cells as grey dots, whereas the marker(+) traced cells are depicted as yellow dots and marker(-) traced cells as magenta dots. The schematic image was acquired from Atlas of the Mouse Spinal Cord (Watson and Paxinos, 2009). For starter and traced cells in *Glra3*-Cre(-) mice and the cervical, thoracic and sacral divisions of the spinal cord in *Glra3*-Cre(+) mice, please see Figure 11.

Figure 12. Lumbar *Glra3*-Cre(+) neurons receive monosynaptic input from several brain areas. (A–D) Schematic illustrations of the mono- and pre-synaptic traced mCherry(+) cells found in several brain areas (n cells=89 from 7 out of the ten mice; 5 females, 5 males; 3 females and 4 males had traced mCherry(+) cells). The brain areas with more than one mCherry(+) cell or the brain areas with mCherry(+) cells in more than one mouse are shown in the figure. The coloring of the dots represents the different mice and the schematic images were acquired from The Mouse Brain Atlas *in Stereotaxic Coordinates* (Paxinos and Franklin, n.d.). **(A)** Traced mCherry(+) cells in the ipsilateral and contralateral primary and secondary motor cortices (M1, M2) (bregma: -0.94–(-)1.22mm; 2 females) and in the ipsilateral somatosensory cortex, barrel field (S1BF) (bregma: 0.38–(-)1.34mm; 1 male). **(B)** mCherry(+) cells were observed in the contralateral p1 reticular formation (p1Rt) (bregma:-3.08–(-)3.16mm; 3 females). **(C)** The contralateral localization of mCherry(+) cells in the magnocellular and parvicellular parts (RMC and RPC) (bregma: -3.08–(-)4.04mm; 3 females). **(D)** The ipsilateral and contralateral localization of traced mCherry(+) cells in the oral and caudal part of the pontine reticular nucleus (PnO and PnC) (bregma: -4.24–(-

)5.23mm; 2 females, 2 males). **(E)** mCherry(+) cells were bilaterally localized in the gigantocellular vestibular nucleus (Gi) (bregma: -5.88–(-)6.97mm; 2 females, 2 males). Scale bars: 100μm.

Figure 13. The spinal *Glra3*-Cre populations receive monosynaptic information from multiple subgroups of primary afferents. (A–K) Co-expression of markers (yellow) in lumbar DRG mCherry(+) traced cells (magenta). The cyan dotted circles indicate mCherry(+)marker(+) and the magenta dotted circles show examples of mCherry(+)marker(-) cells. **(A)** NF200: 2 females, 2 males. **(B)** TRKA: 2 females, 2 males. **(C)** CGRP: 1 female, 2 males. **(D)** IB4: 3 females, 1 male. **(E)** TH: 4 females, 2 males. **(F)** *Mrgprd*: 1 female, 2 males. **(G)** *Mrgpra3*: 1 female, 2 males. **(H)** SST: 2 females, 1 male. **(I)** *Nppb*: 1 female, 2 males. **(J)** *Trpv1*: 1 female, 2 males. **(K)** *Trpm8*: 1 female, 2 males. Scale bars: 100⌈m. **(L)** Bar plot of the proportion of marker co-expression in mCherry(+) DRG cells. **(M)** Bar plot of the occurrence of mCherry(+) in marker-expressing DRG cells. The results are shown as total percentages of overlap. **(N)** Schematic illustration of a root stimulation combined patch-clamp recording, where red dots indicate *Glra3*-Cre(+) neurons. The traces in the middle are representative patch-clamp recordings of monosynaptic inputs from different afferent fibers. **(O)** Venn diagram illustrating the distribution of monosynaptic inputs from the different afferent fiber subtypes. The overlapping areas denote neurons that received monosynaptic inputs from multiple afferent fiber subtypes. For separate channels, see Figure 14.

Figure 14. Spinal *Glra3*-Cre traced mCherry(+) co-localization with the marker genes and tested proteins, relating to Figure 13. Separate channel view of the different DRG markers from

1444 Figure 9. **(A–K)** The lumbar *Glra3*-Cre traced mCherry(+) cells in the DRG (magenta). **(A'–K')**

1445 Marker genes and proteins. Scale bars: 100μm.

Tables

Table 1. Membrane and firing properties of *Glra3*-Cre:*tdTomato* neurons

Firing pattern	**Tonic (n=36)**	**Delayed (n=9)**	**Phasic (n=12)**	**Irregular (n=7)**	**Single (n=5)**	**Total (n=69)**
Resting membrane potential (mV)	-59.37±1.54	**-72.33*±3.15**	-56.33±2.78	-58.14±1.83	-52.40±3.17	-59.91±1.22
Input resistance (MΩ)	1010.6±119.1	732.78±123.53	796.33±119.4	718.71±150	649.60±110.31	879.43±70.08
Capacitance (pF)	56.79±7.12	79.22±7.38	48.42±6.84	51.71±7.54	26.40±6.23	55.07±4.34
Rheobase (pA)	21.24±2.82	23.33±3.51	19.00±4.55	20.43±2.89	41.40±12.82	22.56±2.03
AP threshold (mV)	-31.38±1.55	-30.84±2.73	-28.34±2.88	-36.69±1.65	-25.14±6.51	-30.94±1.12
AP rising time (ms)	1.08±0.12	1.35±0.08	1.98±0.35	1.39±0.12	1.27±0.32	1.34±0.10
AP peak (mV)	21.43±2.69	28.43±2.77	20.80±5.15	17.16±5.16	19.59±7.68	21.71±1.85

**: Delayed AP group showed lower resting membrane potential (numbers in bold red) compared to all the other groups in two tailed one-way ANOVA followed by Turkey's Multiple Comparison Test.*

1449 **Table 2. Brain areas containing spinal lumbar *Glra3*-Cre(+) retrogradely traced neurons.**

Sex	Animal ID	Bregma	Brain Structure Abbreviation	Brain Structure	Number of cells	Ipsi/Contra
Female	1	-3.08–(-4.04)	RMC	Red nucleus, magnocellular part	5	Contra
		-3.08	p1Rt/REth	p1 reticular formation/ retroethmoid nucleus	1	Contra
		-3.16	p1Rt	p1 reticular formation	1	Contra
		-3.52	RMC/RPC	Red nucleus, magnocellular part/ red nucleus, parvicellular part	1	Contra
		-3.80	PaR	Pararubal nucleus	1	Contra
		-5.80	IRt	Intermediate reticular nucleus	2	Contra
		-5.88	MVeMC	Medial vestibular nucleus, magnocellular part	1	Ipsi

		-5.88	MVePC	Mmedial vestibular nucleus, parvicellular part	2	Contra
		-6.12	Gi	Gigantocellular vestibular nucleus	1	Contra
		-6.96	MVe	Medial vestibular nucleus	1	Contra
Female	2	-0.94	M1	Primary motor cortex	3	Contra
		-0.94	M2	Secondary motor cortex	3	Contra
		-1.22	M1/M2	Primary motor cortex/ secondary motor cortex	1	Ipsi
		-3.08	p1Rt	p1 reticular formation	2	Contra
		-3.16	RMC/RPC	Red nucleus, mangocellular part/ red nucleus, parvicellular part	2	Contra
		-3.40	RMC	Red nucleus, magnocellular part	2	Contra
		-4.04	LPAG	Lateral periaqueductal gray	1	ipsi

-4.04	Su3	Supraoculomotor periaqueductal gray	1	Ipsi
-4.24	mRT	Mesencephalic reticular formation	1	Contra
-4.24	PTg/PnO	Reticulotegmental nucleus of the pons/ pontine reticular nucleus, oral part	1	Contra
-4.48–(-4.60)	PnO	Pontine reticular nucleus, oral part	7	1 ipsi, 6 contra
-5.20	PnC	Pontine reticular nucleus, caudal part	2	1 ipsi, 1 contra
-5.20	PO	Paraolivary nucleus	1	Contra
-5.68	SuVe	Superior vestibular nucleus	1	Contra
-5.88	SuVe/LVe	Superior vestibular nucleus/lateral vestibular nucleus	1	Contra
-5.88–(-7.08)	Gi	Gigantocellular vestibular nucleus	5	2 ipsi, 3 contra
-6.24–(-7.08)	SpVe	spinal vestibular nucleus	3	1 ipsi, 2 contra
-6.96	C1	C1 adrenaline cells	1	Ipsi
-7.08	Sol	Solitary tract	1	Ipsi

Female	3	-1.22	M1	Primary motor cortex	1	Contra
		-1.22	M2	Secondary motor cortex	1	Contra
		-3.08	p1Rt	p1 reticular formation	3	Contra
		-3.28	mRt	Mesencephalic reticular formation	2	Ipsi
		-3.28	RMC	Red nucleus, magnocellular part	1	Contra
		-3.28	RPC	Red nucleus, parvicellular part	5	Contra
		-4.24–(-4.60)	PnO	Pontine reticular nucleus, oral part	2	Contra
		-4.60	Pa4	Paratrochlear nucleus	1	
		-5.20	PnC	Pontine reticular nucleus, caudal part	2	Contra
		-5.20	PnR	Pontine raphe nucleus	1	Ipsi
		-5.88	MVeMC	Medial vestibular nucleus, magnocellular part	1	Contra
Male	4	-0.7	RSD	Retrosplenial dysgranular cortex	2	Contra

		-1.34	S1BF	Primary somatosensory cortex, barrel	1	Ipsi
		0.38	S1BF/S1ULp	Primary somatosensory cortex, barrel/ Primary somatosensory cortex, upper lip	1	Ipsi
		-0.7	S1HL	Primary somatosensory cortex, hindleg	1	Contra
		-6.97	Gi	Gigantocellular vestibular nucleus	1	Ipsi
Male	5	-5.23	PnC	Pontine reticular nucleus, caudal part	2	Contra
		-6.64	Gi	Gigantocellular vestibular nucleus	1	Contra
Male	6	-4.16	mRt	Mesencephalic reticular formation	1	Contra
		-6.0	LVe	Lateral vestibular nucleus	1	Contra
		-7.78	MdV	Medullary reticular nucleus, ventral part	1	Contra

Male	7	-5.20	PnC	Pontine reticular nucleus, caudal part	1	Contra
		-5.88	LPGi	Lateral paragigantocellular nucleus	1	

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