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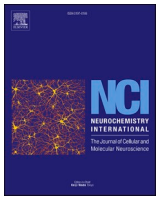

Differential synaptic inhibition and serotonin 5-HT$_7$ receptor-mediated modulation in identified dorsal horn neurons

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ABSTRACT

Serotonergic modulation of pain transmission in the spinal cord involves the activation of multiple receptor types, including 5-HT$_7$ receptors. Activation of spinal 5-HT$_7$ receptors appears to have a predominant anti-nociceptive effect in various animal models. Although the serotonergic modulation of dorsal horn circuits has been extensively investigated, information about the specific effects of serotonergic receptors on identified neuron types remains limited. To address this, we have employed transgenic mice expressing channelrhodopsin-2 (ChR2) in inhibitory neurons, under the control of the vesicular GABA transporter (VGAT) promoter. Post-synaptic inhibitory responses (oIPSCs) induced by optogenetic stimulation of spinal cord slices displayed distinct properties in superficial dorsal horn VGAT+ and VGAT- neurons (inhibitory and putative excitatory neurons, respectively). While oIPSCs recorded from VGAT + neurons showed GABA- and glycine-mediated components of similar amplitudes, oIPSCs from VGAT- neurons were predominantly mediated by glycine. Consistently, immunofluorescence staining for the glycine transporter GlyT2 in mice expressing dTomato in GAD2 neurons revealed that GlyT2+ boutons primarily contact putative excitatory interneurons, which are negative for GAD2. Activation of 5-HT$_7$ receptors by the agonist LP-211 significantly enhanced both the frequency of spontaneous inhibitory currents and the amplitude of oIPSCs in VGAT- neurons. In minimal optical stimulation experiments, application of LP-211 reduced the number of synaptic failures and increased the quantal content of oIPSCs, indicating presynaptic modulation mediated by 5-HT$_7$ receptors. Our results suggest that enhanced synaptic inhibition of dorsal horn excitatory interneurons may contribute to the role of 5-HT$_7$ receptors in suppressing pain transmission at the spinal cord level.

1. Introduction

The spinal cord dorsal horn acts as the first integration center for somatosensory input within the central nervous system. Somatic sensory signals, conveyed by primary afferent fibers of A- and *C*-type, are relayed to dorsal horn neuronal circuits. These circuits comprise excitatory (glutamatergic) and inhibitory (GABAergic and/or glycinergic) interneurons and projection neurons.

In the dorsal horn of rodents, inhibitory interneurons constitute approximately 25 % of the neurons in laminae I-II and 40 % in lamina III (Polgár et al., 2013; Hughes and Todd, 2020). In laminae I-III, about one third of inhibitory interneurons are exclusively GABAergic, while the remaining two-thirds release both GABA and glycine. In deeper laminae, most neurons are also both GABA- and glycinergic, with a strong dominance of glycine (Miranda et al., 2022, 2023). Under physiological conditions, synaptic inhibition regulates the transmission of sensory information to higher centers and prevents activation of nociceptive neurons by tactile fibers. The vesicular GABA transporter (VGAT), expressed in GABAergic, glycinergic, and mixed GABAergic-glycinergic synapses, plays a key role in concentrating GABA and glycine within synaptic vesicles (McIntire et al., 1997; Chaudhry et al., 1998; Dumoulin et al., 1999). Accumulation of glycine in presynaptic terminals also

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require the glycine transporter 2 (GlyT2) that works synergistically with VGAT (Aubrey et al., 2007). Both transporters intervene in the regulation of GABAergic and glycinergic synaptic networks (Dumoulin et al., 1999; Poyatos et al., 1997). In chronic pain conditions, the efficacy of such inhibition is decreased, leading to the development of central sensitization, hyperalgesia, and allodynia (reviewed in Hughes and Todd, 2020; Stachowski and Dougherty, 2021). Consistently, selective optogenetic suppression of VGAT expressing inhibitory interneurons induces mechanical hypersensitivity of wide dynamic range neurons in the dorsal horn (Fujiwara et al., 2024).

Descending projections from the brainstem provide an additional source of GABA and glycine to the dorsal horn. Notably, approximately 80 % of the fibers descending from the rostral ventral medulla (RVM) release these neurotransmitters, while the remaining 20 % release serotonin (Jones et al., 1991; Antal et al., 1996; Kato et al., 2006). Serotonergic fibers that project to the spinal cord originate from RVM and the caudal pons, including the nucleus raphe magnus, the nucleus paragigantocellularis, and the ventral portion of the nucleus gigantocellularis (Kwiat and Basbaum, 1992).

In the dorsal horn, serotonin acts on a wide range of receptor subtypes, such as 5-HT_{1A}, 5-HT_2, 5-HT_3, and 5-HT_7. Among these, 5-HT_7 receptors are expressed at various sites, including inhibitory interneurons, particularly within the deeper laminae (Doly et al., 2005; Brenchat et al., 2010; Lin et al., 2015). Activation of spinal 5-HT_7 receptors has been shown to produce a primarily analgesic effect, as demonstrated in numerous behavioral studies using various animal models of pain (reviewed in Bardoni, 2023). Notably, this analgesic effect is attenuated by spinal administration of the $GABA_A$ receptor antagonist bicuculline (Viguier et al., 2012, 2013). Consistent with these findings, we have recently shown that 5-HT_7 receptors modulate both excitatory and inhibitory synaptic transmission in the dorsal horn, with a predominant enhancement of inhibitory signaling (Comitato et al., 2022).

Despite extensive research on serotonergic modulation in dorsal horn circuits—both *in vitro* and *in vivo*—data on the specific effects of serotonergic receptors, particularly 5-HT_7 receptors, in distinct neuronal populations remain limited. To address this, we tested the effect of a selective 5-HT_7 agonist, LP-211, on a mouse line in which channelrhodopsin-2 (ChR2) is expressed in neurons under the control of the vesicular GABA transporter (VGAT) promoter. Consequently, in these mice, optical stimulation selectively activates all types of inhibitory GABA and/or glycinergic neurons or inhibitory axon terminals in the dorsal horn and allows the functional identification of the recorded neuron as inhibitory or putative excitatory.

2. Materials and methods

Ethical approval

All experiments involving animals were approved by the Italian Ministry of Health following the Guide for the Care and Use of Laboratory Animals and the EU and Italian regulations on animal welfare. All procedures were designed to minimize animal discomfort and to use the fewest number of animals needed for statistical analysis.

2.1. Animals

The electrophysiological experiments were conducted on young adult transgenic mice (P20–P35, N = 39) known as B6.Cg-Tg(Slc32a1-COP4pH134R/EYFP)8Gfng/J, purchased from The Jackson Laboratory, USA; stock #014548). These mice incorporate a BAC transgene that expresses Channelrhodopsin 2 (ChR2) and enhanced yellow fluorescent protein (EYFP) under the control of the VGAT promoter.

Immunoflurescence experiments were conducted on both VGAT-ChR2-YFP (N = 3) and *Gad2tdTomato* (N = 3) male mice (>60 days). *Gad2tdTomato* is a transgenic line expressing the red fluorescent protein dTomato in inhibitory interneurons and were obtained by crossing Gad2Cre mice (The Jackson Laboratory, stock #028867) with Rosa26LSL-tdTomato Ai14 reporter mice line (The Jackson Laboratory; stock 007914).

All animals were housed in plastic cages with soft bedding and maintained on 12 h light/12 h dark cycle at room temperature-controlled conditions. Food and water were available *ad libitum*.

2.2. Drugs

All the components of Krebs' and intracellular solutions were obtained from Sigma-Aldrich (Merck Group, Darmstadt, Germany). LP-211 tartrate was synthesized according to Leopoldo et al. (2008). Aliquots of 1 mM stock solution in dimethyl sulfoxide (DMSO) were initially prepared and then diluted to 1 μM in recording Krebs' solution on the day of the experiment.

Strychnine hydrochloride was obtained from Sigma-Aldrich (Merck Group, Darmstadt, Germany), while SB-269970 hydrochloride and bicuculline methobromide were purchased from Hello Bio (Dunshaughlin, Ireland).

2.3. Spinal cord slice preparation

Spinal cord slices were obtained following the procedure described previously (Salio et al., 2017; Comitato et al., 2022). Briefly, the animals were anesthetized with isoflurane and decapitated; the spinal cord and vertebrae were rapidly removed and placed in ice-cold dissecting Krebs' solution (composition in mM: 95 NaCl, 50 sucrose, 2.5 KCl, 1.25 NaH_2PO4, 26 $NaHCO_3$, 25 glucose, 6 $MgCl_2$, 1.5 $CaCl_2$, and 1 kynurenic acid, pH 7.4, 320 mOsm), bubbled with 95 % O_2 and 5 % CO_2. The lumbar spinal cord was isolated, embedded in an agarose block (low melting point agarose 3 %, Thermo Fisher Scientific, Waltham, MA, United States), and transverse slices (400–500 μm thick) were obtained using a vibrating microtome (Campden Instruments, Loughborough, UK). The slices were maintained in oxygenated incubation Krebs' solution (in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose, 6 $MgCl_2$, and 1 $CaCl_2$, pH 7.4, 320 mOsm) at 32 °C for 20 min and then used for recording.

2.4. Patch-clamp recording

The patch-clamp recording in whole-cell configuration was performed on lamina I and II outer (IIo) neurons at room temperature. The slices were perfused at 2 ml/min with recording Krebs' solution (in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose, 1 $MgCl_2$, and 2 $CaCl_2$, pH 7.4, 320 mOsm). Current-clamp recordings and voltage-clamp recordings of optically-evoked inhibitory postsynaptic currents (oIPSCs) were performed by using a potassium-based intracellular solution with the following composition (in mM): 120 potassium methanesulfonate, 10 NaCl, 10 ethylene glycol bis(2-aminoethyl ether) tetraacetic acid (EGTA), 1 $CaCl_2$, 10 hydroxyethyl piperazineethanesulfonic acid (HEPES), 5 adenosine triphosphate (ATP)-Mg, 0.5 guanosine triphosphate (GTP)-Na, pH adjusted to 7.2 with KOH, and osmolarity 300 mOsm. To reduce noise and enhance the detection of currents, spontaneous inhibitory currents (sIPSCs) were recorded using a cesium-based intracellular solution with the following composition (in mM): 130 cesium methanesulfonate, 10 sodium methanesulfonate, 10 EGTA, 1 $CaCl_2$, 10 HEPES, 2 ATP-Mg, pH adjusted to 7.2 with CsOH, and osmolarity 300 mOsm.

The data were recorded and acquired using a MultiClamp 700A amplifier, a Digidata 1550 digitizer, and pClamp 10 software (Molecular Devices, Sunnyvale, CA, United States). The sampling rate was 10 kHz, and the data were filtered at 2 kHz. Series resistance was not compensated, and cells with a resistance higher than 30 MOhm were discarded. Junction potentials were corrected off-line. Recordings in current-clamp were performed at the beginning of the experiments. Resting potential

was determined within the first 5 min of recording, and cells with a membrane potential more positive than −50 mV were discarded. The neuronal firing pattern was determined by applying at least 10 current steps (amplitude: 10–20 pA; duration: 500 ms). Between steps, the membrane potential was held at approximately −60 mV (near resting potential) and at approximately −80 mV to better reveal the delayed firing pattern, as previously described (Yasaka et al., 2010; Bardoni et al., 2019). Inhibitory postsynaptic currents were recorded in voltage-clamp at −30 mV to minimize large potassium currents, which are activated at more depolarized potentials when using a potassium-based intracellular solution. Recordings began a few minutes after switching to −30 mV to minimize baseline fluctuations caused by inactivating currents. The holding potential was also set to −30 mV for sIPSC recordings using a cesium-based intracellular solution.

Spontaneous IPSCs were analyzed offline using pClamp10 software and Minianalysis (Synaptosoft, United States). The responsivity of individual cells to LP-211 was assessed by performing the Kolmogorov–Smirnov test on cumulative distributions of inter-event intervals. Optically-evoked IPSCs were analyzed using pClamp10 software.

To identify neurons sensitive to LP-211, the method described by Dickie and Torsney (2014) was applied. In detail, linear regression analysis was performed on control data to calculate 95 % prediction bands. A neuron was classified as a responder if the oIPSC peak amplitude fell above the upper limit of the 95 % prediction bands for at least 3 min during LP-211 application (administered for 5 min).

For the quantal content analysis, 30–50 consecutive traces of oIPSCs were recorded, and minimal light stimulation was applied to ensure a sufficient number of failures. Using the method of failures

[m = ln(N/N0)] in the Poisson model, quantal content (m) was calculated, where N is the total number of stimuli, and N0 is the number of failures where stimulation did not evoke a synaptic response (Bang et al., 2021, 2022).

2.5. Optical stimulation

Activation of the channelrhodopsin 2 (ChR2) channel in spinal cord slices from VGAT-ChR2-eYFP mice was achieved through optical stimulation of the slice. The ChR2-mediated current was evoked in VGAT-expressing inhibitory interneurons, by applying a 500-ms blue light (470 nm) stimulus, generated by a High power LED Collimator LCS-0470-03-22 and controlled by BioLED light source control module (both from Mightex, Pleasanton, CA, USA; estimated focal plane power density: 200 mW/mm-^{2}, through a 40× water-immersion objective). To evoke ChR2-mediated current, light was presented as 500 ms pulses with an inter-trial interval of 20 s, which was predicted to correspond to an effective 5 mW/mm^{-2} average power (Stujenske et al., 2015). The pulses were triggered by Digidata 1550 digitizer (Molecular Devices, Sunnyvale, CA, United States) and typically applied between 50 and 90 % of the maximum light intensity. Neurons lacking the ChR2-mediated plateau phase of the response were identified as putative excitatory neurons.

Optically stimulated inhibitory postsynaptic currents (oIPSCs) were evoked at the light intensity that produced an oIPSC of maximum amplitude (between 10 and 50 % of the maximum light intensity). Light pulses had a duration of 10 ms and were applied every 20 s. Minimal optical stimulation was achieved by administering light stimuli of 1 ms duration at 5–10 % of maximum intensity at a frequency of 1 Hz. This stimulation protocol evoked oIPSCs along with synaptic failures.

2.6. Imaging methods

For Immunofluorescence experiments, mice were transcardially perfused under deep pentobarbital anesthesia (30 mg/kg) with Ringer's solution followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (PB). Lumbar spinal cord segments were dissected out and post-fixed for two additional hours in the same fixative. They were then transversally cut with a vibratome (Leica VT1000S; Leica Microsystems) at a thickness of 70 μm.

Free-floating spinal cord sections were pre-incubated in 0.02 M PBS containing 5 % normal goat serum or 6 % bovine serum albumin, for 30 min at room temperature. They were then incubated overnight in primary antibodies at 4 °C: rabbit anti-VGAT (1:500; Synaptic Systems, catalog number: 131003) or guinea-pig anti-GlyT2 (1:500**;** Synaptic Systems - catalog number: 272004). After washing in PBS, sections were incubated for 1 h in the appropriate secondary anti-species antibodies: anti-rabbit Alexa Fluor 488 (1:500, Invitrogen - A11034); anti-guinea-pig biotinylated antibody (1:250; Vector Labs - BA-7000) followed by ExtrAvidin FITC (1:250; Sigma - E2761). Slices were mounted on microscope slides using Fluoroshield mounting medium (Sigma - F6182). Immunofluorescence controls consisting of omission of primary antibodies were routinely performed.

Immunofluorescence was acquired using a Leica TCS SP8 confocal laser scanning microscope and LasX Software (Leica Microsystems). Images were acquired with HC Pl FLUOTAR 20x/0.55 DRY objective and HC PL APO 63×/1.40 OIL objective with Hybrid Detectors (HyD; Leica). Appropriate lasers (Argon or DPSS 561 nm) were selected based on the excitation spectra of the fluorophores. The pinhole was maintained at 1 airy and scanning speed at 200 Hz. The defined settings for a specific fluorophore (laser intensity, gain, emission band) were applied to all the sections with the same staining. A Z-stack was performed per section, with a Z-Step Size of 1.27 μm (as optimized by the LasX, based on the Z-resolution of the objective).

The number of inhibitory synapses contacting putative inhibitory/excitatory interneurons was estimated by counting the number of GlyT2-immunoreactive/VGAT- immunoreactive puncta surrounding lamina I-II cell somata. Specifically, three non-adjacent images from each confocal stack acquisition were selected; the perimeter of all visually identified GAD2+ (inhibitory) and GAD2- (excitatory) interneurons in each optical section was measured; the number of GlyT2-immunoreactive or VGAT- immunoreactive contacts surrounding inhibitory/excitatory interneuron somata in all the three selected optical sections were counted and the mean value per neuron calculated; the linear density of GlyT2 and VGAT contacts was calculated as the ratio between the number of GlyT2 or VGAT contacts and the interneuron's perimeter in microns (multiplied by 100; i.e. number of contacts per 100 μm of membrane).

2.7. Statistical analysis

Electrophysiological data were obtained from a total of 39 mice, 25 males and 14 females. Since there were no differences in the results that were disaggregated by sex, results from both sexes are pooled. Data analysis was performed on a total of 84 lamina I-IIo neurons. Comparisons between groups were performed by using paired t-tests or one way repeated measures ANOVA (Bonferroni post-hoc test). Non-parametric tests were applied when the data were not normally distributed.

The data were expressed as the mean ± SEM, and differences were considered significant for $P < 0.05$. Graphs and statistical analysis were generated using Sigmaplot 16 software (Graphiti, Palo Alto, CA, USA).

The analysis of data from immunofluorescence experiments was performed by using ImageJ software (NIH, Bethesda, MD). Statistical analysis was performed by using the GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Since data display a Gaussian distribution, unpaired *t*-test was used.

3. Results

3.1. Functional characterization of dorsal horn neurons from VGAT-ChR2-YFP mice

Patch-clamp recordings were obtained from dorsal horn neurons primarily located in lamina I and IIo from VGAT-ChR2-YFP mice.

Optostimulation of the spinal cord slice evoked a Channelrhodopsin 2 (ChR2)-mediated current in inhibitory interneurons expressing the vesicular transporter VGAT (VGAT+, Fig. 1A and B). This current, recorded in voltage-clamp at −70 mV, exhibited the characteristic plateau phase after the initial peak (Fig. 1B). An inhibitory postsynaptic current evoked by optical stimulation (oIPSC) was sometimes superimposed on the ChR2 current. This current, mediated by GABA and glycine, appeared as inward due to the reversal potential of chloride being −60 mV under our recording conditions. Putative excitatory interneurons not expressing VGAT (VGAT-) were identified by the absence of the ChR2 current. The majority of these neurons exhibited an inward oIPSC at −70 mV (Fig. 1C).

The firing patterns of VGAT- and VGAT + neurons were assessed at the beginning of the experiment at both −80 and −60 mV. VGAT + neurons predominantly exhibited a tonic firing pattern at both membrane potentials. In contrast, VGAT- neurons mostly fired with a delayed pattern at both −60 and −80 mV (Fig. 1D and E). At −80 mV, the delayed pattern was more frequent compared to −60 mV, likely due to the stronger impact of the potassium I_A current (Yasaka et al., 2010; Melnick, 2011; Sinha et al., 2021).

The analysis of the oIPSCs recorded in voltage clamp at −30 mV revealed differences in amplitude and composition between VGAT+ and VGAT- neurons (Fig. 2). These oIPSCs were mediated by both $GABA_A$ and glycine receptors, as they were completely blocked by bicuculline and strychnine (Fig. 2A and B). oIPSCs recorded from VGAT + neurons tended to show lower amplitudes compared to those recorded from VGAT- neurons. The glycine-mediated component was larger in VGAT- neurons compared to VGAT + neurons, where the GABA and glycine-components had, on average, similar amplitudes (Fig. 2C–E; VGAT-: paired *t*-test, P=<0.01, n = 19; VGAT+: paired *t*-test, P = 0.79, n = 11). The presence of large glycinergic IPSCs in VGAT- neurons located in lamina I and IIo was quite surprising, as GABA is generally considered the predominant inhibitory neurotransmitter in these areas.

3.2. *VGAT+ and GlyT2 + boutons principally contact excitatory interneurons in the superficial dorsal horn*

Vesicular GABA transporter VGAT is highly concentrated in

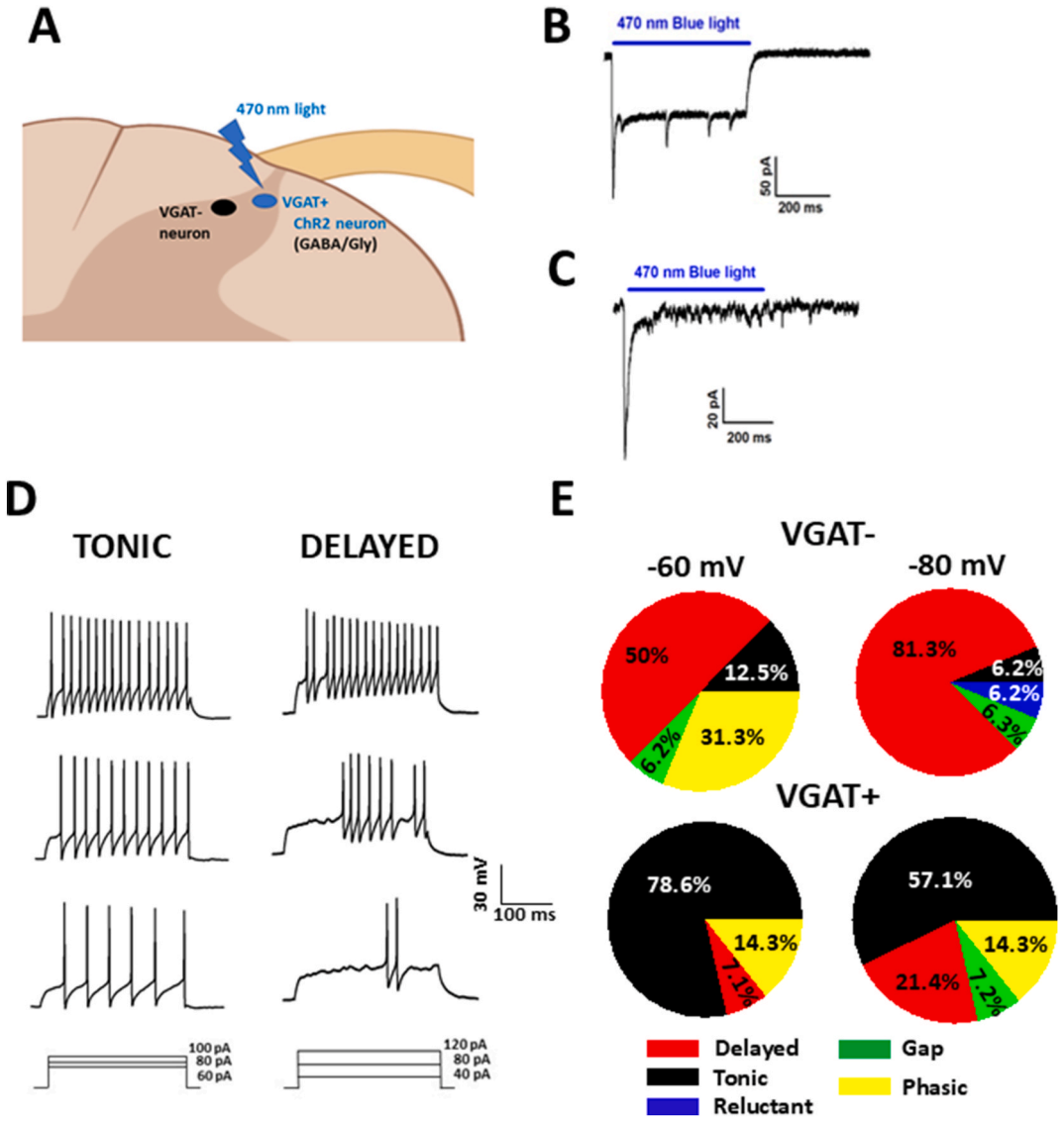

Fig. 1. Excitability properties of inhibitory and putative excitatory lamina I-IIo neurons. A. Schematic image of the spinal cord slice. Optical stimulation activates VGAT-expressing inhibitory interneurons. **B.** Example of a Channelrhodopsin (ChR2)-mediated current recorded from a VGAT + neuron at −70 mV. **C.** In a VGAT- neuron optical stimulation evokes only an inhibitory postsynaptic current (oIPSC). **D.** Examples of tonic and delayed firing patterns obtained in current-clamp at −60 mV and evoked by current steps. **E.** Percentage expression of the different firing patterns recorded at −60 and −80 mV (n = 14, VGAT+; n = 16, VGAT-). VGAT + neurons mostly exhibit a tonic firing pattern, while VGAT- show a prevalent delayed pattern.

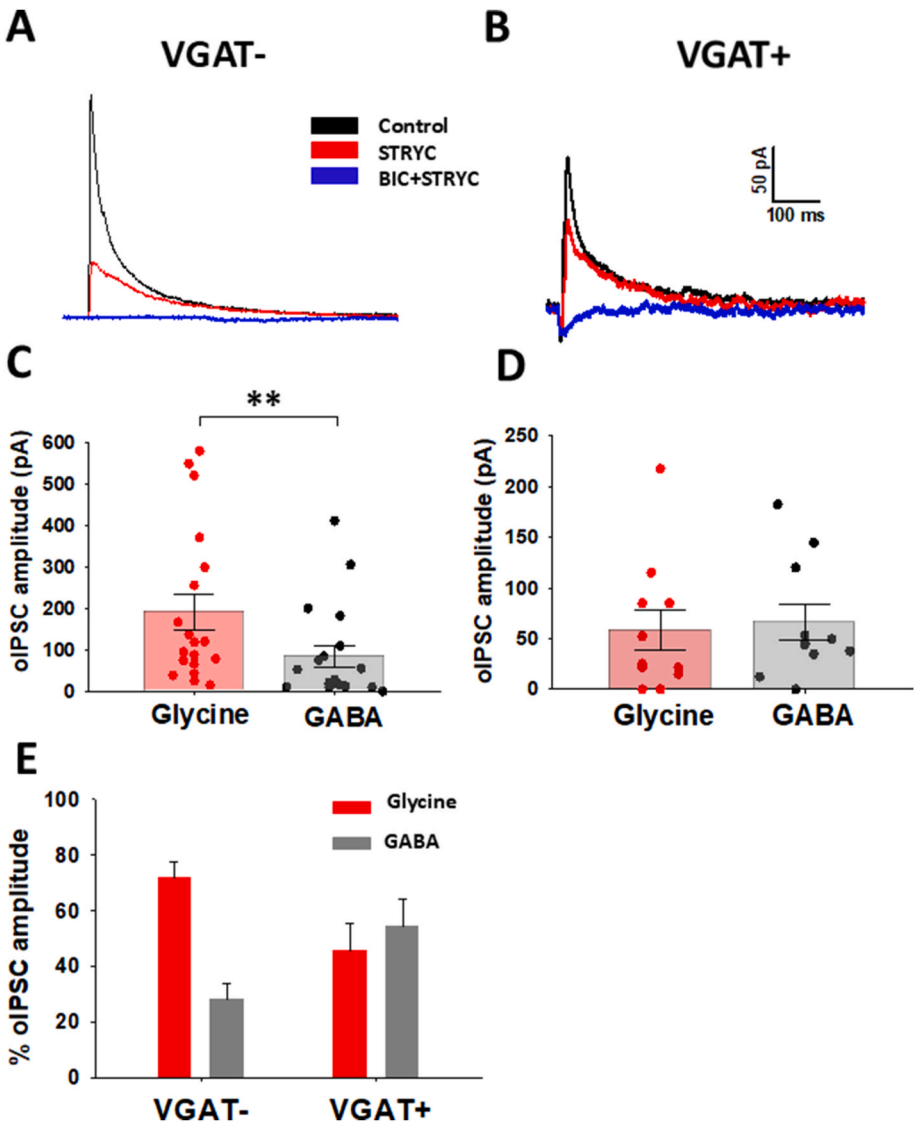

Fig. 2. Inhibitory synaptic responses in VGAT positive and negative neurons are differently mediated by GABA and glycine. **A, B.** Examples of oIPSCs recorded from a VGAT- (A) and a VGAT+ (B) neuron at −30 mV. Both IPSCs were completely blocked by strychnine (1 μM) and bicuculline (10 μM), indicating that they were mediated by glycine and $GABA_A$ receptors. In the VGAT + neuron a small ChR2 current is visible in the presence of the two blockers (blue trace). ***C-E.*** oIPSCs recorded from VGAT- neurons exhibit a prevalent glycinergic component, while in VGAT + neurons glycine- and GABA-mediated oIPSCs have similar amplitudes (VGAT-: **P < 0.01, paired *t*-test, n = 19; VGAT+: P > 0.05, paired *t*-test, n = 11).

GABAergic, glycinergic, and mixed GABAergic-glycinergic axon terminal (McIntire et al., 1997; Chaudhry et al., 1998; Dumoulin et al., 1999). To confirm that VGAT is co-expressed with the glycinergic transporter GlyT2, a marker of glycinergic terminals, we preformed immunofluorescence staining for VGAT and GlyT2 in the spinal dorsal horn of VGAT-ChR2-eYFP mice. Both VGAT and GlyT2 were found at high concentrations in axons and pre-synaptic terminals scattered throughout the entire spinal cord, with a predominance in the dorsal horn. We observed numerous VGAT- positive boutons that were also GlyT2-positive, while others were single labeled for VGAT or GlyT2 (Fig. 3A). To quantify the number of GlyT2+ and VGAT + synapses at inhibitory and excitatory neurons we used a transgenic mouse expressing dTomato in GAD2 neurons, which allows an unambiguous visual identification of the majority of inhibitory interneurons in the dorsal horn (Liu et al., 2021). Both GlyT2 and VGAT boutons make contacts with inhibitory GAD2+ neurons and putative excitatory GAD2- interneurons (Fig. 3B and C). We found that the linear density of GlyT2 terminals surrounding GAD2- cell bodies was almost twice as high as compared to GAD2+ cells (44.9 ± 0.4 puncta per 100 μm in GAD2-*vs* 26.5 ± 0.4 puncta per 100 μm in GAD2+; P < 0.0001; Fig. 3D). Also, the linear density of VGAT puncta was higher in GAD2- cells, but only 20 % more than GAD2+ neurons (35.4 ± 0.5 puncta per 100 μm in GAD2-*vs* 29.2 ± 0.5 puncta per 100 μm in GAD2+; P < 0.0001; Fig. 3E).

All together, these findings suggest that a higher number of glycinergic terminals surround excitatory neurons as compared to inhibitory ones, consistent with electrophysiological results.

3.3. *Activation of 5-HT_7 receptors potentiates spontaneous inhibitory transmission predominantly in VGAT- neurons*

The serotonergic 5-HT_7 receptors have recently been shown to increase the frequency of spontaneous postsynaptic currents (sIPSCs) in dorsal horn lamina II neurons (Comitato et al., 2022). However, it was unclear whether this effect differed between inhibitory and excitatory interneurons. To investigate this, we tested the effect of the specific 5-HT_7 receptor agonist LP-211 (1 μM) on sIPSCs recorded at −30 mV from VGAT+ and VGAT- neurons. Responsive neurons were identified using the Kolmogorov-Smirnov test on the cumulative distribution of

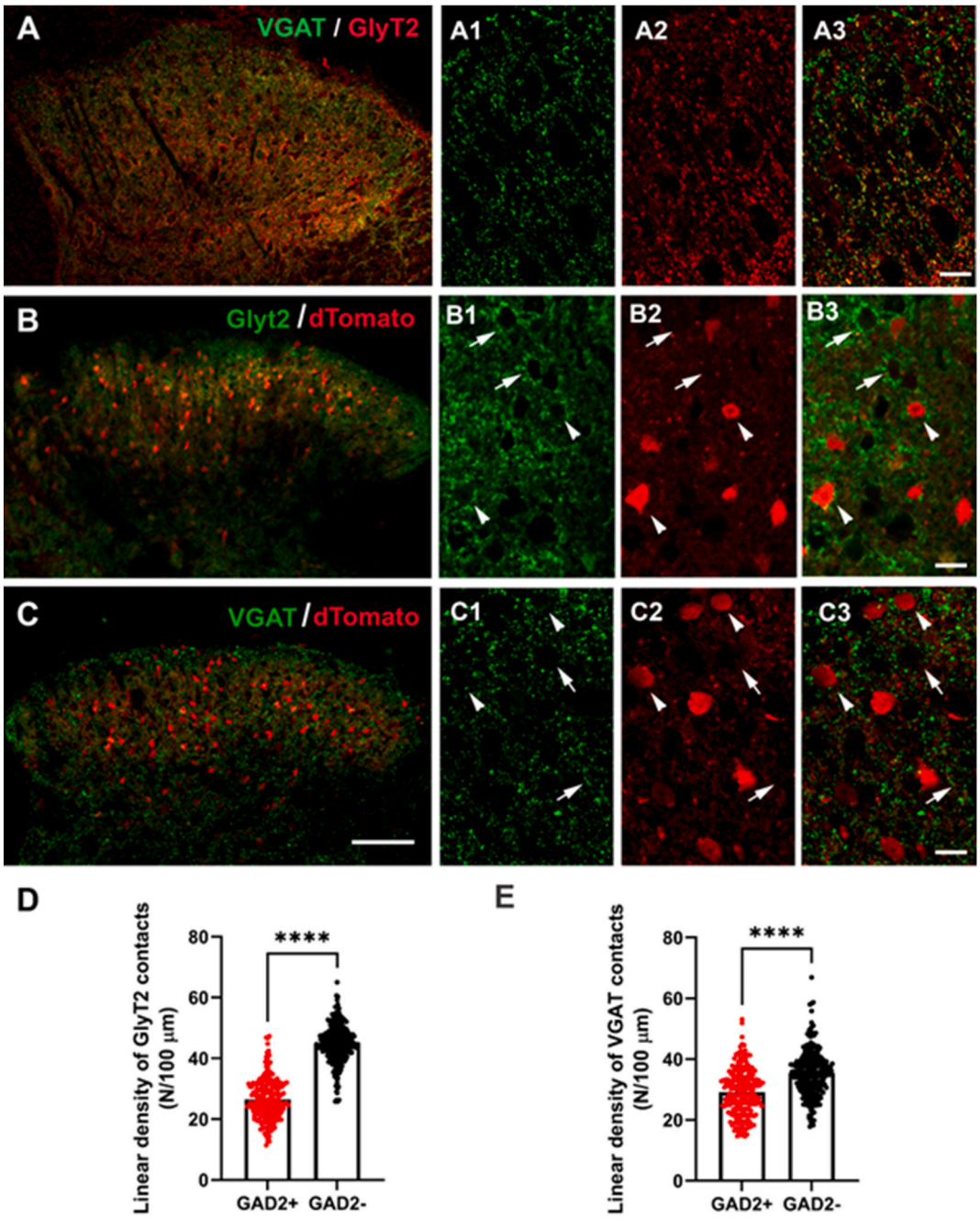

Fig. 3. Distribution of VGAT and GlyT2 in the spinal dorsal horn of VGAT-ChR2-eYFP and *Gad2tdTomato* mice. **A.** Distribution of VGAT (green) and GlyT2 (red) immunoreactivity in the spinal dorsal horn of VGAT-ChR2-eYFP mice. At higher magnification, note that numerous VGAT- positive boutons are also GlyT2-positive (yellow, A3), while others are single labeled for VGAT (green, A1) or GlyT2 (red, A2). **B.** Distribution of GlyT2 immunoreactivity in the spinal dorsal horn of *Gad2tdTomato* mice. GlyT2 immunoreactive dots are apposed to both putative excitatory GAD2- interneurons (arrows, B3), and inhibitory GAD2+ interneurons (arrowheads, B3). **C.** Distribution of VGAT immunoreactivity in the spinal dorsal horn of *Gad2tdTomato* mice. VGAT immunoreactive puncta are apposed to both putative excitatory GAD2- neurons (arrows, C3), and inhibitory GAD2+ interneurons (arrowheads, C3). Scale bar: A–C: 200 µm; A1-A3: 50 µm; B1–C3: 25 µm. **D.** Linear density of GlyT2 contacts on inhibitory GAD2+ neurons (red dots) and excitatory GAD2- neurons (black dots) calculated as the ratio between the GlyT2 contacts and the interneuron's perimeter (multiplied by 100; i.e. number of contacts per 100 µm). Each dot represents a cell (n = 229 GAD2+ cells; n = 249 GAD2- cells; ****P < 0.0001, unpaired *t*-test). **E.** Linear density of VGAT contacts on inhibitory GAD2+ neurons (red dots) and excitatory GAD2- neurons (black dots) calculated as the ratio between the VGAT contacts and the interneuron's perimeter (multiplied by 100; i.e. number of contacts per 100 µm). Each dot represents a cell (n = 229 GAD2+ cells; n = 241 GAD2- cells; ****P < 0.0001, unpaired *t*-test).

inter-event intervals for each cell. This analysis revealed a significantly higher percentage of responsive neurons in the VGAT- population (11/17) compared to VGAT+ neurons (only 2 out of 10) (Fisher's exact test, P = 0.04; Fig. 4B and C). Considering both responsive and non-responsive neurons from both populations, LP-211 induced a significant increase in sIPSC frequency only in VGAT- neurons (Fig. 4D; VGAT-, control: 0.74 ± 0.26 Hz; LP-211: 0.93 ± 0.32, Wilcoxon Signed Rank test, P < 0.001, n = 17; VGAT+, control: 0.33 ± 0.11 Hz; LP-211: 0.29 ± 0.10, Wilcoxon Signed Rank test, P = 0.57, n = 10). The average sIPSC amplitude was not affected by LP-211, in either VGAT negative or VGAT positive neurons (Fig. 4E; VGAT-, paired *t*-test, P = 0.93, n = 17; VGAT+, Wilcoxon Signed Rank test, P = 0.77, n = 10).

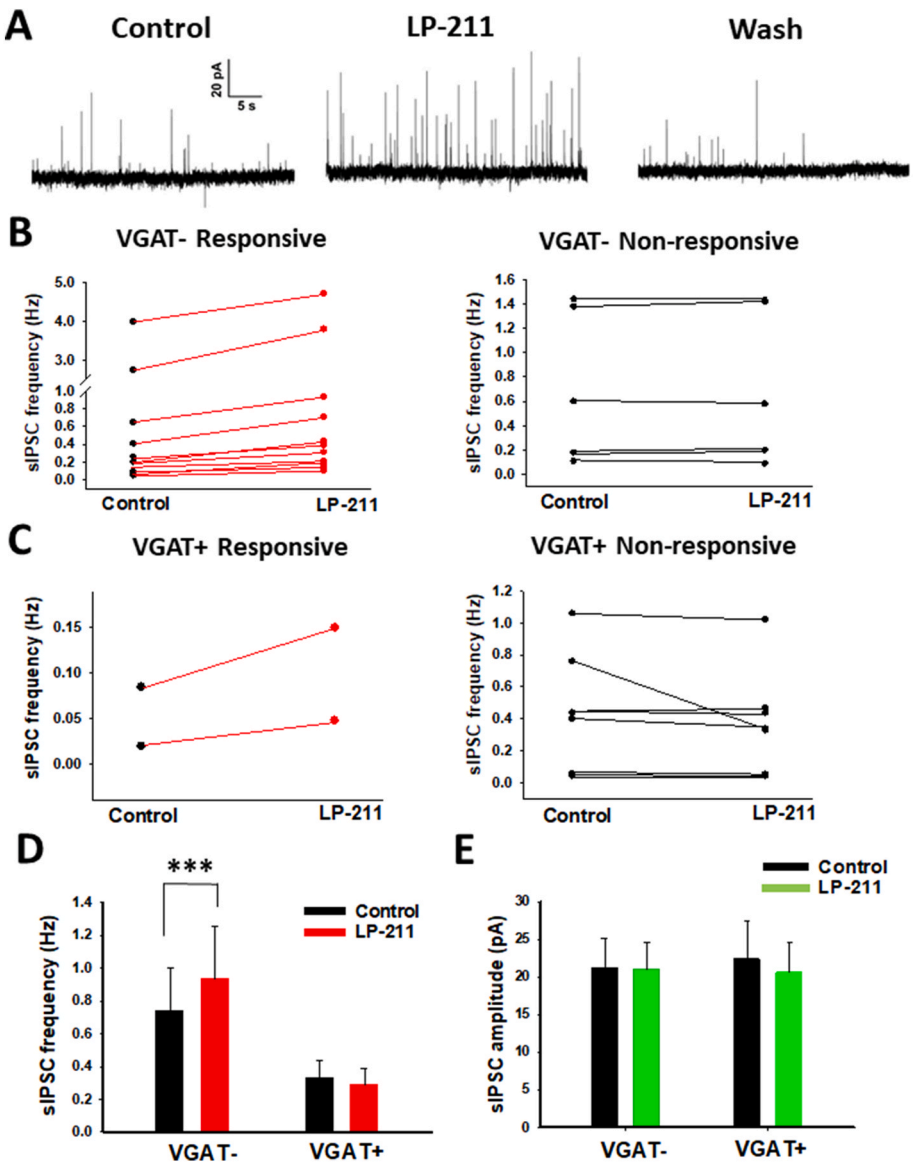

Fig. 4. Activation of 5-HT$_7$ receptors by LP-211 enhances the frequency of spontaneous inhibitory postsynaptic currents. **A**. Examples of spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from a VGAT- neuron at −30 mV. LP-211 (1 μM) induced a reversible increase of sIPSC frequency. **B, C.** sIPSC frequencies recorded in VGAT- neurons and VGAT + neurons in control and LP-211. Eleven VGAT- neurons out of 17 showed a significant increase in the presence of LP-211, as assessed by the Kolmogorov-Smirnov test on inter-event intervals (B). Only 2 out of 10 VGAT + neurons responded to LP-211 (C). **D.** Considering both responsive and non-responsive neurons, LP-211 significantly enhanced sIPSC frequency only in VGAT- neurons, (***P < 0.001, Wilcoxon Signed Rank test). **E.** sIPSC amplitude was not significantly affected by LP-211 application in both neuron populations (VGAT-: paired *t*-test, P = 0.93, n = 17; VGAT+: Wilcoxon Signed Rank test, P = 0.77, n = 10).

LP-211 caused a significant frequency increase of spontaneous excitatory synaptic currents (sEPSCs) in 2 out of 12 VGAT- neurons and in 1 out of 5 VGAT + neurons, where these currents were detectable. However, considering the entire neuron populations, no significant effects of LP-211 on sEPSCs were determined, either in VGAT- neurons (control: 0.15 ± 0.02 Hz, LP-211: 0.15 ± 0.03 Hz, Wilcoxon Signed Rank test, P = 0.3, n = 10), or in VGAT + cells (control: 0.08 ± 0.03 Hz, LP-211: 0.13 ± 0.08 Hz, paired *t*-test, P = 0.37, n = 5).

These results suggest that the activation of 5-HT$_7$ receptors effectively increases spontaneous inhibitory transmission in the superficial dorsal horn, with a minor effect on glutamate release. The effect on inhibitory transmission is more prominent in putative excitatory interneurons and, since the sIPSC amplitude was not affected, it is likely exerted at the presynaptic site.

3.4. Inhibitory synaptic currents evoked by optical stimulation are enhanced by 5-HT$_7$ receptor activation

The modulatory action of LP-211 on inhibitory synaptic transmission was then tested on optically-evoked oIPSCs. In these experiments, we used a light intensity sufficient to evoke an IPSC at maximum amplitude. As shown in Fig. 5A, 1 μM LP-211 increased the oIPSC amplitude in a reversible way. This effect was observed in subpopulations of VGAT-positive and VGAT- negative neurons. Neurons were classified as responsive if the oIPSC peak amplitude fell above the upper 95 % prediction band for at least 3 min of LP-211 treatment (as shown by the example in Fig. 5B, data inside the red circle). Neurons where this did not occur were classified as non-responsive. Using these criteria, it was determined that LP-211 was effective on 8 cells out of 19 in VGAT- neurons, and in 4 out 20 neurons in VGAT + population. In responsive neurons, the mean peak amplitude of oIPSCs was increased by 37.6 ±

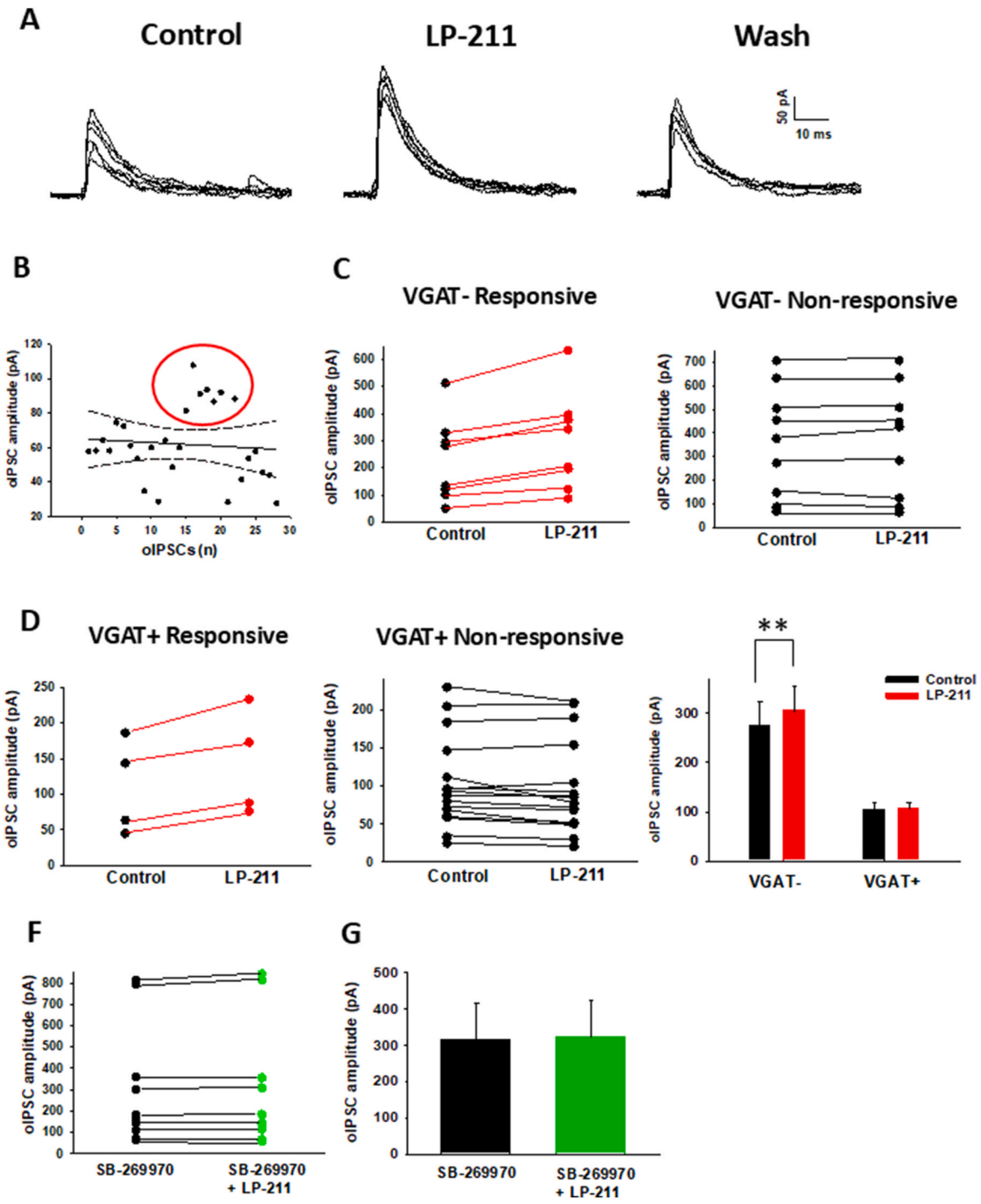

Fig. 5. Optically-evoked IPSCs are potentiated by LP-211 in a subpopulation of lamina I-IIo neurons. A. Traces representing oIPSCs recorded at −30 mV from a VGAT- neuron. LP-211 potentiated oIPSC amplitude in a reversible way. **B.** Example of a neuron classified as LP-211 responder, because the oIPSC amplitudes fell above the 95 % prediction bands (dashed lines) during LP-211 application (dots inside the red circle). oIPSCs were evoked every 20 s. **C, D.** oIPSC amplitudes recorded in control and LP-211 from VGAT- and VGAT + neurons. LP-211 induced a significant increase of oIPSC amplitude in 8 out of 19 VGAT- neurons and only in 4 out 20 VGAT + neurons. **E.** LP-211 significantly increased the mean amplitude of oIPSCs only in VGAT- neurons, considering both responsive and non-responsive neurons (**P < 0.01, paired *t*-test). **F, G.** In the presence of the 5-HT$_7$ antagonist SB-269970 (1 μM), LP-211 failed to potentiate the amplitude of oIPSCs (P > 0.05, paired *t*-test, n = 9).

7.5 % in VGAT- neurons and by 38.0 ± 10.9 % in VGAT + neurons. Considering both responsive and non-responsive neurons from both populations, LP-211 induced a significant increase in oIPSC amplitude only in VGAT- neurons (Fig. 5D; VGAT-: paired *t*-test, P < 0.01, n = 19; VGAT+: paired *t*-test, P = 0.78, n = 20).

Thus, similar to its effect on spontaneous transmission, LP-211 also potentiated IPSCs evoked by optical stimulation, acting predominantly on VGAT- interneurons. As shown in Fig. 5F and G, the effect of LP-211 was specifically mediated by 5-HT$_7$ receptors, as it was not observed in the presence of the 5-HT$_7$ antagonist SB-269970 (1 μM; paired *t*-test, P = 0.28, n = 9).

3.5. 5-HT$_7$ receptors modulate IPSCs evoked by minimal opto-stimulation through a presynaptic mechanism

To further investigate whether a presynaptic mechanism was involved in the potentiation exerted by 5-HT$_7$ receptors on inhibitory transmission, we used a protocol of minimal opto-stimulation (see Methods). This approach allowed us to evoke oIPSCs along with synaptic failures, as shown in Fig. 6A. These experiments were conducted exclusively on VGAT- neurons, which had been shown to be more sensitive to LP-211. Application of the 5-HT$_7$ agonist potentiated oIPSC amplitude in all 9 neurons tested, and the effect was reversible upon washout (Fig. 6B and C; Friedman Repeated Measures Analysis of Variance on Ranks, Tukey post-hoc test, control vs LP-211 and LP-211 versus wash: $P < 0.01$; control versus wash, $P = 0.97$).

The activation of 5-HT$_7$ receptors increased the mean oIPSC amplitude by 67.8 ± 18.0 %, whereas the percentage of failures decreased from 32.2 ± 5.2 % to 13.7 ± 6.2 %. Quantal analysis was performed by using the method of failures and the quantal content (m) was determined by $m = \ln(N/N0)$, as described in the Methods. Application of LP-211 on oIPSCs evoked by minimal stimulation significantly increased the quantal content (Fig. 6D; one way repeated measures ANOVA, Bonferroni post-hoc test: control vs LP-211 and LP-211 versus wash: $P \leq 0.001$; control versus wash, $P = 0.46$). The decrease of synaptic failures and increase of quantal content suggest that activation of 5-HT$_7$ receptors by LP-211 effectively enhances the probability of GABA and glycine release at the presynaptic site. In line with a predominant glycinergic component in putative excitatory VGAT- neurons, IPSCs evoked by minimal optical stimulation were primarily mediated by glycine receptors, as demonstrated by their sensitivity to strychnine (Fig. 6E; paired *t*-test, $P < 0.01$, $n = 8$).

4. Discussion

In this study, we used the transgenic mice VGAT-ChR2-eYFP, in which channelrhodopsin-2 (ChR2) and EYFP are driven by the vesicular GABA transporter and expressed in both GABAergic and glycinergic neurons. This animal model allows for both the activation of inhibitory interneurons and the identification of inhibitory (VGAT+) and putative excitatory (VGAT-) neurons, based on the presence or absence of the ChR2-mediated current. Optical stimulation of spinal cord slices

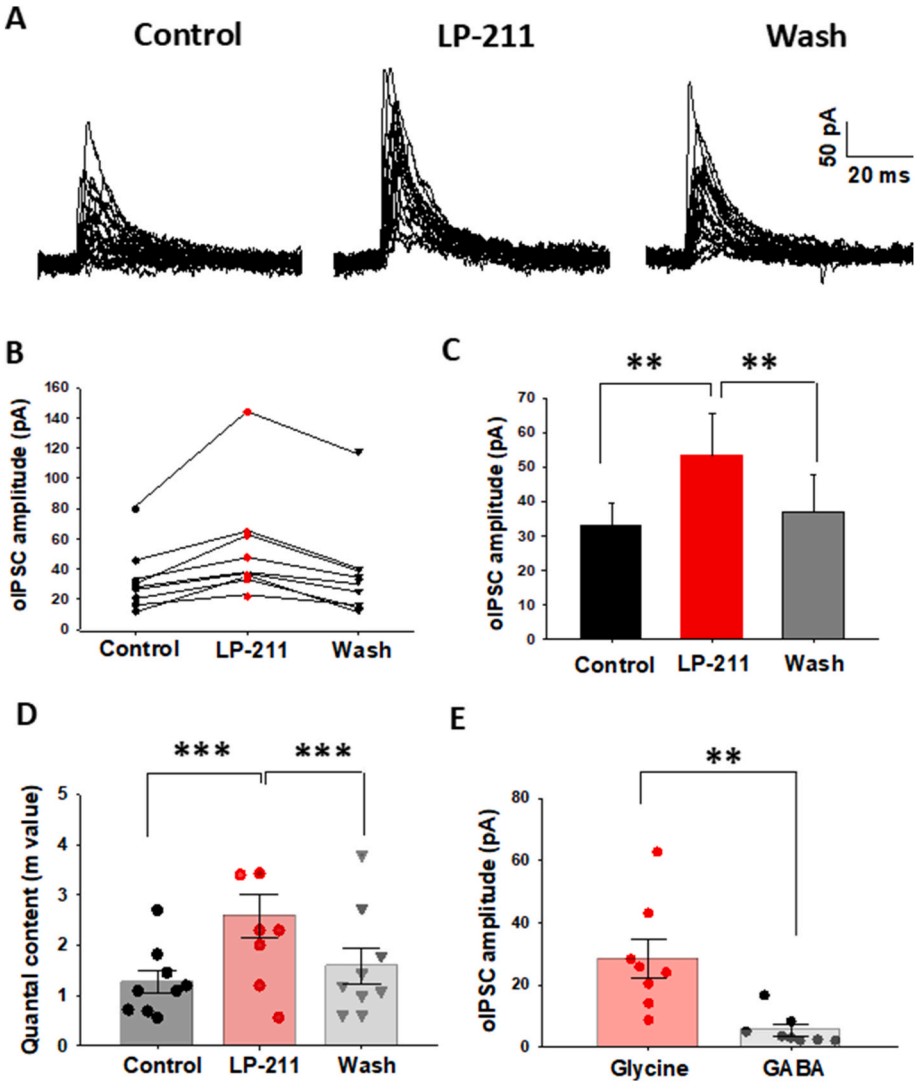

Fig. 6. Activation of 5-HT$_7$ receptors facilitates oIPSCs evoked by minimal optical stimulation in VGAT- neurons A. Examples of oIPSCs evoked by minimal optical stimulation in a VGAT- neuron held at −30 mV. Some synaptic failures are visible only in control and during wash. **B, C**. LP-211 significantly increased the amplitudes of oIPSCs evoked by minimal stimulation in all the 9 neurons tested (**$P < 0.01$, Friedman Repeated Measures Analysis of Variance on Ranks, Tukey post-hoc test**). D.** Quantal content (m), determined using the method of failures, was also significantly increased by LP-211 (***$P \leq 0.001$, One Way Repeated Measures ANOVA, Bonferroni post-hoc test). **E.** oIPSCs were predominantly mediated by glycine receptors (**$P < 0.01$, paired *t*-test, $n = 8$).

obtained from these mice evoked oIPSCs, which were mostly mediated by both $GABA_A$ and glycine receptors and blocked by their respective antagonists bicuculline and strychnine. The co-application of the two antagonists achieved a complete block of the optically-evoked synaptic currents, confirming the selective expression of ChR2 in inhibitory interneurons. These results confirm that VGAT-ChR2-YFP mice represent a valuable model for stimulating and investigating inhibitory interneurons, as previously shown in the spinal cord and other central nervous system areas (see, for example, Xie and Manis, 2014; Caggiano et al., 2014; Adamek et al., 2022; Bang et al., 2022).

We have characterized the features of action potential discharge initiated by depolarizing step injection in VGAT+ and VGAT- neurons. Our data indicates a predominance of tonic firing in VGAT + neurons and delayed firing in VGAT- neurons. These findings are consistent with previous studies using transgenic mice where excitatory and inhibitory neurons were labeled by fluorescent proteins (Daniele and MacDermott, 2009; Takazawa and MacDermott, 2010; Cui et al., 2011; Punnakkal et al., 2014; Browne et al., 2020; Liu et al., 2021). Specifically, Browne et al. (2020) reported that tonic firing was the prevalent response among VGAT + neurons, while delayed and phasic patterns were more frequent in VGAT- neurons. These neurons exhibited a higher presence of the potassium A-type current, which contributes to the generation of the delayed firing pattern. Accordingly, our recordings from VGAT- neurons revealed a higher incidence of the delayed firing at −80 mV compared to −60 mV, likely due to the greater removal of A-type current inactivation at the more hyperpolarized potential (Yasaka et al., 2010; Melnick, 2011; Sinha et al., 2021; reviewed in Rivera-Arconada et al., 2025).

VGAT positive and negative neurons exhibited differences not only in the excitability properties, but also in the nature of their synaptic inhibition. Optically-evoked IPSCs in VGAT- putative excitatory neurons showed a predominant component mediated by glycine receptors, while in VGAT + neurons the glycinergic and GABAergic IPSCs had similar amplitudes.

These data were also supported by our morphological analysis in *Gad2-tdTomato* mice. Indeed, we found that excitatory (GAD2-) neurons receive a higher number of both VGAT+ and GlyT2+ contacts. Previous studies evidenced the importance of VGAT and GlyT2 for GABAergic/glycinergic inhibitory transmission (Eulenburg et al., 2005; Betz et al., 2006) and the involvement of GlyT2 in Gad- immunoreactive boutons (Mackie et al., 2003). Interestingly, we showed that relative higher amount of purely glycinergic input (GlyT2+) at excitatory neurons is more pronounced than that of VGAT + boutons, which identified both glycinergic and GABAergic synapse. These pieces of evidence confirm our functional data, indicating that the higher number of inhibitory contacts at excitatory neurons is mainly due to the glycinergic components.

Data in the literature regarding the relative contributions of glycine and GABA to synaptic inhibition of excitatory and inhibitory neurons in the dorsal horn are often controversial. Earlier studies indicated a prevalence of synaptic inhibition mediated by GABA in neurons located in dorsal horn superficial laminae, either released alone or co-released with glycine (Lu and Perl, 2003; Ferrini et al., 2007; Inquimbert et al., 2007; Takazawa and MacDermott, 2010, 2017; Zheng et al., 2010). More recently, a significant contribution of glycine-mediated transmission in lamina I and outer lamina II has been proposed. Miranda et al. (2022) have demonstrated that pure glycinergic terminals are present in a substantial number in superficial dorsal horn, primarily originating from neurons located in deeper laminae. In laminae I-III, GlyT2 immunostained glycinergic terminals target various populations of dorsal horn excitatory and inhibitory neurons, as well as specific subsets of primary afferent axon terminals, as non-peptidergic C and non-nociceptive Aβ fibers. Accordingly, the presence of substantial glycinergic components in the postsynaptic responses of lamina II neurons has been reported by some more recent electrophysiological studies performing electrical or optical stimulation of inhibitory interneurons in spinal cord slices (Yasaka et al., 2007; Foster et al., 2015; Imlach et al., 2016; Liu et al., 2021). Consistent with these observations, we have recorded large IPSCs mediated by glycine in lamina I and II outer, particularly in putative excitatory neurons. Interestingly, loss of glycinergic inhibition at excitatory neurons has been associated with development of neuropathic pain symptoms after nerve injury (Imlach et al., 2016). Since glycinergic neurons of the dorsal horn are most abundant in lamina III and deeper (Hossaini et al., 2007; Polgár et al., 2013), these responses are likely due to the stimulation of neurons located in the deep dorsal horn, which send their axons to more superficial laminae.

A recent study (Miranda et al., 2023) reported that about one third of the glycinergic terminals in laminae I-II of spinal cord lumbar region originate from the brain stem. This suggests that some of the optically-evoked inhibitory responses in our preparation may result from the stimulation of VGAT- expressing fibers descending from supraspinal sites. Notably, dorsal horn neurons exhibiting a delayed firing pattern (likely excitatory interneurons) have been shown to receive a glycine-dominant descending input from the brain stem (Otsu and Aubrey, 2022).

Activation of 5-HT_7 receptors using the selective agonist LP-211 enhanced both spontaneous and optically evoked inhibitory synaptic transmission. As previously shown (Comitato et al., 2022), LP-211 application significantly increased the frequency of spontaneous inhibitory currents in a subset of dorsal horn neurons. 5-HT_7 receptor-mediated potentiation of inhibitory transmission has been reported also in other central nervous system areas, such as the hippocampus, amygdala and dorsal raphe nucleus (Tokarski et al., 2011; Kusek et al., 2015, 2021).

Spontaneous IPSCs recorded from putative excitatory neurons exhibited a stronger response to LP-211 compared to sIPSCs generated by inhibitory interneurons. Similarly, 5-HT_7 receptor activation more potently enhanced optically-evoked IPSCs in VGAT- neurons, which primarily express glycinergic components. The facilitation of inhibitory synaptic transmission by LP-211 was blocked by the selective 5-HT_7 antagonist SB-269970, confirming the involvement of these receptors.

Multiple cellular mechanisms may contribute to the 5-HT_7 receptor-mediated potentiation of inhibitory transmission in the dorsal horn. Activation of these receptors may enhance the excitability of inhibitory interneurons by increasing the hyperpolarization-activated cation current Ih and/or inhibiting potassium currents (Chapin and Andrade, 2001; Bickmeyer et al., 2002; Siwiec et al., 2020; Goaillard and Vincent, 2002). Furthermore, as observed in both the hippocampus and dorsal horn, 5-HT_7 receptors located on axon terminals may directly modulate neurotransmitter release. This is supported by their ability to increase the frequency of spontaneous IPSCs even in the presence of tetrodotoxin in a subset of neurons (Tokarski et al., 2011; Comitato et al., 2022). Additional evidence from the minimal optical stimulation experiments in this study shows that LP-211 reduces the rate of synaptic failures and increases the quantal content of IPSCs in VGAT- neurons, suggesting a direct modulatory role of 5-HT_7 receptors at presynaptic terminals.

Although several studies have examined the location of 5-HT_7 receptors in the dorsal horn (Doly et al., 2005; Brenchat et al., 2010; Lin et al., 2015), a comprehensive characterization of their expression remains elusive, largely due to the limited availability of selective 5-HT_7 receptor antibodies. Brenchat et al. (2010) reported that 5-HT_7 receptors are highly expressed on inhibitory interneurons, particularly in laminae III-IV, where glycinergic neurons are more abundant. This observation is consistent with our results, which show a pronounced effect of LP-211 on VGAT- neurons that receive substantial glycinergic input. The effect of LP-211 could also be exerted on axon terminals belonging to descending fibers; however, no evidence has yet been presented regarding the expression of 5-HT_7 receptors on these fibers.

An antinociceptive role for spinal 5-HT_7 receptors has been reported in several studies obtained using various animal pain models (reviewed in Bardoni, 2023). The modulation of synaptic inhibitory transmission by 5-HT_7 receptors has been proposed as a potential underlying mechanism. Specifically, in rats with constriction injury of the sciatic nerve,

activation of 5-HT$_7$ receptors exhibited an anti-hyperalgesic effect, which was blocked by intrathecal administration of the GABA$_A$ antagonist bicuculline (Viguier et al., 2012, 2013). Additionally, 5-HT$_7$ receptor activation was found to alleviate mechanical allodynia and prevent the decrease in spinal GABA levels induced by sciatic nerve ligation (Lin et al., 2015).

5. Conclusions

Using optogenetic, electrophysiological, and immunohistochemical approaches, we have observed differential contributions of GABAergic and glycinergic transmission onto inhibitory and excitatory interneurons located in superficial dorsal horn. Furthermore, we have demonstrated that the activation of 5-HT$_7$Rs by the specific agonist LP-211 enhances inhibitory transmission, predominantly onto excitatory interneurons, through a presynaptic mechanism. These finding are consistent with the predominant inhibitory action of 5-HT$_7$Rs on the activity of dorsal horn networks.

CRediT authorship contribution statement

Chiara Salio: Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Data curation, Conceptualization. **Francesco Ferrini:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Data curation, Conceptualization. **Andrea Bighinati:** Writing – review & editing, Investigation, Conceptualization. **Enza Lacivita:** Writing – review & editing, Investigation, Conceptualization. **Marcello Leopoldo:** Writing – review & editing, Investigation, Conceptualization. **Rita Bardoni:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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