METABOTROPIC GLUTAMATE RECEPTORS AND NEUROLOGICAL/PSYCHIATRIC DISORDERS

EDITED BY: Enza Palazzo, Volker Neugebauer and Sabatino Maione
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METABOTROPIC GLUTAMATE RECEPTORS AND NEUROLOGICAL/PSYCHIATRIC DISORDERS

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Considering that neurological and psychiatric illnesses do not still have efficient therapies and are becoming increasingly widespread, the search for novel targets appears fundamental. Neuroinflammation, alterations in adult neurogenesis and excitotoxicity, all associated with glutamate transmission dysfunction, are key components of the pathogenetic mechanisms underlying neuropsychiatric illnesses. Counteracting the neurotoxic actions of glutamate through the modulatory actions of metabotropic glutamate receptors (mGluRs), represents a rational intervention that offers tolerability compatible with clinical therapy. Their suitability as targets for developing novel therapies is also based on the fact that mGluRs are found abundantly expressed in synapses of the central nervous system (CNS) that are considered critical in certain neurological and psychiatric disorders. Moreover, recently there has been an exponential development of selective ligands, especially positive and negative allosteric modulators, which by binding to less conserved transmembrane domain sequences ensure, besides a good permeability in the CNS, an adequate selectivity for each mGluR subtype. The eBook "Metabotropic Glutamate Receptors and Neurological/Psychiatric Disorders", collects contributes (original and review articles) on the beneficial effects of positive and negative allosteric modulators of mGluRs in animal models of neurological and psychiatric diseases from renown experts in the field. The hope is that the effort of both Editors and Contributors of this eBook confirms the unlimited potential of mGluRs in CNS diseases and generates a collaborative discussion on the state of art, overall trends, novel strategies, and future direction of this attractive field of research.

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EDITORIAL
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Editorial: Metabotropic Glutamate Receptors and Neurological/Psychiatric Disorders

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Keywords: mGluRs, neurologcial disorders, psychiatric disorders, neuroinflammation, pharmacological manipulation

Editorial on the Research Topic

Metabotropic Glutamate Receptors and Neurological/Psychiatric Disorders

Neurological and psychiatric disorders significantly impact the quality of life, present devastating symptoms, cause severe disabilities, and are becoming more and more common. No efficient therapies are available at the moment. Thus, revealing novel targets and consequent interventions appears fundamental. Although the term "neurological and psychiatric disorders" encompasses a large number of heterogeneous illnesses, common underlying mechanisms are emerging. Alterations in adult neurogenesis and development of neuroinflammation appear indeed shared hallmarks of both neurodegenerative and psychiatric illnesses. Neurogenesis and neuroinflammation are in turn associated with glutamate, whose excessive or prolonged exposure leads to death of neurons, a key mechanism underlying neurodegenerative diseases. In addition, glutamate hyper- or dys-function has been implicated in the neuropathology of a number of other disorders. Therefore, strategies to control glutamate functions may have therapeutic potential.

Metabotropic glutamate receptors (mGluRs) provide an exciting toolbox to modulate neuronal responses to the pathological actions of glutamate; they are broadly distributed throughout the central nervous system and act on sites and synapses that are considered critical for certain neuropsychiatric disorders. As a consequence, mGluR manipulations produce a variety of responses depending on their different localization on synaptic elements, cell population (neurons or glia), and peripheral or central nervous system sites. In this Research Topic, we aimed for a state of the art summary of our current knowledge about the effect of mGluR manipulations in animal models of neurological and psychiatric diseases. To do so we were fortunate to recruit several renowned experts in the field to share their points of view on the most influential discoveries in this exciting area of research, with particular emphasis on the role and benefits of positive and negative allosteric modulators for mGluR as potential interventions in neurological and psychiatric disorders.

The role of mGluRs in chronic pain is addressed in three contributions. The article by Pereira and Goudet describes the role of mGluRs in pain control and therapeutic strategies aimed at their modulation throughout the pain neuraxis. Mazzitelli et al. review the potential usefulness of group II mGluRs for pain control because of their generally inhibitory synaptic and cellular action and their location on peripheral, spinal, and supraspinal neural elements involved in pain processing and pain modulation. The involvement of the mGluR5 subtype in medial prefrontal cortex (mPFC)

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activity changes associated with pain and depression is addressed by Chung et al. The mPFC represents the executive center for reciprocal interactions between pain and depression, but the role of mGluR5 in these interactions is still controversial.

The involvement of mGluR5 in social behavior and anxiety, however, has been investigated in detail by Ramos-Prats et al. using mGluR5 negative allosteric modulators and mGluR5 knockout strategies. Continuing with the spectrum of neuropsychiatric disorders, Matrisciano et al. addresses the role of mGluR2/3 in a mouse model of prenatal stress-induced schizophrenia, where activation of mGluR2/3 reverted the molecular and behavioral anomalies associated with the early phase of schizophrenia. The therapeutic potential of mGluR7 in chronic psychosis is the focus of the article by Cieślik et al. that describes studies to address the pharmacokinetic and pharmacodynamic properties and *in vivo* effects of negative allosteric modulators for mGluR7 in animal models of schizophrenia. The authors conclude that the mGluR7 receptor is a putative target for novel antipsychotic therapies.

The therapeutic potential of mGluR7 is not limited to psychotic disorders but extends to neurodevelopmental disorders, including intellectual, learning, communication, and motor disabilities, autism spectrum, and attention-hyperactivity disorders. Fisher et al. provide evidence that mutation or decreased expression of mGluR7 is associated with symptoms overlapping those of neurodevelopmental disorders; conversely, positive modulation of mGluR7 proved beneficial in preclinical studies. Vergassola et al. studied the interaction between mGluR1 and $GABA_B$ receptor on GABAergic and glutamatergic cortical terminals. This previously unknown receptor-receptor interaction, which occurs at pre-synaptic level, opens up an unexplored panorama to be exploited for the indirect modulation of inhibitory and excitatory drives in opposite directions, which may have protective and beneficial effects in all neuropsychiatric pathologies associated with "hyperglutamatergism." The role of mGluRs in neuroinflammation is addressed in two review articles that provide the reader with the basics needed to appreciate the seemingly unlimited potential of mGluRs in CNS diseases. Spampinato et al. focuses on mGluRs expressed on glial cells, microglia, astrocytes, and oligondendrocytes, all important players in the development and maintenance of neuroinflammation. Crupi et al. describe the role of each mGluR group and subtype in neuroinflammation-dependent neurological disorders.

And so we hope that this Research Topic highlighting the unique multi-faceted functions of mGluRs in peripheral and central nervous system disorders will make an original contribution to the field that is useful to basic scientists and clinicians interested in understanding CNS mechanisms of neurological and psychiatric disorders and developing new therapies.

AUTHOR CONTRIBUTIONS

EP, VN, and SM contributed and collaborated profitably in the realization and organization of the Research Topic and in the drafting of the editorial article.

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ORIGINAL RESEARCH
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Presynaptic mGlu1 Receptors Control $GABA_B$ Receptors in an Antagonist-Like Manner in Mouse Cortical GABAergic and Glutamatergic Nerve Endings

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Mouse cortical GABAergic synaptosomes possess presynaptic inhibitory $GABA_B$ autoreceptors. Accordingly, (±)baclofen (3 μM) inhibits in a CGP53423-sensitive manner the 12 mM KCl-evoked release of preloaded [^{3}H]GABA. Differently, the existence of presynaptic release-regulating metabotropic glutamate type 1 (mGlu1) heteroreceptors in these terminals is still matter of discussion, although confocal microscopy unveiled the existence of mGlu1α with $GABA_{B1}$ or $GABA_{B2}$ proteins in cortical VGAT-positive synaptosomes. The group I mGlu agonist 3,5-DHPG failed to modify on its own the 12 mM KCl-evoked [^{3}H]GABA exocytosis from cortical nerve endings, but, when added concomitantly to the $GABA_B$ agonist, it significantly reduced the 3 μM (±)baclofen-induced inhibition of [^{3}H]GABA exocytosis. Conversely, the mGlu1 antagonist LY367385 (0.03–1 μM), inactive on its own on GABA exocytosis, amplified the 3 μM (±)baclofen-induced inhibition of [^{3}H]GABA overflow. The (±)baclofen-induced inhibition of [^{3}H]GABA exocytosis was more pronounced in cortical synaptosomes from *Grm1*$^{crv4/crv4}$ mice, which bear a spontaneous mutation of the *Grm1* gene leading to the functional inactivation of the mGlu1 receptor. Inasmuch, the expression of $GABA_{B2}$ receptor protein in cortical synaptosomal lysates from *Grm1*$^{crv4/crv4}$ mice was increased when compared to controls. Altogether, these observations seem best interpreted by assuming that mGlu1 coexist with $GABA_B$ receptors in GABAergic cortical synaptosomes, where they control GABA receptors in an antagonist-like manner. We then asked whether the mGlu1-mediated control of $GABA_B$ receptors is restricted to GABAergic terminals, or if it occurs also in other subpopulations of nerve endings. Release-regulating $GABA_B$ receptors also exist in glutamatergic nerve endings. (±)baclofen (1 μM) diminished the 12 mM KCl-evoked [^{3}H]D-aspartate overflow. Also in these terminals, the concomitant presence of 1 μM LY367385, inactive on its own, significantly amplified the inhibitory effect exerted by (±)baclofen on [^{3}H]D-aspartate exocytosis. Confocal microscopy confirmed the colocalization of mGlu1 with $GABA_{B1}$ and $GABA_{B2}$ labeling in vesicular glutamate type1

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transporter-positive particles. Our results support the conclusion that mGlu1 receptors modulate in an antagonist-like manner presynaptic release-regulating GABA$_B$ receptors. This receptor–receptor interaction could be neuroprotective in central disease typified by hyperglutamatergicity.

Keywords: mGlu1 receptor, GABA$_B$ receptor, receptor–receptor interaction, *Grm1*$^{crv4/crv4}$ mice, release

INTRODUCTION

Dimerization of G protein-coupled receptors (GPCRs) is a necessity for signal transduction, leading from agonist binding to G protein activation. Homodimers originate from the association of two units of single receptor proteins, while heterodimers involve different receptor proteins. Metabotropic glutamate (mGlu) receptors exist as either homo or heterodimers (Doumazane et al., 2010; Nicoletti et al., 2011), while GABA$_B$ receptors are heterodimers (Pin and Bettler, 2016).

GABA$_B$ receptors have a widespread distribution in the central nervous system (CNS) where they mediate the inhibition of chemical transmission. They preferentially locate presynaptically, close to the site of transmitter release, and contribute to control synaptic plasticity. GABA$_B$ receptors exist as auto-receptors on GABAergic nerve terminals (Pittaluga et al., 1987) and as heteroreceptors on non-GABAergic terminals (i.e., the glutamatergic and the peptidergic nerve endings, Bonanno and Raiteri, 1993).

Release-regulating mGlu1 receptors also locate presynaptically in CNS (Pittaluga, 2016) where they control glutamate (Musante et al., 2008) noradrenaline (Longordo et al., 2006) and acetylcholine (Feligioni et al., 2003) release. mGlu1 receptor proteins are largely expressed in GABAergic interneurons. In particular, evidence in the literature demonstrate that the mGlu1 receptor protein exists in GABAergic neurons in the cortex, in the striatum, in the hippocampus and in the cerebellum (Pellegrini-Giampietro, 2003; Ferraguti et al., 2008). The effects that follow mGlu1 receptors activation/inactivation suggest they could have a main role in controlling synaptic plasticity (Battaglia et al., 2001; Pellegrini-Giampietro, 2003). Agonist acting at mGlu1 receptors depress synaptic transmission in the CA1 region of the rat hippocampus (Gereau and Conn, 1995; Morishita et al., 1998), while, in rat corticostriatal slices, it inhibits GABA-mediated inhibitory postsynaptic currents (Battaglia et al., 2001). Because of these actions, antagonists acting at mGlu1 receptors are propose to be neuroprotective (see for a review Pellegrini-Giampietro, 2003). Clear evidence of the presynaptic release-regulating activity of mGlu1 receptors in GABAergic nerve terminals, however, are so far incomplete and deserve further investigation. 3,5-DHPG was reported to increase the spontaneous GABA release from rat parietal-cortical synaptosomes (Bragina et al., 2015), but it failed to affect the release of GABA elicited by a mild depolarizing stimulus from mouse cortical and hippocampal GABAergic nerve endings (Musante et al., 2010; Rossi et al., 2013; Zucchini et al., 2013; Pittaluga, 2016).

Evidence in the literature evidentiate that mGlu1 and GABA$_B$ receptor proteins are co-expressed and physically associate in selected CNS regions (Ige et al., 2000; Tabata et al., 2004; Luján and Shigemoto, 2006; Rives et al., 2009; Tadavarty et al., 2011). Furthermore, activation of GABA$_B$ receptors was shown to increase calcium responses generated by mGlu1 receptors, consistent with the functional cross-talk of the two GPCRs (Hirono et al., 2001; Tabata et al., 2004). Conversely, whether mGlu1 receptors could affect GABA$_B$-mediated responses was not so far investigated.

The present study aimed at confirming the existence of mGlu1 heteroreceptors in cortical GABAergic nerve endings, and, concomitantly, at highlighting if these receptors could influence the release-regulating activity of colocalized presynaptic GABA$_B$ autoreceptors. Based on previous observations showing that mGlu1 receptors could not modify *on its own* the depolarization-evoked release of preloaded [^{3}H]GABA, we posited that cortical synaptosomes could represent an appropriate model to highlight the mGlu1/GABA$_B$ receptor–receptor interaction. The working hypothesis is that, if present in GABAergic nerve endings, the activation of presynaptic mGlu1 receptors could elicit an intra-terminal cascade of events *insufficient* "*per se*" to alter GABA exocytosis, but *sufficient* to modulate intraterminal processes which may affect the functions of other proteins, including the GABA$_B$ subunits (Longordo et al., 2006). The study was also extended to glutamatergic terminals to investigate whether mGlu1 receptor modulates presynaptic release-regulating GABA receptors also in non-GABAergic terminals.

MATERIALS AND METHODS

Animals

Mice (male, strain C57BL/6J) were obtained from Charles River (Calco, Italy) and were housed in the animal facility of DIFAR, Pharmacology and Toxicology Section, under environmentally controlled conditions (temperature = 22°C, humidity = 40%) on a 12-h light/dark cycle with food and water *ad libitum*. Breeding procedures were in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and the ARRIVE guidelines.

Grm1^{crv4} mice with the spontaneous recessive *crv4* mutation were also used. The *crv4* mutation occurred in the BALB/c/Pas inbred strain and consisted of an intronic insertion of a retrotransposon LTR (Long Terminal Repeat) fragment that disrupted the *Grm1* gene splicing, causing the absence of mGlu1 receptor protein. *Grm1*$^{crv4/crv4}$ homozygous mice presented mainly with motor coordination deficits and bone defects (Conti et al., 2006; Musante et al., 2017). Affected (*Grm1*$^{crv4/crv4}$) and control [*Grm1*$^{+/+}$, *wild type (WT)*] mice were maintained on the same genetic background by intercrossing *Grm1*$^{crv4/+}$ mice. The

animals were housed at the animal facility of the IRCCS A.U.O. San Martino-IST (Genoa, Italy). The procedures for breeding and genotyping of *Grm1*$^{crv4/crv4}$ mice were reviewed and approved by the Animal welfare ethical committee of the IRCCS-AOU San Martino-IST National Cancer Research Institute (Genoa, Italy), and definitive approval obtained by the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol number 371). To obtain the genotype of the mouse progeny, DNA was extracted from ear clippings according to the manufacturer's protocol (KAPA Mouse Genotyping Kits). *Crv4* mutation was detected by DNA polymerase chain reaction (PCR) amplification using specific primers as previously described (Musante et al., 2010; Rossi et al., 2013).

All the mice were euthanized by cervical dislocation, followed by decapitation, and the cortices were rapidly removed. The experimental procedures were carried out at the animal facility of DIFAR, Pharmacology and Toxicology Section, and approved by the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol number 02/10/06/2015-OPBA), according to the Guidelines for Animal Care and Use of the National Institutes of Health and according to the Society's Policies on the Use of Animals and Humans in Neuroscience Research. In line with the 3Rs rules (replacement, refinement, and reduction), any effort was made to reduce the number of animals to obtain statistically reliable results. All experiments were performed using adult animals (3–8 months of age).

Preparation of Synaptosomes

Mouse cortical purified synaptosomes were prepared as previously described (Summa et al., 2013). Briefly, the mouse cortex was homogenized in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with Tris-(hydroxymethyl)-amino methane (TRIS, final concentration 0.01 M) with a glass/Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged (1000 x *g* for 5 min) to remove nuclei and debris, and the supernatant was gently layered on a discontinuous Percoll gradient (6, 10, and 20% v/v in Tris-buffered sucrose). After centrifugation at 33,500 x *g* for 5 min, he layer between 10 and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation (20,000 × g for 16 min). Synaptosomes were resuspended in a physiological medium having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.4.

Release Experiments

Synaptosomes were incubated for 15 min at 37°C in a rotary water bath in the presence of [^{3}H]GABA (f.c: 20 nM) or [^{3}H]D-aspartate ([^{3}H]D-Asp, f.c.: 50 nM). Fifty micrometer amino-oxyacetic acid was added during the incubation to avoid GABA catabolism. Identical portions of the synaptosomal suspension were then layered on microporous filters at the bottom of parallel thermostated chambers of a Superfusion System (Raiteri et al., 1974; Pittaluga, 2016; Ugo Basile, Comerio, Varese, Italy). Synaptosomes were then superfused at 0.5 ml/min with physiological medium. Synaptosomes were equilibrated during 36 min of superfusion and starting from t = 36 min superfusate fractions were collected as follows to quantify tritium release: two 3-min samples (basal release), one before (t = 36–39) and one after (t = 45–48 min) a 6-min fraction (t = 39–45 min; evoked release). Synaptosomes were exposed for 90 s, starting from t = 39 min, to high KCl solution (12 mM, NaCl substituting for an equimolar concentration of KCl, Zucchini et al., 2013), in the absence or in the presence of GABA$_B$ receptor and/or mGlu1 receptor agonists and antagonists, as well as protein kinase C (PKC) inhibitor.

The amount of radioactivity released into each superfusate fraction was expressed as percentage of the total synaptosomal radioactivity. The 12 mM KCl -evoked tritium overflow was evaluated by subtracting the neurotransmitter content in the first and in the third fractions collected (basal release, b1 and b3) from that in the 6-min fraction collected during and after the depolarization pulse (evoked release, b2). In all the figures, data are reported as the mean ± SEM of independent determinations obtained in different experiments run in triplicate (at least three superfusion chambers for each experimental condition).

Confocal Microscopy and Colocalization

Mouse cortical synaptosomes were fixed with 2% paraformaldehyde, permeabilized with 0.05% Triton X-100 phosphate-buffered saline (PBS) and incubated overnight at 4°C with the following primary antibodies diluted in 3% albumin PBS: rabbit anti-mGlu1a (1:500), mouse anti-GABA$_{B1}$ (1:500), mouse anti-GABA$_{B2}$ (1:500), guinea pig anti-vesicular GABA transporter (VGAT, 1:300), and guinea pig anti-vesicular glutamate transporter type 1 (VGLUT1; 1:500) as indicated. Synaptosomes were then washed in PBS and incubated for 1 h at room temperature with the following secondary antibodies: donkey anti-mouse AlexaFluor-647, goat anti-guinea pig AlexaFluor-488, goat anti-rabbit AlexaFluor-555 as appropriate. Finally, synaptosomes were applied onto coverslips (Musante et al., 2008). Fluorescence images (512 × 512 pixels) were acquired by a Leica TCS SP5 confocal microscope, through a 63X/1.4 NA objective. Bleed-through of emission spectra was avoided by sequential channel acquisition. The evaluation of colocalized proteins was performed as previously described (Summa et al., 2013), by using the "Colocalization threshold" plugins (WCIF Colocalization Plugins, Wright Cell Imaging Facility, Toronto Western Research Institute, Canada) in the ImageJ 1.51w software (Wayne Rasband, NIH, Bethesda, MD, United States).

Immunoblot Analysis

Cortical synaptosomes from *Grm1*$^{crv4/crv4}$ and WT mice of the same breeding and age were homogenized in lysis buffer [10 mM Tris, pH 8.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1 mM Ethylenediaminetetraacetic acid (EDTA), 5% β-mercaptoethanol]. The protein concentration of the homogenates was determined using the Bradford method. Twenty microgram of total protein was separated on a 4–15% precast polyacrylamide gel (Bio-Rad) by means of SDS–polyacrylamide gel electrophoresis. The concentration of proteins in each sample was on the linear portion of the curve. A triplicate analysis was performed for each lysate sample. Electroblotted proteins were monitored using Naphtol blue black

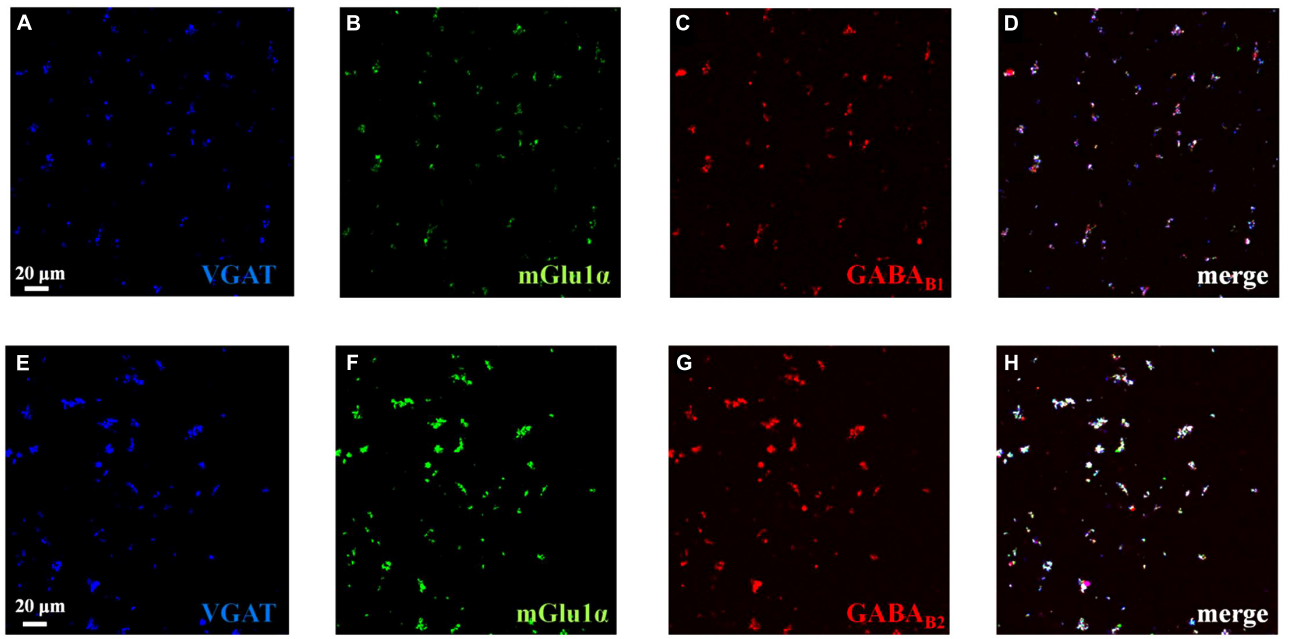

FIGURE 1 | Identification and colocalization (white, **D** and **H**, merge) of GABA$_{B1}$ and of GABA$_{B2}$ and of mGlu1 receptor proteins and vesicular GABA transporter (VGAT) in mouse cortical synaptosomal particles. GABAergic synaptosomes were identified as VGAT-immunopositive particles (blue, panels **A** and **E**) and they were analyzed for the GABA$_{B1}$ receptor protein content (red, panel **C**), for the GABA$_{B2}$ receptor protein content (red, panel **G**) and for the mGlu1α receptor protein content (green, panels **B** and **F**). The figure shows representative images of five independent experiments carried out in different days.

staining. Membranes were then incubated with the following antibodies: mouse monoclonal anti-mGlu1 receptor antibody (1:2500); mouse monoclonal anti-GABA$_{B1}$ receptor antibody (1:500); mouse monoclonal anti-GABA$_{B2}$ receptor antibody (1:500); mouse monoclonal anti-Gapdh antibody (1:10000). After incubation with peroxidase-coupled secondary antibodies, protein bands were detected by using a Western blotting detection system (ECL Advance^{TM}). Bands were detected and analyzed for density using an enhanced chemiluminescence system (Versa-Doc 4000; Bio-Rad), and QuantityOne software (Bio-Rad). All of the protein bands used were normalized for Gapdh level in the same membrane.

Statistical Analysis

For data handling/statistics and for graph drawing Sigma plot 10 data analysis and graphing software package was used. Analysis of variance was performed by ANOVA, followed by Dunnett's or Tukey's multiple-comparisons test; direct comparisons were performed by Student's *t*-test or by Mann Whitney test, as indicated. The level of significance was set at $p < 0.05$.

Chemicals

[2,3-^{3}H]D-aspartate (specific activity 11.3 Ci/mmol) and [^{3}H]GABA (specific activity 30.0 Ci/mmol) were from Perkin Elmer (Boston, MA, United States). (±)-baclofen, LY367385, (RS)-3,5 DHPG, and CGP 52432 were purchased from Tocris Bioscience (Bristol, United Kingdom). GF109203X, aminooxyacetic acid, naphtol blue black staining, horseradish peroxidase-coupled anti-mouse and anti-rabbit secondary antibodies were from Sigma (Milan, Italy). Donkey anti-mouse AlexaFluor-647, goat anti-guinea pig AlexaFluor-488, goat anti-rabbit AlexaFluor-555 were from Life Technologies Corporation (Carlsbad, CA, United States). Mouse anti-GABA$_{B1}$ and mouse anti-GABA$_{B2}$ antibodies were from Santa Cruz Biotechnology (Dallas, TX, United States). Rabbit anti-mGlu1 antibody, used in confocal analysis, was from Abcam (Cambridge, United Kingdom) and mouse anti-mGlu1 monoclonal antibody, used in Western blotting, was from BD Biosciences (San Jose, CA, United States). Guinea pig anti-vesicular glutamate transporters type 1 antibody was from Millipore (Temecula, CA, United States). Guinea pig anti-VGAT was from AlomoneLabs (Jerusalem, Israel). Bradford assay was from Bio-Rad (Segrate, Milan, Italy). KAPA Mouse Genotyping Kits were from Kapa Biosystems (Woburn, MA, United States). ECL Advance^{TM} was from Amersham Biosciences (Piscataway, NJ, United States).

RESULTS

Mouse Cortical GABAergic Nerve Endings Possess mGlu1, GABA$_{B1}$, and GABA$_{B2}$ Receptor Proteins

We performed confocal analysis to detect the presence of mGlu1α, GABA$_{B1}$, and GABA$_{B2}$ immunopositivity in purified cortical synaptosomes that express the VGAT protein, here used as a selective marker of GABAergic particles. We identified a large colocalization (93 ± 3%) of GABA$_{B1}$ (red, **Figure 1C**) or of GABA$_{B2}$ (89 ± 2%) immunostaining

(**Figure 1G**, red) with VGAT in hippocampal particles (blue, **Figures 1A,E**, respectively). Furthermore, VGAT-positive cortical synaptosomes (blue, **Figures 1A,E**, respectively) efficiently stained for mGlu1α receptor protein (green, **Figures 1B,F**, 66 ± 3%). Finally, we analyzed the colocalization of mGlu1α receptor protein (green, **Figures 1B,F**) with GABA$_{B1}$ and GABA$_{B2}$ subunits (red, **Figures 1C,G**, respectively). A diffuse colocalization of mGlu1α immune-positivity with GABA$_{B1}$ and with GABA$_{B2}$ subunit proteins was observed. The impossibility to perform a triple-labeling quantification, however, does not allow speculating the percentage of colocalization of the mGlu1 and GABA$_B$ receptor proteins in the GABAergic synaptosomal particles, although there is a high overlapping of the mGlu1α with either the GABA$_{B1}$ and GABA$_{B2}$ stainings in the VGAT-positive particles (**Figures 1D,H**, merge, white).

Presynaptic Release-Regulating GABA$_B$ Autoreceptors and mGlu1 Heteroreceptors Functionally Interact in Mouse Cortical GABAergic Nerve Endings

Presynaptic release-regulating GABA$_B$ autoreceptors exist in both rat (Pittaluga et al., 1987) and mouse (Lin et al., 1995) cortical synaptosomes. The activation of these receptors hampers the [^{3}H]GABA exocytosis elicited by 12 mM KCl. Accordingly, 3 μM (±)baclofen significantly reduced the 12 mM KCl-evoked overflow of preloaded [^{3}H]GABA from superfused mouse cortical synaptosomes (Bonanno et al., 1997). The (±)baclofen-induced effect was prevented by 0.1 μM of the GABA$_B$ antagonist CGP52342 (12 mM KCl/3 μM (±)baclofen: 68.67 ± 3.47%; 12 mM KCl/3 μM (±)baclofen/0.1 μM CGP52342: 95.45 ± 4.56%, result expressed as percent of residual exocytosis, $n = 5$, $p < 0.05$, see also Raiteri, 2008). CGP52342 alone did not modify the KCl-evoked release of [^{3}H]GABA from cortical synaptosomes (not shown).

Figure 2 shows that the broad spectrum group I agonist 3,5-DHPG (30 μM, i.e., a drug concentration able to fully activate mGlu1 receptor subtypes, Musante et al., 2008) does not affect the 12 mM KCl-evoked release of the radiolabelled transmitter from cortical synaptosomes.

We asked whether activating or inactivating mGlu1 ligands could modulate the release-regulating activity of presynaptic GABA$_B$ autoreceptors. To this aim, experiments were carried out to quantify the impact of the mGlu1 receptor agonist 3,5-DHPG on the (±)baclofen-induced inhibition of [^{3}H]GABA exocytosis. The mGlu1 agonist slightly, although significantly, reduced the 3 μM (±)baclofen-induced inhibition of the 12 mM KCl-evoked [^{3}H]GABA overflow from superfused mouse cortical synaptosomes (**Figure 2**). Conversely, the mGlu1 antagonist LY367385 (0.03–1 μM) significantly amplified the inhibitory effect exerted by 3 μM (±)baclofen on the 12 mM KCl-evoked exocytosis of [^{3}H]GABA. At the maximal concentration applied, the mGlu1 antagonist failed to affect on its own the release of tritium evoked by high KCl (**Figure 3**).

Impact of *Grm1* Mutation on the GABA$_B$ Autoreceptors Controlling GABA Release in Mouse Cortical GABAergic Nerve Endings

We then examined the 12 mM KCl-evoked overflow of preloaded [^{3}H]GABA and its modulation by presynaptic release-regulating GABA$_B$ autoreceptors in cortical synaptosomes from *Grm1*$^{crv4/crv4}$ mice, the mouse mutants bearing a genetic mutation that inactivate the mGlu1 receptor coding gene (*Gmr1*, Conti et al., 2006; Bossi et al., 2018). The release of [^{3}H]GABA elicited by the mild depolarizing stimulus was not affected by the genetic mutation (WT mice, 12 mM KCl-evoked [^{3}H]GABA overflow: 5.19 ± 0.53, $n = 6$; *Grm1*$^{crv4/crv4}$ mice, 12 mM KCl-evoked [^{3}H]GABA outflow: 5.13 ± 0.48, $n = 6$; n.s.; data expressed as KCl-evoked tritium overflow).

Differently the inhibition of the [^{3}H]GABA exocytosis elicited by (±)baclofen (1–10 μM) was significantly reinforced in *Grm1*$^{crv4/crv4}$ mouse cortical synaptosomes when compared to *w.t.* mice (**Figure 4**).

Impact of the *Grm1* Mutation *crv4* on the GABA$_{B1}$ and GABA$_{B2}$ Receptor Proteins Expression in Mouse Cortical Nerve Endings

According to results obtained from release experiments, an altered expression of GABA$_{B1}$ receptor, GABA$_{B2}$ receptor or both could account for functional changes in GABA$_B$-mediated control of GABA exocytosis. Thus, we quantified the amount of GABA$_{B1}$ and GABA$_{B2}$ subunit proteins in cortical synaptosomes isolated from *Grm1*$^{crv4/crv4}$ and WT mice. **Figure 5** shows that the *Grm1* inactivating mutation did not cause a significant change in the GABA$_{B1}$ subunit content when compared to WT mice. Differently, a significant enhancement of the amount of GABA$_{B2}$ subunit was observed in *Grm1*$^{crv4/crv4}$ mice lacking mGlu1 receptors when compared to WT mice.

PKC-Dependent Intraterminal Pathway Links mGlu1 and GABA$_B$ Receptors in Mouse Cortical GABAergic Nerve Endings

GABA$_B$ receptors desensitize and desensitization often occurs because of an enhanced phosphorylation of the GABA$_B$ proteins themselves. In particular, PKC activity attenuates the release-regulating activity of GABA$_B$ receptors by promoting receptor desensitization, through the phosphorylation of the GABA$_{B1}$ subunits and the dissociation from the *N*-ethylmaleimide-sensitive fusion (NSF) protein (Terunuma et al., 2010 and references therein). Activation of mGlu1 receptors triggers PKC-dependent phosphorylative pathways. We asked whether the mGlu1-mediated modulation of the GABA$_B$-induced inhibition of GABA release relies on PKC-mediated events. To this aim, synaptosomes were exposed to GF109203X (0.1 μM), a PKC

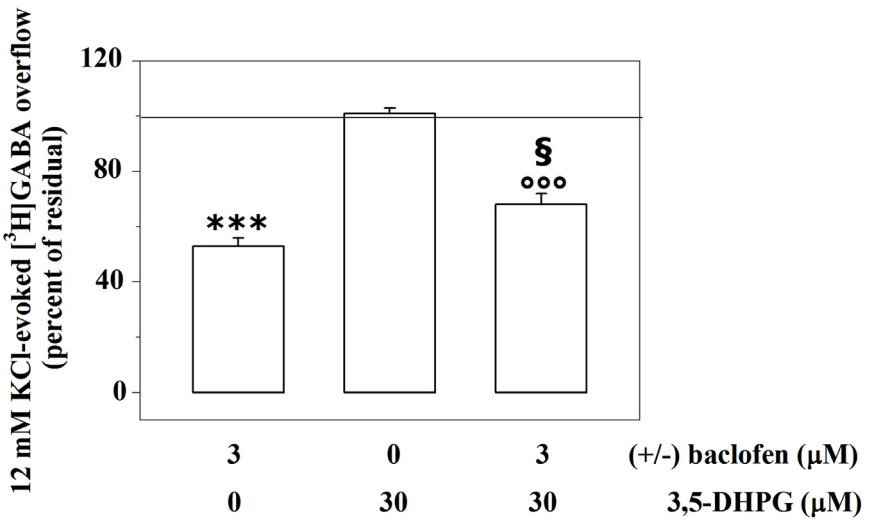

FIGURE 2 | Presynaptic release-regulating GABA$_B$ autoreceptors and mGlu1 heteroreceptors functionally cooperate to control GABA exocytosis from mouse cortical GABAergic nerve endings. Effects of 3 μM (±)baclofen and 30 μM 3,5-DHPG alone or concomitantly added on the 12 mM KCl-induced [^{3}H]GABA overflow from mouse cortical nerve terminals. Results are expressed as percentage of the 12 mM KCl-induced [^{3}H]GABA overflow (percent of residual). Data are the means ± SEM of five experiments run in triplicate. ***$p < 0.001$ versus the 12 mM KCl-induced tritium overflow; °°°$p < 0.001$ versus the 12 mM KCl/30 μM 3,5-DHPG-induced tritium overflow; § $p < 0.05$ versus the 12 mM KCl/3 μM (±)baclofen-induced tritium overflow.

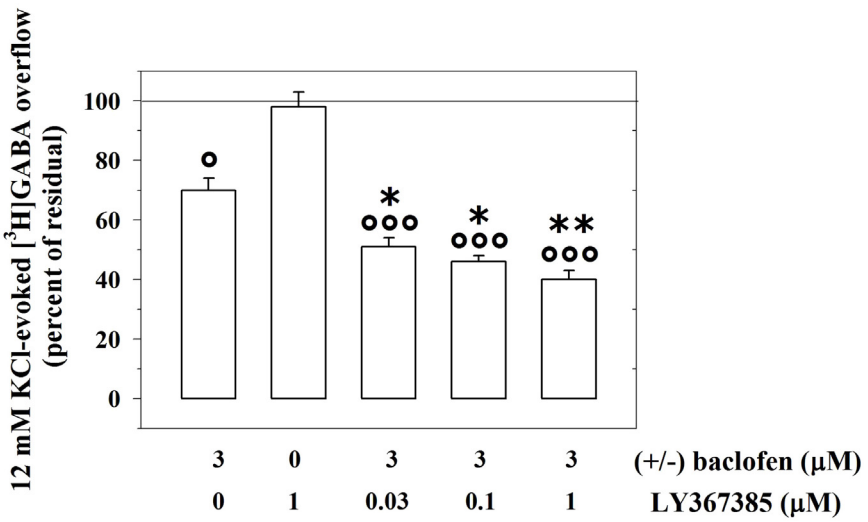

FIGURE 3 | The mGlu1 receptor antagonist LY367385 favors the GABA$_B$ autoreceptor-mediated control of preloaded [^{3}H]GABA from mouse cortical GABAergic nerve endings. Effect of 3 μM (±)baclofen in the absence or in the presence of LY367385 (0.03–1 μM) on the release of preloaded [^{3}H]GABA elicited by 12 mM KCl. Results are expressed as percentage of the 12 mM KCl-induced [^{3}H]GABA overflow (percent of residual). Data are the means ± SEM of seven experiments run in triplicate. °$p < 0.05$ versus the 12 mM KCl-induced tritium overflow; °°°$p < 0.001$ versus the 12 mM KCl-induced tritium overflow; *$p < 0.05$ versus the 12 mM KCl/3 μM (±)baclofen-induced tritium overflow; **$p < 0.01$ versus the 12 mM KCl/3 μM (±)baclofen-induced tritium overflow.

selective blocker, and the impact of 3 μM (±)baclofen on the 12 mM KCl-evoked [^{3}H]GABA overflow was analyzed. **Figure 6** shows that the concomitant presence of the PKC inhibitor caused a huge significant reinforcement of the 3 μM (±)baclofen-induced inhibition of tritium overflow when compared to control condition. The PKC blocker failed to affect on its own the 12 mM KCl-evoked [^{3}H]GABA exocytosis.

Presynaptic Release-Regulating GABA$_B$ Heteroreceptors and mGlu1 Autoreceptors Functionally Interact in Mouse Cortical Glutamatergic Nerve Endings

We asked whether the mGlu1-GABA$_B$ receptor–receptor interaction is restricted to the GABAergic nerve endings or,

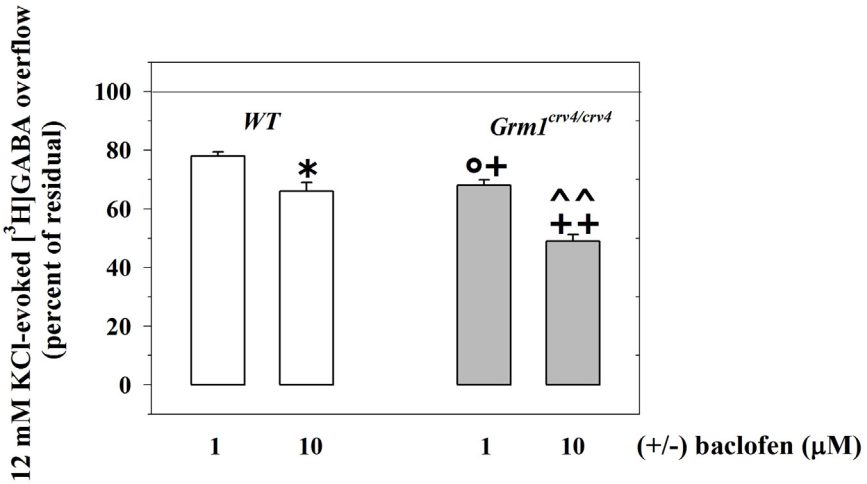

FIGURE 4 | The mGlu1 genetic mutation affect the release-regulating activity of presynaptic GABA$_B$ autoreceptors controlling [^{3}H]GABA exocytosis from cortical GABAergic nerve endings. Synaptosomes from the cortices of control (*WT,* white bar) animals and of *Grm1*$^{crv4/crv4}$ mice (gray bar) were exposed to 12 mM KCl in the absence or in the presence of (±)baclofen (1–10 μM). Results are expressed as percentage of the 12 mM KCl-induced [^{3}H]GABA overflow (percent of residual). Data are the media ± SEM of five experiments run in triplicate (three superfusion chambers for each mouse strain). *$p < 0.05$ versus the 12 mM KCl-evoked tritium overflow from *WT* cortical synaptosomes; $^+p < 0.05$ versus the 12 mM KCl-evoked tritium overflow from *Grm1*$^{crv4/crv4}$ cortical synaptosomes; $^{++}p < 0.01$ versus the 12 mM KCl-evoked tritium overflow from *Grm1*$^{crv4/crv4}$ cortical synaptosomes; °$p < 0.05$ versus the 12 mM KCl/1 μM (±)baclofen-evoked tritium overflow from *w.t.* cortical synaptosomes; ^^$p < 0.01$ versus the 12 mM KCl/10 μM (±)baclofen-evoked tritium overflow from *w.t.* cortical synaptosomes.

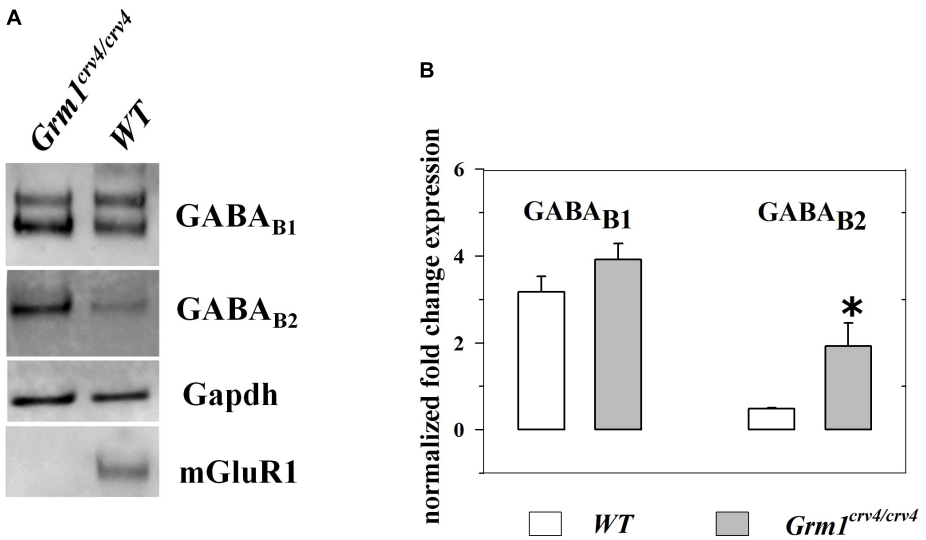

FIGURE 5 | Expression of GABA$_{B2}$ receptor proteins is significantly increased in cortical synaptosomes of *Grm1*$^{crv4/crv4}$ mice. Western blotting analyses were performed to determine the levels of GABA$_{B1}$ and GABA$_{B2}$ receptor expression in cortical synaptosomes ($n = 4$) of *Grm1*$^{crv4/crv4}$, and age-matched *WT* mice. **(A)** Examples of immunoreactive bands obtained from cortical synaptosomes (20 μg proteins/lane) from *WT* and mutated mice. **(B)** Quantification of GABA$_{B1}$ and GABA$_{B2}$ receptor expression in cortical synaptosomes. The relative expression level of GABA$_{B1}$ and GABA$_{B2}$ receptor is expressed as the ratio of GABA$_{B1}$ and GABA$_{B2}$ receptor to the glyceraldeide-3-phosphate dehydrogenase (*Gapdh*) protein. Data represent the mean ± SEM (percentage *versus WT* mice). *$p < 0.05$ versus *WT* cortical synaptosomes.

alternatively, if it also occurs in other subpopulations of nerve endings. Presynaptic release-regulating GABA$_B$ heteroreceptors exist in cortical glutamatergic terminals. By acting at these receptors, (±)baclofen, inhibits significantly the 12 mM KCl-induced [^{3}H]D-Aspartate ([^{3}H]D-Asp) exocytosis (Raiteri, 2008). Accordingly, 1 μM (±)baclofen significantly reduced the 12 mM KCl-evoked overflow of preloaded [^{3}H]D-Asp (**Figure 7**). Furthermore, these terminals also possess mGlu1 autoreceptors, whose activation potentiates the 12 mM KCl-evoked release of [^{3}H]D-Asp (Musante et al., 2008). **Figure 7** shows that, when concomitantly added to 1 μM (±)baclofen, LY367385 (0.1 μM) significantly reinforced the inhibitory effect exerted

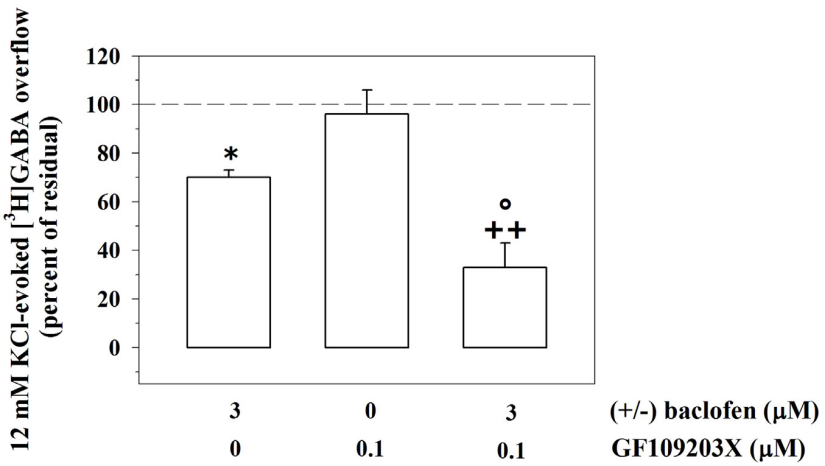

FIGURE 6 | The release-regulating activity of presynaptic GABA$_B$ autoreceptors in cortical GABAergic nerve endings depends on PKC-mediated intraterminal processes. Effects of the PKC inhibitor GF109203X (0.1 μM) on the 12 mM KCl in the absence or in the presence of 3 μM (±)baclofen. Results are expressed as percentage of the 12 mM KCl-induced [^{3}H]GABA overflow (percent of residual). Data are the means ± SEM of four experiments run in triplicate. $^*p < 0.05$ versus the 12 mM KCl-induced tritium overflow; $^{++}p < 0.01$ versus the 12 mM KCl/0.1 μM GF109203X- induced tritium overflow; $^\circ p < 0.05$ versus the 12 mM KCl/3 μM (±)baclofen-induced tritium overflow.

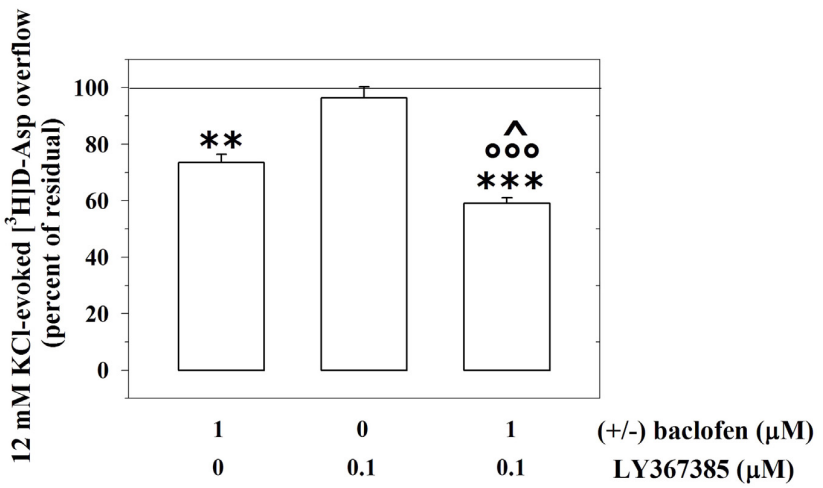

FIGURE 7 | The mGlu1 receptor antagonist LY367385 amplifies the GABA$_B$ heteroreceptor-mediated control of [^{3}H]D-aspartate release from glutamatergic mouse cortical nerve endings. Effect of 1 μM (±)baclofen in the absence or in the presence of LY367385 (0.1 μM) on the release of preloaded [^{3}H]D-aspartate ([^{3}H]D-Asp) elicited by 12 mM KCl. Results are expressed as percentage of the 12 mM KCl-induced [^{3}H]D-Asp overflow (percent of residual). Data are the means ± SEM of five experiments run in triplicate. $^{**}p < 0.01$ versus the 12 mM KCl-induced tritium overflow; $^{***}p < 0.001$ versus the 12 mM KCl-induced tritium overflow; $^{\circ\circ\circ}p < 0.001$ versus the 12 mM KCl/0.1 μM LY367385-induced tritium overflow; $\hat{}p < 0.05$ versus the 12 mM KCl/1 μM (±)baclofen-induced tritium overflow.

by the GABA$_B$ agonist on the 12 mM KCl-evoked overflow of preloaded [^{3}H]D-Asp. At the concentration applied LY367385 did not modify the release of tritium evoked by high KCl (see also Musante et al., 2008).

mGlu1, GABA$_{B1}$, and GABA$_{B2}$ Receptor Proteins Colocalize in Mouse Cortical Glutamatergic Nerve Endings

Confocal microscopy was also performed by labeling cortical synaptosomes with VGLUT1 antibody (blue, **Figures 8A,E**), to highlight glutamatergic nerve endings, and with anti-mGlu1α antibody (green, **Figures 8B,F**) and with antibodies recognizing the GABA$_{B1}$ (red, **Figure 8C**) and the GABA$_{B2}$ (green, **Figure 8G**) receptor proteins. Synaptosomal preparations efficiently stained for all the antibodies tested and the colocalizations of VGLUT1 and mGlu1α receptor proteins, of VGLUT1 and GABA$_{B1}$ receptor proteins and of VGLUT1 and GABA$_{B2}$ receptor protein were analyzed. Merging of the appropriate image pairs revealed that a large percentage of VGLUT1-positive particles expressed mGlu1 receptor proteins (46 ± 7%), as well as GABA$_{B1}$ (52 ± 7%) and GABA$_{B2}$

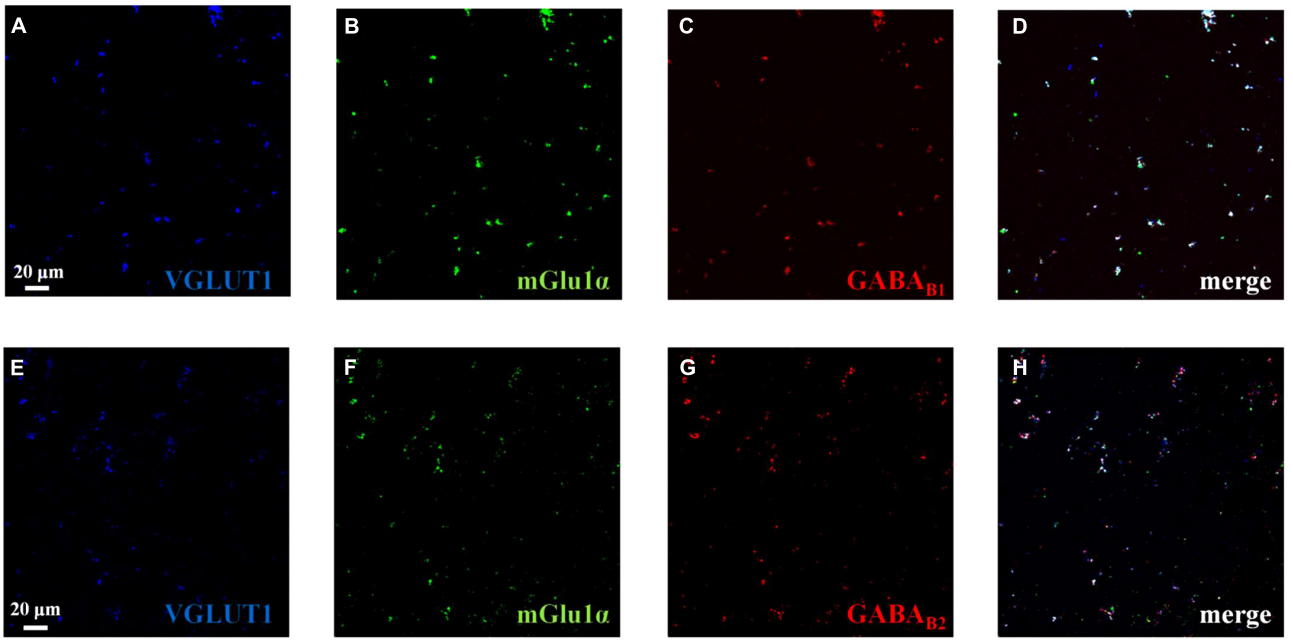

FIGURE 8 | Identification and colocalization (white, **D** and **H**, merge) of GABA$_{B1}$, of GABA$_{B2}$ and of mGlu1 receptor proteins and of VGLUT1 in mouse cortical synaptosomal particles. Glutamatergic synaptosomes were identified as VGLUT1-immunopositive particles (blue, panels **A** and **E**) and they were analyzed for the GABA$_{B1}$ receptor protein content (red, panel **C**), for the GABA$_{B2}$ receptor protein content (red, panel **G**) and for the mGlu1 receptor protein content (green, panels **B** and **F**). The figure shows representative images of four independent experiments carried out in different days.

(45 ± 6%) receptor subunits. Confocal analysis also unveiled a colocalization of mGlu1α staining with GABA$_{B1}$ and GABA$_{B2}$ immuno-positivities. Again, the triple-labeling quantification of the percentage of colocalization of the mGlu1 and GABA$_B$ receptor proteins in the glutamatergic synaptosomes cannot be proposed. However, the merged images (**Figures 8D,H**, merge, white) indicates a high overlapping of the mGlu1α with either the GABA$_{B1}$ and GABA$_{B2}$ stainings in the VGLUT1-positive particles.

DISCUSSION

The existence and the role of mGlu1 receptors controlling presynaptically the release of GABA has been argument of discussion for several years. GABAergic interneurons were proposed to possess mGlu1 receptors the activation of which alters inhibitory transmission, also at GABAergic autapses. This conclusion was also supported by the observations that mGlu1 antagonists are neuroprotectant and that neuroprotection is abolished by increasing the $[GABA]_{out}$ by means of GABA uptake inhibitors (Baude et al., 1993; Gereau and Conn, 1995; Morishita et al., 1998; Battaglia et al., 2001). Most of these studies, however, failed to prove the existence of presynaptic release-regulating mGlu1 receptors in GABAergic nerve endings. Rather, some of them proposed the existence of postsynaptic mGlu1 receptors promoting the endogenous production of cannabinoids that retrogradally modulate GABA release (Alger, 2002; Diana et al., 2002; Gerdeman et al., 2002; Pellegrini-Giampietro, 2003; Ferraguti et al., 2008). The complexity of the scenario is now further implemented by our results which suggest the existence of presynaptic mGlu1 receptors coupled in an "antagonist-like manner" to presynaptic GABA$_B$ receptors.

Neurotransmitters are usually analyzed individually, for their releasing activity, unmindful that they also trigger receptor-mediated events that control the function(s) of other, by-standing, receptors. The complexity that originates from these converging actions is referred to as "metamodulation" and have a huge impact on synaptic transmission in CNS (Marchi et al., 2015). A useful approach to study "metamodulation" is the technique of the "*up-down superfusion of a thin layer of synaptosomes*" (Raiteri et al., 1974; Raiteri and Raiteri, 2000; Pittaluga, 2016). By assuring the rapid removal of any substances endogenously released, this technique prevents the presence of the biophase, then impeding the onset of indirect events due to endogenous compounds acting at presynaptic receptors (including those produced postsynaptically, i.e., the endocannabinoids). This approach represents therefore a method of choice to highlight the functional cross-talk linking presynaptic receptors (Pittaluga et al., 2000, 2005; Musante et al., 2011; Summa et al., 2011; Grilli et al., 2012; Di Prisco et al., 2016). By a functional point of view, the receptor–receptor interaction can be evidentiated in release studies as change(s) in transmitter release efficiency observed when exposing concomitantly synaptosomes to exogenous ligands acting at the colocalized receptors (Longordo et al., 2006; Luccini et al., 2007; Olivero et al., 2018). In general, it is proposed that two receptors coexist and functional couple when the releasing

activity due to their concomitant activations differs quantitatively from the sum of the releasing effects elicited by each receptor (Pittaluga and Raiteri, 1992).

The fact that mGlu1 ligands cannot modify GABA exocytosis (Pittaluga and Raiteri, 1992; Musante et al., 2010; Zucchini et al., 2013) hugely simplifies the system and led us to propose the cortical GABAergic terminals as an appropriate model to investigate the consequences of the mGlu1/GABA$_B$ receptor–receptor interaction. In release studies, we confirmed that neither 3,5-DHPG nor LY367385 caused changes to GABA exocytosis. The two ligands, however, significantly influenced the control of GABA exocytosis elicited by presynaptic GABA$_B$ autoreceptors. In particular, the mGlu1 agonist significantly reduced the (±)baclofen-mediated inhibition of GABA exocytosis, while the orthosteric mGlu1 selective antagonist reinforced it. On the basis of the above considerations, these observations were predictive of the existence of presynaptic mGlu1 heteroreceptors on GABAergic nerve endings and of their functional cross-talk with GABA$_B$ autoreceptors.

The efficacy of the orthosteric antagonist in controlling GABA$_B$-mediated signaling deserves some comments. The lack of biophase makes unlike the possibility that the mGlu1 antagonist can compete with the endogenous glutamate for the binding at the presynaptic mGlu1 heteroreceptors on GABAergic terminals. The possibility, however, exists that, in cortical synaptosomes, mGlu1 heteroreceptors could have adopted a constitutive active conformation (De Blasi et al., 2001). This conformation would assure a productive coupling of the receptor to the associate G protein and the propagation of the mGlu1-mediated signaling, despite the absence of the agonist in the biophase (Musante et al., 2008; Rossi et al., 2013). If this is the case, the binding of the orthosteric antagonist would force a conformational change of the receptor protein, interrupting the coupling of mGlu1 receptors with the G proteins and the associated intraterminal cascade of events that, we speculate, could reverberate on the co-localized GABA$_B$ receptor protein (see below).

Interestingly, the genetic deletion of mGlu1 receptor proteins affects the (±)baclofen-mediated inhibition of GABA exocytosis in a way that is reminiscent of the impact of mGlu1 antagonist on the GABA$_B$ receptor. In particular, the GABA$_B$-mediated inhibition of GABA release in cortical nerve endings from *Grm1*$^{crv4/crv4}$ mice is more efficient when compared to *WT* mice, but it is largely comparable to that observed in the presence of the mGlu1 orthosteric antagonist. To note, in cortical synaptosomes from mutant mice, the expression of the GABA$_{B2}$ receptor subunit, i.e., the subunit that dictates the affinity of (±)baclofen at GABA receptor (Møller et al., 2017 and references therein), is largely increased when compared to *WT* mice. This observation could give the rationale for the changes in the agonist efficacy observed in mutant mice; further studies are required to correctly address this point.

To summarize, the observations depicted so far seem best interpreted by assuming that: (i) mGlu1 heteroreceptors exist in GABAergic terminals; (ii) they colocalize with GABA$_B$ autoreceptors; (iii) the activation of mGlu1 receptors influences the GABA$_B$-mediated control of GABA exocytosis; (iv) the genetic deletion of mGlu1 receptors affect the expression and the presynaptic release-regulating activity of GABA receptors.

The second result of our study is that the "mGlu1 to GABA$_B$" receptor-receptor cross–talk is not restricted to GABAergic nerve endings, but rather represents a wide-spread event that occurs also in other subfamilies of cortical nerve endings, i.e., the glutamatergic ones. Actually, also in cortical glutamatergic synaptosomes, the blockade of the presynaptic mGlu1 autoreceptors reinforced the inhibitory tune exerted by (±)baclofen at inhibitory presynaptic GABA$_B$ heteroreceptors.

In both synaptosomal populations, the "enabling" modulatory effect exerted by mGlu1 antagonists on GABA$_B$ receptors occurs because of the co-existence of the two receptors on the same nerve endings, as confirmed by confocal microscopy showing the overlapping of mGlu1α, GABA$_{B1}$, and GABA$_{B2}$ immunostainings in either the VGAT-containing or the VGLUT1-positive synaptosomal particles. For the sake of clarity, the mGlu1/GABA$_B$ receptor–receptor interaction was already reported in the literature. In particular, in Purkinje cells, a GABA$_B$-mediated control of mGlu1-induced signaling was described, which relied on the physical association of mGlu1 receptor protein with the GABA$_B$ receptor complex. The assembly of the receptor complex was independent on G protein-mediated mechanisms, but dependent on external calcium ions. The final outcome was an increased sensitivity of mGlu1 receptors to glutamate (Dittman and Regehr, 1996, 1997; Vigot and Batini, 1997; Tabata et al., 2004). Unfortunately, in the present case, the role of external calcium in dictating the mGlu1-GABA$_B$ receptor–receptor interaction cannot be investigated, since the removal of the cation from the superfusion medium abrogates *per se* the transmitter exocytosis.

On the basis on the main features of the receptor(s) involved in the receptor-receptor cross-talk, some speculations on the molecular event(s) underlying the receptor-receptor cross-talk can be proposed. It is known that activation of mGlu1 receptors preferentially leads to the translocation of phospholipase C, hydrolysis of membrane phosphoinositide and accumulation of diacylglycerol and calcium ions in the cytosol, which in turn activate PKC-mediated processes, including GABA$_B$ receptor desensitization. Actually, the phosphorylation of the carboxy terminus of the GABA$_{B1}$ subunit assure its dissociation from the NSF protein and the desensitization of the receptor (Terunuma et al., 2010). Blockade of the PKC-dependent phosphorylative processes should therefore be expected to slow GABA$_B$ receptor desensitization, reinforcing its inhibitory control on transmitter exocytosis, as indeed observed with the selective PKC blocker GF109209X. On the basis of these considerations, we propose that the mGlu1-GABA$_B$ receptor-receptor cross-talk involves a PKC-dependent intraterminal phosphorylative pathway which modulate GABA$_B$ receptor desensitization.

CONCLUSION

The results from our study highlight a functional cross-talk linking excitatory glutamatergic receptors (the mGlu1 receptor subtype) and inhibitory GABAergic receptor (the GABA$_B$

complex). We provide evidence that the two receptors colocalize in both glutamatergic and GABAergic terminals and that the mGlu1 receptors tune in an antagonist-like manner the efficiency of the presynaptic release-regulating GABA$_B$ receptors.

Synaptic efficiency depends on the equilibrium between the excitatory and the inhibitory inputs on neurons. mGlu1 antagonist or negative mGlu1 allosteric modulator (NAM) would reinforce the paracrine effect of GABA at GABA$_B$ heteroreceptors located on glutamatergic nerve endings, positively tuning the excessive glutamate release that often characterize central neurological diseases. Concomitantly, mGlu1 antagonist and mGlu1 NAM would reduce GABA exocytosis from GABAergic terminals, because of the reinforcement of the inhibitory tone of GABA$_B$ autoreceptors at this level. The "enhancement" of the paracrine GABAergic control of glutamate release from nerve endings would compensate for the diminished spillover of GABA at GABA$_B$ heteroreceptors Although further studies are required to define the impact of "*in vivo*" administration of mGlu1 ligands on the presynaptic GABA$_B$-mediated control of both transmitters, our findings improve the knowledge of the complex homeostatic mechanisms of control of the excitatory/inhibitory balance in CNS.

AUTHOR CONTRIBUTIONS

APi designed the experiments, supervised the execution of the research activity and the statistical analysis, and wrote the manuscript. GO, MV, and FC performed release experiments. CU performed confocal microscopy. APu and SB made available to the study the *Grm1*$^{crv4/crv4}$ mice, and performed genotyping of these animals and Western blot analysis in both *Grm1*$^{crv4/crv4}$ and *WT* mice. GO, MV, FC, SB, APu, and CU approved the final version of the manuscript and agreed to be accountable for all the aspects of the work.

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An Appraisal of the Influence of the Metabotropic Glutamate 5 (mGlu5) Receptor on Sociability and Anxiety

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Amongst the many neurotransmitter systems causally linked to the expression of social behavior, glutamate appears to play a pivotal role. In particular, metabotropic glutamate 5 (mGlu5) receptors have received much attention as its altered function has been reported in several mouse models of autism spectrum disorders and mental retardation. Inhibition of the activity of mGlu5 receptors by means of genetic or pharmacological manipulations improved social deficits in some of these animal models. However, in normal wild-type (WT) mice, pharmacological blockade of mGlu5 receptors yielded inconsistent results. The aim of our study was to investigate the actual contribution of decreased or absent mGlu5 receptor function in sociability and anxiety-like behavior as well as to explore the impact of mGlu5 receptor ablation on the pattern of brain activation upon social exposure. Here we show that *Grm5*−/− mice display higher social preference indexes compared to age-matched WT mice in the three-chambered social task. However, this effect was accompanied by a decreased exploratory activity during the test and increased anxiety-like behavior. Contrary to mGlu5 receptor ablation, the mGlu5 receptor negative allosteric modulator 3-((2-methyl-1,4-thiazolyl)ethynyl)pyridine (MTEP) induced anxiolytic effects without affecting social preference in WT mice. By mapping c-Fos expression in 21 different brain regions known to be involved in social interaction, we detected a specific activation of the prefrontal cortex and dorsolateral septum in *Grm5*−/− mice following social interaction. C-Fos expression correlation-based network and graph theoretical analyses further suggested dysfunctional connectivity and disruption of the functional brain network generated during social interaction in *Grm5*−/− mice. The lack of mGlu5 receptors resulted in profound rearrangements of the functional impact of prefrontal and hippocampal regions in the social interaction network. In conclusion, this work reveals a complex contribution of mGlu5 receptors in sociability and anxiety and points to the importance of these receptors in regulating brain functional connectivity during social interaction.

Keywords: social behavior, glutamate receptors, anxiety, brain networks, MTEP

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INTRODUCTION

The group I metabotropic glutamate 5 (mGlu5) receptor couples to Gαq/11 proteins to activate a number of intracellular signaling cascades (Hermans and Challiss, 2001; Nicoletti et al., 2011) and regulates synaptic activity and plasticity (Manahan-Vaughan and Braunewell, 2005; Homayoun and Moghaddam, 2010). This receptor is abundantly expressed in telencephalic brain areas

(Ferraguti and Shigemoto, 2006) involved in learning and memory, emotions and in the control of movement and it was found to contribute to a variety of behaviors ranging from cognition to sensory-motor gating and novelty-induced locomotion (Lu et al., 1997; Kinney et al., 2003; Brody et al., 2004; Jew et al., 2013).

A large body of evidence has implicated an altered mGlu5 receptor signaling or expression in the pathology of several neuropsychiatric disorders, including autism, schizophrenia, and anxiety (Nicoletti et al., 2011; Bhakar et al., 2012; D'Antoni et al., 2014; Matosin et al., 2017; Ferraguti, 2018). In particular, enhanced mGlu5 receptor activity has been suggested as one of the underlying mechanisms contributing to several symptoms of fragile X (FX) syndrome, the most common inherited form of intellectual disability (Bhakar et al., 2012). FX-like phenotypes, such as impaired sociability, could be corrected in Fmr1 knock-out mice by reducing the activity of mGlu5 receptors using both genetic and chronic pharmacological treatments (Dölen et al., 2007; Thomas et al., 2011; Bhakar et al., 2012; Gantois et al., 2013). Likewise, altered mGlu5 receptor function was reported in other mouse models of autism spectrum disorders (ASD) and mental retardation (Burket et al., 2011; Chung et al., 2015; Tian et al., 2015; Tao et al., 2016; Vicidomini et al., 2017). The "mGlu5 receptor theory of FX" was recently tested in several phase II, placebo controlled, clinical trials, which, however, did not achieve significant efficacy in the primary end point of improvement on behavioral symptoms (Berry-Kravis et al., 2016; Youssef et al., 2018). Despite the big disappointment and conjectures on the validity of the theory, a number of caveats characterizing these trials may explain why they have failed. First and foremost, the scales of treatment response used in these studies could be biased by caregiver or family involvement in ratings, thus lacking truly quantitative and objective measures of behavioral and cognitive performance. These assessment scales are also known to be subject to a strong placebo effect. Moreover, the study duration and the doses of mGlu5 receptor antagonists utilized might have been inadequate based on preclinical animal data. These results, besides highlighting the difficulty of translating findings from animal models to humans, also call for a new appraisal of the role of mGlu5 receptors in distinct behaviors.

For instance, the influence of mGlu5 receptor antagonism on different aspects of social behavior in wild-type (WT) rodents has been poorly investigated and the current literature contains a number of inconsistent results. The negative allosteric modulator (NAM) 3-((2-methyl-1,4-thiazolyl)ethynyl)pyridine (MTEP) was shown to induce social isolation in rats (Koros et al., 2007), whereas both AFQ056/Mavoglurant and 2-methyl-6-(phenylethynyl)pyridine (MPEP), when administered systemically, elicited no substantial effects on sociability in WT mice (Gantois et al., 2013; Chung et al., 2015). It remains, therefore, unclear whether the complex effects of mGlu5 receptor antagonism in social behavior are due to the limited specificity of systemic pharmacological approaches, species differences in receptor occupancy or pharmacokinetic differences of distinct mGlu5 receptor antagonists (Anderson et al., 2003).

Another complex aspect in the study of mGlu5 receptor in social behavior is its controversial interaction with anxiety. Besides ASD, social function is severely affected in patients with anxiety disorders. The high co-morbidity between social deficits and anxiety can be partially explained by the shared circuitry underlying both anxiety-related and social behavior (for review see: Allsop et al., 2014). Several studies have reported anxiolytic properties of different mGlu5 receptor antagonists, both in animal research and in humans (for review see: Ferraguti, 2018). Nonetheless, to which extent mGlu5 receptor modulation simultaneously regulate anxiety and social behavior in healthy and pathological conditions has not been investigated in depth. Only one study has addressed the role of mGlu5 receptors in the acquisition, expression and extinction of social anxiety in rodent models (Slattery et al., 2017). Overall, the role of mGlu5 receptors in social behavior remains unclear. In the present study, we sought to reassess the effect of mGlu5 receptor ablation or mGlu5 receptor negative allosteric modulation in social preference and anxiety-like behavior, using the classical three-chambered social task (Moy et al., 2004; Nadler et al., 2004) and the light-dark test, respectively. Furthermore, we have investigated the influence of mGlu5 receptors on brain activity patterns upon social and non-social investigation using c-Fos expression as a marker of neuronal activation. Based on these data, we further explored interregional functional connectivity using network analysis to understand at the anatomical level where mGlu5 receptors may regulate functional brain connectivity during social exploration.

MATERIALS AND METHODS

All procedures involving animals were approved by the Austrian Animal Experimentation Ethics Board and were performed in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123). Every effort was taken to minimize the number of animals used. mGlu5 receptor knock-out (*Grm5*−/−) mice (Lu et al., 1997), were backcrossed to C57BL/6J from Charles River Laboratories (Sulzfeld, Germany) for at least 10 generations. Because *Grm5*−/− female mice have deficits in maternal care, breeding was carried out using homozygous male *Grm5*−/− and heterozygous females. Animals were weaned at 4 weeks of age and grouped housed in a climate-controlled facility on a 12 h/12 h light/dark cycle with lights on at 07:00 AM, with water and food *ad libitum*. Genotyping was performed from ear punches and determined by PCR. Since the breeding strategy or postnatal mothering of Grm5−/+ mice were shown not to influence the offspring behavioral phenotype (Brody and Geyer, 2004), age-matched wild type (WT) C57BL/6J mice (purchased from Charles River Laboratories, Sulzfeld) were selected, instead of WT littermates, as control animals for Grm5−/− mice as well as for pharmacological experiments. C57BL/6J mice were allowed to acclimatize for at least 2 weeks before any experimental procedure. Only adult (11–18 weeks old) male mice were used. All experiments were performed during the light cycle. Prior to all experiments, animals were acclimatized to the testing room for at least 2 days.

For pharmacological experiments, mice were injected i.p. with MTEP (Hello Bio; Bristol, United Kingdom; 10 mg/kg diluted

in 4% dimethylsulfoxide in saline) or vehicle. Mice were tested 5 min or 1-h post i.p. injection of MTEP. Dose and time post i.p. injection were chosen based on previous studies on behavioral activity and receptor occupancy of MTEP (Anderson et al., 2003; Busse et al., 2004; Nagel et al., 2015).

Three-Chambered Social Task

Social behavior was assessed by means of a modified three-chambered social task apparatus (Moy et al., 2004; Nadler et al., 2004). The chamber was an opaque glass rectangular box (75 cm long × 30 cm wide × 35 cm tall) divided into three equal compartments, connected through small rectangular doors (7 cm × 7 cm) allowing free access into each chamber. Different illumination conditions were used for pharmacological (infrared light; Lux < 5) and non-pharmacological experiments (Lux < 30). The procedure involved two phases: habituation and sociability. The test mouse was first placed in the middle chamber and allowed to explore all three chambers for 10 min. After this habituation period, a novel unfamiliar mouse (sex, strain and age-matched) was placed into a mesh cylinder (15 cm tall, 7 cm diameter) in the least explored side chamber, whereas an identical empty mesh cylinder was placed in the opposite chamber. The mesh cylinder allowed for air exchange, visual, olfactory and auditory interaction, but prevented fighting. The test mouse was then allowed to explore the chambers for 10 min (sociability). Measurements during the test phase included: distance traveled, latency to explore side chambers at the beginning of the test, time spent in each chamber and in close proximity to the mesh cylinders (<5 cm). Tracking and scoring was performed using Ethovision XT 10 software (Noldus; RRID:SCR_000441). The social preference index for conspecific chamber time was calculated as follows:

$$\frac{(Tc - To)}{(Tc + To)} \times 100$$

Tc = Time spent in conspecific chamber
To = Time spent in object chamber

whereas the social preference index for time in close interaction was calculated as follows:

$$\frac{(Tnc - Tno)}{(Tnc + Tno)} \times 100$$

Tnc = Time spent in close proximity to the conspecific
Tno = Time spent in close proximity to the object

Object Interaction Task

Object interaction was assessed in an identical apparatus as described above. Under infrared light conditions, mice were first allowed to explore the three chambers of the empty apparatus for 10 min. After this habituation, an empty mesh cylinder was placed in the most preferred side chamber and mice were allowed to freely explore the apparatus for 30 min. Tracking and scoring was performed using Ethovision XT 10 software (Noldus; RRID:SCR_000441). Time spent in the object chamber was scored and expressed as the percentage of total test time.

Light-Dark Test

Anxiety-like behavior was tested using the light-dark test. The apparatus (TSE Systems, Bad Homburg, Germany) consisted of a dark (<10 Lux) "safe" compartment and an illuminated aversive compartment (400 Lux). The compartments were connected by a small opening (7 cm × 7 cm wide) located in the center of the partition at floor level. Animals were individually placed in the apparatus facing the opening to the dark compartment and allowed to freely explore the apparatus for 10 min. Time spent in the light compartment was measured using Ethovision (Noldus). In pharmacological experiments, mice tested 5 min after injection in the three-chambered social task were re-used after 48 h in the light-dark test using a counterbalanced design. Conversely, independent mouse cohorts were used for the 1 h post-injection experiments.

Forced Social and Object Interaction for c-Fos Induction

Adult male WT and *Grm5−/−* mice were single-housed for 72 h prior to the test. Mice from each genotype were divided into three groups (n = 7/group): A group exposed to an empty mesh cylinder (object) in the home cage for 10 *min*, a second group exposed in the home cage to a mesh cylinder enclosing an unfamiliar sex and age-matched mouse (conspecific) for 10 min and a control group maintained undisturbed in the home cage (HC). After the test, the cylinder was removed and the mice left in their home cages undisturbed. Mice were then perfused 2 h after the end of the experimental manipulation. Tracking and scoring of the time spent in close proximity (<3 cm) with the object or conspecific was performed using the Ethovision XT 10 software (Noldus). Measurement of exploration time of the novel object or novel conspecific was obtained from an independent batch of mice from those used for c-Fos quantification.

Immunocytochemistry

Mice were deeply anesthetized by i.p. injection of Thiopental (150 mg/kg) and were perfused with a fixative made of 4% w/v paraformaldehyde and 15% v/v of a saturated solution of picric acid in phosphate buffer (PB) 0.1 M pH 7.4, for 12 min. Brains were removed from the skull, washed in 0.1 M PB and sliced coronally in 50 μm-thick sections on a vibratome (VT1000S, Leica Microsystems, Vienna, Austria).

Immunocytochemistry was performed as previously described (Sreepathi and Ferraguti, 2012). Briefly, free-floating sections were first washed with Tris-buffered saline (TBS; 0.9% NaCl, pH 7.4) and then incubated in 20% normal horse serum in TBS and 0.3% Triton X100 (TX) for 1 h at room temperature (RT, 21–23°C). After blocking, sections were incubated with a polyclonal goat primary antibody against c-Fos (1:300; Santa Cruz Biotechnology, Santa Cruz, CA, United States, catalog #sc-52, lot #F1112) for ∼72 h at 6°C. After three washes in TBS, the biotinylated secondary antibody (horse anti-goat IgG 1:500, Vector Laboratories, Burlingame, CA, United States, catalog #BA-9500,) was applied overnight at 6°C at a dilution of 1:500 in a buffer with the same composition as for the primary antibody. The sections were then washed and incubated in Vectastain

elite ABC complex (diluted 1:100; Vector Laboratories) in TBS at RT for 1 h. Subsequently, the sections were washed in TB several times, pre-incubated with 3,3′-diaminobenzidine (DAB; 0.5 mg/ml) for 10 min and then H_2O_2 was added to the solution at a final dilution of 0.003% for 2–5 min. Sections were then washed with TBS, mounted in gelatin onto glass slides, air-dried, and then treated with graded ethanol (50, 70, 90, 95, and 100%) and butyl acetate. Finally, slides were coverslipped with Eukitt (Agar Scientific Ltd., Stansted, United Kingdom).

The following brain structures, relevant for social behavior (Kim et al., 2015), were selected for c-Fos quantification and identified based on the mouse brain atlas of Franklin and Paxinos (2007): medial orbital cortex (MO; bregma between +2,8 and +2,22 mm), prelimbic cortex (PrL; bregma between +2,34 and +1,54 mm), infralimbic cortex (IL; bregma between +1,94 and +1,54 mm), accumbens nucleus shell (AcbSh; bregma between +1,42 and +1,18 mm), accumbens nucleus core (AcbC; bregma between +1,42 and +1,18 mm), lateral septal nucleus intermedial part (LSI; bregma between +0,62 and +0,14 mm), lateral septal nucleus dorsal part (LSD; bregma between +0,62 and +0,14 mm), piriform cortex (Pir; bregma between +0,98 and +0,50 mm), medial septal nucleus (MS; bregma between +0,98 and +0,50 mm), medial preoptic nucleus medial part (MPOM; bregma between +0,0,02 and −0,22 mm), paraventricular thalamic nucleus (PV; bregma between −0,22 and −0,58 mm), paratenial thalamic nucleus (PT; bregma between −0,22 and −0,58 mm), reuniens thalamic nucleus (RE; bregma between −0,46 and −0,70 mm), basolateral amygdala (BLA; bregma between −0,94 and −1,46 mm), dorsal hippocampus (CA1, CA2, CA3, DG, bregma between −1,58 and −1,94 mm), lateral hypothalamic area (LH; bregma between −2,18 and −2,46 mm), posteromedial cortical amygdaloid area (PMCo; bregma between −2,18 and −2,46 mm) and periaqueductal gray (PAG; bregma between −2,92 and −3,16 mm). The number of c-Fos positive cells/area was semi-automatically counted with the Neurolucida software (Version 11, MBF Bioscience, RRID:SCR_001775) coupled to an Olympus BX51 Microscope by an experimenter blinded to the treatment condition and genotype. Each brain area was analyzed bilaterally across at least three sections using a sampling window (200 μm × 200 μm) placed always in the same position within the selected area.

Brain Network Construction and Graph Theoretical Analysis

Network analyses were performed as previously described (Tanimizu et al., 2017a). Briefly, Pearson *r*-values from interregional c-Fos expression data from home cage, object-exposed and conspecific-exposed groups from both genotypes were obtained and used to generate correlation matrices. In order to compare average correlations between groups/genotypes, *r*-values were transformed to Fischer *Z*-values, statistics were calculated, and values were retransformed to r values for graph representation. To characterize the generated social and object interaction networks in both genotypes, positive ($r > 0.60$) interregional c-Fos correlations with a significance level of $p < 0.05$ were used to generate unweighted network graphs. Community clustering to generate weighted network graphs was performed based on modularity optimization, according to Newman and Girvan (2004). Finally, participation coefficient and within-community *z* scores were calculated as defined in Guimerà and Amaral, 2005 and plotted as described by Tanimizu et al. (2017a) in order to visualize the main hubs in the generated networks. Interregional correlation matrices were obtained with Prism 7 software (GraphPad Software Inc., RRID:SCR_002798). Network construction and visualization were performed in R (version 3.3.3) using the igraph (version 1.1.2; Csardi and Nepusz, 2006) and brainGraph (version 1.0.0) packages.

Data Analysis

Data were analyzed with the Prism 7 software (GraphPad Software Inc.) using two-tailed Student's *t*-tests or analysis of variance (one-way or two-way ANOVA, factorial or repeated measures). Whenever an ANOVA resulted significant, Holm–Sidak *post hoc* comparisons were applied to analyze the effects of group, genotype, treatment, time and chamber in the behavioral experiments. Two-way ANOVA followed by a *post hoc* Newman–Keuls comparison was used to analyze the effects of genotype and groups in the c-Fos mapping experiment. Two-way ANOVA followed by a *post hoc* Bonferroni comparison was used to analyze differences in network density. Data were considered significant when $p < 0.05$.

RESULTS

Effects of mGlu5 Receptor Ablation on Sociability and Anxiety-Like Behavior

We investigated the role of mGlu5 receptors in sociability using the classical three-chambered social task apparatus, where sociability is measured as the preference for interacting with an enclosed conspecific placed in one of the side chambers as compared to a novel object (an empty cage) placed in the opposite side chamber. At first, we examined germ-line *Grm5*−/− mice and compared them to age-matched WT C57BL/6j mice. During the sociability test, both *Grm5*−/− and WT control mice displayed sociability, spending significantly more time in the social chamber than in the novel object chamber [2-way ANOVA: chamber $F(2,90) = 133$, $p < 0.001$; chamber × genotype $F(2,90) = 8.06$, $p < 0.001$; novel object vs. novel mouse chamber: WT: $p < 0.001$; *Grm5*−/−: $p < 0.001$] (**Figures 1A,B**). However, *Grm5*−/− mice spent less time than WT mice investigating the novel object chamber ($p < 0.05$) and spent significantly more time in the middle chamber during the test ($p < 0.05$). Similarly, time in close proximity to the novel mouse was higher than for the novel object for both genotypes [2-way ANOVA: close interaction $F(1,60) = 49.7$ $p < 0.001$, and genotype $F(1,60) = 6.97$, $p < 0.05$] (**Figure 1C**), whereas the overall time in close interaction with the conspecific or object did not differ between genotypes [2-way ANOVA: close interaction × genotype $F(1,60) = 2.52$ $p = 0.11$]. *Grm5*−/− mice showed a higher social preference index for both conspecific chamber time [*t*-test: $t(1,30) = 2.18$, $p < 0.05$] (**Figure 1D**) and time in close interaction with the conspecific [*t*-test: $t(1,30) = 3.178$, $p < 0.01$]

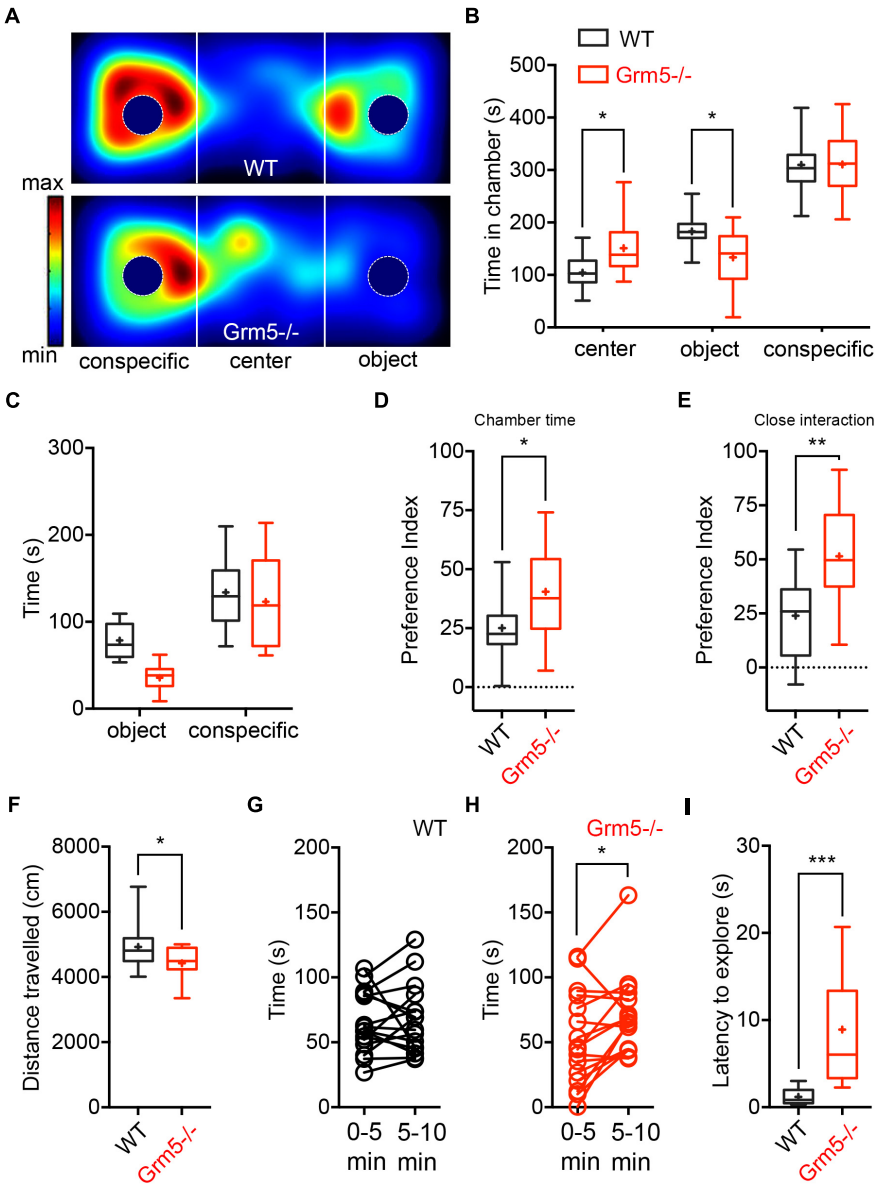

FIGURE 1 | Effects of mGlu5 receptor ablation on sociability. **(A)** Representative heat maps showing time spent by WT mice (upper panel) and *Grm5–/–* (lower panel) mice at each location of the three-chambered apparatus during the test. **(B)** Time spent (s) in the different compartments of the three-chambered apparatus by *Grm5–/–* (n = 16) and WT mice (n = 16) during the test. **(C)** Time spent (s) in close proximity to the social (conspecific) and non-social (object) stimulus. **(D)** Preference index derived from the numerical difference between time spent in conspecific and object chamber divided by total time spent × 100; and **(E)** index derived from the numerical difference between time spent in close proximity to conspecific and object divided by total time spent in close proximity × 100. **(F)** Distance traveled during the test by *Grm5–/–* and WT mice. **(G)** Time spent in close proximity to the conspecific during the first and last 5 min of the test by WT and **(H)** *Grm5–/–* mice. **(I)** Latency (s) to explore side chambers at the beginning of the three-chambered social task was longer for *Grm5–/–* mice. Boxplots represent median, upper and lower quartiles with 10th and 90th percentile whiskers. Mean is represented with a cross. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(**Figure 1E**) when compared to control mice. During the test we observed that the total distance traveled by *Grm5−/−* mice was significantly lower than the control animals [t-test: $t(1,30) = 2.32$, $p < 0.05$] (**Figure 1F**). Since *Grm5−/−* mice are known to display normal locomotion (Chiamulera et al., 2001), this effect could be attributed to a reduced exploratory activity. Unlike WT control, *Grm5−/−* mice explored more actively the novel conspecific during the last 5 min of the test [0–5 vs. 5–10 min: WT, paired t-test: $t(1,15) = 0.25$, $p = 0.79$; *Grm5−/−*: $t(1,15) = 2,68$, $p < 0.05$] (**Figures 1G,H**). *Grm5−/−* mice also exhibited a longer latency to explore the side chambers of the apparatus at the beginning of the test [t-test: $t(1,30) = 4.39$, $p < 0.001$] (**Figure 1I**). These findings suggest that gene-targeted deletion of *Grm5* leads to enhanced social interaction, as measured by the higher social preference index, but also to a reduced exploratory activity or enhanced anxiety

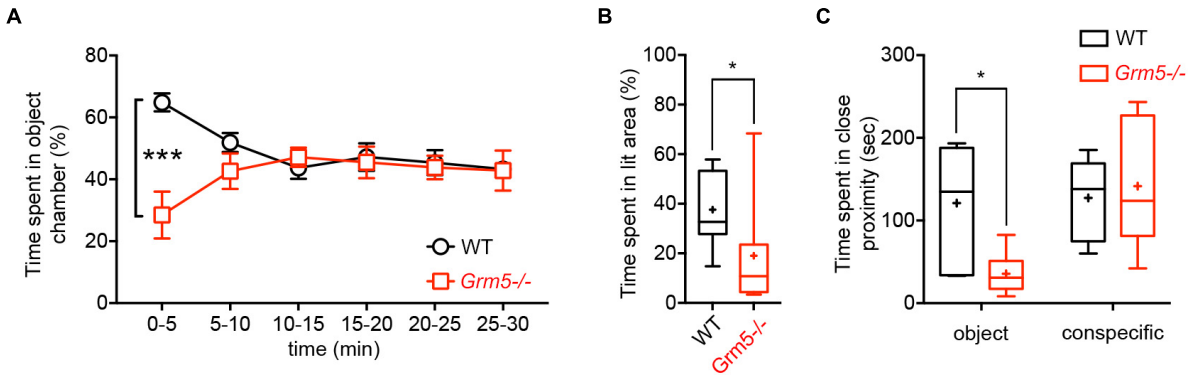

FIGURE 2 | Effects of mGlu5 receptor ablation on non-social exploration and anxiety-like behavior. **(A)** Time spent in object chamber (%) by WT and *Grm5*–/– mice during a 30 min object interaction test in the three-chambered apparatus (n = 9/genotype). Points represent mean ± SEM. **(B)** Time spent in the lit area (%) by WT and *Grm5*–/– mice in a Light-dark Test (n = 12/genotype). **(C)** Time spent in close proximity to an object (empty mesh cylinder) or to an encaged conspecific during the Forced Social or Object interaction test (n = 6–7/group). Boxplots represent median, upper and lower quartiles with 10th and 90th percentile whiskers. Mean is represented with a cross. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

as suggested by the reduced distance traveled and high latency to explore the side chambers. To assess whether the lower time spent in the object chamber was due to the putative anxiogenic phenotype or an exploration deficit, we performed an additional experiment in which mice were presented only with the novel object (i.e., the empty enclosure), while the opposite chamber of the apparatus was left empty. In the first 5 min of a 30 min session, *Grm5*−/− indeed explored significantly less the object chamber than WT mice [2-way repeated measures ANOVA: time $F(5,85) = 0.36$; genotype $F(1,17) = 3.66$; time × genotype $F(5,85) = 7.38$ $p < 0.001$; 5 min: WT vs. *Grm5*−/−: $p < 0.001$] (**Figure 2A**). Conversely, in the remaining time of the session the two genotypes showed no difference in time spent in the object chamber (**Figure 2A**), therefore, showing no generalized deficit in exploration. These findings strongly suggest that *Grm5*−/− mice have an axiogenic phenotype.

Thus, we specifically tested these mice for measures of anxiety-like behavior using the light-dark-box test. *Grm5*−/− mice spent indeed significantly less time in the bright side of the box as compared to WT mice [t-test: $t(1,22) = 2.42$, $p < 0.05$] (**Figure 2B**).

Taken together, these results indicate that *Grm5*−/− mice show an anxiogenic phenotype although with a paradoxically enhanced sociability as measured with social ratios.

Pharmacological Blockade of mGlu5 Receptors Is Anxiolytic Without Affecting Sociability

We next assessed the effects of mGlu5 receptor negative allosteric modulation in WT C57BL/6J mice in sociability and anxiety. We assessed the effects of MTEP (10 mg/kg) at two different time points, when MTEP receptor occupancy should be at its peak (5–15 min post-i.p. injection) and when it should have returned at baseline levels (1 h post-i.p. injection) (Anderson et al., 2003). Vehicle- and MTEP-treated animals displayed sociability, spending more time in the conspecific chamber than in the novel object chamber both at 5 min [2-way ANOVA: chamber $F(2,72) = 233$, $p < 0.001$; treatment $F(1,72) = 0.002$, $p > 0.05$; and chamber × treatment $F(2,72) = 4.58$, $p < 0.05$; object chamber vs. conspecific chamber: WT $p < 0.001$, *Grm5*−/− $p < 0.001$] and 1 h post-injection [2-way ANOVA: chamber $F(2,66) = 196.8$, $p < 0.001$; treatment $F(1,66) = 0.001$, $p > 0.05$; chamber × treatment $F(2,66) = 0.59$, $p > 0.05$] (**Figures 3A,G**). MTEP had no effects on the overall time spent in the novel conspecific or novel object chamber either at 5 min (WT vs. *Grm5*−/−: $p > 0.05$) or at 1 h post-injection (WT vs. *Grm5*−/−: $p > 0.05$) (**Figures 3A,G**). However, MTEP reduced the amount of time spent interacting closely with the conspecific and object in a non-specific manner at 5 min [2-way ANOVA: treatment $F(1,48) = 13.6$, $p < 0.05$; close interaction $F(1,48) = 84.9$, $p < 0.05$; treatment × close interaction $F(1,48) = 1,93$, $p = 0.17$] (**Figure 3B**) and at 1 h post-injection [2-way ANOVA: treatment $F(1,44) = 6.14$, $p < 0.05$; close interaction $F(1,44) = 54.8$, $p < 0.05$; treatment × close interaction $F(1,44) = 0.53$, $p = 0.47$] (**Figure 3H**). Social preference indexes for both conspecific chamber time and time in close interaction with the conspecific did not differ between vehicle and MTEP-treated animals both at 5 min [chamber: t-test: $t(1,24) = 0.102$, $p > 0.05$; close interaction: t-test: $t(1,24) = 0.312$, $p > 0.05$] (**Figures 3C,D**) and 1 h post-injection [chamber: t-test: $t(1,22) = 0.95$, $p > 0.05$; close interaction: t-test: $t(1,22) = 0.36$, $p > 0.05$] (**Figures 3I,J**). These findings indicate that the interaction with a conspecific is not altered by acute MTEP treatment. The lowered active exploration of the conspecific in mice treated with MTEP was accompanied by a marked decrease in object exploration and an increase in locomotor activity during the test, as shown by the distance traveled during the test at 5 min post-injection [t-test: $t(1,24) = 4.56$, $p < 0.001$] (**Figure 3E**) as well as by a statistical trend toward significance at 1 h post injection [t-test: $t(1,22) = 1.77$, $p = 0.09$] (**Figure 3K**).

We then tested whether the reduced active exploration of the object and conspecific during the test was due to the proposed anxiolytic activity of MTEP (Klodzinska et al., 2004;

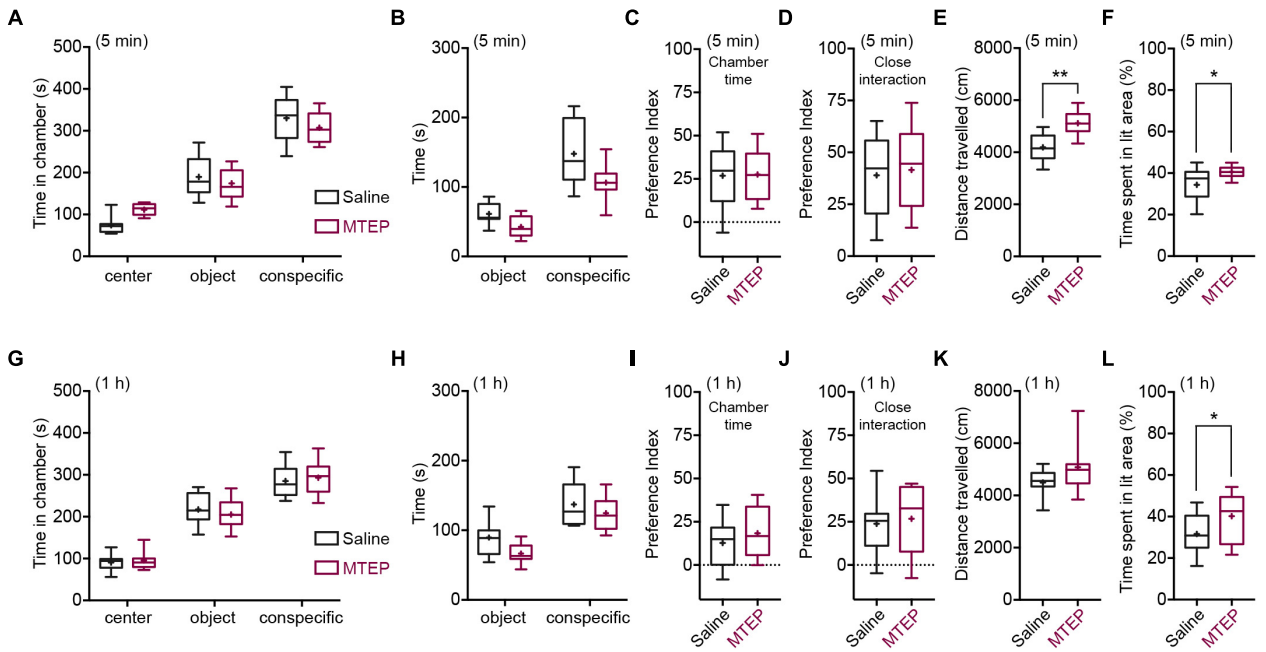

FIGURE 3 | Pharmacological blockade of mGlu5 receptors is anxiolytic without affecting sociability. Time spent (s) in the different compartments of the three-chambered apparatus by MTEP- and vehicle-treated (4% DMSO in saline) mice during the test, at 5 min (MTEP $n = 13$; vehicle $n = 13$) **(A)** or 1 h post-injection (MTEP $n = 12$; vehicle $n = 12$) **(G)**. Time spent (s) in close proximity with the conspecific and object at 5 min **(B)** or 1 h post-injection **(H)**. Preference index derived from the numerical difference between time spent in conspecific and object chamber divided by total time spent × 100 at 5 min **(C)** or 1 h post-injection **(I)**. Index derived from the numerical difference between time spent in close proximity to conspecific and object divided by total time spent in close proximity × 100 at 5 min **(D)** or 1 h post-injection **(J)**. Both social preference indexes did not differ between MTEP and vehicle-treated mice at any post-injection time. At 5 min **(E)** post-injection, but not at 1 h **(K)**, MTEP-treated mice traveled a longer distance than vehicle-treated mice. In the light-dark test, both at 5 min (MTEP $n = 13$; vehicle $n = 13$) **(F)** and 1 h (MTEP $n = 14$; vehicle $n = 14$) **(L)** post-injection, mice treated with MTEP displayed anxiolytic-like activity, spending more time (%) in the lit area of the box than vehicle-treated mice. Boxplots represent median, upper and lower quartiles with 10th and 90th percentile whiskers. Mean is represented with a cross. $^{*}p < 0.05$, $^{**}p < 0.01$.

Stachowicz et al., 2007; Lee et al., 2017, 2018; but see Ade et al., 2016). Similar to sociability, we tested the effects of MTEP at both 5 and 1 h post-injection in the light-dark test. MTEP-treated animals spent significantly more time in the bright side of the box, both at 5 min [$t(1,24) = 2.55$, $p < 0.05$] (**Figure 3F**) and at 1 h post-injection [$t(1,26) = 2.08$, $p < 0.05$] (**Figure 3L**). Taken together, these results confirm the anxiolytic action of MTEP and suggest that mGlu5 receptor NAM does not influence sociability in WT mice when administered acutely.

Aberrant Brain Region-Specific Activation After Social Exposure in *Grm5−/−* Mice

In order to investigate whether the increased sociability observed in Grm5−/− mice could result from different patterns of brain activation upon exposure to social and non-social cues, we exposed WT and *Grm5−/−* mice in their home cage to either a novel conspecific or novel object and quantified the expression of the immediate early gene c-Fos. We used three independent experimental groups for each genotype: a group exposed to an age-matched conspecific enclosed into a wire mesh cage (conspecific group), a group exposed only to the wire mesh cage (object group), and a third control group left undisturbed in the home cage (HC). Similar to the three-chambered social task, Grm5−/− mice displayed a reduced exploration of the novel object in comparison to WT [2-way ANOVA: cue $F(1,22) = 6.05$; genotype $F(1,22) = 2.45$; cue × genotype $F(1,22) = 4.80$, $p < 0.05$; WT vs. *Grm5−/−* object: $p < 0.05$] and a similar exploration of the novel conspecific (**Figure 2C**). Two hours after the exposure, mice were perfused and processed for immunocytochemistry. Twenty-one brain regions, previously reported to be activated after social interaction (Kim et al., 2015), were preselected for c-Fos analysis. In mice kept undisturbed in their home cage, no significant differences between WT and *Grm5−/−* mice were detected in the number of c-Fos+ neurons in any of the areas analyzed (see **Table 1** for statistical significance, **Figure 4** and **Supplementary Figure S1**). This suggests that under resting conditions basal activity in the set of brain areas that we have analyzed is not altered by the lack of mGlu5 receptors. Conversely, compared to the HC group a significant increase in the number of c-Fos+ cells was observed in most of the areas analyzed with the exception of AcbC, LSI, CA1, CA2, and PMCo in WT mice after exposure to a novel object; AcbC, LSI, CA2, and PMCo in WT mice after exposure to a conspecific and AcbC, LSI, CA1, and PMCo in *Grm5−/−*mice after exposure to a novel object (see **Table 1**).

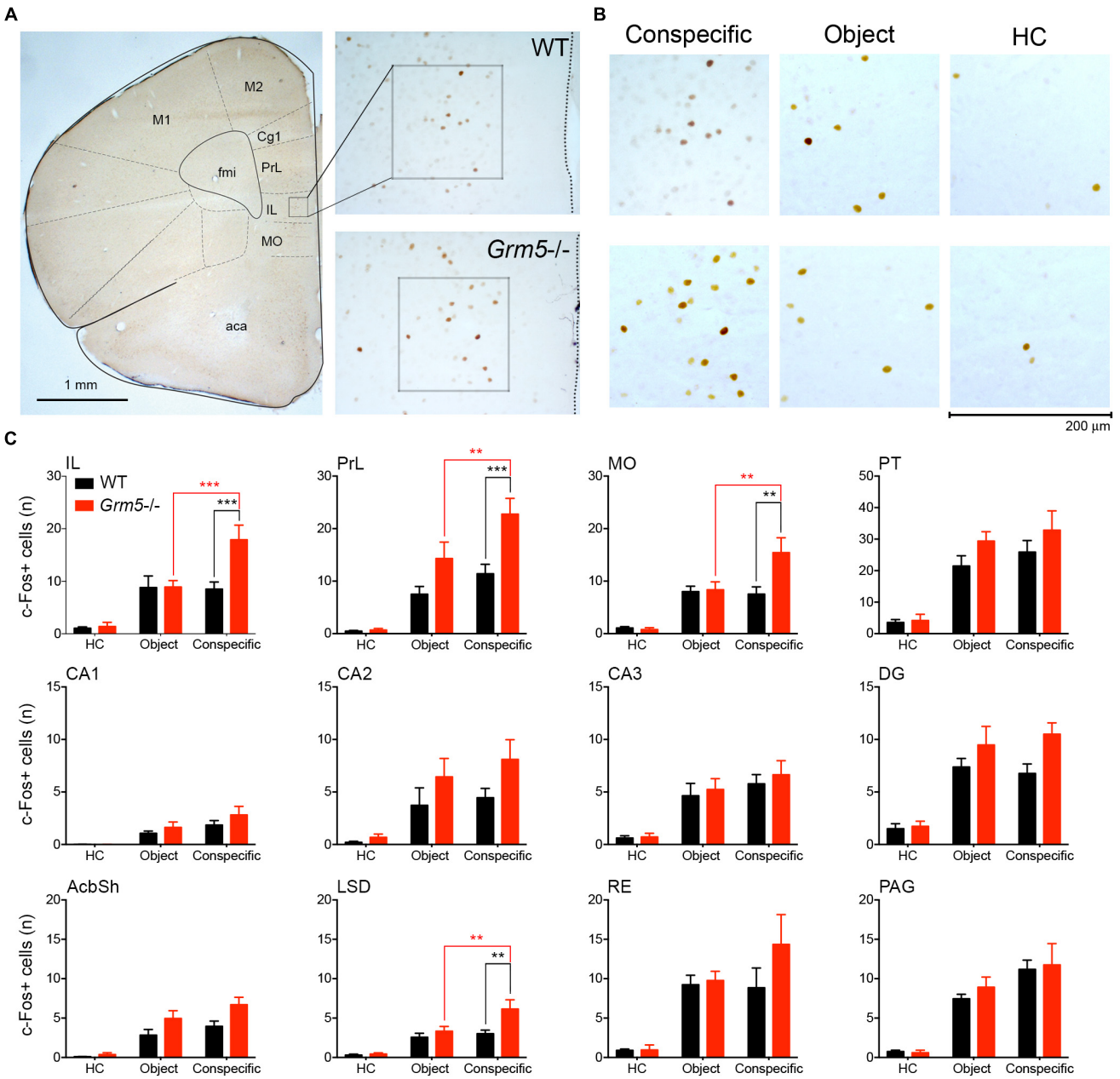

FIGURE 4 | Aberrant brain region-specific activation after social exposure in *Grm5–/–* mice. Number of c-Fos+ cells in WT and *Grm5–/–* mice following 10 min home cage exposure to a conspecific enclosed in a wire mesh cage (conspecific group), a wire mesh cage only (object group) or left undisturbed in home cage (HC, home cage group). **(A)** The micrograph on the left side shows a coronal section immunostained for c-Fos obtained from the frontal lobe of a WT mouse exposed to a novel conspecific in its home cage. A 200 µm × 200 µm sampling window placed in the IL is shown. On the right side, the micrographs show a magnified view of the IL area from where the sampling window was taken in WT (upper panel) and *Grm5–/–* mice (lower panel). **(B)** Representative images of c-Fos immunostaining in the IL in each experimental condition. Scale bar, 200 µm. **(C)** Histograms of the number of c-Fos+ cells for each experimental group in 12 selected brain regions. $N = 7$ in each experimental group. Bars represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, following two-way ANOVA with Neuman–Keuls *post hoc* comparisons whenever interaction group × genotype resulted significant (see **Table 1**). Aca, anterior commissure anterior part; AcbSh, accumbens nucleus shell; CA1-3, dorsal hippocampal areas; Cg1, cingulate cortex area 1; DG, dentate gyrus; fmi, forceps minor of the corpus callosum; IL, infralimbic cortex; LSD, lateral septal nucleus dorsal part; M1, primary motor cortex; M2, secondary motor cortex; MO, medial orbital cortex; PAG, periaqueductal gray; PrL, prelimbic cortex; PT, paratenial thalamic nucleus; RE, reuniens thalamic nucleus.

A significant group × genotype interaction in the 2-way ANOVAs was observed only in 4 brain areas, namely IL, MO, PrL, and LSD (see **Table 1** and **Figure 4**). The interaction with a conspecific triggered higher activation as compared with the object in *Grm5−/−* mice, whereas no statistical significant differences were observed between object and conspecific

TABLE 1 | Statistical summary data of **Figure 3**.

		MO	PrL	IL	AcbC	AcbSh	LSD	LSI	MS	MPOM	Pir	PT	RE	LH	BLA	PV	CA1	CA2	CA3	DG	PMCo	PAG
Interaction $F(2,36)$	F	4.6	3,9	5,1	1,7	1,7	3,4	1,8	0,7	3,2	0,8	0,6	1,1	0,04	0,9	1,5	0,6	0,7	0,08	1,5	0,3	0,2
	p	*	*	*	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Group $F(2,36)$	F	26	34,4	26,5	13,4	28,8	24,4	11,8	42,6	40,4	31,3	30,2	16,1	40,7	22,8	51,6	14,1	11,2	19,7	30,7	20,1	34,1
	p	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Genotype $F(1,36)$	F	4.6	13,9	5,8	3,5	9,1	7,2	3,4	1,3	15,0	0,6	3,2	1,5	0,3	0,5	4,3	1,9	4,6	0,5	5,9	0,9	0,3
	p	*	***	**	ns	**	*	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	*	ns	*	ns	ns
HC WT vs. HC *Grm5−/−*	p	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HC WT vs. Obj WT	p	**	*	*	ns	*	*	ns	***	**	**	**	*	***	*	***	*	ns	*	**	ns	**
HC WT vs. Consp WT	p	**	**	**	ns	**	***	ns	***	***	***	***	*	***	***	***	*	ns	*	**	**	***
HC *Grm5−/−* vs. Obj *Grm5−/−*	p	**	**	*	ns	***	**	ns	***	**	**	*	*	***	**	***	ns	*	**	***	ns	***
HC *Grm5−/−* vs. Consp *Grm5−/−*	p	***	**	***	***	***	***	***	**	***	***	***	***	***	***	***	***	**	***	***	***	***
Obj WT vs. Consp WT	p	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Obj *Grm5−/−* vs. Consp *Grm5−/−*	p	**	**	***	*	Ns	**	*	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns
Obj WT vs. Obj *Grm5−/−*	p	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Consp WT vs. Consp *Grm5−/−*	p	**	***	***	*	*	**	*	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. *Two-way ANOVAs followed by Newman–Keuls comparisons.*

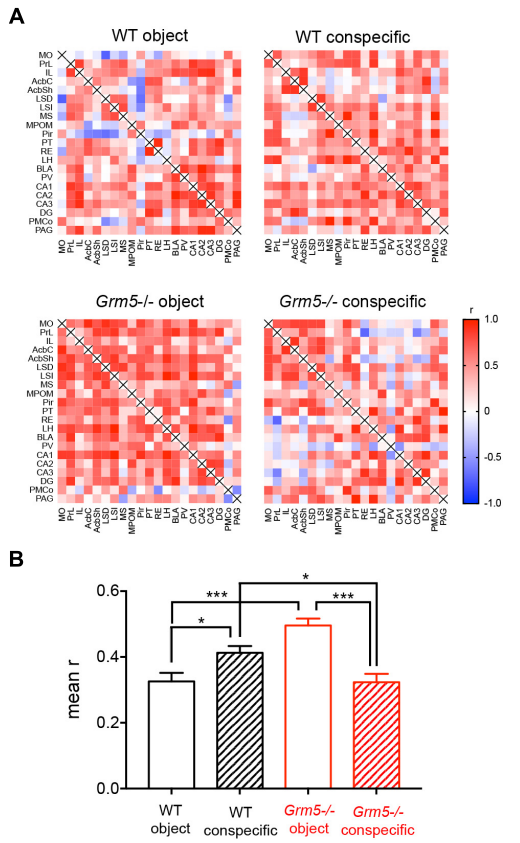

FIGURE 5 | Altered functional connectivity of multiple brain regions during social exposure in *Grm5–/–* mice. **(A)** Matrices displaying interregional correlations of c-Fos expression in WT and *Grm5–/–* mice exposed to an object (wire mesh cage) or to an enclosed conspecific. Colors represent the correlation strength based on Pearson's *r* scale. Warmer colors show stronger correlations. **(B)** Mean *r* calculated from each genotype and experimental condition matrix. Bars represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

exposed-groups in WT mice. These four brain areas had also a higher number of c-Fos+ cells in *Grm5−/−* mice as compared to WT mice when exposed to the novel conspecific, but not to the novel object (see **Table 1** and **Figure 4**). The arousal produced by the exposure to the novel object or conspecific in our paradigm may have masked in WT mice distinct pattern of c-Fos activation. On the other hand, the lack of mGlu5 receptors was able to induce region-specific changes in the number of c-Fos+ cells specifically related to social interaction. We, thus, reasoned that social interaction, contrary to non-social, rather than producing a higher degree of activation, namely number of c-Fos+ cells per area, it enhances functional connectivity among a set of brain regions. In addition, the high activation of prefrontal areas and LSD observed in *Grm5−/−* mice, could underlie a disrupted activity coordination. To explore this possibility, we analyzed the functional connectivity generated during social or object investigation in WT and *Grm5−/−* mice.

Altered Functional Connectivity of Multiple Brain Regions During Social Exposure in *Grm5*−/− Mice

In order to infer interactions between neural elements, we computed correlation coefficients across subjects using our c-Fos expression data set (Horwitz et al., 1995; Tanimizu et al., 2017a,b; Rogers-Carter et al., 2018). This allowed us to obtain an approximation of the strength of the coordinated activity changes among brain regions following social and non-social interactions in *Grm5*−/− and WT mice. We first computed inter-regional correlations for each experimental group (**Figure 5A**). As shown in **Figure 5B**, changes in network density upon social investigation were observed both in WT and *Grm5*−/− mice [2-way ANOVA: genotype $F(1,836) = 6.50$, $p < 0.05$; group $F(1,836) = 6.66$, $p < 0.01$; group × genotype $F(1,836) = 47.51$, $p < 0.001$]. A higher functional connectivity (mean r) was observed upon conspecific as compared to object interaction in WT mice ($p < 0.05$). Conversely, in *Grm5*−/− mice functional connectivity was higher upon non-social interaction ($p < 0.001$). Interaction with a conspecific led to higher functional connectivity in WT mice as compared to *Grm5*−/− mice ($p < 0.05$).

These results suggest that in WT mice social investigation leads to a higher functional connectivity than upon interaction with an object. On the contrary, the lack of mGlu5 receptors inverts the strength of the functional connectivity toward non-social interaction.

Social Interaction Network Hubs Are Rearranged in *Grm5*−/− Mice

Based on interregional matrices (**Figure 5A**), we generated network graphs (**Figure 6A**), where nodes represent brain regions and edges represent above-threshold (Pearson's $r > 0.6$) significant ($p < 0.05$) correlations (**Figure 6A**). We further applied graph theoretical analysis to the network graphs to explore if social investigation induces changes to the relative weight of any of the identified nodes and whether they are affected by the lack of mGlu5 receptors (**Figure 6B**). We then computed the within-community z score and participation coefficient for each node (**Figure 6C**). In correlation-based networks, the within-community z score measures how well connected a region is to its own community and the participation coefficient of a node constitutes a measure of the degree to which a node is linked with nodes in other communities (Guimerà and Amaral, 2005). The participation coefficient, therefore, denotes "hubness" (Power et al., 2013; Rogers-Carter et al., 2018). Nodes displaying a high value of within-community z score and participation coefficient are thought to be key hubs, crucial for coordinating other nodes and, thus, the overall activity of the network (Tanimizu et al., 2017a). Our analysis identified the RE, AcbSh, PAG, and CA3 as key hubs in the social interaction functional network and the PT in the object functional network in WT mice. In addition, the hippocampal regions CA1, CA2, and DG as well as the IL appear as key regions in coordinating the activity between communities in the social interaction network given their high participation coefficient. Of note is the differential participation of the PrL and IL in the object and conspecific functional networks, respectively. In *Grm5*−/− mice, we observed a complete derangement in the role of nodes in both the non-social and social functional networks. In these animals, while the hippocampus and mPFC lost their coordinating role in conspecific network activity, the lateral septum increased it. Moreover, the PT transferred its role as a key hub from the non-social to the social functional network.

DISCUSSION

Here, we reassessed how genetic ablation and pharmacological blockade of mGlu5 receptors affect sociability and anxiety-like behavior in mice. We show that germ line deletion of mGlu5 receptors leads to an anxiogenic phenotype accompanied by a paradoxical enhancement of sociability. Conversely, negative allosteric modulation of this receptor reduced anxiety-like behavior in the light-dark test, consistent with previous studies (Klodzinska et al., 2004; Stachowicz et al., 2007; Lee et al., 2017, 2018), without influencing sociability in WT mice. We further determined how the lack of mGlu5 receptors affects the pattern of brain activation following social and non-social interaction by quantifying the number of neurons expressing the IEG c-Fos. A computational approach was then used to model the potential impact of mGlu5 receptor ablation on functional connectivity of brain areas relevant for social interaction. Our c-Fos quantification revealed a restricted activation, limited to the MO, PrL, IL, and LSD, in *Grm5*−/− mice following interaction with a conspecific as compared to a novel object, although the anxiogenic phenotype of these animals could have in part influenced our analysis. On the other hand, the lack of wide-ranging changes in cFos expression between social and non-social interactions in control mice might have been confounded by the high arousal state induced by the alteration of the home cage environment. Alternatively, our computational analysis suggests that social interaction, rather than inducing broad changes in c-Fos expression, leads to an increased functional connectivity of specific brain regions important for social behavior.

As a first step toward understanding the role of mGlu5 receptors in social preference, we examined the behavior of *Grm5*−/− mice in the three-chambered social task. We observed that ablation of the mGlu5 receptor enhances sociability, as indicated by the social preference index. This was accompanied by an increase in anxiety-like behavior, that was initially observed as a delayed exploration of the side chambers and a reduced distance traveled in the three-chambered social task. This was then confirmed by the light-dark test in which *Grm5*−/− mice spent less time in the lit compartment than WT mice. The anxiogenic phenotype observed in *Grm5*−/− mice appears at odds with the widely accepted anxiolytic action of mGlu5 receptor antagonists (see for review: Ferraguti, 2018) and the reduced stress-induced hyperthermia previously reported for these mice (Brodkin et al., 2002). *Grm5*−/− mice were previously shown to explore more the center of a novel arena in the open field test, but were found to behave similarly to control animals in the light-dark and elevated plus-maze tests (Olsen et al., 2010).

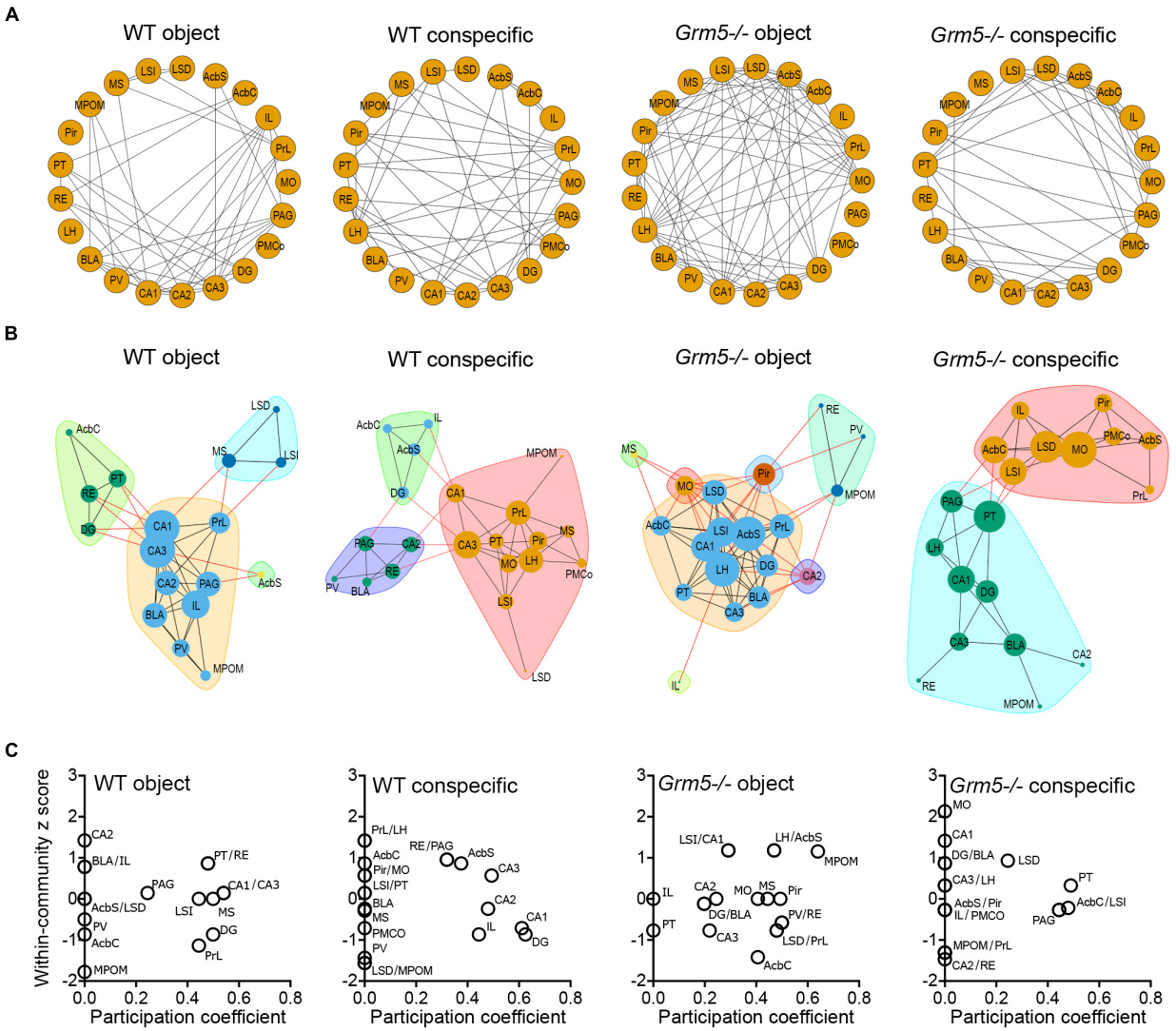

FIGURE 6 | Social interaction network hubs are rearranged in *Grm5–/–* mice. **(A)** Network graphs showing above-threshold ($r > 0.6$) significant positive correlations ($p < 0.05$) between the different brain regions in each experimental condition and genotype. **(B)** Networks identified for each experimental condition and genotype. The width of the edges is proportional to the correlation strength of above-threshold significant correlations and the node size is proportional to the degree (number of edges associated with a given node) of the node in a given network. Colors in the network represent the different communities identified via modularity optimization. **(C)** Within-community *z* scores and participation coefficients of each brain region in the different networks generated following interaction with an object or conspecific in WT and *Grm5–/–* mice.

However, Inta et al. (2013) reported an age-dependent anxiogenic phenotype in *Grm5−/−* mice using the light-dark test, consistent with our findings. These controversies could be explained by differences in the anxiety tests used, e.g., conflict-based vs. physiological measurement, or by procedural variations. Since *Grm5−/−* mice have a normal locomotor activity (Chiamulera et al., 2001), we can exclude that the reduced time spent in the lit compartment of the light-dark test could have resulted from a motor impairment.

Inhibition of mGlu5 receptor activity through systemic administration of antagonists was found to rescue the impaired social behavior typical of the BTBR inbred mouse strain and of different mouse models of ASD (Silverman et al., 2012; Gantois et al., 2013; Chung et al., 2015). On the other hand, inconsistent effects of mGlu5 antagonists were reported in WT rodents, e.g., MTEP induced social isolation in rats (Koros et al., 2007), whereas MPEP increased sniffing and extended time spent interacting in Balb/c mice, but reduced sniffing in Swiss Webster mice (Burket et al., 2011).

In our study, the three-chambered social task revealed increased social preference in *Grm5−/−* mice, based on the preference index, despite the total time spent in the social chamber was similar to that of WT animals. However, given the increased anxiety-like behavior showed by *Grm5−/−* mice, the sociability expressed by these animals in the three-chambered social task might have been underestimated. Selective ablation of

the mGlu5 receptor in parvalbumin-positive neurons resulted in higher duration of social interaction bouts (Barnes et al., 2015), whereas their ablation in cortical glutamatergic principal cells did not produce any detectable effect (Jew et al., 2013). The increased sociability in germline *Grm5*−/− mice may, therefore, primarily result from a role of mGlu5 receptors at inhibitory circuits. Further studies should explore the pathways and neurons at which mGlu5 receptors regulate sociability.

Our pharmacological study shows that negative allosteric modulation of mGlu5 receptors with MTEP had no effect on sociability despite it reduced active exploration of both the conspecific and object. These non-specific effects of MTEP on social and non-social interactions could explain the reported social isolation in rats after MTEP treatment (Koros et al., 2007). The complex effects on measures of sociability observed in mice (Burket et al., 2011), on the other hand, may depend on the known off-target effects of MPEP (Lea and Faden, 2006). The anxiolytic action elicited by MTEP in WT mice in our study, although modest, confirms that the absence of an effect on sociability does not result from a lack of activity of the drug.

Both mGlu5 receptor NAMs MPEP and MTEP have been described as potent anxiolytics in different rodent models (see for review: Ferraguti, 2018). However, their anxiolytic properties on WT mice appear to greatly depend on strain, dose and delay between administration and testing. MTEP was found to be anxiolytic at 3 mg/kg and anxiogenic at 30 mg/kg in the light-dark test in C57BL/6j (Lee et al., 2018). Whether this differential effect on anxiety is due to an inverted U-shaped dose-response activity or to potential unspecific effects of the highest dose of MTEP remains to be explored.

From these findings, it could be concluded that deviations in any direction of mGlu5 receptor function may lead to impairments in both social and anxiety-like behaviors.

To understand at the anatomical level where mGlu5 receptors regulate brain activity during social exploration, we have analyzed the expression of c-Fos in a selected set of brain areas previously reported to be activated upon social interaction (Kim et al., 2015). Our study shows that in *Grm5*−/− mice, MO, PrL, IL, and LSD were selectively activated upon interaction with a conspecific, suggesting that mGlu5 receptors dampen neuronal activity in these brain regions during social behavior, possibly by activating interneurons and facilitating feedforward inhibition (Pollard et al., 2014). Future studies will have to unveil the expression and role of mGlu5 receptors in the different neuronal types in these brain areas and their specific contribution to social behavior.

Our functional connectivity analysis of 21 brain regions relevant for social behavior suggested a disruption of the network density in *Grm5*−/− during exploration of both social and non-social stimuli. Moreover, the networks generated during social and non-social interactions, as well as the role of individual brain regions in coordinating network activity, such as hippocampal and prefrontal areas, dramatically changed in *Grm5*−/− mice. In line with previous findings investigating prolonged social interactions (Tanimizu et al., 2017a), we observed high participation coefficients of the hippocampus and mPFC in WT mice exposed to a conspecific. It should be taken into account, however, that c-Fos correlation-based networks suffer from several limitations. Inclusion of different brain regions into the network can lead to rearrangements of the communities, measures of centrality and roles of individual nodes. Thus, with current computational models it is hard to compare the role of single nodes between differently generated networks or even between similar experiments that include different brain regions into the network. Nonetheless, c-Fos based functional connectivity and network analysis can serve as a promising tool for hypothesis generation (Vetere et al., 2017; Rogers-Carter et al., 2018), although promising key hubs will have to be validated experimentally, e.g., using chemo- or opto-genetic approaches.

The hippocampus and mPFC, together with the amygdala, appear as key regions underlying sociability circuits (Felix-Ortiz and Tye, 2014; Felix-Ortiz et al., 2016). The CA2 region, in particular, has been recently proposed as a critical hub for socio-cognitive memory processing (Hitti and Siegelbaum, 2014), independent of other hippocampus-dependent behaviors such as spatial memory. In our study, the CA2 region was shown to display largely different roles in the functional networks generated following a 10 min interaction with a conspecific or an object, sufficient time to allow for social and non-social memory formation (Tanimizu et al., 2017a,b). In the network generated upon interaction with a conspecific in *Grm5*−/− mice, the CA2 region was the most isolated node in the network. Therefore, it would be of interest to determine whether manipulation of mGlu5 receptors activity in this region affects social recognition memory as predicted by our functional network analysis.

To our knowledge, only one study has attempted to address the contribution of mGlu5 receptors in a specific brain region to sociability, so far. In line with our findings that the lack of mGlu5 receptors increases activity in the LSD upon social interaction, Mesic et al. (2015) suggested that selective removal of mGlu5 receptors in this brain area impaired expression of sociability but not social novelty.

In conclusion, our work shows that while the mGlu5 receptor NAM MTEP is anxiolytic upon systemic acute administration, the lack of these receptors, as in germ line *Grm5*−/− mice, results in an anxiogenic phenotype. Similarly, while sociability is not affected by pharmacological blockade of mGlu5 receptors, their lack leads to an apparent increased sociability. Whether this is the result of complex and perhaps opposite effects at different brain regions or developmental adaptations remains to be established. Indeed, a further note of caution concerns data obtained with germ line knockouts as adaptive changes may influence behavior differently from manipulations carried out in adult animals. Further studies in which mGlu5 receptor activity is abolished or modulated in a time-controlled and region- and/or cell-type specific manner are, therefore, warranted. Our c-Fos expression and network analyses offers candidate areas for such specific targeting.

Taken together our findings support the relevance of mGlu5 receptors in modulating anxiety-like behavior and sociability. The paradoxical increased social preference within an overall anxiogenic phenotype, as in the germ line Grm5−/− mice, shows remarkable analogies with Williams syndrome (Meyer-Lindenberg et al., 2006; Barak and Feng, 2016). Future studies

should address whether mGlu5 function is altered in this rare neurodevelopmental disorder extending the implication of these receptors beyond ASD.

AUTHOR CONTRIBUTIONS

AR-P and FF conceived and designed the project. AR-P, JK, MZ, EP, and GS were involved with experimental and analytical aspects of the manuscript. AR-P performed data analyses, functional connectivity, and graph theoretical analysis. AR-P and FF wrote the manuscript. All contributing authors commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2019.00030/full#supplementary-material

FIGURE S1 | Histograms of the number of c-Fos+ cells for each experimental group in the other 9 selected brain regions. $N = 7$ in each experimental group. Bars represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, following two-way ANOVA with Neuman–Keuls *post hoc* comparisons whenever interaction group × genotype resulted significant (see **Table 1**). AcbC, accumbens nucleus core; BLA, basolateral amygdala; LH, lateral hypothalamic area; LSI, lateral septal nucleus intermedial part; MPOM, medial preoptic nucleus medial part; MS, medial septal nucleus; Pir, piriform cortex; PMCo, posteromedial cortical amygdaloid area; PV, paraventricular thalamic nucleus.

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Metabotropic Glutamate Receptor 5 in the Medial Prefrontal Cortex as a Molecular Determinant of Pain and Ensuing Depression

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Pain and depression affect one another, and this bidirectional interaction implies the existence of common or interacting neural pathways. Among the neural circuits relevant to negative affection, the medial prefrontal cortex (mPFC) is known to be involved in both pain and depression. Persistent stress from physical pain and mental distress can evoke maladaptive changes in mPFC circuits to induce depression. Conversely, the unpleasant mood condition alters mPFC circuits to distort the appraisal of aversion and make individuals vulnerable to pain. In this article, recent findings regarding mPFC in chronic pain and/or depression are reviewed, with particular focus on the metabotropic glutamate receptor 5 (mGluR5). Although the involvement of mGluR5 within the mPFC in both pain and depressive disorders has been extensively studied, there are controversies regarding changes in the activity of the mPFC during chronic pain and depression, and the functional roles of mGluR5 on altered mPFC activity. We discuss alterations in the availability of mGluR5 in the mPFC in these disorders, its role in behavioral manifestations, and its possible influence on cellular subpopulations that mediate dysfunction in the mPFC. We also propose molecular mechanisms that may cause expressional changes in mGluR5 within the mPFC circuitry.

Keywords: chronic pain, depression, medial prefrontal cortex, prelimbic cortex, metabotropic glutamate receptor 5

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INTRODUCTION

Chronic pain patients often develop negative mood symptoms such as depression (Turk et al., 2010; Radat et al., 2013). Conversely, patients with depressive disorders are more susceptible to pain symptoms compared with normal individuals (Gupta et al., 2007; Wise et al., 2007; de Heer et al., 2014). The clinical manifestation of comorbid pain and depression implies that common or interacting neural circuits underlie the persistence of physical pain and negative moods. Conceptually, the long-term presence of physical pain would act as a persistent stress, and the chronic unavoidable stress would, in turn, alter the neural circuits that perceive the self-state and decide the coping strategy. Previous studies involving human subjects and animal models have revealed the critical role of the prefrontal cortex in concurrent pain and negative moods (Romero-Sandoval, 2011; Lemogne et al., 2012; Chung et al., 2017; Ong et al., 2018). The medial prefrontal cortex (mPFC) processes information about the external and internal environment to appraise the

present state, predict future outcomes, and to make decisions. The stress-induced alteration of mPFC activity would change the levels of cognitive flexibility that affect the subjective perception of the self-state and following decisions impacting behavioral coping strategies.

The neural circuits within the mPFC undergo various changes during the development of chronic pain and/or depression, and these alterations play a key role in the persistence of the disorder (Lemogne et al., 2012; Marsden, 2013; Guida et al., 2015; Wang G.Q. et al., 2015; Zhang et al., 2015; Davis et al., 2016; Chung et al., 2017). The molecular alterations related to glutamatergic transmission have been of particular interest, including metabotropic glutamate receptor (mGluR)-mediated neuronal changes. Among the mGluRs, mGluR5 is one of the most studied receptor in various neurological disorders including chronic pain and mood disorders (Kolber, 2015; Pillai and Tipre, 2016; Sengmany and Gregory, 2016; Esterlis et al., 2018), given its known role in plastic changes in neural circuits (Bordi and Ugolini, 1999; Ribeiro et al., 2010). mGluR5 flexibly controls neuronal firing, and is not only responsible for physiological experience-dependent neuronal plasticity but also maladaptive changes in neural circuits which lead to neurological disorders. The activation of mGluR5 influences synaptic transmission and the intrinsic excitability of neurons. mGluR5 is densely expressed in the mPFC, and expression levels in the mPFC are altered in conditions of chronic pain or depressive disorders (Matosin et al., 2014; DeLorenzo et al., 2015; Lee K.W. et al., 2015; Chung et al., 2017; Esterlis et al., 2017).

However, there have been discrepancies in the previous reports regarding the direction of altered mPFC activity, the expressional changes of mGluR5 within the mPFC (mPFC-mGluR5), and their functional consequences. In this article, we discuss chronic pain-induced hypo- or hyper-excitability of mPFC pyramidal neurons, and their roles in pain and depression. Furthermore, we offer our perspective on the issue of contrasting reports, the specific mPFC neuronal subpopulation that may be affected by mGluR5 alteration, and possible underlying molecular mechanisms.

ACTIVITY CHANGES IN THE mPFC IN CHRONIC PAIN STATES

Numerous animal model studies have found that the activity of mPFC neurons is altered in the pain state, and that the altered activity of pyramidal and/or GABAergic neurons is associated with increased pain perception. In rodents, the mPFC consists of three subregions: anterior cingulate cortex (ACC); prelimbic cortex; and infralimbic cortex. Although the role of hyperactive ACC activity on abnormal pain is well established (Rainville et al., 1997; Hsieh et al., 1999; Seminowicz et al., 2009; Koga et al., 2015; Santello and Nevian, 2015), controversies remain regarding the actions of the prelimbic and infralimbic subregions on pain perception. In this article, we primarily focus on the prelimbic subregion, as the prelimbic cortex rather than infralimbic cortex is implicated in the emotional dimension of pain (Jiang et al., 2014).

Many researchers have associated hypoactive prelimbic pyramidal neurons with increased pain perception and attenuation of pain modulatory function. Previous studies have demonstrated that pyramidal neuronal activity in the prelimbic cortex is decreased in several animal models of chronic pain, and that increasing its activity ameliorates pain. According to electrophysiological analyses, reduction of prelimbic pyramidal neuronal activity was associated with decreased intrinsic excitability of pyramidal neurons (Wang G.Q. et al., 2015; Radzicki et al., 2017), reduced excitatory (glutamatergic) inputs (Kelly et al., 2016; Cheriyan and Sheets, 2018), and increased inhibitory (GABAergic) inputs to neurons (Ji et al., 2010; Zhang et al., 2015; Kelly et al., 2016; Kiritoshi et al., 2016; Cheriyan and Sheets, 2018).

Among these mechanisms, the increased influence of GABAergic neurons on pyramidal neurons has been actively studied. The increase in inhibitory inputs to pyramidal neurons is due to the loss of available endocannabinoid in pyramidal neurons (Kiritoshi et al., 2016), and the increased activity of GABAergic neurons themselves (Zhang et al., 2015). Kiritoshi et al. (2016) found that in a model of arthritis, mGluR5-mediated production of 2-arachidonyl glycerol (2-AG) is impaired in mPFC infralimbic pyramidal neurons. Due to the lack of 2-AG, presynaptic CB1 receptor-mediated suppression of GABA release is disrupted, which leads to abnormally enhanced inhibition to postsynaptic pyramidal neuron. Considering the study of prelimbic neurons in the same model (Ji et al., 2010), this breakdown of mGluR5-endocannabinoid signaling might occur in the prelimbic pyramidal neurons as well. The increase of GABAergic neuronal activity has been reported in both of arthritic and neuropathic pain models. GABAergic neurons that inhibit prelimbic pyramidal neurons receive more excitatory signals in the chronic pain state compared with normal (Ji et al., 2010; Zhang et al., 2015). This indicates increased synaptic influences from the presynaptic glutamatergic excitatory neurons to the postsynaptic GABAergic inhibitory neurons. However, the mechanisms underlying increased synaptic transmission to GABAergic neurons remain unclear, and it has not been studied whether the intrinsic excitability of GABAergic neurons is also increased in the chronic pain state.

On the other hand, some studies have found increased mPFC neuronal activity in the pain state. These investigations showed that pharmacological deactivation of activity reduces chronic pain symptoms. In a study by Wu et al. (2016) the excitability of prelimbic layer 5 pyramidal neuron was increased in a complete Freund's adjuvant (CFA)-induced inflammatory pain model in mice, in contrast to the reduced intrinsic excitability (with enhanced glutamatergic transmission) of the layer 2/3 pyramidal neurons in CFA model rats (Wang G.Q. et al., 2015). Matos et al. (2015) demonstrated that the intrinsic excitability of layer 2/3 pyramidal neurons located in the ACC and prelimbic cortex was enhanced in animals with neuropathic pain, due to an increase in hyperpolarization-activated, cyclic nucleotide-gated (HCN) channel activation. With the increased open probability of HCN, the manifestation of persistent firing induced by mGluR5 activation is facilitated. The administration of an HCN channel blocker decreased neuronal excitability in the slice condition,

and reduced cold but not mechanical allodynia *in vivo*. Fan et al. (2018) showed that hyperresponsive prelimbic neurons are critically involved in enhanced behavioral responses to noxious stimuli after chronic pain. The previous experience of CFA-induced chronic pain enhanced prelimbic neuronal activation following formalin assaults, and inhibiting prelimbic activity reversed aggravated formalin pain. The authors suggested that increased prelimbic cortex neuronal activity might facilitate pain via increased inhibition of periaqueductal gray (PAG) neurons.

The inconsistencies indicate that the changes in synaptic transmission and intrinsic excitability are highly specific to mPFC subregion, cell type, cortical layer, the cause and the duration of the pain. In neuropathic pain models, excitability of layer 5 pyramidal neurons was reduced in the prelimbic cortex (Radzicki et al., 2017; Cheriyan and Sheets, 2018), but not infralimbic cortex (Cheriyan and Sheets, 2018). In contrast, the activation of prelimbic neurons was increased in an inflammatory pain model (Dale et al., 2018; Fan et al., 2018), with increased excitability of layer 5 pyramidal neurons (Wu et al., 2016). Interestingly, intrinsic excitability was reduced in the layer 2/3 pyramidal neurons in the same inflammatory pain model (Wang G.Q. et al., 2015). Also, it is suggestive that the changes are specific to the separate subpopulations of mPFC neurons which have connections to different brain regions (Lee M. et al., 2015; Kelly et al., 2016; Cheriyan and Sheets, 2018; Kiritoshi and Neugebauer, 2018).

Although further studies are needed to clarify apparent contradictions between a hyperactive and hypoactive mPFC in the chronic pain state, research to date has commonly observed alterations in excitatory and/or inhibitory influences on pyramidal neurons, and these disturbances are critically involved in the pain itself and the ensuing affective and cognitive disorders. Interestingly, multiple studies have described mGluR5 as a molecular mediator of altered mPFC pyramidal neuronal activity in the chronic pain state, but with inconsistent descriptions of its functional roles.

THE EFFECT OF mPFC-mGluR5 BLOCKADE ON THE MODULATION OF PAIN AND DEPRESSION

There are conflicting reports regarding the effect of mGluR5 blockade in the mPFC on pain modulation (Ji and Neugebauer, 2011; Giordano et al., 2012; Palazzo et al., 2014; David-Pereira et al., 2016; Chung et al., 2017). A previous study demonstrated that application of mGluR5 antagonist to the prelimbic and infralimbic cortex facilitated neuropathic pain induced by spared nerve injury (SNI) surgery in mice (Giordano et al., 2012). In contrast, other studies have reported that the administration of mGluR5 antagonist to the infralimbic or prelimbic cortex produced analgesic effects in an animal model of arthritis (David-Pereira et al., 2016) or spinal nerve ligation (SNL)-induced neuropathic pain (Chung et al., 2017). It is noteworthy that pain facilitation occurred when mGluR5 was blocked in the mPFC contralateral to the peripheral pain (Giordano et al., 2012), whereas pain suppression was induced when the administration was targeted to the mPFC ipsilateral to the peripheral pain (David-Pereira et al., 2016) or to the bilateral mPFC (Chung et al., 2017). Interestingly, in the SNL-induced neuropathic pain study, mGluR5 was increased in the prelimbic subregion of the mPFC ipsilateral – but not contralateral – to the peripheral nerve injury (Chung et al., 2017). The ipsilesional increase in prelimbic mGluR5 was observed in the deep layer, presumably layer 5/6, in which long-range GABAergic and transcallosal neurons are abundant (Kawaguchi and Kubota, 1997; Lee et al., 2014; Saffari et al., 2016; Anastasiades et al., 2018). This raises the unique prospect that interhemispheric and inter-layer connectivity may be involved in disease manifestation and may explain the inconsistent results between studies. The study of Kiritoshi et al. (2016) further implicates the involvement of GABAergic influences. According to the study, activation of mGluR5 in the mPFC pyramidal neuron fails to suppress presynaptic GABA release in chronic pain state due to loss of endocannabinoid signaling. Activation of mPFC-mGluR5 could induce analgesic effect only when the CB1 receptor was coactivated with a CB1 agonist treatment. Thus, the inconsistency might be stem from the differential changes in the interacting molecules which affect the modulation of synaptic transmission.

In the field of depression research, previous studies investigating non-pain depression have reported inconsistent results regarding the alteration of prefrontal mGluR5 expression. A few studies have reported lower prefrontal mGluR5 levels in patients with major depressive disorder (MDD) compared with healthy controls (Deschwanden et al., 2011; Esterlis et al., 2017), whereas other studies have reported comparable or even higher levels of prefrontal mGluR5 (DeLorenzo et al., 2015; Gray et al., 2015). This inconsistency is likely due to differences in sex, age, time of onset, and pathophysiological differences among patients (DeLorenzo et al., 2015). In fact, Gray et al. (2015) reported that female MDD patients exhibited higher levels of mGluR5 gene expression in the PFC subregion, whereas male MDD patients exhibited lower levels. In a postmortem study by Deschwanden et al. (2011) which reported lower levels of mGluR5 in the PFC of MDD patients, 80% of the subjects were male. Interestingly, a preclinical study reported that male – but not female – rats exhibited higher levels of prefrontal mGluR5 in the depressive condition induced by chronic mild prenatal stress (Wang Y. et al., 2015). Other studies have reported that male mice with mGluR5 deletion exhibit antidepressive-like behavior, suggesting that the activity of mGluR5 primarily facilitates depression (Witkin et al., 2007; Lee K.W. et al., 2015).

In contrast to such inconsistencies regarding expressional change in mPFC-mGluR5 in the depressive state, it is generally accepted that the administration of an mGluR5 antagonist exerts an anti-depressive effect (Palucha and Pilc, 2007; Pilc et al., 2008; Chaki et al., 2013; Hashimoto et al., 2013; Hughes et al., 2013; Fuxe and Borroto-Escuela, 2015; Kato et al., 2015; Lee K.W. et al., 2015; Lindemann et al., 2015; Park et al., 2015). mGluR5 antagonist treatment induces anti-depressant-like effects in animal models of stress-induced depression and chronic pain-induced depression. A recent study reported that the mGluR5 antagonist-induced anti-depressive effect is mediated via blockade of mGluR5 in GABAergic – but not

glutamatergic – neurons in the mPFC (Lee K.W. et al., 2015). Lee K.W. et al. (2015) showed that mGluR5 conditional knockout in GABAergic neurons resulted in an anti-depressive effect. Conversely, mGluR5 knockout in glutamatergic neurons induced depressive-like effects. The results from these conditional knockout mice suggest that the activation of mGluR5 in the mPFC GABAergic neurons induces depression, presumably via inhibition of glutamatergic neurons. The administration of mGluR5 antagonist *in vivo* resulted in an increase in mPFC glutamatergic neuronal activity (Lee K.W. et al., 2015) and exogenous activation of mGluR5 produced GABAergic inhibition (Ji and Neugebauer, 2011), supporting the concept. Furthermore, depressive-like effects induced by glutamatergic neuronal mGluR5 knockout could be overcome by disinhibition (i.e., blocking mGluR5 in GABAergic neurons).

THE CAUSAL ROLE OF mPFC-mGluR5 UPREGULATION IN PAIN AND ENSUING DEPRESSION

PET studies of mGluR5 in chronic pain patients have yet to be reported, and the possible differences of mPFC-mGluR5 according to sex, age, education level, and social class of pain patients are unknown. In the preclinical level, alteration of mGluR5 was investigated in the brains of male rats with neuropathic pain in a previous neuroimaging study (Chung et al., 2017). The study demonstrated that increased mGluR5 availability in the prelimbic subregion of the mPFC is responsible for amplified pain as well as depression-like behavior. The administration of mGluR5 antagonist to the prelimbic cortex of nerve-injured animals induced analgesic and antidepressant-like effects. Conversely, the naïve animals developed mechanical allodynia-like and negative mood symptoms, such as depression and anxiety, after lentiviral overexpression of mGluR5 in the bilateral prelimbic cortex. These data support a causal role for mGluR5 upregulation in the mPFC in amplified pain and negative mood symptoms.

Although the behavioral consequences of mPFC-mGluR5 upregulation have been revealed as such, the cellular roles in these circuits remain elusive. Normally, excitatory manipulation of mPFC pyramidal neuronal activity induces an analgesic effect and ameliorates pain. Administration of ionotropic glutamate receptor (iGluR) agonists to the mPFC activated the endogenous analgesic action of the PAG (Ong et al., 2018). Alternatively, mPFC pyramidal neuronal activation increased mPFC-Nucleus accumbens (NAc) activity to reduce pain perception (Lee M. et al., 2015). The action of mGluR5 activation is generally excitatory to neurons; however, there is a gap between the pain-suppressing actions of mPFC-iGluR5 activation and mPFC-mGluR5 deactivation.

One possible explanation is that increased prelimbic mGluR5 levels eventually result in a reduction in the firing of excitatory pyramidal mPFC neurons, which project to the PAG or the NAc. Previous studies have reported that the neuronal excitability of mPFC-PAG projection neurons is decreased in chronic pain states, and the increased activity of inhibitory interneurons is responsible for the reduced activity of pyramidal neurons (Zhang et al., 2015; Cheriyan and Sheets, 2018). Interestingly, glutamate stimulation elicited inhibitory inputs to mPFC-PAG projection neurons (Cheriyan and Sheets, 2018). Although the causal relationship between mGluR5 upregulation and increased inhibitory neuronal activity has not been extensively studied, the blockade of mPFC-mGluR5, or of inhibitory neuronal activity, drives mPFC circuits in the same direction, i.e., they induce the analgesic and anti-depressive effects.

There are several conceivable hypotheses. First, mGluR5 upregulation in the chronic pain state may occur predominantly in inhibitory neurons in the mPFC (**Figure 1A**). Second, apart from this explanation, mGluR5 may be upregulated in excitatory neurons that preferentially excite inhibitory interneurons within the local circuits (**Figure 1B**). In the mPFC, the activity of local GABAergic neurons is influenced by the glutamatergic excitatory inputs they receive, and the frequency – but not the amplitude – of the excitatory transmission to the GABAergic neurons were increased in the neuropathic pain state (Zhang et al., 2015). Third, alternatively, activated mGluR5 in the pyramidal synapse may induce glutamatergic long-term depression in pyramidal neurons via interaction with other molecular signals (Otani et al., 1999; Zhong et al., 2008; Ghoshal et al., 2017; **Figure 1C**). For example, a recent study found that coactivation of mGluR5 and the M1 muscarinic acetylcholine receptor in the mPFC exerted a long-lasting decrease in excitatory transmission and concurrent enhancement of GABAergic inhibitory tone (Ghoshal et al., 2017). In this scenario, pyramidal neuronal increase in mGluR5 would not be able to modulate the presynaptic release of GABA because mGluR5 signaling in the mPFC pyramidal neurons fails to engage 2-AG mediated endocannabinoid signaling in chronic pain states (Kiritoshi et al., 2016). It has been reported that in the chronic pain state, mGluR5 activation could not increase pyramidal neuronal activity unless the inhibition from the GABAergic neuron is blocked by treatment with a GABA antagonist or activation of the CB1 receptor in GABAergic neurons (Kiritoshi et al., 2016). Fourth, mGluR5 upregulation may occur in glial cells surrounding the synapses of a specific subpopulation of mPFC neurons (**Figure 1D**). Glial mGluR5 serves to evoke complex and bi-directional effects on the modulation of neuronal activity, and upregulated mGluR5 in cortical astrocytes contributes to synaptic remodeling during the chronic neuropathic pain state (Kim et al., 2016; Ishikawa et al., 2018).

Either way, upregulated mGluR5 in the chronic pain state may be working in a direction that increases inhibitory influences on mPFC pyramidal neurons. In fact, mPFC glutamatergic neuronal activity was increased in response to the mGluR5 antagonist treatment *in vivo* (Lee K.W. et al., 2015). mPFC pyramidal neurons comprise several subtypes with different firing properties, expressing receptors, and projecting brain regions, and subserve distinct functions. Therefore, the subpopulation-specific upregulation of mGluR5 and its relevance to pain and depression should be investigated in future studies.

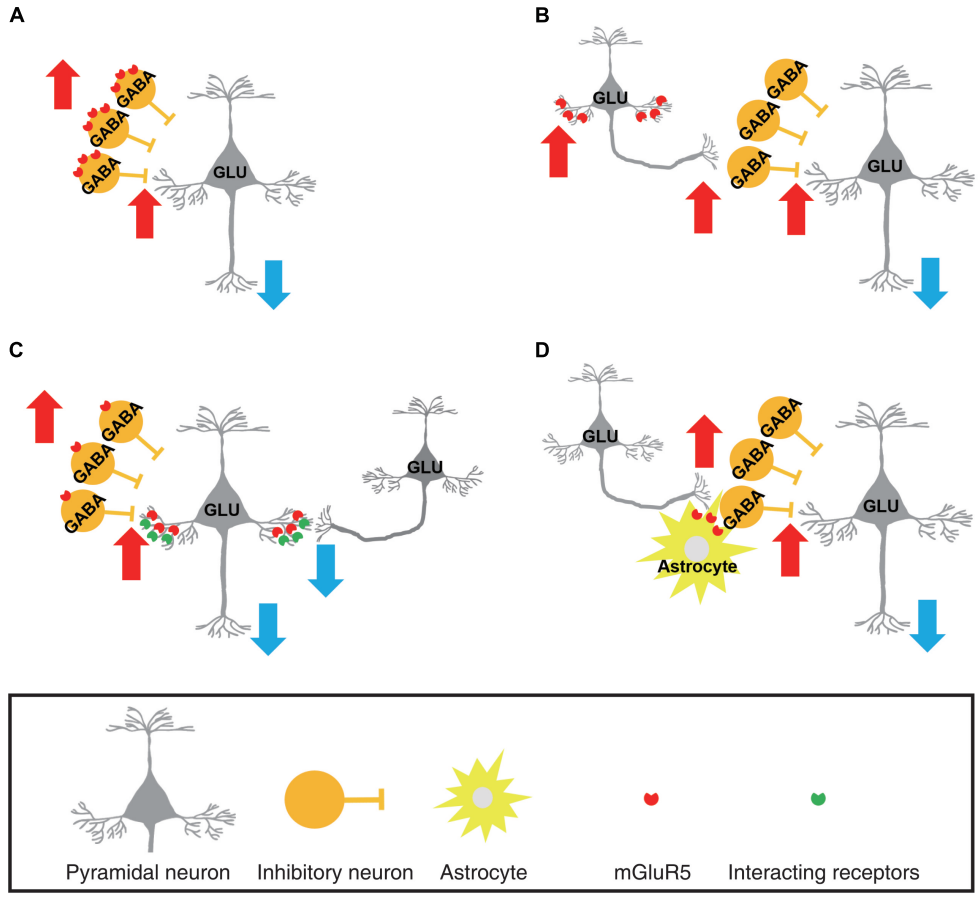

FIGURE 1 | Schematic drawings representing possible cellular subsets of which metabotropic glutamate receptor 5 (mGluR5) upregulation occurs in the chronic pain state. In these scenarios, enhanced mGluR5 levels in the layer 5/6 medial prefrontal cortex (mPFC) were assumed to result in suppression of prelimbic pyramidal neurons. **(A)** mGluR5 is upregulated in the GABAergic inhibitory interneurons. As a result, GABAergic neuronal activity is increased. **(B)** mGluR5 is increased in the specific subpopulation of pyramidal neurons that preferentially excite local GABAergic neurons. **(C)** Layer 5/6 pyramidal neurons express more mGluR5. However, the increased mGluR5 suppresses, rather than facilitates, pyramidal neuronal activity. This is achieved by an enhancement of long-term depression in excitatory synapses and concurrent loss of presynaptic modulation by mGluR5. **(D)** mGluR5 upregulation occurs in astrocytes surrounding the synapses of a specific subpopulation of mPFC neurons. The increased glial mGluR5 serves to enhance excitatory influences on GABAergic neurons. (GLU, glutamatergic neuron, GABA, GABAergic neuron).

BIOLOGICAL PROCESSES UNDERLYING mPFC-mGluR5 UPREGULATION: POSSIBLE MOLECULAR MECHANISMS

The biological processes that mediate the alteration of mPFC circuits and the cellular mechanisms underlying upregulation of mPFC-mGluR5 are largely unknown. A previous study revealed that depression is critically related to mPFC-mGluR5, and the expression level is regulated by protein p11 (Lee K.W. et al., 2015). As mentioned, the lentiviral knock-down of mGluR5 in excitatory neurons in the mPFC facilitated depressive-like behavior, whereas inhibitory neuron-specific mGluR5 knock-down exerted an anti-depressive effect. Similar results were obtained from the conditional knockout of protein p11 in excitatory or inhibitory neurons. It is worth noting that although p11 could be a strong candidate to be a mediator of mPFC-mGluR5 alteration, p11-mediated changes would predominantly occur in a specific subtype of prelimbic layer 2/3 pyramidal neurons, with decreased expression in response to chronic stress (Seo et al., 2017). Thus, the molecular mechanisms underlying upregulated mGluR5 in the prelimbic area of animals with chronic pain (Chung et al., 2017) remain vague.

A possible candidate is dopamine 2 receptor (D2R)-mediated control of mGluR5 expression. From the perspective of a reinforcement learning paradigm, dopamine signaling plays a key role in motivated approach or avoidance behavior following aversion. Because D2Rs have a strong binding affinity compared with the dopamine 1 receptor (D1R), persistent dopamine would be mainly occupied by D2R in the nervous system. When negative prediction error occurs by salient event (aversion), dopaminergic neurons would cease firing, resulting in transient dopamine depletion in the relevant brain regions (Bromberg-Martin et al., 2010; Glimcher, 2011). Theoretically,

the reinforcement learning mechanisms induced by these types of negative prediction errors are to correct one's expectancies and behaviors to avoid determinants responsible for aversion. However, some determinants, such as intractable somatic pain or psychological distress are essentially unavoidable. Long-term experiences of unavoidable, persistent distress would alter the prefrontal circuits that assess the internal state to decide coping strategies (Roy et al., 2014; Shrestha et al., 2015; Aliczki et al., 2016; Zhang et al., 2017). It is well established that chronic pain, as well as depression, leads to hypodopaminergic tone in the brain (Niikura et al., 2010; Belujon and Grace, 2017; Ong et al., 2018). Because the baseline occupancy of dopamine is higher in D2Rs, the reduction in spontaneous dopamine level predominantly affects the D2R, leading to receptor deactivation (Bromberg-Martin et al., 2010). The deactivation of D2R due to somatic pain or psychological distress has been implicated in both animal and human studies. Interestingly, D2R deactivation could increase mGluR5 availability in the striatum (Mao and Wang, 2016). According to the previous study, pharmacological deactivation of D2R resulted in an increase in mGluR5 trafficking to the synaptic membrane, which is mediated by the Src kinase family Fyn (Mao and Wang, 2016). The researchers reported that the same regulation of Fyn, which is induced by D2R deactivation, was also observed in the mPFC (Mao and Wang, 2017). Hence, it is plausible that mPFC-mGluR5 increase in chronic neuropathic pain is related to D2R deactivation, although further study is needed to elucidate the occurrence of the phenomenon in the mPFC.

CONCLUDING REMARKS

Experiencing unavoidable stress, such as somatic pain or psychological distress, alters mPFC circuitry and distorts the perception of the self-state and ensuing decisions regarding coping strategies. We propose that mGluR5 in the mPFC may be a common mediator for both pain and depression. mGluR5 levels would regulate the gain for the assessment of internal state, guide the appraisal of the external state, and be involved in updating the expectancies of behavioral outcome in the mPFC. Increased mPFC-mGluR5 levels during the pathological state would inhibit the activity of pyramidal neurons and reduce the flexibility of the system, resulting in the loss of control of the appropriate processing of the information.

AUTHOR CONTRIBUTIONS

GC conceived the idea, performed the bibliographical research, wrote the draft, and revised the manuscript. SJK and SKK supervised and revised the manuscript.

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frontiers in Molecular Neuroscience

REVIEW
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Group II Metabotropic Glutamate Receptors: Role in Pain Mechanisms and Pain Modulation

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Glutamate is the main excitatory neurotransmitter in the nervous system and plays a critical role in nociceptive processing and pain modulation. G-protein coupled metabotropic glutamate receptors (mGluRs) are widely expressed in the central and peripheral nervous system, and they mediate neuronal excitability and synaptic transmission. Eight different mGluR subtypes have been identified so far, and are classified into Groups I–III. Group II mGluR2 and mGluR3 couple negatively to adenylyl cyclase through Gi/Go proteins, are mainly expressed presynaptically, and typically inhibit the release of neurotransmitters, including glutamate and GABA. Group II mGluRs have consistently been linked to pain modulation; they are expressed in peripheral, spinal and supraspinal elements of pain-related neural processing. Pharmacological studies have shown anti-nociceptive/analgesic effects of group II mGluR agonists in preclinical models of acute and chronic pain, although much less is known about mechanisms and sites of action for mGluR2 and mGluR3 compared to other mGluRs. The availability of orthosteric and new selective allosteric modulators acting on mGluR2 and mGluR3 has provided valuable tools for elucidating (subtype) specific contributions of these receptors to the pathophysiological mechanisms of pain and other disorders and their potential as therapeutic targets. This review focuses on the important role of group II mGluRs in the neurobiology of pain mechanisms and behavioral modulation, and discusses evidence for their therapeutic potential in pain.

Keywords: glutamate, pain, nociception, metabotropic glutamate receptor, mGluR, analgesia

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Abbreviations: ACPD, (1S,3S)-1-aminocyclopentane-1,3-dicarboxylic acid; AP-5, (-)-2-amino-5-phosphonopentanoic acid; APDC, (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate; APICA, (RS)-1-amino-5-phosphonoindan-1-carboxylic acid; BINA, 4-[3-[(2-cyclopentyl-6,7-dimethyl-1-oxo-2,3-dihydroinden-5-yl)oxymethyl]phenyl]benzoic acid; CBiPES hydrochloride, *N*-(4′-cyano-[1,1′-biphenyl]-3-yl-*N*-(3-pyridinylmethyl)-ethanesulfonamide hydrochloride; CBIPES, *N*-(4′-cyano-biphenyl-3-yl)-*N*-(3-pyridinylmethyl)-ethanesulfonamide hydrochloride; DGC-IV, (2S,1′R,2′R,3′R)-2-(2,3-dicarboxycyclopropyl)glycine; HDAC, histone deacetylase; L-CCG-I, (2S, 1S, 2S)-2-(carboxycyclopropyl)glycine; LAC, L-acetylcarnitine; LY341495, (1S,2S)-2-[(2S)-2-amino-3-(2,6-dioxo-3H-purin-9-yl)-1-hydroxy-1-oxopropan-2-yl]cyclopropane-1-carboxylic acid; LY379268, (-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate; LY354740, 2-aminobicyclo[3.1.0]hexane 2,6-dicarboxylate; LY487379, 2,2,2-trifluoro-*N*-[4-(2-methoxyphenoxy) phenyl]-*N*-(3-pyridinylmethyl)ethanesulfonamide; NAAG, *N*-acetylaspartylglutamate; NAC, *N*-acetylcysteine; 2-PMPA, 2-(phosphonomethyl)pentanedioic acid; SLx-3095-1, (+/-)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate; TTX, tetrodotoxin; ZJ-11, (S)-2-[3-[(S)-1-carboxy-3-(methylsulphanyl)propyl]ureido]pentanedioic acid; ZJ-17, (S)-2-[3-[(S)-1-carboxy-2-(4-hydroxyphenyl)ethyl]ureido]pentanedioic acid; ZJ-43, (S)-2-[3-[(S)-1-carboxy-3-methylbutyl]ureido]pentanedioic acid.

METABOTROPIC GLUTAMATE RECEPTORS

The glutamatergic system provides excitatory neurotransmission throughout the central nervous system (CNS), and dysfunction of this system seems is correlated with several disorders such as schizophrenia, depression, and pain states. The interaction of glutamate with its ligand-gated cation channels (NMDA, AMPA, and kainate) mediates fast transmission and cell signaling while the activation of metabotropic glutamate receptors (mGluRs) stimulates intracellular pathways linked to various effector systems involved long-lasting modifications. First evidence for the ability of glutamate to stimulate the production of inositol phosphate (Sladeczek et al., 1985) formed the foundation for the cloning of the first mGluR (Masu et al., 1991). Numerous studies have greatly expanded the field. mGluRs belong to the G-protein coupled receptors (GPCRs) superfamily, and are classified in three groups based on sequence homology, signal transduction pathways and pharmacological agent selectivity (Schoepp et al., 1999; Neugebauer, 2007, 2015; Niswender and Conn, 2010; Nicoletti et al., 2011; Yin et al., 2014).

- Group I includes mGluR1 and mGluR5 coupled to $G_{q/11}$ (Masu et al., 1991), and therefore their activation leads mainly to increased intracellular levels of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) via stimulation of phospholipase C_β, although some evidence suggests the action on additional effector systems, such as mammalian target of rapamycin (mTOR) and the mitogen-activated protein kinase/extracellular receptor kinase (MAPK/ERK) pathways, key components of some form of synaptic plasticity (Hou and Klann, 2004; Page et al., 2006).
- Group II consists of mGluR2 and mGluR3, which are $G_{i/o}$ coupled and promote the inhibition of adenylyl cyclase (Tanabe et al., 1992) and voltage-dependent calcium channels, as well as the activation of voltage-dependent potassium channels (Niswender and Conn, 2010; Nicoletti et al., 2011; Muguruza et al., 2016).
- Group III comprises mGluR4, mGluR7 and mGluR8, which similarly to group II mGluRs are negatively linked to $G_{i/o}$ type proteins, and mGluR6, which is positively coupled to a cGMP phosphodiesterase (Neugebauer, 2008; Niswender and Conn, 2010).

The activity of those receptors can be modulated by pharmacological manipulations of the orthosteric binding sites with agonists, antagonists, or inverse agonists. The issue with the orthosteric ligands is the conservation of the binding site that makes it difficult to develop selective molecules for specific receptors. Another approach is the use of allosteric modulators that bind to specific sites of the receptors different from the orthosteric ones, and as a consequence they modulate the affinity of the receptor for its endogenous ligand. This binding is saturable such that no further effect is possible when all sites are occupied; better selectivity can be achieved because the allosteric binding sites present a lower evolutionary conservation compared to the orthosteric sites, and allosteric modulation can produce positive or negative effects based on the intrinsic activity of the compound (Conn et al., 2009; Montana and Gereau, 2011; Wood et al., 2011). Current research efforts are focused on the development of new positive and negative allosteric modulators (PAM and NAM) as potential therapeutic tools and strategies.

GROUP II METABOTROPIC GLUTAMATE RECEPTORS

The group II mGluRs are found throughout the nervous system, including regions and circuits critically involved in nociceptive signaling and pain modulation as well as in emotional processing (Gu et al., 2008; Wright et al., 2013). They contribute to and modulate synaptic transmission and neuroplasticity, acting at the preterminal region away from the active zone of the synapse as autoreceptors on glutamatergic neurons or heteroreceptors on GABAergic neurons mediating a negative feedback signal (Nicoletti et al., 2011). As perisynaptic receptors, they are located on the pre-synaptic membrane distant from the synaptic cleft, where they can be activated by substantial synaptic glutamate release or astrocytic glutamate (Muguruza et al., 2016; Maksymetz et al., 2017). However, some evidence suggests that mGluR2 and mGluR3 are also expressed post-synaptically. Whereas mGluR2 seems to be present exclusively on neurons, mGluR3 is also found on glia cells (Muguruza et al., 2016). It is becoming clear now that group II mGluRs interact closely with other mGluRs, which has important functional implications. For example, mGluR2 forms a heterodimeric complex with mGluR4 that regulates the efficacies of mGluR2 and mGlu4 allosteric modulators (Yin et al., 2014), while mGluR3 and mGluR5 interact synergistically in the CNS through cross-talk of signaling pathways rather than by heterodimer interactions (Di Menna et al., 2018).

Therapeutic usefulness of compounds acting on group II mGluRs has been suggested for amyotrophic lateral sclerosis (Battaglia et al., 2015), schizophrenia, depression, anxiety (Patil et al., 2007; Fell et al., 2011; O'brien et al., 2014; Muguruza et al., 2016), drug addiction (Moussawi and Kalivas, 2010), Parkinson's disease (Dickerson and Conn, 2012), and pain states (Neugebauer, 2007; Montana and Gereau, 2011; Neugebauer, 2013; Chiechio, 2016). The specific contribution of mGluR2 or mGluR3 subtypes has been challenging to determine because of the close similarity of the proteins that makes it difficult for pharmacological approaches to target these subtypes selectively. In order to better understand the role of individual mGluR subtypes, considerable effort has been dedicated to the development of more selective and CNS penetrant NAMs and PAMs, together with the development of mGluR knockout (KO) mice. While there is strong evidence for an over-activation of the glutamatergic system in pain states (Neugebauer, 2007; Zhou et al., 2011; Guida et al., 2015), and mGluRs in particular (Neugebauer, 2007; Montana and Gereau, 2011; Kolber, 2015), the role of the group II mGluRs and their subtypes in pain mechanisms and pain modulation is less well understood.

PHARMACOLOGICAL AGENTS TARGETING mGluR2/3

Orthosteric Ligands

A number of molecules, classified according to their intrinsic activity and receptor selectivity, have been developed and tested in order to clarify the contribution of group II mGluRs to disease mechanisms and pathological conditions (**Table 1**). Most of the currently available compounds have effects also on other glutamate receptors (ionotropic or metabotropic) and do not differentiate between mGluR2 and mGluR3. For example, DCG-IV is a very potent and selective group II mGluR agonist but has also NMDA agonist effects (Zhou et al., 2011), while L-CCG-I is a potent but not very selective group II mGluR agonist and (2R,4R)-APDC (APDC) is highly selective group II mGluR2/3 agonist (Brabet et al., 1998; Schoepp et al., 1999). (1S,3S)-ACPD (ACPD) is the most selective mGluR2/3 agonist among the isomers of (±)-*cis*-ACPD (Hölscher et al., 1997). More selective and highly potent group II agonists, such as LY2934747, LY389795, LY354740, LY404039, LY379268 (also in the form of disodium salt), have been developed with good efficacy in animal models (Nicoletti et al., 2011, 2015; Caulder et al., 2014; Yin and Niswender, 2014; Maksymetz et al., 2017) including for pain states (Neugebauer et al., 2000; Simmons et al., 2002; Li and Neugebauer, 2006; Neugebauer, 2007; Chiechio and Nicoletti, 2012; Yin and Niswender, 2014; Johnson et al., 2017). SLx-3095-1 is the racemate (± isomers HCl salt) of the agonist LY379268 (– isomer) (Yamamoto et al., 2007). EGLU, LY341495 (also produced as disodium salt), and APICA are selective mGluR2/3 antagonists (Niswender and Conn, 2010; Yin and Niswender, 2014). Recently discovered LY3020371 seems to be even more selective for mGluR2/3 among all the mGluRs with potent effects in rat and human synaptosome preparations as well as in *in vivo* assays (Witkin et al., 2017).

Allosteric Modulators

The development of selective NAMs and PAMs is now beginning to allow the targeting of mGluR2 and mGluR3 (Dhanya et al., 2010; Sheffler et al., 2011b; Bollinger et al., 2017). Only PAMs selectively binding to mGluR2, but not mGluR3, are available such as BINA, LY487379 hydrochloride, and CBIPES hydrochloride (Johnson et al., 2005; Dhanya et al., 2010; Sheffler et al., 2011b). These mGluR2 PAMs attenuated the ketamine-induced release of histamine in the medial prefrontal cortex (mPFC) (Fell et al., 2010) and decreased ketamine- or phencyclidine-induced hyperlocomotion (Sheffler et al., 2011b), suggesting antipsychotic activity of mGluR2. Selective NAMs are available for mGluR3 (LY2389575, VU0477950, and VU0650786) and more recently for mGluR2 (VU6001966) (Bollinger et al., 2017). LY2389575 established a key role of mGluR3 in neuroprotection against β-amyloid induced toxicity (Caraci et al., 2010; Sheffler et al., 2011a), suggesting that pharmacological activation of mGluR3 with PAMs may be a possible therapeutic strategy in Alzheimer's disease. VU0477950 revealed a crucial role of mGluR3 in cognitive functions in mPFC-dependent fear extinction learning (Walker et al., 2015). VU0650786, an even more selective mGluR3 NAM, implicated the synergistic interaction of mGluR3 and mGluR5 in the generation of synaptic plasticity (long-term depression of excitatory transmission in cortical neurons) (Engers et al., 2015; Di Menna et al., 2018). Recently, VU6001966 emerged as a NAM for mGluR2 without any activity at the other mGluRs and with high CNS penetration (Bollinger et al., 2017; Di Menna et al., 2018).

PHARMACOLOGICAL MANIPULATION OF mGluR2/3 IN PAIN: BEHAVIORAL STUDIES

Pharmacological activation of group II mGluRs generally has antinociceptive effects in preclinical studies in rats and mice (Varney and Gereau, 2002; Neugebauer, 2007; Montana and Gereau, 2011; Chiechio, 2016). The activation of mGluR2/3 by systemically (intraperitoneally, i.p.) applied agonists (LY354740, LY379268, and LY389795) decreased nociceptive behavior in the late phase of the formalin test, a relatively acute pain model, in a dose-dependent manner, and the effect was reversed by a group II antagonist (LY341495) (Simmons et al., 2002). Systemic mGlu2/3 activation also decreased mechanical allodynia of neuropathic rats (spinal nerve ligation model, SNL), but had no effect in the tail flick test or the paw withdrawal latency test (acute thermal pain models) (Simmons et al., 2002). Importantly, the antinociceptive effect of systemically (i.p.) applied LY379268 in the formalin pain model was lost in mGluR2, but not mGluR3, knock-out mice (Zammataro et al., 2011), suggesting an important role of mGluR2. Recently, oral application of a prodrug (LY2969822) for the selective mGluR2/3 agonist LY2934747 has been reported to have antinociceptive effects in various preclinical models of inflammatory (formalin, capsaicin, complete Freund's adjuvant [CFA]), postsurgical (plantar incision), visceral (colorectal distension), and neuropathic (SNL) pain (Johnson et al., 2017).

Peripheral

Subcutaneous injection of a group II mGluR agonist (APDC) into the plantar surface of the hindpaw did not change baseline mechanical and thermal sensitivity but blocked prostaglandin E_2 (PGE_2)-induced thermal hyperalgesia, PGE_2- or carrageenan-induced mechanical allodynia, and nociceptive responses in both phases of the formalin test; these antinociceptive effects were inhibited by co-application of a group II mGluR antagonist (LY341495) (Yang and Gereau, 2002, 2003; Yamamoto et al., 2007). Interestingly, group II antagonists (LY341495 and APICA) alone prolonged the mechanical allodynia in the PGE2 and carrageenan models and increased nociceptive behaviors in the capsaicin model, supporting the hypothesis of endogenous mGluR2/3 activation in inflammatory pain conditions (Yang and Gereau, 2002, 2003; Carlton et al., 2011). The data are consistent with antinociceptive effects of peripheral group II mGluR activation, although there may be species differences (Sheahan et al., 2018; see the sections "Peripheral" and "Clinical Trials and Potential Clinical Uses").

TABLE 1 | Drugs acting on mGluR2/3 tested in pain models.

	Compounds	Selectivity	Route of application	Pain model	Effect	Behaviors tests	Neural activity
Orthosteric agonists	DCG-IV	mGluR2/3, NMDA	i.th.	Spinal nerve ligation	Inhibition	Allodynia, mechanical hyperalgesia	
	L-CCG-I	Potent but not selective for mGluR2/3	Intra-PAG	Formalin	Inhibition	Paw-licking, lifting, shaking, flicking	
					Facilitation	Hot plate	
			Intra-spinal	Capsaicin	Inhibition		Spinothalamic tract – neuronal activity
	(1S,3S)-ACPD	mGluR2/3	i.th.	Carrageenan		Inhibition	Spinal dorsal horn – neuronal activity
	(2R,4R)-APDC	mGluR2/3	s.c.	PGE_2-sensitization, carrageenan, formalin	Inhibition	Thermal hyperalgesia, allodynia	
			i.th.	Capsaicin	Inhibition	Allodynia	
				Capsaicin, Complete Freund's adjuvant	No effect	Thermal hyperalgesia, allodynia	
			Ex vivo (skin nerve preparation and cultured DRG)	Capsaicin, PGE_2-sensitization	Inhibition		Neuronal activity and calcium signals
	LY379268	mGluR2/3	i.p.	Formalin	Inhibition	Paw-licking	
				Spinal nerve ligation	Inhibition	Allodynia	
			Ex vivo (mPFC slice)	Kaolin-carrageenan-induced monoarthritis			mPFC – pyramidal output, excitatory transmission
	SLx-3095-1	mGluR2/3	*Ex vivo* (amygdala slice)	Formalin	Inhibition		Amygdala – neuronal activity, excitatory transmission
	LY354740	mGluR2/3	i.p.	Formalin	Inhibition	Paw-licking	
			Intra-amygdala and *ex vivo* (amygdala slice)	Kaolin-carrageenan-induced monoarthritis			Amygdala – neuronal activity, excitatory transmission
	LY389795	mGluR2/3	i.p.	Formalin	Inhibition	Paw- licking	
	LY2934747	mGluR2/3	Oral	Formalin, capsaicin, complete Freund's adjuvant, plantar incision postsurgical, visceral (colorectal distension)	Inhibition	Allodynia, thermal and mechanical hyperalgesia	
			i.v.	Spinal nerve ligation	Inhibition	Allodynia	Spinal dorsal horn – neuronal activity
Orthosteric antagonists	EGLU	mGluR2/3	Intra-PAG	Formalin	No effect	Hotplate	
			Intra-reticular thalamic nucleus	Complete Freund's adjuvant	Inhibition	Ankle-bend score	

(Continued)

TABLE 1 | Continued

	Compounds	Selectivity	Route of application	Pain model	Effect	Behaviors tests	Neural activity
			Intra-amygdala	Kaolin-carrageenan-induced monoarthritis	Facilitation		Amygdala – neuronal activity, excitatory transmission
	APICA	mGluR2/3	s.c.	PGE2-sensitization, carrageenan, capsaicin	Facilitation	Allodynia, paw-licking, flinching	
			Ex vivo (skin nerve preparation and cultured DRG)	Capsaicin			Nociceptive fiber activity; calcium signals
	LY341495	mGluR2/3	s.c.	PGE2-sensitization, carrageenan, capsaicin	Facilitation	Allodynia, paw-licking, flinching	
			i.th.	Complete Freund's adjuvant	Inhibition	Allodynia	
					No effect	Thermal hyperalgesia	
			Ex vivo (skin nerve preparation and cultured DRG)	Capsaicin	Facilitation		Nociceptive fiber activity; calcium signals
			Ex vivo (mPFC slice)	Kaolin-carrageenan-induced monoarthritis			mPFC – pyramidal output, excitatory transmission

See the sections "Pharmacological Manipulation of mGluR2/3 in Pain: Behavioral Studies" and "Pharmacological Manipulation of mGluR2/3 in Pain: Electrophysiological Studies" for references.

Spinal

Intrathecal (i.th.) administration of a selective group II mGluR agonist (APDC) in the absence of tissue damage had no effect on mechanical thresholds (von Frey test) and thermal paw withdrawal latencies but inhibited capsaicin-induced mechanical allodynia without affecting thermal hyperalgesia (Soliman et al., 2005). However, intrathecal APDC had no effect in an inflammatory pain model induced by subcutaneous (s.c.) CFA injection into the hindpaw (Zhang et al., 2009). In neuropathic rats (SNL model) intrathecal application of a group II mGluR agonist (DCG-IV) decreased mechanical allodynia (von Frey test) and mechanical hyperalgesia (paw withdrawal threshold to noxious pressure stimuli) in a dose-dependent way, and these antinociceptive effects were blocked by a group II mGluR antagonist (EGLU) (Zhou et al., 2011). Interestingly, intrathecal application of DCG-IV had a pronociceptive effect in sham rats, which was reversed by an NMDA receptor antagonist (AP-5), suggesting that this effect was mediated by the activation of NMDA receptors (Zhou et al., 2011). Intrathecal application of a group II mGluR antagonist (LY341495) ameliorated mechanical allodynia, but not thermal hyperalgesia, in the CFA-induced inflammatory pain model; the antinociceptive effect was potentiated by a glial cells inhibitor (fluorocitric acid) (Zhang et al., 2009). These mixed and somewhat inconsistent effects of group II mGluR compounds may be due to a lack of subtype-specificity and/or reflect rather complex functions of group II mGluRs in spinal nociceptive processing.

Brainstem

In the periaqueductal gray (PAG), activation of group II mGluRs had pronociceptive effects under normal conditions but antinociceptive effects in an acute pain model. Microinjection of a group II agonist (L-CCG-I) into the dorsolateral PAG dose-dependently inhibited the nociceptive responses (lifting, licking, shaking and flicking the injected paw) in the late phase of the formalin test (acute pain model) (Maione et al., 2000), but had a dose-dependent pronociceptive effect in the hot plate test, decreasing the latency of the nociceptive responses (licking the paw; jumping) (Maione et al., 1998). Both effects were counteracted by the intra-PAG administration of a group II mGluR antagonist (EGLU) (Maione et al., 1998, 2000). EGLU alone had no effect in the hotplate test (Maione et al., 1998). Together with microdialysis data showing that L-CCG-I increased serotonin release in the PAG in a $GABA_A$ receptor dependent way (Maione et al., 1998), these results were interpreted to suggest that group II mGluRs in the PAG promote an antinociceptive effect mainly by decreasing GABA release to potentiate the activity of the descending antinociceptive pathway following persistent noxious stimulation (Maione et al., 2000).

Brain

In the thalamus group II mGluRs mediate the presynaptic inhibition of GABAergic inhibitory transmission from the reticular thalamic nucleus to the somatosensory ventrobasal

thalamus (VB) (Salt and Turner, 1998) to facilitate sensory processing through an action on mGluR2 (Copeland et al., 2012) possibly on astrocytes (Copeland et al., 2017). Stereotaxic administration of a group II mGluR antagonist (EGLU) into the reticular thalamic nucleus, but not other thalamic nuclei, had an antinociceptive effect in an arthritis pain model (complete Freund's adjuvant-induced monoarthritis in the ankle joint), reducing the ankle-bend test scores, possibly through a mechanism that involves blocking the disinhibition of somatosensory thalamic relay neurons (Neto and Castro-Lopes, 2000).

Subtype Selective Interventions

Behavioral effects of negative and positive allosteric modulators for mGluR2 and mGluR3 remain to be determined in pain conditions, but the contribution of individual subtypes is being addressed using alternative approaches.

N-acetylcysteine (NAC) has been used to probe mGluR2 function in pain models. NAC promotes the activity of the L-cystine/L-glutamate membrane exchanger (Sxc-), a crucial antiporter for the release of glutamate from astrocytes for the endogenous activation of perisynaptic mGluR2/3 (Kalivas, 2009). NAc may therefore be used to increase endogenous activation of these receptors (Chiechio and Nicoletti, 2012). Systemic (i.p.) application of NAC inhibited nocifensive behaviors in the tail flick test (Truini et al., 2015) and in the second phase of the formalin test (Bernabucci et al., 2012), and decreased mechanical hypersensitivity in an inflammatory pain model (subcutaneous CFA in the hindpaw) and in a neuropathic pain model (chronic constriction injury, CCI) (Bernabucci et al., 2012). The effects of NAC were blocked by an mGluR2/3 antagonist (LY341495) (Bernabucci et al., 2012). The antinociceptive effect of NAC in the formalin pain model was lost in mGluR2, but not mGluR3, knockout mice (Bernabucci et al., 2012), which points to an action on mGluR2.

Drug-induced potentiation of the transcription of GRM2, the gene encoding for mGluR2, has been used to assess antinociceptive effects of increased expression of mGluR2 in dorsal root ganglia and spinal dorsal horn (see Chiechio and Nicoletti, 2012). Indeed, *epigenetic drugs* such as LAC (Chiechio et al., 2002) and HDAC inhibitors (Chiechio et al., 2009) showed antinociceptive effects in different pain models. Systemic (s.c.) application of LAC decreased mechanical and thermal hypersensitivity in a neuropathic pain model (CCI) through increased expression of mGluR2 but not mGluR3 (Chiechio et al., 2002). Systemic (s.c.) application of HDAC inhibitors reduced the nociceptive response in the second phase of the formalin test by up-regulation of mGluR2 expression (Chiechio et al., 2009). Spinal (i.th.) administration of HDAC inhibitors attenuated the pronociceptive effect of estrogen on visceral sensitivity (increased visceromotor response to colorectal distension) and increased mGluR2 but not mGluR3 expression (Cao et al., 2015), which is consistent with a predominant action on mGluR2.

For the study of mGluR3 function, the neuropeptide *N-acetylaspartylglutamate (NAAG)* has been tested as a preferential activator of mGluR3 (Neale et al., 2000; Neale, 2011). Consistent with its wide distribution throughout the nervous system, local peripheral (s.c.) application of NAAG inhibited mechanical allodynia in the carrageenan-induced hindpaw inflammatory pain model (Yamamoto et al., 2007) and intracerebroventricular (i.c.v.) administration was antinociceptive in both phases of the formalin pain test (Yamamoto et al., 2008). Another strategy to target mGluR3 is to increase NAAG levels with NAAG peptidase inhibitors such as ZJ-11, ZJ-17 and ZJ-43, ZJ-45 or 2-PMPA, to block NAAG degradation (Neale et al., 2005). NAAG peptidase inhibitors administered systemically or peripherally or locally into CNS regions had antinociceptive effects in models of inflammatory and neuropathic pain. Systemic (intravenous, i.v., or i.p.) application decreased nociceptive behaviors (flinching) in both phases of the formalin pain test (Yamamoto et al., 2004; Nonaka et al., 2017) and had antiallodynic effects in a neuropathic pain model (partial sciatic nerve ligation) without affecting baseline mechanical and thermal sensitivity in the von Frey and hot plate tests, respectively (Yamamoto et al., 2004). Peripheral (s.c.) injection also decreased both phases of the formalin pain test and had anti-allodynic effects in the carrageenan pain model (Yamamoto et al., 2007). Spinal (i.th.) application inhibited nocifensive responses (flinching) in both phases of the formalin pain test and mechanical allodynia in the partial sciatic nerve ligation model, but had no effect in the von Frey and hot plate tests (Yamamoto et al., 2004). Microinjections into PAG or rostral ventromedial medulla (RVM) inhibited nociceptive behaviors (flinching) in both phases of the formalin pain test but had no effect in the hot plate test (Yamada et al., 2012). Injections into the locus coeruleus had similar antinociceptive effects in the formalin test (Nonaka et al., 2017). Intracerebroventricular administration also reduced nociceptive behaviors in both phases of the formalin pain test response (Yamamoto et al., 2008). Antinociceptive effects were blocked with a group II mGluR antagonist (LY341495) where tested in these studies. While there has been some controversy regarding the selective activation of mGluR3 with NAAG and peptidase inhibitors (for Discussion, see Neale, 2011) studies from mGluR2 and mGluR3 knockout mice provide strong evidence for mGluR3 mediated effects (Olszewski et al., 2017).

PHARMACOLOGICAL MANIPULATION OF mGluR2/3 IN PAIN: ELECTROPHYSIOLOGICAL STUDIES

Effects of group II mGluR agonists or antagonists on pain-related neuronal activity were studied in primary sensory neurons, spinal dorsal horn, and a few brain regions (amygdala and mPFC). Drugs were typically administered locally and there is surprisingly little information available for neuronal effects of systemic drug application and their site(s) of action. To the best of our knowledge, only a recent study showed inhibitory effects of a systemically (i.v.) applied mGluR2/3 agonist (LY2934747) on extracellularly recorded background activity and electrically evoked (C-fiber stimulation) wind-up discharges of dorsal horn

neurons in neuropathic rats (SNL model) (Johnson et al., 2017).

Peripheral

A group II agonist (APDC) inhibited extracellularly recorded activity (action potentials) of nociceptive fibers evoked by capsaicin or by an inflammatory soup, and blocked the inflammatory soup- or forskolin-induced sensitization of heat responses in an *in vitro* rat skin-nerve preparation (Du et al., 2008). APDC had no effect on baseline heat or mechanical thresholds or discharges (Du et al., 2008; Carlton et al., 2011). APDC reversed the PGE_2-induced hyperexcitability of cultured mouse and human primary sensory (dorsal root ganglia, DRG) neurons (Davidson et al., 2016). Interestingly, APDC blocked the PGE_2-induced sensitization of capsaicin responses (calcium influx) in cultured mouse, but not human, DRG neurons (Yang and Gereau, 2002; Sheahan et al., 2018) through a mechanism that involved Gi dependent inhibition of adenylyl cyclase (Yang and Gereau, 2002).

Group II mGluR antagonists (LY341495 or APICA) enhanced the capsaicin-induced action potentials of nociceptive fibers in the skin nerve preparation or calcium signals in DRG neurons, but had no effect alone, suggesting that group II mGluRs act endogenously to reverse hypersensitivity (Carlton et al., 2011). In presence of excess extracellular glutamate in the skin nerve preparation, the blockade of the group II mGluRs also increased activity and heat responses of nociceptive fibers, which was interpreted to suggest that GluR2/3 activation by the exogenous glutamate decreased nociceptor activity and this activity-dependent autoinhibition of nociceptive signal transmission to the CNS would modulate pain sensitivity (Carlton et al., 2011).

Spinal

Intrathecal administration of a group II agonist, ACPD, inhibited electrically evoked C-fiber responses of dorsal horn neurons recorded extracellularly in anesthetized rats with a carrageenan-induced hindpaw inflammation (3 h postinduction), but had mixed (excitatory or inhibitory) effects in normal animals (Stanfa and Dickenson, 1998). Administration of group II mGluR agonists (LY379268 and L-CCG-I) into the spinal dorsal horn by microdialysis decreased the central sensitization of primate (*Macaca fascicularis*) spinothalamic tract cells induced by intradermal capsaicin (30 min postinduction), but had no effect on the responses of non-sensitized neurons to innocuous and noxious cutaneous mechanical stimuli (Neugebauer et al., 2000). Information about the role of spinal group II mGluRs and their subtypes nociception and pain models is surprisingly thin, and given the mixed results of behavioral studies (see the section "Spinal") the spinal cord may not be the main target of their overall beneficial effects related to pain.

Brain

Actions of group II mGluR compounds have been studied in the amygdala, a key player in emotions, emotional aspects of pain and pain modulation (Neugebauer et al., 2004; Thompson and Neugebauer, 2017), and in the mPFC, a center for executive functions and behavioral control related to negative emotions and pain (Neugebauer et al., 2009; Ong et al., 2018). In anesthetized rats, stereotaxic administration (microdialysis) of a group II mGluR agonist (LY354740) into the central nucleus of the amygdala (CeA) targeting its laterocapsular division (CeLC), which is also referred to as the "nociceptive amygdala," decreased the responses of CeLC neurons to innocuous and noxious mechanical stimuli under normal conditions, but became more potent in an arthritis pain model (kaolin-carrageenan-induced monoarthritis in the knee) (Li and Neugebauer, 2006). The agonist effects were blocked by co-administration of a group II antagonist (EGLU), which by itself had no effect under normal conditions but increased the evoked responses to noxious stimulation of the arthritic knee in the pain model (Li and Neugebauer, 2006). This would be consistent with endogenous activation and gain of function of mGluR2/3 in the amygdala in a pain condition. Patch-clamp recordings of CeLC neurons in rat brain slices showed that a group II mGluR agonist (LY354740) inhibited excitatory synaptic inputs (EPSCs) from the parabrachial area, which provide nociceptive information to the amygdala (Neugebauer et al., 2004; Thompson and Neugebauer, 2017), under normal conditions, but became more potent in the arthritis pain condition (Han et al., 2006; Kiritoshi and Neugebauer, 2015). LY354740 decrease frequency, but not amplitude, of miniature EPSCs in the presence of TTX, suggesting a presynaptic site of action on the glutamatergic terminals (Han et al., 2006; Kiritoshi and Neugebauer, 2015). EGLU blocked the agonist effect, but had no effect on its own, which is similar to the lack of significant effects of another group II mGluR antagonist (LY341495) at the presumed parabrachial (PB)-CeLC synapse in mouse brain slices (Adedoyin et al., 2010), suggesting that the endogenous activation observed in the *in vivo* condition (see above) may be lost in the reduced brain slice preparation (Han et al., 2006; Kiritoshi and Neugebauer, 2015).

In the infralimbic mPFC, rat brain slice physiology experiments found that group II mGluRs decreased the output of principal layer V pyramidal cells as the result of an inhibitory action on glutamatergic synapses under normal conditions and in an arthritis pain model (kaolin-carrageenan-induced monoarthritis in the knee), and that this system was tonically active under both conditions (Kiritoshi and Neugebauer, 2015; Thompson and Neugebauer, 2017). Specifically, a selective group II mGluR agonist (LY379268) decreased synaptically evoked spiking of pyramidal cells in brain slices from normal and arthritic rats by inhibiting direct excitatory inputs (EPSCs) as well as glutamate-driven feedforward inhibitory transmission (IPSCs) (Kiritoshi and Neugebauer, 2015; Thompson and Neugebauer, 2017). Abnormally enhanced synaptic inhibition of mPFC output in pain conditions has been linked to cognitive dysfunction (Ji et al., 2010; Kiritoshi et al., 2016) and loss of cortical control of amygdala function (Ji and Neugebauer, 2014; Kiritoshi and Neugebauer, 2018). Effects of LY379268 on EPSCs preceded those on IPSCs, resulting in a net inhibitory effect on pyramidal output. Spontaneous and miniature (in TTX) analyses of EPSCs and IPSCs showed that LY379268 acted presynaptically on glutamatergic, but not GABAergic, terminals. The effects of LY379268 were blocked by a selective group II mGluR antagonist

(LY341495) that by itself increased synaptically evoked spiking of pyramidal cells under normal conditions and in the pain model (Kiritoshi and Neugebauer, 2015; Thompson and Neugebauer, 2017), suggesting endogenous activation of mGluR2/3. It has been speculated that failure to release this inhibitory tone to enhance mPFC output could be a mechanism of pain persistence due to a lack of cortical control (Kiritoshi and Neugebauer, 2015; Thompson and Neugebauer, 2017).

Subtype Selective Interventions

In mouse amygdala brain slices, exogenous NAAG and a NAAG peptidase inhibitor (ZJ-43) (see the section "Subtype Selective Interventions") have been tested in order to elucidate the specific contribution of mGluR3. Under normal conditions, NAAG and ZJ-43 inhibited excitatory transmission (EPSCs) at the presumed PB-CeLC synapse (see the section "Brain") similarly to an mGluR2/3 agonist (SLx-3095-1) (Adedoyin et al., 2010), which is consistent with an mGluR3 effect. The effect of ZJ-43 was blocked by a group II mGluR antagonist (LY341495). In the formalin pain model (brain slices taken 24 h postinduction), ZJ-43 was much less efficacious than SLx-3095-1 in inhibiting EPSCs, suggesting a decreased release of NAAG or an increased contribution of mGluR2 rather than mGluR3 in the pain condition (Adedoyin et al., 2010).

CLINICAL TRIALS AND POTENTIAL CLINICAL USES

Pain conditions affect millions of people, and pain management can be challenging and often is insufficient with currently available tools. Based on several lines of evidence from preclinical studies, drugs acting on mGluR2/3 may be useful for pain relief, but so far have not advanced to clinical trials as analgesic candidates, perhaps because of concerns about the translation from animal models to the human condition (Davidson et al., 2016). However, despite concerns for example about the development of tolerance in some rodent studies, there is no evidence for a loss of efficacy on repeat dosing of group II mGluR agonists in humans (Johnson et al., 2017). In fact, significant anxiolytic efficacy of an oral prodrug (LY544344) of an mGluR2/3 agonist (LY354740) was observed in patients with generalized anxiety (Dunayevich et al., 2008). An oral prodrug (LY2140023, pomaglumetad methionil) of an mGluR2/3 agonist (LY404039) had significant antispychotic efficacy in schizophrenia patients (Patil et al., 2007) or in subgroups of schizophrenia patients (Kinon et al., 2015; Nisenbaum et al., 2016). In these studies, drug effects were maintained or enhanced following several weeks dosing (Johnson et al., 2017). It should be noted that NAC, which has been linked to the endogenous activation of mGluR2/3 (see the section "Subtype Selective Interventions"), given orally to healthy human subjects, decreased thermal pain ratings to laser stimuli and amplitudes of laser-evoked brain potentials without affecting thermal pain thresholds (Truini et al., 2015).

CONCLUSION

Preclinical studies suggest that group II mGluRs play a significant role in the modulation of nociception and pain conditions. There is some evidence to suggest distinct roles of mGluR2 and mGluR3 subtypes in different neural circuits and regions, but this remains to be determined more thoroughly with the availability of more selective compounds such as allosteric modulators. These new tools are also useful for the analysis of the pathophysiological mechanisms of pain conditions. Effectiveness of mGluR2/3 compounds in clinical studies on conditions other than pain may support their therapeutic potential for the management of pain (Johnson et al., 2017).

AUTHOR CONTRIBUTIONS

MM collected information and provided first draft of manuscript. EP and SM provided valuable input to the manuscript. VN conceived the project and finalized the manuscript.

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Epigenetic Alterations in Prenatal Stress Mice as an Endophenotype Model for Schizophrenia: Role of Metabotropic Glutamate 2/3 Receptors

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Mice subjected to prenatal restraint stress (*PRS* mice) showed biochemical and behavioral abnormalities consistent with a schizophrenia-like phenotype (Matrisciano et al., 2016). PRS mice are characterized by increased DNA-methyltransferase 1 (DNMT1) and ten-eleven methylcytosine dioxygenase 1 (TET1) expression levels and exhibit an enrichment of 5-methylcytosine (5MC) and 5-hydroxymethylcytosine (5HMC) at neocortical GABAergic and glutamatergic gene promoters. Activation of group II metabotropic glutamate receptors (mGlu2 and−3 receptors) showed a potential epigenetically-induced *antipsychotic* activity by reversing the molecular and behavioral changes observed in PRS mice. This effect was most likely caused by the increase in the expression of growth arrest and DNA damage 45-β (Gadd45-β) protein, a molecular player of DNA demethylation, induced by the activation of mGlu2/3 receptors. This effect was mimicked by clozapine and valproate but not by haloperidol. Treatment with the selective mGlu2/3 receptors agonist LY379268 also increased the amount of Gadd45-β bound to specific promoter regions of reelin, BDNF, and GAD67. A meta-analysis of several clinical trials showed that treatment with an orthosteric mGlu2/3 receptor agonist improved both positive and negative symptoms of schizophrenia, but only in patients who were early-in-disease and had not been treated with atypical antipsychotic drugs (Kinon et al., 2015). Our findings show that PRS mice are valuable model for the study of epigenetic mechanisms involved in the pathogenesis of schizophrenia and support the hypothesis that pharmacological modulation of mGlu2/3 receptors could impact the early phase of schizophrenia and related neurodevelopmental disorders by regulating epigenetic processes that lie at the core of the disorders.

Keywords: mGlu2/3 receptors, schizophrenia, clozapine, epigenetics, prenatal stress

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HIGHLIGHTS

- Prenatal restraint stress (*PRS*) in mice showed epigenetic changes and behavioral abnormalities consistent with a schizophrenia-like phenotype.
- Prenatal stress (PRS) represents a suitable non-pharmacological model to study schizophrenia and to develop novel antipsychotics.
- Activation of mGlu2/3 receptors corrects the altered epigenetic and behavioral changes induced by prenatal stress in mice.
- Both clozapine and the mGlu2/3 receptor agonist LY379268 acted as epigenetic agents targeting specifically DNA methylation reversing the molecular and behavioral alterations in PRS mice.

INTRODUCTION

Schizophrenia is a major psychotic disorder which affects one percent of the world's population and usually leads to a severe mental disability (Ribe et al., 2015). All marketed antipsychotic drugs antagonize D2 dopamine and 5-HT_{2A} serotonin receptors, showing good clinical efficacy in improving positive symptoms, and moderate activity in improving negative symptoms of schizophrenia. None of these drugs has significant activity on cognitive symptoms associated with schizophrenia, with the possible exception of clozapine (Lieberman et al., 2005). Treatment of cognitive and negative symptoms remains an unmet need in the treatment of schizophrenia, and this encourages the identification and validation of novel drug targets. Etiology of schizophrenia is still unknown despite the recent progresses made possible by molecular genetics and functional neuroimaging. It is generally believed that schizophrenia is not caused by a single factor, but results from the convergence of genetics and environmental factors. Recently, an imbalance between GABA and glutamate neurotransmission has been suggested as a key mechanism underlying the pathophysiology of schizophrenia. Drugs that block the activity of NMDA receptors present on cortical-limbic GABAergic interneurons, such as ketamine or phencyclidine (PCP) are capable to replicate the full range of psychotic symptoms, including hallucinations (Kristiansen et al., 2007; Meltzer et al., 2011). Thus, novel antipsychotic drug development should focus on the GABA and glutamate systems, which act upstream of the dopamine circuit, and are primarily involved in the pathophysiology of the disorder (**Figure 1**). A hypofunction of the NMDA receptors on GABAergic interneurons, particularly fast-spiking, parvalbumin-positive chandelier and basket cells, leads to an overactivity of pyramidal neurons and to an impairment of network oscillations that underlie multiple domains of cognitive function (Homayoun and Moghaddam, 2007; Gonzalez-Burgos and Lewis, 2008). The release of GABA is crucial for the normal firing of pyramidal neurons in the prefrontal cortex and for the equilibrium of the subcortical regions fundamental for optimizing cognitive and emotional function (Benes et al., 2007). Schizophrenia is a chronic devastating disorder that leads to a severe disability at relatively young age. It has a peculiar pathological course starting with the prodromal phase followed by a first episode, which occurs around adolescence or young adult age (Millan, 2012). Evidence suggests that epigenetic changes, occurring during early development as a result of the combination of a predisposing genetic background, in shaping the premorbid phase of the disease, and environmental factors, acting as "second hits," precipitate the onset of schizophrenia (Guidotti et al., 2005). We and others have studied the epigenetic hallmarks of schizophrenia in postmortem human brain tissue. Moving from these findings, we have investigated whether the same epigenetic changes occur in the brain of mice subjected to prenatal stress at different stages of postnatal development. The purpose of this review is to provide an update (i) of our current findings and knowledge of the topic of *neuroepigenetics* in schizophrenia, (ii) of the role of metabotropic glutamate 2/3 receptors in prenatally stressed mice (PRS mice) as potential targets for novel antipsychotics; and (iii) to show our more recent observations on the epigenetic effects induced by the mGlu2/3 receptors agonist, LY379268, and by clozapine.

EPIGENETIC CHANGES IN SCHIZOPHRENIA

Neuroepigenetic dysregulations were detected in the hippocampus and cortex of brain of patients affected by schizophrenia (Numata et al., 2014; Dong et al., 2015). *Epigenetics* is defined as modifications of the genome, heritable during cell division, that do not involve a change in DNA sequence. Epigenetic mechanisms are considered to mediate gene-environment interplay during the entire lifespan. Several clinical evidence support a role of altered epigenetic mechanisms underlying embryonic, postnatal, and adult neurogenesis (Roth et al., 2011). Aberrations in the epigenetic regulation machinery have been hypothesized in neurodevelopmental disorders, such as schizophrenia and autism spectrum disorders (Zhubi et al., 2017). A growing body of evidence from Dr. Guidotti's group (Matrisciano et al., 2012, 2013, 2016) and other researchers (Meaney and Szyf, 2005; Benes et al., 2007; McGowan and Szyf, 2010) suggest that epigenetic modifications of DNA (promoter methylation) and chromatin remodeling induced by environmental factors, including stress, may contribute to the complex phenotypes of neuropsychiatric disorders, such as schizophrenia. DNA methyltransferases (DNMT1 and 3a) (the enzymes that transfer a methyl group from S-adenosylmethionine to carbon 5 of the cytosine pyrimidine ring embedded in cytosine-phospho-guanine [CpG] islands containing promoters), and ten-eleven translocation hydroxylase (TET 1,2,3), (the enzymes that catalyze the conversion of 5MC to 5HydroxyMC), are important components of the DNA- methylation/demethylation pathways regulating the expression of key molecules involved in brain development and maturation. Importantly, the prefrontal cortex GABAergic interneurons of schizophrenia patients express an increase in DNMT1 and 3a, and an increase in TET1 associated with deficits in GABAergic function (Guidotti et al., 2011). This includes the downregulation of the

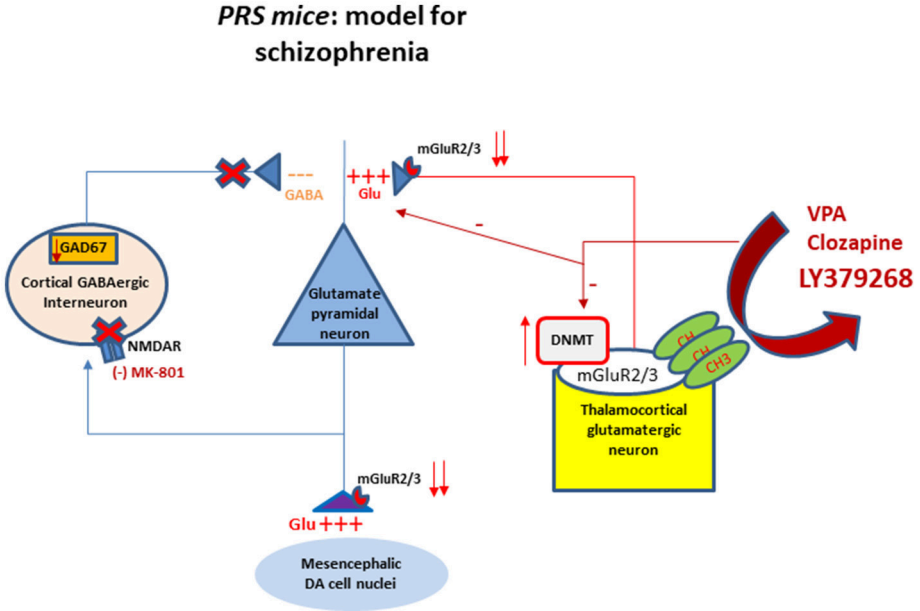

FIGURE 1 | Schematic representation of the interactions between GABAergic and glutamatergic neurotransmission in cortical-limbic structures of PRS mice. The cartoon shows altered DNA promoter hypermethylation (increase in DNMT) occurring at the mGlu2/3 receptors gene promoter and their decreased expression at presynaptic level of thalamocortical glutamatergic neurons. The downregulation of mGlu2/3 receptors at the axon terminal of thalamocortical glutamatergic neurons results in the hyperactivation of glutamatergic pyramidal neurons. This activation is facilitated by a decrease of GABAergic feedback inhibition on pyramidal neurons. The hypofunction of GABAergic interneurons is mediated by a downregulation of NMDA receptor function as suggested by the behavioral hypersensitivity to small doses of NMDA receptor blocker MK-801 (Matrisciano et al., 2013). The same fibers project to subcortical areas causing an excessive firing and dopamine release. The cartoon shows also the mGlu2/3 receptors at presynaptic level of the thalamocortical fibers as potential target for pharmacological interventions such as the mGlu2/3 receptor agonist LY379268, valproate and clozapine to restore the normal balance between GABA and glutamate through epigenetic mechanisms. DA, dopaminergic; DNMT, DNA methytransferase; NMDA, N-methyl-D-aspartate; CH3, methyl group.

glutamic acid decarboxylase 67 (GAD), reelin, GABA reuptake transporters and brain derived neurotrophic factor (BDNF), which are essential for neurogenesis, neurodevelopmental neuronal migration and synaptic plasticity. In addition to alterations of GABAergic transmission (Veldic et al, 2007; Lewis and González-Burgos, 2008; Kundakovic et al., 2009; Marek, 2010; Niswender and Conn, 2010; Marín, 2012), there are evidence of altered glutamatergic transmission involving both ionotropic glutamate receptors such as NMDA receptors present post-synaptically on GABAergic interneurons (**Figure 1**) and metabotropic glutamate receptors such as mGlu2/3 receptors located at least in part pre-synaptically on thalamocortical glutamate axon terminals [Benes et al., 2007; Stansley and Conn, 2018; **Figure 1**] where they modulate the glutamatergic transmission by restoring the hyperactivity of the cortical pyramidal neurons (**Figure 1**). Clinically, while NMDA receptor agonists are not fully considered as antipsychotic drugs due to their potential excitotoxicity effects and neuronal damage, metabotropic glutamate 2/3 receptors represent a suitable target for glutamatergic tone regulation.

Taken together, these observations suggest that the epigenetic hypothesis of schizophrenia is supported by the following evidence obtained in postmortem human brain: (i) alterations of glutamic acid decarboxylase 67 (gene symbol = GAD1), DNMT and reelin in GABAergic neurons, and (ii) of brain derived nerve growth factor (BDNF), vesicular glutamate transporter (VGLUT1) and (iii) alterations in mGlu2/3 receptors in glutamatergic neurons (Guidotti et al., 2011; Nicoletti et al., 2011).

THE *PRS* AS A SUITABLE ANIMAL MODEL TO STUDY NEURODEVELOPMENTAL DISORDERS

Prenatal or early-life stress, through changes in the epigenetic mechanisms, has been considered a predisposing factor for major neuropsychiatric disorders including schizophrenia, bipolar disorders, and autism spectrum disorder. Time- and spatial-dependent neurodevelopmental cues associated with neuronal differentiation and synaptic plasticity support the hypothesis that these disorders might originate even before birth. Interestingly, we have reported that adult offspring of mice exposed to repeated episodes of restraint stress during pregnancy, named *PRS mice*, exhibit a schizophrenia-like behavioral phenotype characterized by hyperactivity, stereotyped and compulsive behavior, deficits in social interaction and pre-pulse inhibition (PPI), altered fear conditioning, object recognition, and hypersensitivity to N-methyl D-aspartate (NMDA) receptor blockers (Matrisciano et al., 2016). This behavioral phenotype recapitulates positive and negative symptoms, as well as cognitive dysfunction displayed

by patients affected by schizophrenia (Matrisciano et al., 2013). PRS mice also show a deficit in cortical GABAergic innervation, which is expected to cause abnormal synchronization of the firing rate of pyramidal neurons, a putative electrophysiological substrate of cognitive dysfunction in psychotic patients and neurodevelopmental animal models of SZ (Gonzalez-Burgos and Lewis, 2008). In addition to alterations of GABAergic system, PRS mice show molecular disruption in chromatin remodeling at genes expressed in glutamatergic neurons, such mGlu2/3 receptors (**Figure 1**). These molecular changes in PRS mice are similar to those observed in the brain of schizophrenia patients, suggesting a strong correlation between the aberrant epigenetic GABAergic/glutamatergic mechanisms and psychotic symptoms. **Table 1** summarize the behavioral and molecular features observed in *PRS mice* and *Schizophrenia patients*.

ROLE OF METABOTROPIC GLUTAMATE 2/3 RECEPTORS IN SCHIZOPHRENIA

Disruption in the glutamatergic system is considered to play a key role in the pathophysiology of schizophrenia (Akbarian et al., 1995). It has been reported that *patients* affected by schizophrenia respond only partially to standard "monoaminergic" antipsychotic drugs (Lieberman et al., 2008; Meltzer, 2013). The lack of a full recovery from negative and cognitive symptoms gave the impetus to investigate different molecular targets including mGlu2 and mGlu3 receptors. mGlu receptors, which belong to class C of the G protein-coupled receptors form a family of eight subtypes traditionally subdivided into three groups based on sequence homology, intracellular signaling and pharmacological profile. mGlu1 and mGlu5 receptors (group I) are coupled to Gq/11 and their activation stimulates polyphosphoinositide hydrolysis with ensuing formation of inositol-1,4,5-trisphosphate and diacylglycerol. These receptors are localized in the peripheral portions of postsynaptic densities [reviewed by (Nicoletti et al., 2011)]. mGlu2 and mGlu3 receptors are coupled to Gi/o and their activation inhibits adenylyl cyclase activity and modulate the activity of calcium and potassium channels. Both receptors are localized in axon terminals where they negatively modulate neurotransmitter release [(Nicoletti et al., 2011); **Figure 1**]. However, recent findings indicate that mGlu3 receptors are also localized in postsynaptic densities, where they boost mGlu5 receptor signaling (Di Menna et al., 2018). mGlu4, mGlu7, and mGlu8 receptors (group III) are also coupled to Gi/o and are found in presynaptic terminals close to the active zone of neurotransmitter release (Nicoletti et al., 2011). Symptoms of schizophrenia are thought to be associated, at least in part, with hyperactive and dysregulated glutamatergic neurotransmission in key brain regions, such as the thalamus, prefrontal cortex, and limbic system. Based on this evidence, pharmacological activation of mGlu2/3 receptors may ameliorate the schizophrenia symptoms through a decrease in glutamate release thereby reducing synaptic firing due to the particular synaptic distribution of these receptors and causing neuroprotective effects. Interestingly, PRS mice showed a decrease in the expression of mGlu2 and mGlu3 receptor mRNA and proteins in the frontal cortex. This decrease manifest at birth and, at least for mGlu2 receptors, persisted in adult life associated with an increase in the DNMT binding to the gene promoter [for more details see (Matrisciano et al., 2013)] suggesting an epigenetic regulation of the receptors induced by prenatal stress and it may reflect a key factor for the pathogenesis of the disease.

In schizophrenia research, particular attention has been paid to group-II mGlu receptors on the basis of genetic and pharmacological data (Gregory and Conn, 2015).

An initial hypothesis was that activation of mGlu2/3 receptors could improve psychotic symptoms by inhibiting glutamate release (Battaglia et al., 1997), and, therefore, restraining the hyperactivity of pyramidal neurons associated with schizophrenia. However, this mechanism may also amplify the defect in glutamate-mediated activation of GABAergic interneurons, thus worsening the "glutamatergic hypofunction" that underlies cognitive dysfunction in schizophrenia. The evidence that activation of mGlu2 receptors inhibits electrophysiological responses mediated by 5-HT_{2A} receptors at thalamo-cortical synapses (Aghajanian and Marek, 2000) shed new light into the defensive role played by mGlu2 receptors in schizophrenia. Javier Gonzales-Maeso and his Associates have consistently shown that mGlu2 and 5-HT_{2A} receptors form functional multimeric complexes, in which mGlu2 receptors negatively modulate 5-HT_{2A} receptor signaling (González-Maeso et al., 2008). Interestingly, opposite changes in the expression of mGlu2 and 5-HT_{2A} receptors were found in postmortem brain tissue from patients affected by schizophrenia, with the physiological balance between the two receptors being shifted toward 5-HT_{2A} receptors (Muguruza et al., 2013). This is nicely consistent with the reduced expression of mGlu2 receptors in the prefrontal cortex found across the postnatal development of PRS mice, which show a schizophrenia-like phenotype in the adult life (Matrisciano et al., 2016). Interestingly, treatment with

TABLE 1 | Epigenetic and behavioral deficits found in schizophrenia patients and in PRS mice.

Schizophrenia	PRS mice
+ Positive (stereotype behaviors)	+ stereotype behaviors
+ Negative (SI deficits) symptoms	+ SI deficits symptoms
+ sensitivity to NMDAR blockers	+ sensitivity to NMDAR blockers
+ cognitive symptoms	+ information processing deficit (PPI, fear conditioning)
Reduction of the GAD67, RELN, BDNF expression levels	Reduction of the GAD67, RELN, BDNF expression levels in frontal cortex
Increase of DNMT1, 3A and TET1 expression levels	Increase of DNMT1, 3A and TET1 expression levels in frontal cortex
Increase in 5MC and 5HMC enrichment at Gadl, Reln and Bdnf promoters	Increase in 5MC and 5HMC enrichment at Gadl, Reln and Bdnf promoters

(+): presence. Abbreviations: BDNF, brain-derived neurotrophic factor; DNMT1, DNA-methyltransferase 1; TET1, ten-eleven methylcytosine dioxygenase 1; SI, social interaction, NMDA, N-methyl D-aspartate; PPI, pre-pulse inhibition; GAD67, glutamic acid decarboxylate; 5HMC, 5-hydroxymethylcytosine; 5MC, 5methylcytosine

atypical antipsychotics down-regulates mGlu2 receptors in the prefrontal cortex as a result of an epigenetic mechanism that, in this particular case, is mediated by histone deacetylation at the *Grm2* gene promoter (Kurita et al., 2012). This contributes to explain why schizophrenic patients who had received a previous treatment with atypical antipsychotics failed to respond to pomeglumetad (Kinon et al., 2015), and raise the interesting possibility that acetylating drugs (e.g., inhibitors of histone deacetylases) may boost the activity of mGlu2 receptor agonists or positive allosteric modulators (PAMs) in the clinic. The mGlu2-centric scenario in the treatment of schizophrenia is supported by a large number of preclinical studies showing that the behavioral effects of orthosteric mGlu2/3 receptor agonists in behavioral tests that are predictive of antipsychotic activity are abrogated in mGlu2 receptor knockout mice, and that selective mGlu2 receptor PAMs display robust antipsychotic-like activity in rodents (Gerwirtz and Marek, 2000; Schoepp and Marek, 2002; Egan et al., 2004; Benneyworth et al., 2007; Patil et al., 2007; Conn et al., 2008). However, the following observations bring to a re-evaluation of mGlu3 receptors in schizophrenia research: (i) mice lacking mGlu3 receptors display a psychotic-like phenotype (Lainiola et al., 2014), and show developmental abnormalities in cortical GABAergic transmission (M. Cannella et al., unpublished observations); (ii) polymorphic variants of GRM3 are consistently associated with schizophrenia [reviewed by Maj et al., 2016], whereas no variants of GRM2 have been associated with psychiatric disorders; and, (iii) as reported above, mGlu3 receptors boost mGlu5 receptor signaling (Di Menna et al., 2018), and mGlu5 receptors are candidate drug targets in the treatment of schizophrenia [reviewed by Foster and Conn, 2017]. It is noteworthy that expression of mGlu3 receptors was also reduced in the prefrontal cortex of PRS mice, although this reduction was significant at 1 and 9 days of postnatal life, but not after weaning (Matrisciano et al., 2013). In clinical studies, systemic treatment with pomeglumetad methionyl, an oral prodrug of the mGlu2/3 receptor agonist, LY404039, showed antipsychotic activity in specific subgroups of population investigated, such as early-in-onset episodes and no history of atypical antipsychotic drug treatment which is known to epigenetically down-regulate mGlu2 receptors in mice, supporting the hypothesis that the additional preclinical studies and the identification of an appropriate target subgroup with altered glutamatergic tone are required to study these compounds (Kinon et al., 2015).

THE *EPIGENETIC* "ENDOPHENOTYPICAL" MOUSE MODEL FOR SCHIZOPHRENIA (PRS): ROLE OF METABOTROPIC GLUTAMATE 2/3 RECEPTORS

Urged by the need to use a neurodevelopmental animal model to study the epigenetic status at each neurodevelopmental stage of schizophrenia, we investigated the molecular and behavioral abnormalities found in the brain of the offspring of dams stressed during pregnancy (PRS mice). PRS mice showed a marked and long-lasting increase in DNMT enzymes (both 1 and 3a), and TET enzymes and a significant increase in 5-methylcytosine (5MC) and 5-hydroxymethylcytosine (5HMC) in the promoters of putative schizophrenia-related genes, such as *bdnf*, *gad1*, *reln*, and the early inducible gene, *GADD45* (growth arrest DNA demage), associated with an alteration in these gene expression.

To the best of our knowledge, the PRS model represents a promising model to study the *natural course* of major psychosis including schizophrenia compared, for example, to the *phencyclidine* (PCP) model which is a well-established pharmacological-induced model for schizophrenia reflecting the positive symptoms through the blockade of the NMDA receptors. Schizophrenia and autism spectrum disorders are considered diseases of *neurodevelopment*, characterized by a natural course, starting with a prodromal phase, first episode during adolescence or early adulthood, followed by relapses/remitted periods and eventually leading to brain function deterioration that ensues over subsequent adult years. Hence, the epigenetic history of such complex neurodevelopmental disorders cannot be adequately studied only in the postmortem brains of chronic SZ patients. We then have focused on studying the epigenetic signature of schizophrenia in offspring of PRS mice.

We also used *PRS* mice for the study of the role played by mGlu2 and mGlu3 receptors in the pathophysiology of schizophrenia based on clinical findings with pomeglumetad methionyl, an oral prodrug of the mGlu2/3 receptor agonist, LY404039. This drug showed an efficacy similar to the comparator, olanzapine, on positive and negative symptoms of schizophrenia in a phase-2 clinical trial (Patil et al., 2007), but not in subsequent trials. However, an exploratory analysis of all clinical studies confirmed the antipsychotic activity of pomeglumetad in schizophrenic patients who were early-in-disease or had not been treated with atypical antipsychotic drugs (Kinon et al., 2015).

We found that expression of mGlu2 and mGlu3 receptors was reduced in the frontal cortex of PRS mice (Matrisciano et al., 2012; Holloway et al., 2013), as a result of an increased binding of DNMT1 and methylcytosine binding protein-2 (MeCP2) to the *Grm2* gene promoter (Matrisciano et al., 2012; **Figure 1**).

As summarized in **Table 1**, PRS adult offspring showed alterations in the epigenetic regulation of schizophrenia-related gene as reelin, GAD67, BDNF, and mGlu2/3 receptors. Behaviorally, adult PRS-mice showed deficits similar to those observed in psychotic patients such as abnormalities in social interaction, locomotor activity, and pre-pulse inibition (PPI). In addition, we found epigenetic abnormalities such as a marked increase in the expression of DNMT1, DNMT3a, and TET, a significant increase in 5-methylcytosine (5MC) and 5-hydroxymethylcytosine (5HMC) in the promoters of putative schizophrenia-related genes, such as *bdnf*, *gad1*, *reln*, and the early inducible gene, *GADD45* (growth arrest DNA demage). Interestingly, the biochemical and behavioral abnormalities of PRS mice were corrected by the treatment with LY379268 (Matrisciano et al., 2012; Holloway et al., 2013), an orthosteric agonist of mGlu2/3 receptors, which shows "therapeutic efficacy" in a range of animal models used to predict antipsychotic activity (Cartmell et al., 1999, 2000; Carter et al., 2004).

TABLE 2 | Comparison of epigenetic and behavioral abnormalities induced by mGlu2/3 receptors agonist LY379268 and clozapine in PRS mice.

Clozapine	LY379268
Increase in Gadd45-β expression	Increase in Gadd45-β expression
Reduction of the overexpression of DNMT1 and TET1 in frontal cortex	Reduction of the overexpression of DNMT1 and TET1 in frontal cortex
Reduction of Gad1, Reln, and Bdnf promoter hypermethylation and increase in their mRNA levels	Reduction of the MeCP2 binding at the mGlu2, Gad1, Bdnf gene promoters
Reversal effects of the binding of DNMT1 to unmethylated target promoters	Reversal effects of the binding of MeCP2 to unmethylated target promoters
Reduction in locomotor hyperactivity and deficits in SI	Reduction in locomotor hyperactivity and deficits in SI

BDNF, brain-derived neurotrophic factor; DNMT1, DNA-methyltransferase 1; TET1, ten-eleven methylcytosine dioxygenase 1. Gadd45 (Growth – arrest and DNA damage; MeCP2, Methylcytosine binding protein 2.

In PRS mice, considered by us as a neurodevelopmental endophenotypical model for schizophrenia, expression levels of epigenetic biomarkers can be assessed at different phases of development in order to further elucidate the underlying pathogenetic mechanisms and predicting treatment responses at specific stages of the disease, with particular attention to early detection and possibly early intervention.

Little is known on the action of antipsychotics on specific epigenetic mechanisms in GABAergic or glutamatergic neurons. Thus, PRS mice represent a valid and suitable model for drug testing and development.

CLOZAPINE AND THE mGlU2/3 RECEPTOR AGONIST LY379268: EPIGENETIC EFFECTS IN THE PRS MOUSE MODEL FOR SCHIZOPHRENIA

Clozapine, the prototype of atypical antipsychotics, is considered the drug of choice in patients with treatment-resistant schizophrenia due, in our opinion, to its unique chromatin remodeling properties. We have shown that clozapine reversed the behavioral deficits and induced chromatin remodeling in PRS mice that are resistant to haloperidol treatment (Dong et al., 2016). We recently studied the epigenetic mechanisms underlying the efficacy as potential antipsychotic-like activity of the mGlu2/3 receptors agonist, LY379268, as compared to the activity of clozapine, in PRS mice. **Table 2** summarizes the epigenetic effects of clozapine and LY379268 in the frontal cortex of PRS mice. Clozapine reversed promoter hypermethylation of schizophrenia-related genes such as *bdnf*, *reln*, and *gad1* (Dong et al., 2016). Interestingly, these effects were shared by valproate, an anti-epileptic drug used for the treatment of bipolar disorder, which is chemically unrelated to clozapine, and induces demethylation of gene promoters presumably as a result of histone acetylation and chromatin opening. Both clozapine and LY379268 were able to reduce the overexpression of DNMT1 and TET found in the frontal cortex of PRS mice. This overexpression is similar to that found in brain tissue of patients affected by schizophrenia (Matrisciano et al., 2016). DNMT enzymes are responsible for the conversion of cytosines into 5-methyl-cytosines, whereas TET enzymes convert the 5MC residues into 5-hydroxymethylcytosines by hydroxylation reaction in a sequence of events of cytosines metabolism. Clozapine and LY379268 were also able to reverse the hypermethylation of schizophrenia-like promoter genes such as *gad1*, *bdnf*, and *reln* and the ensuing increase in their mRNA expression levels. In addition, LY379268 induced a decrease of MECP2 binding at the *mGlu2*, *Gad1*, and *Bdnf* gene promoters, whereas clozapine reversed DNMT binding at the promoters of schizophrenia-related genes. Both clozapine and LY379268 reversed the increase in locomotor activity in PRS mice and the deficits showed by these mice in social interaction tasks. These findings are consistent with the previous evidence that a combined treatment with clozapine and valproate reversed the downregulation of GAD67 expression induced by repeated methionine administration in mice (Guidotti et al., 2014). The same authors showed that the effects of clozapine on DNA-demethylation were mimicked by antipsychotic drugs chemically related to clozapine, such as the dibenzodiazepines, quetiapine and olanzapine, but not by the chemically unrelated risperidone (Guidotti et al., 2011). Thus, a more systematic and comprehensive analysis of the effects of different antipsychotics on the epigenetic signature in PRS mice is warranted. We reported that the strong effect of clozapine on DNA methylation in PRS mice and the lack of effect of clozapine in control mice cannot be considered as secondary to changes in dopaminergic or serotonergic genes such as D2, Htr1a, or Htr2a in the cortex of PRS mice. Of note, a correlation between the methylation state of schizophrenia-related genes and behavioral deficits exists (Dong et al., 2016). The increase in DNMT1 binding to selected *Gad1*, *Reln*, and *Bdnf-ix* regulatory regions in PRS mice was considerably reduced by clozapine treatment whereas haloperidol failed to reduce the increased DNMT1 binding in PRS mice, in agreement with previous results (Matrisciano et al., 2013). Clozapine and LY379268 may exert their antipsychotic activity either indirectly by decreasing DNMT and TET expression levels, and/or more directly by interfering with the DNMT1 or MeCP2 DNA-binding domains. In addition, both clozapine and LY379268 increased the expression levels of Gadd45-β (growth arrest and DNA-damage-inducible protein 45), a member of the Gadd45 family of small nuclear acidic proteins, which it was reported to facilitate DNA de-methyation (Ma et al., 2009; Matrisciano et al., 2011). Taken together, we can speculate that both clozapine and mGlu2/3 receptor agonists act in our model as epigenetic de-methylating agents, and because of that they may regulate processes that lies at the core of the pathophysiology of schizophrenia.

CONCLUSIONS

This review underlies the concept that the *PRS* mouse model has construct and face validity as an experimental epigenetic model of vulnerability for neurodevelopmental disorders such as

schizophrenia, schizoaffective disorders, and autism. This mouse model is highly reproducible and useful for novel anti-psychotic drug screening acting on altered epigenetic mechanisms. Early-life stressors, even during pregnancy, in mice lead to alterations of some molecular players of epigenetic mechanisms that are translated into a schizophrenia-like phenotype. A potential glutamate-based pharmacotherapy for schizophrenia remains, at least in part, a possibility that requires the identification of an appropriate subgroup of patients that satisfy specific criteria such as no previous history of atypical antipsychotic treatments and treatment onset in early phases of the disease. For preclinical studies, PRS mice represent a valid epigenetic "endophenotype" model for drug testing and development and for studying the pathogenesis of the disease. In our opinion, mGlu2/3 receptors, based on the peculiar role as *modulators* of glutamate transmission in the frontal cortex, can still represent a suitable target for novel antipsychotic medications targeting specific high-risk population with dysregulation of brain glutamatergic tone. Then, ligands acting on mGlu2 and 3 receptors, either orthosteric agonists or PAMs, require further experimental studies in PRS mice and other epigenetic models to identify the *optimum* receptors target and time window of intervention in the treatment of psychosis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Molecular Neuroscience

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Negative Allosteric Modulators of $mGlu_7$ Receptor as Putative Antipsychotic Drugs

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The data concerning antipsychotic-like activity of negative allosteric modulators (NAMs)/antagonists of $mGlu_7$ receptors are limited. The only available ligands for this receptor are MMPIP and ADX71743. In the present studies, we used stable cell line expressing $mGlu_7$ receptor and it was shown that both compounds dose-dependently potentiated forskolin elevated cAMP concentration in the T-REx 293 cells, showing their inverse agonist properties. Subsequently, pharmacokinetic studies were performed. Both compounds were given intraperitoneally (i.p.) at the dose of 10 mg/kg and reached Cmax 0.25–0.5 h after administration, and then they declined rapidly, ADX71743 being almost undetectable 2 h after administration, while the concentration of MMPIP was still observed, suggesting that the concentration of MMPIP was more stable. Finally, we investigated the role of both $mGlu_7$ receptor NAMs in animal models of schizophrenia. Behavioral tests commonly used in antipsychotic drug discovery were conducted. Both tested compounds dose-dependently inhibited MK-801-induced hyperactivity (MMPIP at 15 mg/kg; ADX at 5 and 15 mg/kg) and DOI-induced head twitches (MMPIP at 5, 10, 15 mg/kg; ADX at 2.5, 5, 10 mg/kg). Moreover, the same effects were noticed in novel object recognition test, where MMPIP (5, 10, 15 mg/kg) and ADX71743 (1, 5, 15 mg/kg) reversed MK-801-induced disturbances. In the social interaction test, antipsychotic activity was observed only for ADX71743 (5, 15 mg/kg). ADX71743 at the dose 2.5 mg/kg reversed MK-801-induced disruption in prepulse inhibition while MMPIP at 10 mg/kg reversed MK-801-induced disruption in spatial delayed alternation. The present studies showed that $mGlu_7$ receptor may be considered as a putative target for antipsychotic drugs, though more studies are needed due to limited number of available ligands.

Keywords: schizophrenia, metabotropic glutamate receptor 7, antipsychotic, negative allosteric modulators, MMPIP, ADX71743

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INTRODUCTION

Metabotropic glutamate receptors (mGluR) are being extensively studied as new pharmacological targets for central nervous system (CNS) disorders such as depression (Mitsukawa et al., 2006), anxiety (Swanson et al., 2005), schizophrenia (Conn et al., 2009; Nickols and Conn, 2014), neurodegenerative disorders (Gu et al., 2014; Litim et al., 2017), and pain (Acher and Goudet, 2015; Chiechio, 2016). Among these receptors, $mGluR_7$ is one of the most conserved mGluR

which is abundantly expressed in the cerebral cortex (Ohishi et al., 1995; Kinoshita et al., 1998; Dalezios et al., 2002), hippocampus (Ohishi et al., 1995; Kinoshita et al., 1998; Sansig et al., 2001), amygdala (Ohishi et al., 1995; Kinoshita et al., 1998), and basal ganglia (Ohishi et al., 1995; Kinoshita et al., 1998; Kosinski et al., 1999). mGluR$_7$ is localized both presynaptically, where it negatively regulates glutamate and GABA release, and postsynaptically (Schoepp, 2001), where it mediates slow postsynaptic potentials. The affinity of mGlu$_7$ for glutamate is also relatively low (K_i = 869 μM) (Wright et al., 2000) and thus the receptor might play a modulatory role in the CNS, protecting from glutamate overstimulation (Niswender and Conn, 2010). Recent evidence suggests mGlu$_7$ receptor involvement in the pathology of schizophrenia, as several polymorphisms of gene encoding this receptor have been found in different populations, for example, significant transmission distortion of rs17031835 in intron 1 of GRM7 in Indonesian sib-pair families (Ganda et al., 2009), 14 single nucleotide polymorphisms (SNPs) in GRM7 of Han Chinese population (Li et al., 2016) or synonymus polymorphism (371T/C, rs3749380) in exon 1 of GRM7 in Japanese patients with schizophrenia (Ohtsuki et al., 2008). Due to the lack of highly specific, bioavailable compounds mGluR$_7$ activity is yet poorly understood, especially in the context of schizophrenia.

Mitsukawa et al. described the first selective positive allosteric modulator of mGlu$_7$ receptors – AMN082 (Mitsukawa et al., 2005). It was shown that AMN082 possesses antidepressant-like profile in FST and TST, and anxiolytic properties in four plate test and stress-induced hyperthermia (Palucha et al., 2007; Stachowicz et al., 2008). However, it did not exhibit any antipsychotic-like profile and rather enhanced MK-801- or DOI-induced effects, which may suggest a potential beneficial role of antagonists or negative allosteric modulators (NAMs) in animal models of schizophrenia (Wierońska et al., 2012). Additionally, some studies reported off target activity of AMN082 (Sukoff Rizzo et al., 2011).

Up to date, there have been only two mGlu$_7$ receptor NAMs synthetized – MMPIP and ADX71743 (Suzuki et al., 2007; Kalinichev et al., 2013). MMPIP was shown to impair cognition and decrease social interaction in WT mice or rats, bared no effect on spontaneous activity and motor performance (Hikichi et al., 2010) but induced analgesic effects (Palazzo et al., 2016, 2015). Additionally, no antidepressive and anxiolytic effects were described. Moreover, MMPIP did not reverse the pharmacologically induced disruption of prepulse inhibition (PPI, Hikichi et al., 2010). However, the effects of MMPIP might be difficult to explain as it also acts as an inverse agonist (Suzuki et al., 2007).

Another NAM of mGlu$_7$ receptor – ADX71743 – was found to exert an anxiolytic but not antidepressant effect (Kalinichev et al., 2013). When administered to animals, it did not impair their locomotor activity and motor performance. The antipsychotic activity of ADX71743 is not well described and understood as it caused a moderate decrease in amphetamine-induced hyperactivity, but had no effect on DOI-induced head twitches and the conditioned avoidance response (Kalinichev et al., 2013).

Here, we extensively describe the action of MMPIP and AXD71743 both *in vitro* and *in vivo* in the context of schizophrenia. Antipsychotic activity of both compounds was evaluated in number of behavioral tests, such as: MK-801-induced hyperactivity, DOI-induced head twitches, modified forced swim test, social interaction test, PPI, and novel object recognition (NOR) test. Their potential effect on motor performance was assessed in rotarod test. Due to better pharmacokinetic properties, the activity of MMPIP was also tested in spatial delayed alternation test. In order to confirm the profile of interaction of MMPIP and ADX71743 with mGlu$_7$ receptor, the intracellular levels of cAMP were measured. Additionally, pharmacokinetic and electrophysiological studies were performed.

MATERIALS AND METHODS

Animals and Housing

Male Albino Swiss mice (20–25 g) were used in most behavioral tests. Male Wistar rats (200–250 g) were used in spatial delayed alternation test and PPI of the acoustic startle response test. Male C57BL/6J WT and mGlu$_7$ KO mice were used in electrophysiological studies. The animals were kept in a room with 12:12 light–dark cycle at a temperature of 21–22°C. Food and water were provided *ad libitum*. The animals were used only once, none of the animals has been run multiple experiments. All procedures were conducted according to the guidelines of the National Institutes of Health Animal Care and Use Committee and were approved by the II Local Ethics Committee by the Institute of Pharmacology, Polish Academy of Sciences in Krakow.

Drugs

MMPIP [6-(4-Methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo(4,5-c)pyridin-4(5H)-one], ADX71743 [6-(2,4-Dimethylphenyl)-2-ethyl-6,7-dihydro-4(5*H*)-benzoxazolone], MK-801 [(5*S*,10*R*)-(+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate] and DOI (4-Iodo-2,5-dimethoxy-α-methylbenzeneethanamine hydrochloride) were purchased from Tocris Bioscience, Bristol, United Kingdom. For behavioral and pharmacokinetic studies, MK-801 and DOI were dissolved in 0.9% NaCl, MMPIP in 0.5% methylcellulose (Sigma–Aldrich, St Louis, MO, United States) and ADX71743 in small amount of DMSO (Sigma–Aldrich) and then titrated in 20% captisol (Cydex Pharmaceuticals, Lawrence, KS, United States). Final concentration of DMSO in the whole solution was 2%. Control groups received appropriate vehicles. All drugs were administered in a volume of 10 ml/kg when given to mice, and 1 ml/kg when given to rats. The doses of the compounds we used were partially chosen on the basis of the other studies (Hikichi et al., 2010), but mostly were established experimentally. Mostly, the compounds were administered up to the dose of 15 mg/kg; however, in some case when the activity was evident at the lower doses, the dose of 15 mg/kg was not investigated.

cAMP

A homogeneous time-resolved fluorescence (HTRF) cAMP dynamic 2 (Cisbio, Codolet, France) assay was performed as previously described (Chruścicka et al., 2015) with recombinant cell lines. Briefly, HEK 293 T-REx cells stably expressing mGlu$_7$ receptor, were collected and suspended in Hanks-HEPES buffer. The cell suspension was added to compounds solution with 5 μM of forskolin (final concentration). After 5 min incubation in 37°C, 5 μl of cAMP-d2 conjugate in lysis buffer was added and mixed with the 10 μl cell suspension by means of an automated pipetting system (Tecan Evo 200, Tecan, Mannedorf, Switzerland). Next, 5 μl anti-cAMP cryptate conjugate was added and the fluorescence at 620 and 665 nm was read after 1 h (Tecan Infinite M1000). The results are shown as the 665 nm/620 nm ratio multiplied by 10^4. The detected signal was inversely proportional to the concentration of cAMP in the sample. Antagonist activity of ADX71743 or MMPIP are shown as a percentage of the inhibition of L-Glu activity at its EC_{80} concentration. Dose response data from ADX71743 or MMPIP were analyzed with Prism Version 7.03 (GraphPad Software Inc.). Each experiment was performed three times (n = 3), and each data point was in triplicate.

Pharmacokinetic Studies

The method described below was successfully applied to a pharmacokinetic study of ADX71743 and MMPIP in mouse (Albino Swiss) after i.p. injection. Compound ADX71743 and MMPIP were administered to mice at 10 mg/kg i.p. At 0.25, 0.50, 1.0, 2.0, 4.0, 6.0 h, the mice were anesthetized, and the blood was collected from the portal vein to the tubes containing 5% EDTA. The mice were then perfused with 0.1M PBS to remove remaining blood from the body, and the brains were taken out for the analysis. Blood was centrifuged at 2000 rpm for 10 min at 4°C, and the plasma was collected and frozen at −80°C for further analysis.

Plasma and tissue samples from all drug-treated animals were thawed at room temperature prior to use. Standard protocol of sample preparation: 200 μl acetonitrile was added to the eppendorfs with 50 μl of studied plasma samples or tissue homogenate. Samples were mixed for 5 min on a mixer at 25°C and 1400 rpm. Tubes were then centrifuged at 2000 × g for 15 min at 4°C. About 180 μl of each supernatant was transferred into a plate well. Finally, each sample was injected into the column.

In calibration curve – serial dilution method, plasma was spiked with standard at different concentration levels. Acetonitrile was added. Mixed, centrifuged supernatant was taken.

LC-MS Analysis

Chromatographic Conditions

Plasma and tissue samples from all drug-treated animals at selected time points are analyzed using previously developed a non-validated liquid chromatography-tandem mass spectrometry (LC-MS)/MS method. A sensitive and highly selective LC-MS method was used to determine drug concentration in mouse plasma samples or tissue homogenate.

LC/MS analysis was carried on a Bruker amaZon SL mass spectrometer using positive/ negative ion ESI mode. Chromatographic separation was achieved on a Ascentis Express C_{18} column, (5 cm × 2.1 mm, 2.7 μM, Supelco Technologies) at room temperature with a thermostatted column oven. A gradient elution of eluents A [acetonitrile (LiChrosolv, Reag. Ph Eur) +0.1% formic acid (Sigma–Aldrich, 98–100%)] and B (water +0.1% formic acid) was used for separation. The flow rate was set at 1 ml/min. The injection volume was 20 μl, and the time of injection was 4 min.

Mass Spectrometric Conditions

An Ion trap mass spectrometer (Bruker amaZon SL) was equipped with an electrospray source, operating in the positive/negative ion mode. Data were collected and processes using Bruker Quant Analysis software. Quantification of analytes was performed in SIM mode.

Electrophysiology

Mice (wild and KO, approx. 25 g) were housed under a controlled light/dark cycle (light on: 0700–1900) and had free access to standard food and tap water. Mice were anesthetized with isoflurane (Aerrane, Baxter) decapitated, their brains were dissected and immersed in an ice-cold artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl (130), KCl (5), $CaCl_2$ (2.5), $MgSO_4$ (1.3), KH_2PO_4 (1.25), $NaHCO_3$ (26), and glucose (10), bubbled with a mixture of 95% O_2/5% CO_2 to pH 7.4. Frontal cortical slices (bregma 1.9–1.4, 380 μm) were cut in a coronal plane using a vibrating microtome and they were stored at 32°C. A single slice was next transferred to the recording chamber (32°C ± 0.5°C) and superfused at 2.5 ml/min with a ACSF.

A bipolar stimulating electrode (FHC) was placed approx. 2 mm lateral to the midline and approx. 1.0 mm below the pial surface (in layer V) (**Figure 1**). Stimuli (duration: 0.2 ms) were applied at 0.033 Hz using a constant-current stimulus isolation unit (WPI). Field potentials (FPs) were recorded using glass micropipettes filled with ACSF (1–3 MΩ), which were placed approx. 0.2 mm below the cortical surface (in layer II/III). FPs were amplified (Axoprobe 1A, Axon Instruments), A/D converted at 10 kHz and stored using Micro1401 interface and Signal 4 software (CED).

The stimulus–response curves obtained for each slice were fit with the Boltzmann equation: Vi = Vmax/(1 + exp[(u − Uh)/−S]), where Vmax is the maximum FP amplitude; u is the stimulation intensity; Uh is the stimulation intensity evoking FP of half-maximum amplitude; S is the factor proportional to the slope of the curve; "exp" is exponentiation – mathematical operation. The results are expressed as the means ± SEM. Statistical analyses were carried out using t-test (Tokarski et al., 2011).

For each slice, at the beginning of the experiment an input-output curve was generated in ACSF. A stimulus-response (input-output) curve was made for each slice. To obtain the curve, stimulation intensity was gradually increased stepwise

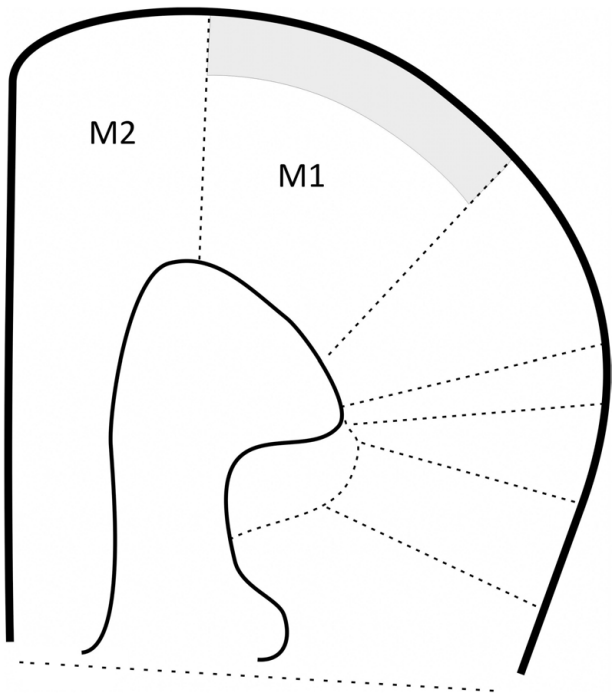

FIGURE 1 | Schematic illustration showing mice cortex coronal slice (Paxinos and Franklin, 2012). Area colored in gray represents recording zone.

(16 steps; 0–100 μA). One response was recorded at each stimulation intensity. Next, standard ACSF was replaced by a solution containing MMPIP or ADX71743, for 20 min, and input-output curves were generated again. Statistical analyzes were carried out using paired *t*-test and ANOVA.

MK-801-Induced Hyperactivity

The locomotor activity was recorded individually for each animal in OPTO-M3 locomotor activity cages (Columbus Instrument) linked online to a compatible PC activity, as described previously by Woźniak et al., 2016b. Each cage (13 cm × 23 cm × 15 cm) was surrounded with an array of photocell beams. Interruptions of these photobeams resulted in horizontal activity defined as ambulation counts. The mice were placed in the locomotor activity cages for acclimatization for 30 min Then, MMPIP (10, 15 mg/kg) or ADX71743 (5, 10 mg/kg) were administered i.p. Both drugs were given 30 min prior to MK-801 injection (0.35 mg/kg, i.p.). The locomotor activity was measured for 60 min immediately after MK-801 administration.

DOI-Induced Head Twitches

The experiment was performed according to previously described procedure (Wierońska et al., 2012, 2013). Immediately after a 30 min acclimatization period, DOI (2.5 mg/kg, i.p.) was administered in order to induce head twitches. The number of head twitches was then counted for 20 min. MMPIP (5, 10, and 15 mg/kg) or ADX71743 (2.5, 5, and 10 mg/kg) were administered i.p. 30 min before DOI. Subsequently the compounds were administered chronically (for 10 days) each at the two active doses (MMPIP 5 and 10 mg/kg and ADX71743 2.5 and 5 mg/kg). The test was performed on 11th day, 30 min after the last administration.

Modified Forced Swim Test

The modified forced swim test was performed according to the method introduced by Noda (Noda et al., 1995, 1997; Wierońska et al., 2015a; Woźniak et al., 2016a). The swim tests were performed in a glass cylinder (height, 20 cm; internal diameter, 15 cm) containing 11 cm of water maintained at 23–24°C. After the acclimation period, the animals underwent the first swim test, where the immobility time was measured during a 3 min period (T_1). On the next day, chronic (13 days) MK-801 administration (0.4 mg/kg, i.p.) was started. After a 1-day break, on the 15th day of experiment, the second swim session was performed and the immobility time during 3-min test was measured again (T_2). The $T_2 - T_1$ difference was reported as the result of the experiment. MMPIP (1, 5, and 15 mg/kg, i.p.) or ADX71743 (5, 10, and 15 mg/kg, i.p.) were administered acutely 30 min before the T_2 session.

Social Interaction Test

The method was adapted from de Moura Linck et al., 2008 and Woźniak et al., 2016b. After the 2-day habituation trial (10 min/day) a pair of mice was placed in the open field for 5 min. The social interactions between two mice were determined based on the total time spent participating in social behavior such as genital investigation, sniffing, chasing, and fighting each other. The total number of social episodes was also measured. The test was video-recorded and viewed by a trained observer. MMPIP (5, 10, and 20 mg/kg, i.p) or ADX71743 (1, 5, and 15 mg/kg, i.p.) were administered 30 min before MK-801 (0.3 mg/kg, i.p.), which was administered 30 min before the test.

Novel Object Recognition Test

The experiment was performed according to Nilsson et al., 2007 with minor modifications (Woźniak et al., 2016b). Following a 2-day habituation period (10 min/day), a training trial was performed, where mice were allowed to explore two identical objects for 5 min. About 1 h later, a test trial was conducted, where one of the familiar object was replaced by a novel object. The animals were then allowed to explore the objects for 5 min. MMPIP (5, 10, and 15 mg/kg, i.p.) and ADX71743 (1, 5, and 10 mg/kg; i.p.) were administered 30 min before MK-801 (0.3 mg/kg, i.p.), which was administered 30 min before the training trial. Time spend exploring (i.e., sniffing or touching) the familiar ($T_{familiar}$) or novel object (T_{novel}) was measured by a trained observer and then the recognition index was calculated for each mouse $[(T_{novel} - T_{familiar})/(T_{familiar} + T_{novel})] \times 100$.

Rotarod Test

The animals were trained for 3 consecutive days at the speed of 18 rpm, one session per day for 3 min. If a mice fell during the habituation period, it was placed back on the apparatus. On the following day, the test trial was performed. After the mice were placed on the apparatus (Mouse Rota-Rod NG, UGO BASILE

S.R.L.) moving at the speed of 12 rpm, the accelerating mode was started (maximum speed – 24 rpm). The latency to fall was measured during 3-min test session. Mice were injected with MMPIP (5, 15, and 30 mg/kg, i.p.) or ADX71743 (5, 15, and 30 mg/kg, i.p.) 30 min before the test trial.

Spatial Delayed Alternation Test

The spatial delayed alternation test was performed using a wooden T-maze, according to Sławińska et al., 2013 and Wierońska et al., 2015b.

During the adaptation phase, lasting 3 days, the animals were allowed to freely explore the maze for 10 min. For the next 2 days, rats were confined to either of the two end-arms and allowed to drink a 10 % sucrose solution there for 10 min twice daily. On the following day, a 2-week training phase was started. The animals performed one training session per day, which consisted of one forced trial (i.e., one of the end-arms was closed) followed by ten free choice trials. During the free choice trial the animal was placed in the starting arm and after the guillotine door was raised, it was allowed to choose to enter one of the end-arms. After the response, the rat was placed back to the starting arm, where it stayed for 10 s. If the chosen end-arm was the opposite to the previously visited one, a correct response was scored, and the animal was closed in the compartment where it was allowed to drink the sucrose solution for 5 s. After an incorrect response, the animal was gently returned to the starting arm. The training phase was carried out until the animals scored 7 correct responses in a training session in 2 consecutive days.

On the day of the test, the animals were injected with MMPIP and/or MK-801, and the aforementioned 10-trial session was repeated. MMPIP was administered at a dose of 5 or 10 mg/kg 30 min prior to MK-801 (0.1 mg/kg) administration. The test was started 30 min after the MK-801 injection.

Prepulse Inhibition

The procedure was performed according to Czyrak et al., 2003. On the day before the experiment, the animals were subjected to a single startle session consisting of two trials, each presented 20 times during the session. During the first trial, a 120 dB, 40 ms pulse was presented, and on the second trial this pulse was preceded by a 75 dB, 20 ms prepulse. On the day of the experiment, the animals were habituated to the background white noise (65 dB) for 5 min (which continued throughout the test), after that the startle session was carried out as described above. Startle response amplitude was defined as the difference between the maximum force detected during a recording window and the force measured immediately before the stimulus onset (the threshold was set at 10 g). For each animal, the amplitudes were averaged separately for each type of trial. The PPI was calculated as the difference between the amplitudes of the pulse (P) and the prepulse + pulse (PP+P), divided by the amplitude of the pulse alone $[([P - (PP + P)]/P) \times 100]$.

MMPIP (5, 10, and 15 mg/kg) and ADX71743 (2.5, 5, and 10 mg/kg) were administered 30 min prior to MK-801 (0.3 mg/kg), which was administered 30 min before the habituation phase.

Statistical Analysis

Statistical analysis was performed using Statistica 12 package (StatSoft Inc., Tulsa, OK, United States). A one-way ANOVA followed by Newman-Keuls *post hoc* test was used to analyze the behavioral experiments and Student's *t*-test for paired samples was used to assess the differences in the amplitude of FPs. Data are presented as mean ± SEM.

RESULTS

cAMP

In order to confirm the NAM profile of ADX71743 and MMPIP, the substances were incubated with 6.26 mM of L-Glu (EC_{80}). Both ADX71743 and MMPIP dose-dependently antagonized L-Glu inhibition of cAMP accumulation in the presence of forskolin, with the IC_{50} values of 0.44 μM (± 0.13) (n = 3) and 0.38 μM (± 0.15) (n = 3) respectively (**Figure 2A**).

In the second set of experiments the cells were incubated with forskolin with increasing concentration of ADX71743 or MMPIP without agonist in order to analyze their inverse agonist properties. Both compounds dose-dependently potentiated forskolin action, elevating cAMP concentration in the T-REx 293 cells (**Figure 2B**). IC_{50} of both substances was very similar – ADX71743 0.22 μM (± 0.07) (n = 3) and MMPIP 0.34 μM (± 0.14) (n = 3). This effect was not observed for an antagonist of mGluR$_7$ – XAP044.

Pharmacokinetics

The concentration of ADX71743 and MMPIP in mouse plasma and brain are shown in **Table 1**. C_{max} was evident in brain and plasma 0.25 h after injection of ADX71743, and 0.5 h after MMPIP administration. **Figure 3** represents comparison between ADX71743 and MMPIP concentrations in the brain in selected time points after administration.

Data presented in **Table 2** showed that ADX71743 and MMPIP had different cytochrome P450 inhibition profile. Weak inhibition ($IC_{50} > 10\mu M$) of cytochrome P450 was observed in case of 1A2, 2B6, 2C9, 2D6 isoforms for both NAM mGluR$_7$ standards. Mild inhibition ($3.3 < IC_{50} < 10$) of isoform 2C19 was determined for ADX71743 standard, while strong inhibition ($IC_{50} < 1.1$) was observed only for MMPIP in case of isoform 3A4 as well as 2C19.

Electrophysiology

Analyses of FPs recorded in slices obtained from WILD mice revealed an increase in the relationship between stimulus intensity and FP amplitude (input–output curve) after MMPIP administration in wild animals (**Figure 4A**), compared to KO ($P < 0.001$, paired *t*-test, **Figure 4B**). Parameters characterizing input-output curves of FPs, calculated using the Boltzmann fits, are summarized in **Table 3A**. The amplitude of FPs was markedly higher over a wide range of stimulation intensities ($P < 0.001$, two-tailed) (**Figure 4A**).

The effect of MMPIP administration (before-after effect) was about 23% higher in WILD group compared to KO animals

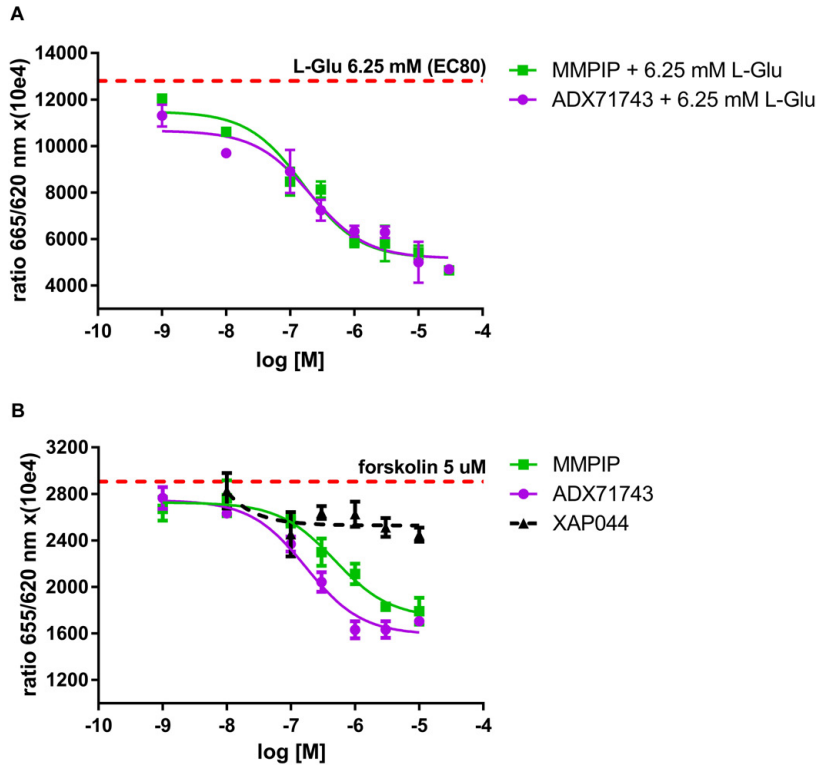

FIGURE 2 | MMPIP and ADX71743 antagonized L-Glu inhibition of cAMP accumulation in presence of 5 μM of forskolin thus confirming their NAM profile **(A)** MMPIP and ADX71743 enhance the action of forskolin in dose-dependent manner increasing cAMP concentration. This effect was not observed for XAP044. The dashed line represents the cAMP level corresponding to 5 μM of forskolin **(B)**. Representative results, data points presented as mean ± SEM.

TABLE 1 | Plasma **(A)** and brain **(B)** concentration of MMPIP and ADX71743 after administration of 10 mg/kg.

(A)

Parameters	ADX71743	MMPIP
Tmax (h)	0.25	0.25
T1/2 (h)	0.90	1.16
Cmax (μmol/L)	3.73	9.85
AUC (μmol/L*h)	1.90	13.52

(B)

Parameters	ADX71743	MMPIP
Tmax (h)	0.25	0.50
T1/2 (h)	0.34	1.75
Cmax (μmol/L)	3.38	5.42
AUC (μmol/L*h)	2.27	8.98

Tmax – time at maximum observed concentration Cmax noted in minutes after administration of drug, T1/2 – terminal elimination half-life after administration, Cmax – maximum drug concentration obtained after administration of a drug between the time of doing and the final observed point, and AUC – the area under the concentration-time curve.

TABLE 2 | *In vitro* profiles, physicochemistry, and ADME.

Parameters	ADX71743	MMPIP
Molecular weight	269.14	333.35
clogD	3.64	1.79
clogD	3.64	1.79
PSA	43.10	68.46
Kinetic solubility in HHB medium	509.87	5.6
Metabolic stability (microsomes, mice)	0.01	49.78
Clint	500.14	38.74
Cytochrome P_{450} (IC_{50}, μM)		
1A2	> 10	> 10
3A4	> 10	< 1.1
2B6	> 10	> 10
2C9	> 10	> 10
2C19	3.3 < IC_{50} < 10	< 1.1
2D6	> 10	> 10

Kinetic solubility (μg/ml), metabolic stability mice microsomes (% remaining after 60 min), and clint (ml/min/mg/ protein).

(123% vs. 100,1%, $P < 0.001$, two-tailed, $t = 6.544$ df = 30, **Figure 4C**).

ADX71743 administration increased the amplitude of recorded FP in wild animals, whereas were ineffective in KO group ($P < 0.002$; $P < 0.49$, paired t-test). Parameters characterizing input-output curves of FPs, calculated using the Boltzmann fits, are summarized in **Table 3B**. The amplitude of FPs increased over a higher ranges of stimulation intensities ($P < 0.001$, paired t-test, two-tailed, $t = 9.426$ df = 23)

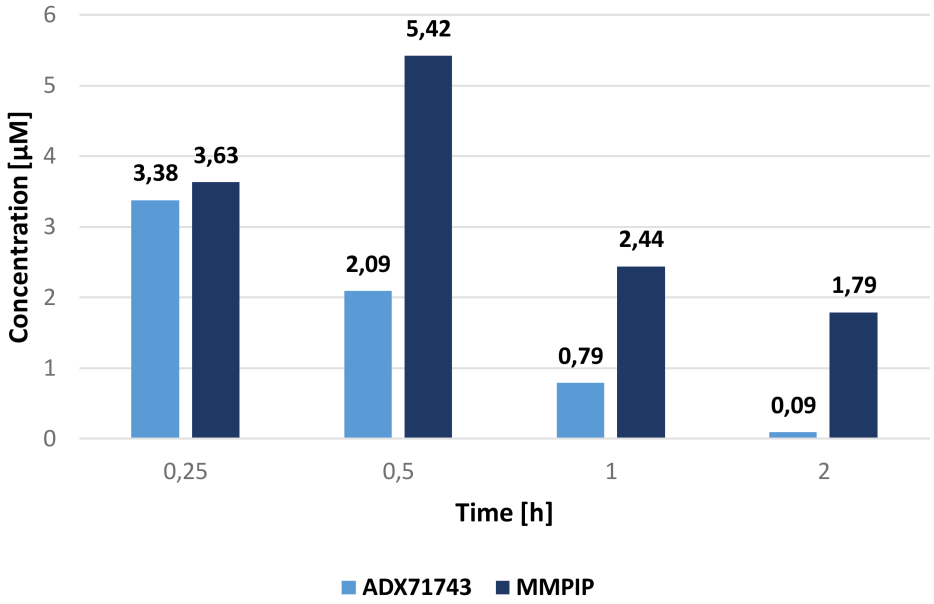

FIGURE 3 | Comparison of ADX71743 and MMPIP concentrations in brain.

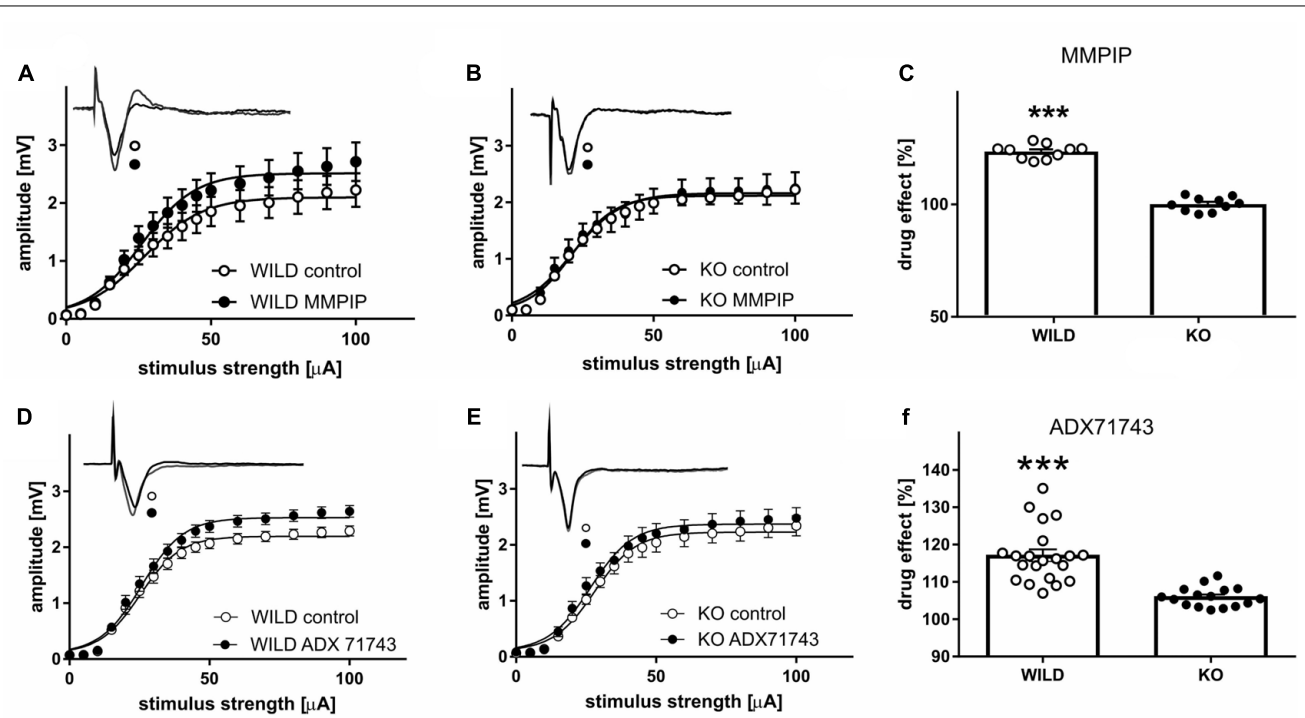

FIGURE 4 | The influence of mGlu$_7$-selective antagonist (MMPIP) on the relationship between stimulus intensity and amplitude of field potentials (FP) in WILD **(A)** and KO mice **(B)**, and the before-after effect of the drug **(C)**. The influence of mGlu$_7$-selective antagonist (ADX71743) on the relationship between stimulus intensity and amplitude of FPs in WILD **(D)** and KO mice **(E)**, and the before-after effect of the drug **(F)**. Filled circles, quadrates: FPs recorded in slices after 20 min MMPIP/ADX administration prepared from WILD mice, open circles/quadrates: control preparations (n = 18). ***$P < 0.001$.

(**Figures 4D,E**). The effect of ADX71743 administration (before-after effect) was higher in WILD group compared to KO animals (116% vs. 106, $P < 0.001$, two-tailed, $t = 5.71$ df = 35, **Figure 4F**).

MK-801-Induced Hyperactivity

One-way ANOVA analysis revealed the statistically significant effects of treatments [$F_{(3.34)} = 23.38$, $P < 0.0001$ (**Figure 5A**)

TABLE 3 | Parameters characterizing input-output curves of FPs, calculated using the Boltzmann fits for MMPIP **(A)** and ADX71743 **(B)**.

(A)

Treatment	Vmax	Uh	S	*n*
Veh	2.11 ± 0.2	27.37 ± 1.6	10.28 ± 0.8	10
Veh MMPIP	2.53 ± 0.3***	26.93 ± 1.6	9.37 ± 0.83	10
KO	2.11 ± 0.18	21.64 ± 1.8	7.3 ± 0.2	4
KO MMPIP	2.16 ± 0.2	22.1 ± 1.4	8.6 ± 1.01	4

(B)

Treatment	Vmax	Uh	S	*n*
Veh	2.195 ± 0.09	25.44 ± 1.2	6.42 ± 0.3	24
Veh ADX 71743	2.531 ± 0.1***	26.07 ± 1.3	6.53 ± 0.3	24
KO	2.225 ± 0.17	27.19 ± 0.93	7.49 ± 0.4	16
KO ADX 71743	2.36 ± 0.18	26.06 ± 1.18	7.12 ± 0.41	16

****P < 0.001. Two-tailed paired t-test, t = 8.084, df = 9.*

****P < 0.001. Two-tailed paired t-test, t = 9.426, df = 23. Vmax is the maximum FP amplitude; Uh is the stimulation intensity evoking FP of half-maximum amplitude; S is the factor proportional to the slope of the curve; and n is the number of slices.*

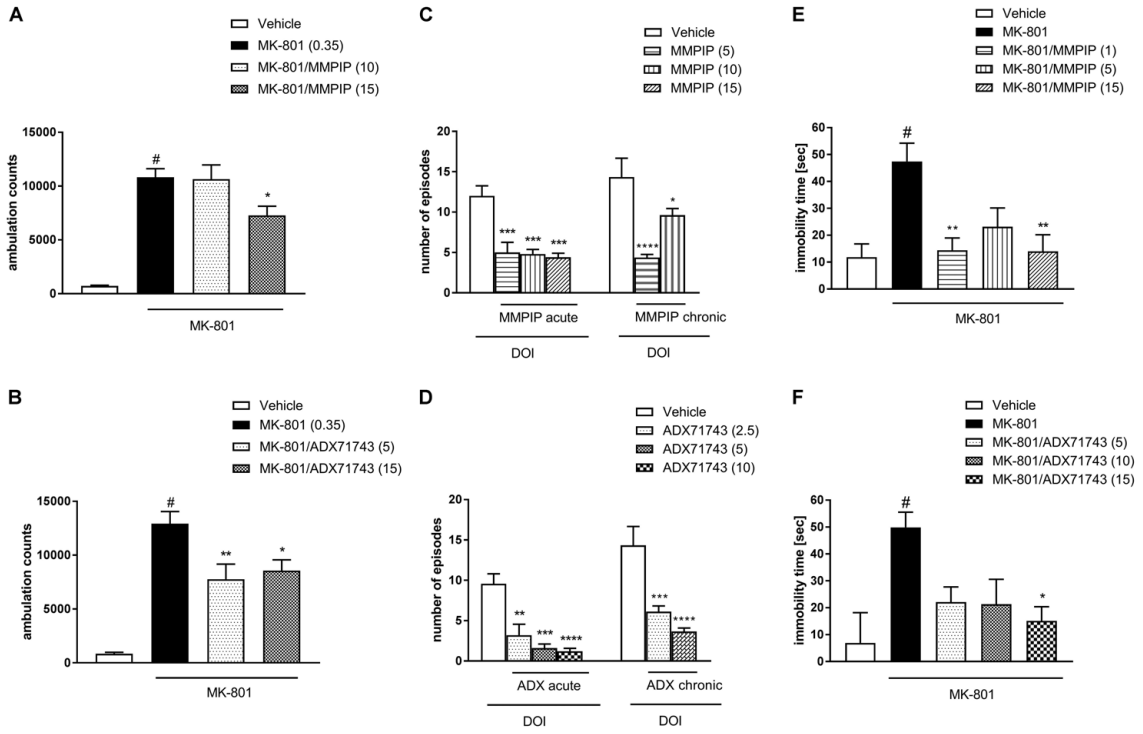

FIGURE 5 | Effects of MMPIP and ADX71743 on MK-801-induced hyperactivity in mice that had been habituated to locomotor activity cages **(A,B)**, DOI-induced head twitches after acute (left panel) or chronic (right panel) administration **(C,D)**, and the immobility time in the modified forced swim test after chronic administration of MK-801 (13 days) **(E,F)**. Doses in mg/kg are indicated in parentheses. Data are presented as means ± SEM. $^{\#}P < 0.001$ comparing to vehicle-treated animals, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ compared with the DOI or MK-801 treated group. Number of animals in each group $n = 7$ **(C,D)** or $n = 10$ **(A,B,E,F)**.

and $F_{(3.32)} = 21.2$, $P < 0.0001$ (**Figure 5B**)]. Neuman-Keuls *post hoc* analysis indicated significant increase in the locomotor activity after MK-801 administration when compared to control groups ($P < 0.0001$) and the significant reversal of MK-801-induced effect after MMPIP administration at the highest dose (15 mg/kg) used in the study ($P < 0.05$). Both doses of ADX71743 (5 and 15 mg/kg) decreased MK-801-induced hyperactivity in a statistically significant way ($P < 0.001$ and $P < 0.05$) when compared to MK-801-treated groups.

DOI-Induced Head Twitches

One-way ANOVA analysis revealed the statistically significant effects of treatments [$F_{(3.16)} = 13.96$, $P < 0.0001$ (**Figure 5C**) and $F_{(3.18)} = 15.75$, $P < 0.0001$ (**Figure 5D**)]. Dunnet's

post hoc analysis indicated that the administration of MMPIP significantly reduced DOI-induced head twitches at all investigated doses 5, 10, and 15 mg/kg ($P < 0.001$) (**Figure 5C**). The effect of ADX71743 was also significant at 2.5 mg/kg ($P < 0.01$), 5 mg/kg ($P < 0.001$), and 10 mg/kg ($P < 0.0001$) (**Figure 5D**).

The compounds showed similar activity after chronic (10 days) administration: MMPIP at the doses 5 and 10 mg/kg and ADX71743 at the doses 2.5 and 5 mg/kg. One-way ANOVA analysis revealed the statistically significant effect of MMPIP treatment [$F_{(2.19)} = 15.67$, $P < 0.0001$] and Dunnet's *post hoc* comparison revealed statistically significant effect of both doses ($P < 0.05$ and $P < 0.0001$). Similarly, the effect of ADX71743 administration was also significant [$F_{(2.17)} = 16.56$, $P < 0.0001$] and Dunnet's *post hoc* comparison revealed the statistical effect of both investigated doses ($P < 0.001$ and $P < 0.0001$) (**Figures 5C,D**).

Modified Forced Swim Test

One-way ANOVA analysis revealed the statistically significant effects of treatments [$F_{(4.44)} = 5.8$, $P < 0.0007$ (**Figure 5E**) and $F_{(4.45)} = 4.3$, $P < 0.005$ (**Figure 5F**)]. Neuman-Keuls *post hoc* analysis indicated significant increase in the immobility time after MK-801 administration when compared to control groups ($P < 0.001$) and the significant reversal of MK-801-induced effect after MMPIP administration at the dose of 1 mg/kg ($P < 0.01$) and 15 mg/kg ($P < 0.01$). The effect of ADX71743 was significant only at the dose of 15 mg/kg ($P < 0.05$) when compared to MK-801-treated group.

Social Interaction Test

One-way ANOVA analysis revealed the statistically significant effects of treatment in the time of interaction [$F_{(4.30)} = 9.20$, $P < 0.0001$ and $F_{(4.45)} = 7.63$, $P < 0.0001$] (**Figures 6A,C**) and the number of episodes [$F_{(4.30)} = 4.2$, $P < 0.007$ and $F_{(4.45)} = 10.44$, $P < 0.0001$] (**Figures 6B,D**). Neuman-Keuls *post hoc* analysis indicated significant reduction of social behaviors after MK-801 administration when compared to control groups ($P < 0.01$). MMPIP had no effect on both measured parameters (**Figures 6A,B**).

ADX71743 at a dose of 5 and 15 mg/kg reversed the effect of MK-801 on the duration ($P < 0.01$ and $P < 0.05$) and number of social episodes ($P < 0.001$ and $P < 0.001$) (**Figures 6C,D**).

Novel Object Recognition Test

One-way ANOVA analysis revealed the statistically significant effects of treatments [$F_{(4.37)} = 3.7$, $P < 0.01$ (**Figure 7A**) and $F_{(4.44)} = 5.99$, $P < 0.0006$ (**Figure 7B**)]. Neuman-Keuls *post hoc* analysis indicated significant reduction of recognition index after MK-801 administration when compared to control groups ($P < 0.01$) and the significant reversal of MK-801-induced effect after MMPIP administration at the doses of 10 ($P < 0.05$) and 15 mg/kg ($P < 0.01$) (**Figure 7A**) and ADX71743 at the doses 1 mg/kg ($P < 0.01$), 5 mg/kg ($P < 0.01$), and 15 mg/kg ($P < 0.001$) (**Figure 6B**) when compared to MK-801-trated animals.

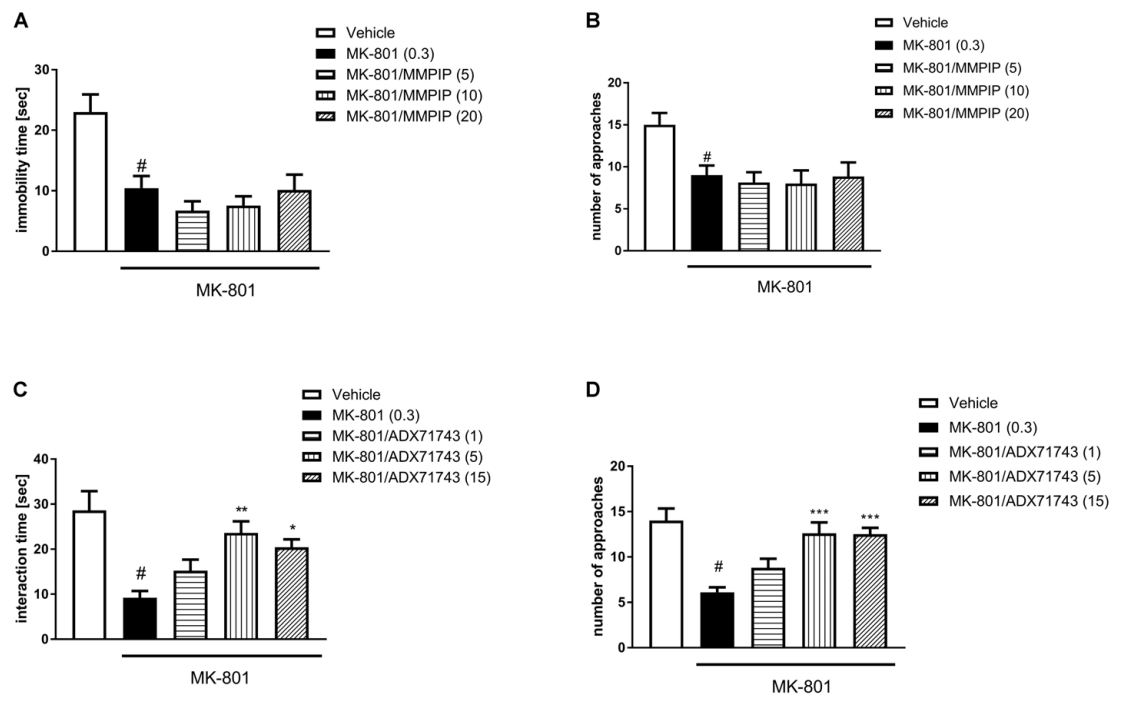

FIGURE 6 | Effects of MMPIP **(A,B)** ADX71743 **(C,D)** on MK-801-induced social interaction deficits. Doses in mg/kg are indicated in parentheses. Data are presented as means ± SEM. $^{\#}P < 0.01$ compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the MK-801-treated group. Number of animals in group varied n = 8–10.

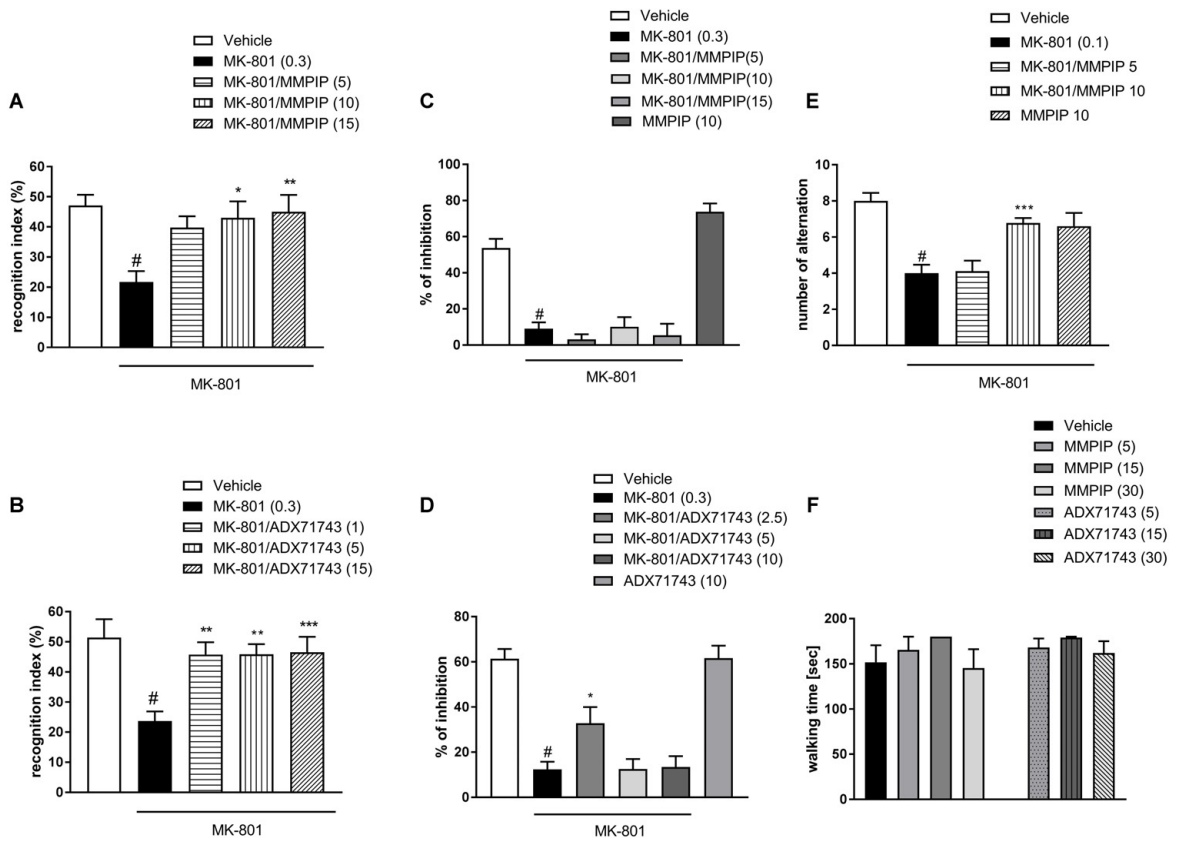

FIGURE 7 | Effects of MMPIP and ADX71743 on MK-801-induced deficits in the NOR test **(A,B)** and prepulse inhibition **(C,D)**. The graph showing the effect of MMPIP on MK-801-induced disruption in spatial delayed alteration test **(E)** and the effect of MMPIP and ADX71743 on rotarod performance **(F)**. Doses in mg/kg are indicated in parentheses. Data are presented as means ± SEM. $^{\#}P < 0.001$ compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the MK-801-treated group. Number of animals in each group n = 8–10.

Prepulse Inhibition

MK-801 (0.3 mg/kg) enhanced the amplitude of the acoustic startle response and markedly attenuated the prepulse-induced inhibition of the acoustic startle response (up to 16% of control). One-way ANOVA analysis revealed statistically significant effect of treatment [$F_{(4.39)}$ = 22.84, $P < 0.0001$ (**Figure 7C**) and $F_{(4.39)}$ = 19.96, $P < 0.000$ (**Figure 7D**)]. Neuman-Keuls *post hoc* comparison revealed that MK-801 inhibited startle response comparing to control groups ($P < 0.0001$). The effect of MK-801 on the prepulse-induced inhibition of the acoustic startle response was not antagonized by the selective NAM of mGlu$_7$ receptor MMPIP in all doses (5, 10, and 15 mg/kg). When given alone, MMPIP (10 mg/kg) attenuated the amplitude of the acoustic startle response and markedly enhanced the prepulse-induced inhibition of the acoustic startle response (up to 137% of control), but the effect was not statistically significant (**Figure 7C**). ADX71743 inhibited MK-801-induced disruption in PPI at the lowest dose 2.5 mg/kg ($P < 0.05$) (**Figure 7D**).

Spatial Delayed Alternation Test

One-way ANOVA analysis revealed a statistically significant effect of treatment [$F_{(4.42)}$ = 10.59, $P < 0.0001$] (**Figure 7E**). Neuman-Keuls *post hoc* analysis indicated significant reduction of choice accuracy after MK-801 administration ($P < 0.0001$). MMPIP at a dose of 10 mg/kg rescued the MK-801–induced cognitive impairments, by improving the choice accuracy ($P < 0.001$) (**Figure 7E**).

Motor Coordination

In the rotarod test, neither MMPIP nor ADX71743 did not induce detectable motor impairments when compared to the control group [$F_{(6.63)}$ = 0.919] (**Figure 7F**).

DISCUSSION

The present paper constitutes the complex study concerning putative antipsychotic-like activity of mGlu$_7$ receptor NAMs. We used two commercially available compounds, MMPIP and ADX71743. The first reports concerning their pharmacological activity were released several years ago, however, the activity of the compounds is still not fully investigated and established.

Here, we have used a variety of techniques that allowed us to investigate the *in vitro* and *in vivo* activity of both compounds, their pharmacokinetics and pharmacological effects.

Using T-REx 293 cell lines (commercially available version of HEK 293 cell line with single FRT site) with inducible expression of mGlu$_7$ receptor the selectivity of the compounds toward mGlu$_7$ receptors was confirmed. Both compounds dose-dependently antagonized L-Glu inhibition of cAMP accumulation in the presence of forskolin and L-glutamate. Similar results were shown earlier for ADX71743 (Kalinichev et al., 2013).

This is the first report showing the activity of MMPIP on HEK line, and thus can be compared with the activity of ADX71743. The earlier studies with MMPIP were performed on CHO lines, and the activity of the compound was investigated in the presence of L-AP4, without the presence of L-glutamate. Additionally, different techniques for intracellular cAMP assessment like AplhaScreen and Phenyx cAMP assays were used to characterize both NAMs which can differ in sensitivity and measurement range (Suzuki et al., 2007; Kalinichev et al., 2013). In the Kalinichev et al.'s (2013) paper, HEK 293 cells and L-glutamate were used. The EC_{50} for L-Glu and IC_{50} for ADX71743 in the presence of EC_{80} the agonist were significantly lower comparing to results obtained by our group. Different method of cAMP measurements and different host cells caused difficulties to collate the biological activity of the two chemicals. Here, we compare the activity of both compounds and our result indicates that the affinity of MMPIP is slightly better than that of ADX71743, although both compounds are very potent. Moreover, we demonstrated inverse agonist action for both MMPIP and ADX71743 in heterologous expression system. Our results and data presented by Suzuki et al. (2007) confirmed the intrinsic activity of mGluR$_7$ that can be showed in the presence of inverse agonist. Moreover, this may have very important biological effect on *in vivo* studies due to dual way of action of ADX71743 and MMPIP. However, we must keep in mind differences which can be observed between species and even between different cell hosts from the same species. For example, the mGluR$_7$ positive allosteric modulator AMN082 activates the receptor in CHO cells, as well its effect can be observed in behavioral studies. In contrast, human cell line HEK 293 expressing mGluR$_7$ does not respond to this compound (Niswender et al., 2010).

Systemic administration of the compounds confirmed that they reach Cmax rapidly, 0.25–0.5 h after administration, followed by a rapid decline. The concentration of ADX71743 was almost undetectable 2 h after administration, while the concentration of MMPIP was still observed. Similar pharmacokinetic profile was described earlier for the compounds; however, the doses of ADX71743 used were much higher (100–150 mg/kg) than that used in our studies, and the compound was administered s.c. (Kalinichev et al., 2013), while MMPIP was administered similarly as in the work of Hikichi et al., 2010. The important thing is that in the present studies several time points were analyzed in contrary to the work of Hikichi et al. (2010) where the concentration of the compound was measured only at one time point, 1 h after administration (Hikichi et al., 2010). Comparing the results obtained for both compounds it may be concluded that the most potent NAM of mGluR$_7$ – ADX71743 exhibits high kinetic solubility, low metabolic stability in mice liver microsomes consistent with high clearance, while MMPIP shows better metabolic stability but lower biological activity as well as solubility.

Subsequently, the specificity of compounds was assessed in electrophysiology experiments, in which, with the use of mGlu$_7$ KO mice, we established that the compounds were active only on the slices obtained from wild type animals and not from mGlu$_7$ KO mice. Then, we compared the activity of the compounds in variety of behavioral models. In our earlier studies, the propsychotic effect of mGlu$_7$ PAM, AMN082, was showed (Wierońska et al., 2012). Therefore, it could be assumed that NAMs of mGlu$_7$ receptor can be proposed as putative antipsychotic agents. To confirm this hypothesis, both compounds were examined in variety of animal tests and models with high predictive validity toward antipsychotic-like efficacy of drugs.

MK-801 induced hyperactivity and DOI-induced head twitches were used as the tests predictive for positive symptoms of schizophrenia. Both compounds reversed MK-801-induced deficit without exerting own effects on spontaneous locomotor activity in active doses. The activity of ADX71743 was more evident in this test, and lower doses of the compound (5 mg/kg) restored MK-801-induced deficit. MMPIP was active only in the highest administered dose, 15 mg/kg. In previous studies, the activity of ADX71743 was showed in amphetamine-induced hyperactivity, but much higher doses were needed to reach significant effect (100 and 150 mg/kg were active). Also, the different route of administration (subcutaneous) was applied in that studies (Kalinichev et al., 2013). The activity of MMPIP has not been investigated in this paradigm so far.

Both investigated compounds also reversed DOI-induced head twitches. DOI, similar to the other 5-HT$_{2A}$ activating agents (i.e., d-lysergic diethylamide acid, LSD), has hallucinogenic potential in humans (Jacobs and Trulson, 1979; Geyer and Vollenweider, 2008; Vollenweider and Kometer, 2010), and in animals, it induces characteristic head twitches (González-Maeso et al., 2008; De Gregorio et al., 2016a), that are reversed by the administration of both typical and atypical neuroleptics (Marona-Lewicka et al., 2005; De Gregorio et al., 2016b). The activity of ADX71743 was observed in lower doses than that observed after MMPIP administration. Again our results differ from the results presented in the studies of Kalinichev et al. (2013) where ADX71743 was active at the dose of 100 mg/kg and higher.

In the next step, the activity of both drugs was investigated in tests for negative symptoms of schizophrenia, such as social interaction and modified forced swim test. The social interaction test resembles social withdrawal observed in schizophrenia patients while modified forced swim test is considered as a model of depressive-like symptoms of schizophrenia (Noda et al., 1995, 1997; de Moura Linck et al., 2008). In both tests, only atypical (e.g., risperidone), and not typical neuroleptics, effectively reverse MK-801-induced deficits (de Moura Linck et al., 2008). It is in line with clinical efficacy of drugs, where only atypical neuroleptics are potent to reverse negative symptoms, although the efficacy of drugs is not always satisfactory.

Here, both compounds were active in modified forced swim test and only ADX71743 reversed MK-801-induced deficits in social interaction test. So again the activity of ADX71743 was better than the activity of MMPIP.

Novel object recognition (NOR) and spatial delayed alteration (SDA) were used as the models of cognition, while PPI reflects attentional deficit associated with schizophrenia. MK-801 disrupts the ability of animals to discriminate between the known, old object, and the novel one in NOR (Nilsson et al., 2007; Grayson et al., 2015), and to make proper choice to obtain the reward in SDA. This disruption of short working memory is antagonized by atypical, but not typical antipsychotics. In NOR, both drugs were active, but the activity of ADX71743 was more evident. Additionally, MMPIP was tested in SDA test and prevented, in all investigated doses, the disruptive effect of MK-801. In the former studies performed in the work of Hikichi et al. (2010), the drug was shown to reduce the recognition index in the NOR test and decreased the location index in the object location test at the doses 10–30 mg/kg. Therefore, it seems that the compound does not possess any procognitive effect when given alone, on contrary it rather disturbs cognitive behaviors, while when given prior MK-801 it prevents the development of disruptive effects of the drug. The other compound, ADX71743, was not investigated in the models of cognition yet. Therefore, this is the first study showing the pro-cognitive activity of the compound in the models of schizophrenia. Here, we also show for the first time that ADX71743 in low doses is potent to prevent MK-801-induced disruption in PPI, while such an activity was not observed for MMPIP, similarly as in previous studies (Hikichi et al., 2010).

In these studies, the activity of compounds was well-investigated in pharmacologically induced animal models of schizophrenia, showing their preventive effect on MK-801-induced disruptions of those behaviors after acute administration. However, it should be taken into consideration that the potential desensitization effect, after repeated administration of two compounds, may be responsible for the failure in neuroleptic efficacy in clinical trials. Therefore both compounds were administered chronically to compare if their efficacy will be similar as after acute administration. The activity of the compounds was tested in DOI-induced head twitches and no tolerance was observed. However, further studies are needed especially with non-pharmacologically induced animal model of schizophrenia to fully characterize the antipsychotic properties of above described compounds.

Considering the putative mechanism of action of mGlu$_7$ NAMs, it must be taken into consideration that mGlu$_7$ receptors are localized mainly on GABAergic terminals (Dalezios et al., 2002). The expression of this receptor on GABAergic neurons is almost 10 times higher than on glutamatergic neurons. Therefore, the receptor predominantly regulates GABA release than glutamate release (Summa et al., 2013). Its activation leads to inhibition of GABA release while its inhibition may contribute to increased release of this neurotransmitter. According to the hypothesis of schizophrenia raised by Conn et al. (2009), the increased release of glutamate due to the loss of inhibitory control over the glutamatergic neurons is the main cause of schizophrenia development. The majority of recent studies concerning antipsychotic activity of mGlu ligands was focused on the inhibition of glutamate release through the activation of the receptors expressed on glutamatergic nerve terminals. Here, it seems that the inhibition of mGlu$_7$ receptors expressed on GABAergic neurons may contribute to the increase of GABA efflux and thus bring back the inhibitory control over the glutamatergic transmission (scheme of the **Figure 8**).

Based on the present studies, it is clear that the investigated compounds may have preventive effect in developing psychotic behaviors. However, to better establish the role of mGlu$_7$ receptor in schizophrenia and putative antipsychotic effects of its inhibition, more work must be undertaken and new ligands with better pharmacokinetic properties acting at mGlu$_7$ receptor should be synthesized. The trend is now open as recently a paper

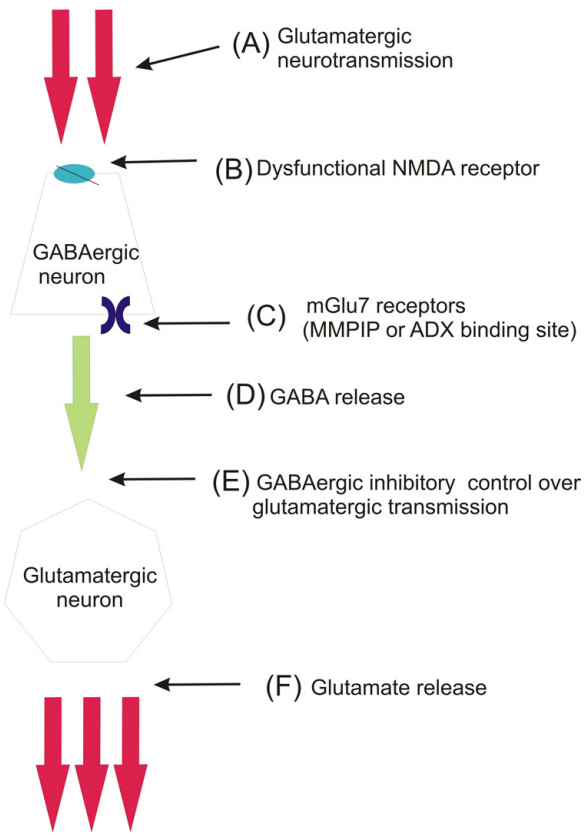

FIGURE 8 | Schematic mechanism involving the role of mGlu$_7$ receptor in schizophrenia pathogenesis (hypothesis partially based on Conn et al., 2009). In normally functioning brain, glutamate **(A)** stimulates GABAergic interneurons via NMDA receptors **(B)** to release GABA **(D)**, which in turn exerts inhibitory control over thalamocortical glutamatergic innervation **(E)**. In schizophrenia this inhibitory control of glutamatergic neurotransmission is lost due to dysfunction of NMDA receptors **(B)** expressed on GABAergic cell bodies. This leads to enhanced glutamate release from thalamocortical glutamatergic neurons **(F)**. The inhibition of mGlu$_7$ receptors, which are expressed presynaptically on GABAergic neurons by MMPIP or ADX **(C)**, leads to activation of GABA release and restores the GABAergic inhibitory control over glutamatergic neurons.

was released, where new compounds inhibiting mGlu$_7$ receptor were proposed (Reed et al., 2017).

ETHICS STATEMENT

All the procedures were conducted in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EU) and Polish legislation acts concerning animal experimentations.

AUTHOR CONTRIBUTIONS

PC and MW performed the DOI-induced head twitches, social interaction, and NOR. PC contributed to the analysis of the results and manuscript writing. KK carried out the pharmacokinetics studies. PB and GB performed the cAMP analysis. AC performed the PPI. BB performed the electrophysiology experiments. PG and EL performed the SDA. AP-P performed the hyperactivity and modified forced swim test. AW performed the analysis of video recordings from behavioral tests (SI, NOR). AP collected the funds and contributed to the discussion. JW performed the data analysis, coordination, wrote the manuscript, and collected the funds.

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REVIEW
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Metabotropic Glutamate Receptor 7: A New Therapeutic Target in Neurodevelopmental Disorders

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Neurodevelopmental disorders (NDDs) are characterized by a wide range of symptoms including delayed speech, intellectual disability, motor dysfunction, social deficits, breathing problems, structural abnormalities, and epilepsy. Unfortunately, current treatment strategies are limited and innovative new approaches are sorely needed to address these complex diseases. The metabotropic glutamate receptors are a class of G protein-coupled receptors that act to modulate neurotransmission across many brain structures. They have shown great promise as drug targets for numerous neurological and psychiatric diseases. Moreover, the development of subtype-selective allosteric modulators has allowed detailed studies of each receptor subtype. Here, we focus on the metabotropic glutamate receptor 7 ($mGlu_7$) as a potential therapeutic target for NDDs. $mGlu_7$ is expressed widely throughout the brain in regions that correspond to the symptom domains listed above and has established roles in synaptic physiology and behavior. Single nucleotide polymorphisms and mutations in the *GRM7* gene have been associated with idiopathic autism and other NDDs in patients. In rodent models, existing literature suggests that decreased $mGlu_7$ expression and/or function may lead to symptoms that overlap with those of NDDs. Furthermore, potentiation of $mGlu_7$ activity has shown efficacy in a mouse model of Rett syndrome. In this review, we summarize current findings that provide rationale for the continued development of $mGlu_7$ modulators as potential therapeutics.

Keywords: neurodevelopmental disorder, ASD, Rett syndrome, $mGlu_7$, *GRM7*, allosteric modulator

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Abbreviations: ADHD, attention deficit hyperactivity disorder; ADX71743, (+)-6-(2,4-dimethylphenyl)-2-ethyl-6,7-dihydrobenzo[*d*]oxazol-4(5*H*)-one; ADX88178, 5-methyl-*N*-(4-methylpyrimidin-2-yl)-4-(1*H*-pyrazol-4-yl)thiazol-2-amine; AMN082, *N,N′*-dibenzhydrylethane-1,2-diamine dihydrochloride; ASDs, autism spectrum disorders; CaM, calmodulin; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; EC50, effective concentration 50; ELFN1, extracellular-leucine-rich repeat fibronectin type III domain containing 1; EPM, elevated plus maze; GABA, γ-aminobutyric acid; GIRK, G protein inwardly rectifying potassium channel; GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-O-[γ-thio]triphosphate; IC50, inhibitory concentration 50; ID, intellectual disability; L-AP4, L-2-amino-4-phosphonobutyric acid; LiCl, lithium chloride; LSP1-2111, (2*S*)-2-amino-4-[hydroxy[hydroxy(4-hydroxy-3-methoxy-5-nitro-phenyl)methyl]phosphoryl]butanoic acid; LSP2-9166, (2S)-2-amino-4-(((4-(carboxymethoxy)-3-(trifluoromethoxy)phenyl)(hydroxy)methyl)(hydroxy)phosphoryl)butanoic acid; LSP4-2022, (2*S*)-2-amino-4-({[4-(carboxymethoxy)phenyl](hydroxy)methyl}(hydroxy)phosphoryl)butanoic acid; LTD, long-term depression; LTP, long-term potentiation; LY341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)propanoic acid; MacMARCKS, macrophage myristoylated alanine−rich C−kinase substrate; MDS, *MECP2* Duplication syndrome; MeCP2, methyl-CpG binding protein 2; mGlu, metabotropic glutamate receptor; MMPIP, 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazolo[4,5-*c*]pyridin-4(5*H*)-one; MRG, mortality factor-related gene; NAM, negative allosteric modulator; NDDs, neurodevelopmental disorders; NK1, neurokinin-1 receptor; OLM, oriens-lacunosum-moleculare; PAM, positive allosteric modulator; PICK1, protein interacting with C kinase 1;

INTRODUCTION

Neurodevelopmental disorders are a group of conditions that present in early life and are characterized by the failure to meet typical developmental milestones. These disorders affect a significant fraction of the population: 15% of children aged 3 to 17 years old were reported to have a developmental disability in the years 2006 to 2008 (Boyle et al., 2011). The current Diagnostic and Statistical Manual of Mental Disorders (DSM-V) categorizes NDDs into six groups: intellectual disabilities (IDs), learning disorders, communication disorders, ASDs, ADHDs, and motor disorders (American Psychiatric Association, 2013). There is often overlap between these groups; for example, 31.6% of patients with ASD also fulfill the diagnostic criteria for ID (Christensen et al., 2016). In addition, NDDs are associated with many co-morbidities, including but not limited to: epilepsy, mood disorders, breathing abnormalities, sleep problems, and gastrointestinal issues (Mannion and Leader, 2013; Doshi-Velez et al., 2014). Individuals with NDDs can struggle to develop interpersonal relationships and face immense challenges in school and in the workforce. Treatment options remain limited and there is a great need to identify novel points of intervention to improve the quality of life of these patients.

A growing body of literature suggests that NDDs arise from complex interactions between the environment and the genome (van Loo and Martens, 2007; Hu et al., 2014). In some cases, NDDs can be traced to genetic abnormalities such as point mutations, gene deletions/duplications, or chromosomal rearrangements. Examples of such disorders include Down syndrome, RTT, Fragile X syndrome, and Angelman syndrome. Although a clear genetic cause is often rare, monogenetic disorders have helped to identify proteins and pathways that are required for proper neuronal development and maintenance. Interestingly, many genes that have been associated with syndromic and non-syndromic NDDs can be clustered into pathways involved in synaptic structure and function (Spooren et al., 2012; Sztainberg and Zoghbi, 2016). In this review, we focus on the metabotropic glutamate receptor 7 (mGlu$_7$), a GPCR that serves as an important regulator of synaptic transmission and plasticity. We will summarize current literature suggesting the involvement of mGlu$_7$ in NDDs and discuss its potential utility as a novel therapeutic target.

METABOTROPIC GLUTAMATE RECEPTORS

mGlu$_7$ is one of eight subtypes of mGlu that are expressed throughout the body. The mGlu receptors are a family of Class C GPCRs that are further divided into three groups based on their sequence homology, signaling pathways, and ligand selectivity. Group I includes mGlu$_1$ and mGlu$_5$, Group II includes mGlu$_2$ and mGlu$_3$, and Group III includes mGlu$_4$, mGlu$_6$, mGlu$_7$, and mGlu$_8$ (Niswender and Conn, 2010). Characteristic of Class C GPCRs, all mGlu receptors consist of a large *N*-terminal ligand binding domain, a cysteine-rich domain, a heptahelical domain, and a C-terminal domain; G proteins interact with intracellular loops and the C-terminus of the receptors.

The large extracellular *N*-terminal ligand binding domain consists of two lobes that sit on top of one another, similar to a Venus flytrap. This structural similarity earned it the name VFD. Glutamate, the endogenous ligand for mGlu receptors, binds to a cleft in between the two lobes of the VFD (Kunishima et al., 2000; Pin et al., 2003; Niswender and Conn, 2010). The mGlu receptors function as constitutive dimers (Pin et al., 2005; El Moustaine et al., 2012), and dimerization primarily occurs at the level of the VFDs (Kunishima et al., 2000; Jingami et al., 2003; Pin et al., 2003; Levitz et al., 2016). The VFDs can exist in three different states within the dimer: open-open, open-closed, and closed-closed. The open-open state is the inactive state, and upon glutamate binding to the cleft of the VFD, the VFD closes and receptor activation occurs. Ligand binding to one VFD results in the open-closed conformation, whereas ligand binding to both VFDs results in the closed-closed conformation (Pin et al., 2005; Muto et al., 2007). It is suggested that glutamate binding to one VFD alone is sufficient for activation (open-closed), but that full activation is achieved when both VFDs are ligand bound (closed-closed) (Kniazeff et al., 2004). Although mGlu$_7$ has been historically predicted to act as a homodimer, it has also been postulated that the receptor enacts some of its function through hetero-dimerization with other receptors, such as mGlu$_8$ (Doumazane et al., 2011; Kammermeier, 2015).

The cysteine-rich domain contains nine cysteine residues linked by disulfide bonds that are critical for propagating signals from the VFDs to the rest of the receptor (Rondard et al., 2006; Muto et al., 2007). After glutamate binding, signals are transduced through the cysteine-rich domain to the heptahelical domain where conformational changes allow for G protein coupling (Kunishima et al., 2000; Tateyama et al., 2004; Binet et al., 2007; Muto et al., 2007; El Moustaine et al., 2012). mGlu$_7$ and the other Group III mGlu receptors couple to $G_{i/o}$, which inhibits adenylyl cyclase activity and reduces intracellular cAMP concentrations (Niswender and Conn, 2010). Furthermore, mGlu$_7$ activation can result in K^+ influx via $G_{\beta\gamma}$-mediated opening of GIRK ion channels, and inhibition of Ca^{2+} currents through N- and P/Q- type calcium channels (Millán et al., 2002, 2003; Martín et al., 2007).

mGlu$_7$ is the most widely expressed mGlu receptor in the CNS with relatively high expression in the amygdala, hippocampus, and hypothalamus (Kinoshita et al., 1998). There are 15 splice variants of *GRM7*, six of which are predicted to be protein coding (Zerbino et al., 2018). The two major isoforms, mGlu$_{7a}$ and mGlu$_{7b}$, differ at their C-termini and it is hypothesized that these distinct C-terminal tails mediate different protein-protein interactions (Dev et al., 2001). While mGlu$_{7a}$ and mGlu$_{7b}$ are primarily expressed in the CNS (Flor et al., 1997; Corti et al., 1998; Kosinski et al., 1999), isoform specificity

PIH, phosphatidylinositol hydrolysis; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PTZ, pentylenetetrazole; RTT, Rett syndrome; SC-CA1, Schaffer Collateral-CA1; SLIN, stratum lucidum interneuron; SNP, single nucleotide polymorphism; VFD, Venus flytrap domain; VU6005649, 3-(2,3-difluoro-4-methoxy-phenyl)-2,5-dimethyl-7-(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidine; VU6010608, 3,4-dimethoxy-*N*-[2-(1*H*-1,2,4-triazol-1-yl)-5-(trifluoromethoxy)phenyl]benzamide; XAP044, 7-hydroxy-3-(4-iodophenoxy)-4*H*-chromen-4-one.

TABLE 1 | Summary of current tool compounds used to study mGlu$_7$.

Name (#)	Type	mGlu$_7$ pEC$_{50}$/pIC$_{50}$	mGlu$_8$ pEC$_{50}$/pIC$_{50}$	mGlu$_4$ pEC$_{50}$/pIC$_{50}$	mGlu$_6$ pEC$_{50}$/pIC$_{50}$	Source
L-AP4 **(1)**	Orthosteric agonist	3.47 (PIH)	6.53 (PIH)	7.00 (PIH)	5.62 (PIH)	Acher et al., 2012;
		3.61 (Ca^{2+})	6.53 (Ca^{2+})	6.89 (Ca^{2+})	6.00 (Ca^{2+})	Selvam et al., 2018
LSP4-2022 **(2)**	Orthosteric agonist	4.34 (Ca^{2+})	4.54 (Ca^{2+})	6.96 (Ca^{2+})	5.36 (Ca^{2+})	Acher et al., 2012; Goudet et al., 2012; Selvam et al., 2018
LSP1-2111 **(3)**	Orthosteric agonist	4.28 (PIH)	4.18 (PIH)	5.66 (PIH)	5.77 (PIH)	Selvam et al., 2018
		4.00 (Ca^{2+})	4.71 (Ca^{2+})	6.05 (Ca^{2+})	5.49 (Ca^{2+})	
LSP2-9166 **(4)**	Orthosteric agonist	5.71 (Ca^{2+})	4.25 (Ca^{2+})	7.22 (Ca^{2+})	Not reported	Acher et al., 2012
VU0422288 **(5)**	Group III PAM	6.85 (Ca^{2+})	6.93 (Ca^{2+})	6.98 (Ca^{2+})	Not reported	Jalan-Sakrikar et al., 2014
VU0155094 **(6)**	Group III PAM	5.80 (Ca^{2+})	6.07 (Ca^{2+})	5.48 (Ca^{2+})	Not reported	Jalan-Sakrikar et al., 2014
ADX88178 **(7)**	mGlu$_{4/8}$ PAM	>4.52 (Ca^{2+})	5.66 (Ca^{2+})	8.46 (Ca^{2+})	>5	Le Poul et al., 2012
ADX71743 **(8)**	mGlu$_7$ NAM	7.20 (human, Ca^{2+})	Inactive	Inactive	Inactive	Kalinichev et al., 2013
		7.06 (rat, Ca^{2+})	Inactive	Inactive	Inactive	
AMN082 **(9)**	Allosteric agonist	6.59 (GTPγS)	>5 (GTPγS)	>5 (GTPγS)	>5 (GTPγS)	Mitsukawa et al., 2005
XAP044 **(10)**	Antagonist	5.26 (cAMP)	4.48 (cAMP)	Inactive	Inactive	Gee et al., 2014
		5.55 to 5.46 (GTPγS)				
LY341495 **(11)**	Orthosteric antagonist	6.00 (cAMP)	6.76 (cAMP)	4.66 (cAMP)	Not reported	Kingston et al., 1998
MMPIP **(12)**	mGlu$_7$ NAM	6.66 (cAMP)	>5 (cAMP)	>5 (cAMP)	Not reported	Suzuki et al., 2007
		7.15 (Ca^{2+})				Niswender et al., 2010
		6.14 (Thallium)				Niswender et al., 2010
VU6010608 **(13)**	mGlu$_7$ NAM	6.12 (Ca^{2+})	>5 (Ca^{2+})	>5 (Ca^{2+})	Inactive (>5)	Reed et al., 2017
VU6005649 **(14)**	mGlu$_{7/8}$ PAM	6.19 (Ca^{2+})	5.59 (Ca^{2+})	>5 (Ca^{2+})	Inactive	Abe et al., 2017

NAM, negative allosteric modulator; PAM, positive allosteric modulator; EC$_{50}$, effective concentration 50; IC$_{50}$, inhibitory concentration 50. Assay type is indicated in parenthesis: PIH, phosphatidylinositol hydrolysis; cAMP, cAMP accumulation; Ca^{2+}, calcium mobilization; GTPγS, GTPγS binding.

has been observed in peripheral tissues such as the testes, trachea, uterus, and salivary gland (Schulz et al., 2002). In the CNS, mGlu$_7$ receptors are primarily localized to presynaptic active zones in neurons where they can act as auto- or hetero-receptors to inhibit the release of their endogenous ligand, glutamate, the main excitatory neurotransmitter or GABA, the main inhibitory neurotransmitter, respectively (Shigemoto et al., 1996; Cartmell and Schoepp, 2000; Dalezios et al., 2002; Somogyi et al., 2003; Niswender and Conn, 2010). Compared to the other Group III mGlu receptors, mGlu$_7$ exhibits an extremely low affinity for glutamate (high μM to mM as opposed to high nM to low mM for the other Group III mGlu receptors). Because of this low affinity, it has been suggested that mGlu$_7$ functions as an "emergency brake" in the case of elevated glutamate levels (Niswender and Conn, 2010). This idea is supported by the observation that mGlu$_7$ knockout mice exhibit spontaneous seizures under certain contexts (Sansig et al., 2001).

CURRENT mGlu$_7$ TOOL COMPOUNDS

Research to investigate mGlu$_7$ biology has been limited, in part, due to the lack of selective tool compounds. Many of the currently existing compounds do not demonstrate high selectivity, desired pharmacokinetic properties, and/or high potency. Here, we review compounds currently available that will be mentioned in subsequent sections (compound properties at Group III mGlu receptors listed in **Table 1** and structures in **Figure 1**).

The development of mGlu$_7$ PAMs and other activators has been a major challenge thus far. Many *in vitro* and *in vivo* studies examining the effects of mGlu$_7$ potentiation have been performed with orthosteric Group III mGlu agonists such as L-2-amino-4-phosphonobutyric acid (L-AP4, **1**), LSP4-2022 (**2**), and LSP1-2111 (**3**). L-AP4 exhibits an *in vitro* potency (EC$_{50}$) of 0.1, 337, and 0.29 μM at mGlu$_4$, mGlu$_7$, and mGlu$_8$, respectively (Acher et al., 2012; Selvam et al., 2018). Similarly, LSP4-2022 exhibits *in vitro* EC$_{50s}$ of 0.11, 11.6, and 29.2 μM at mGlu$_4$, mGlu$_7$, and mGlu$_8$, respectively (Acher et al., 2012; Goudet et al., 2012; Selvam et al., 2018), while a structurally-related analog, LSP1-2111, displays EC$_{50s}$ of 2.2, 53, and 66 μM at each of these receptors (Selvam et al., 2018). In addition to their relatively low potency at mGlu$_7$, these orthosteric agonists have activity at the other Group III mGlu receptors, limiting their utility for the specific exploration of mGlu$_7$ biology. Interestingly, the orthosteric mGlu$_{4/7}$-preferring agonist LSP2-9166 is much more potent at mGlu$_7$ compared to the other agonists described above (EC$_{50s}$ = 0.06, 1.97, 55.6 μM at mGlu$_4$, mGlu$_7$, and mGlu$_8$), but has yet to be investigated further in learning and memory paradigms (Acher et al., 2012; Hajasova et al., 2018; Lebourgeois et al., 2018).

Pan-Group III PAMs such as VU0422288 (**5**), which exhibits EC$_{50s}$ of 108, 146, and 125 nM, for mGlu$_4$, mGlu$_7$, and mGlu$_8$,

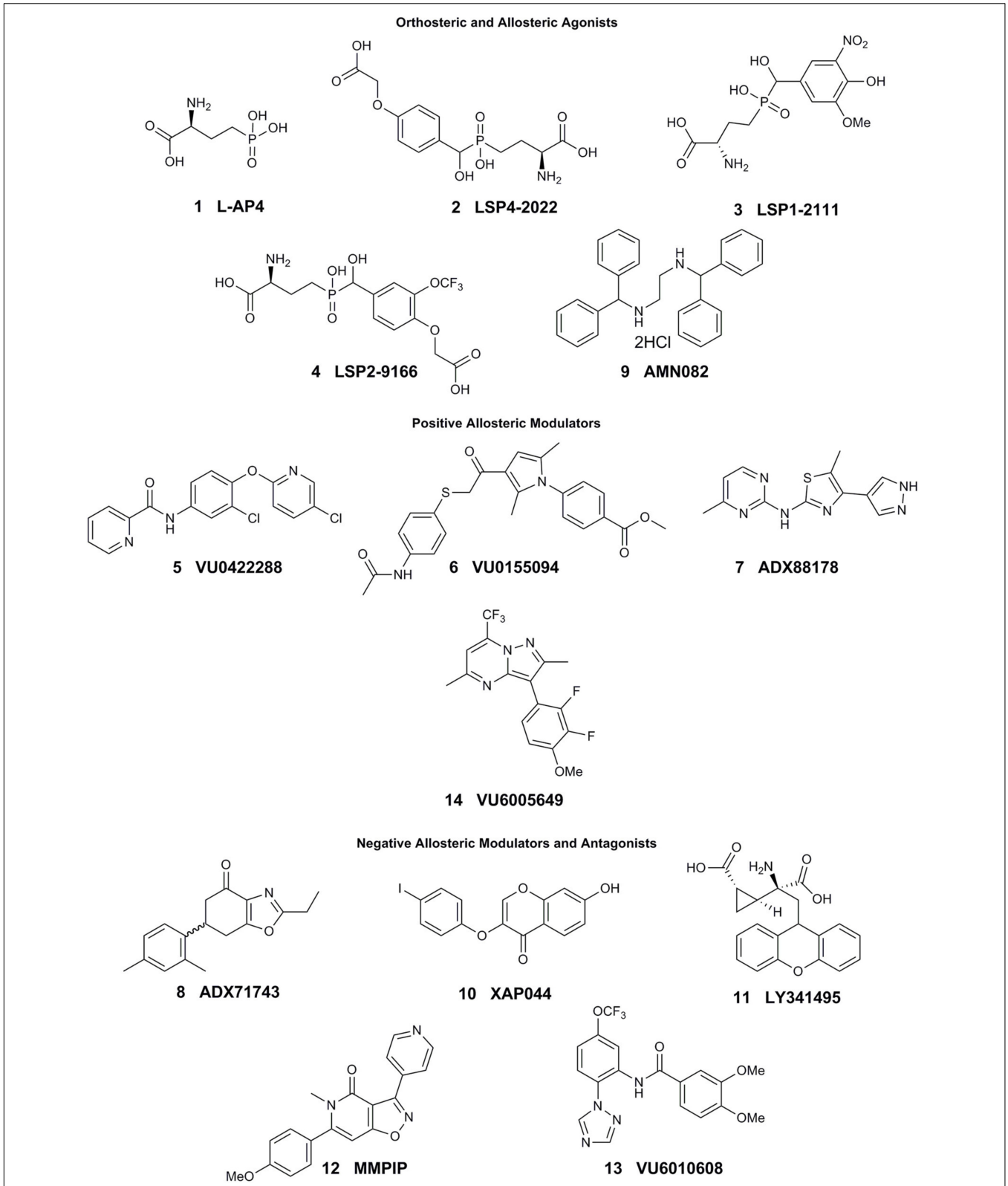

FIGURE 1 | Current tool compounds used to study mGlu$_7$.

respectively, and VU0155094 (**6**), 3.2, 1.5, and 0.9 μM (Jalan-Sakrikar et al., 2014) are also used. Additionally, VU6005649 **14**) is a dual mGlu$_{7/8}$ PAM, with EC_{50} values of 650 nM and 2.6 μM at mGlu$_7$ and mGlu$_8$, respectively. In addition to its activity on mGlu$_8$, VU6005649 displays off-target effects at the neurokinin-1 receptor (NK1). It is believed that these effects on NK1 may mediate sedative effects of this compound, which are observed in both wild-type and mGlu$_7$ knockout animals (Abe et al., 2017). Because many of these tool compounds are not selective, they have been used concomitantly with other molecules, such as the mGlu$_{4/8}$ PAM ADX88178 (**7**) or mGlu$_7$ NAM ADX71743 (**8**), to confirm mGlu$_7$-mediated effects (Le Poul et al., 2012; Kalinichev et al., 2013, 2014; Gogliotti et al., 2017).

To date, only one mGlu$_7$-selective allosteric agonist, AMN082 (**9**, EC_{50} = 260 nM), has been reported in the primary literature (Mitsukawa et al., 2005). AMN082 has been used for animal studies involving learning and memory and plasticity in the amygdala among other areas. However, it has been shown that AMN082 exhibits off-target effects, one of which is predicted to be inhibition of the serotonin transporter (SERT) (Sukoff Rizzo et al., 2011; Ahnaou et al., 2016), somewhat limiting its utility *in vivo* unless coupled with knockout studies.

In contrast to potentiators, there have been several mGlu$_7$ selective antagonists and NAMs reported in the literature. The antagonist XAP044 (**10,** IC_{50} = 5.5 μM) binds within the VFD and has shown efficacy in both *in vivo* and *in vitro* experiments such as anxiety-, depression-, and fear-related behavioral tasks and electrophysiology (Gee et al., 2014). Originally labeled a Group II mGlu receptor antagonist (mGlu$_2$ and mGlu$_3$), LY341495 (**11**) was also found to have efficacy at mGlu$_4$, mGlu$_7$, and mGlu$_8$ with IC_{50s} of 22, 0.99, and 0.173 μM, respectively (Kingston et al., 1998) and has been used to study both groups of mGlu receptor since its discovery.

The mGlu$_7$ NAM 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazolo[4,5-*c*]pyridin-4(5*H*)-one (MMPIP, **12**), reported in 2007, has been used for several studies involving mGlu$_7$ (Suzuki et al., 2007). MMPIP is able to inhibit the response of L-AP4, but its efficacy was later shown to be context-dependent. For example, the potency (IC_{50}) of MMPIP was 70 nM in a calcium mobilization assay utilizing cells expressing $G_{\alpha 15}$ versus 718 nM in a thallium flux assay with cells expressing $G_{\alpha i/o}$, suggesting that its effects may be dependent on cellular background. Further, MMPIP was not effective in blocking an mGlu$_7$-mediated depression of synaptic transmission in electrophysiological studies (Niswender et al., 2010). ADX71743, reported in 2013, exhibits an IC_{50} of 63 and 88 nM at human and rat mGlu$_7$, respectively (Kalinichev et al., 2013). However, it also exhibits low activity at mGlu$_2$ (Kalinichev et al., 2013; Reed et al., 2017) and possesses an electrophilic ketone moiety that could result in covalent modification and subsequent off-target effects. Most recently, Reed et al. (2017) have successfully developed a series of novel, chemically-distinct mGlu$_7$ NAMs based upon a phenylbenzamide scaffold. One of the analogs, VU6010608 (**13**), exhibited modest potency (IC_{50} = 759 nM), but was cleared rapidly in rats (64.2 mL/min/kg) and exhibited low levels of brain penetration, making it challenging for *in vivo* CNS studies (Reed et al., 2017). These existing and emerging tools, coupled with mGlu$_7$ knockout mice, have provided an initial toolbox to begin elucidation of the function of mGlu$_7$ in normal and pathological conditions.

mGlu$_7$ IN SYNAPTIC TRANSMISSION AND PLASTICITY

Inhibition of neurotransmitter release by mGlu$_7$ is believed to be mediated by the inhibition of N-type and P/Q-type calcium channels through interactions with $G_{\beta\gamma}$, PKC, PICK1, and reductions in intracellular cAMP (Millán et al., 2002, 2003; Perroy et al., 2002; Martín et al., 2007). Millan and Colleagues demonstrated that activation of cerebrocortical mGlu$_7$ with L-AP4 inhibited N-type calcium channels in a PKA- and PKC-independent manner, suggesting that the inhibition was caused via interactions with $G_{\beta\gamma}$. These authors also demonstrated that mGlu$_7$-mediated decreases in cAMP could reduce spontaneous glutamate release in the cerebral cortex (Millán et al., 2002). Additionally, Perroy et al. (2000) demonstrated that P/Q-type calcium channels were inhibited via a PKC-dependent pathway, where $G_{i/o}$ and/or $G_{\beta\gamma}$ can stimulate the PLC pathway in cultured cerebellar granule cells. They also showed that the scaffolding protein, PICK1, facilitates the interaction between mGlu$_7$ and PKC, and is required for receptor-mediated P/Q-type calcium channel inhibition in this context (Perroy et al., 2002). In contrast, Martín et al. (2007) demonstrated that mGlu$_7$ inhibited hippocampal P/Q-type calcium channels in a PKC-independent manner. The mGlu$_7$-mediated inhibition of glutamate release is also dependent on interactions with calmodulin (CaM), where activated CaM allows for the displacement of $G_{\beta\gamma}$ from mGlu$_7$ and the subsequent downregulation of calcium influx into the cell via calcium channel inhibition (O'Connor et al., 1999). Moreover, mGlu$_7$'s interaction with MacMARCKS (macrophage myristoylated alanine−rich C−kinase substrate) competitively antagonizes CaM-mediated calcium channel inhibition (Bertaso et al., 2006).

mGlu$_7$'s position within the active zone and its ability to modulate neurotransmitter release has led to numerous studies focused on its role in synaptic plasticity. Two major forms of synaptic plasticity include LTP and LTD, which are persistent changes in synaptic strength that are thought to be correlates of learning and memory (Bliss and Collingridge, 1993; Takeuchi et al., 2014). The role of mGlu$_7$ in synaptic plasticity has been best characterized within the hippocampus at several distinct synapses. mGlu$_7$ was first reported to mediate a form of LTD occurring in stratum radiatum interneurons within area CA3 (Laezza et al., 1999). At excitatory synapses onto interneurons expressing calcium-permeable AMPA receptors, LTP could be induced by high frequency stimulation and blocked by the Group II and Group III mGlu antagonist, LY341495. Further pharmacological experiments confirmed the specific involvement of mGlu$_7$: only a high concentration of L-AP4 depressed synaptic transmission at these synapses and a Group II mGlu agonist showed no effect. A similar form of plasticity was later described at mossy fiber inputs onto SLINs in area CA3 (Pelkey et al., 2005). At SLINs expressing calcium-permeable AMPA receptors,

high frequency stimulation of mossy fibers induced an LTD that required mGlu$_7$ activation and PKC-dependent depression of neurotransmitter release through P/Q-type voltage gated calcium channels (Pelkey et al., 2005, 2006). Interestingly, in slices pre-treated with L-AP4, internalization of mGlu$_7$ receptors revealed the ability of these synapses to undergo LTP instead of LTD in response to the same electrical stimulus. Surface expression of mGlu$_7$, therefore, regulates the direction of plasticity at these synapses, making mGlu$_7$ a "metaplastic switch" that can modulate feedforward inhibition in area CA3.

An additional class of interneurons in which mGlu$_7$-mediated plasticity has been implicated is the OLM interneuron population within the stratum oriens of areas CA3 and CA1. At excitatory inputs onto OLM interneurons, mGlu$_7$ expression is preferentially enriched (Shigemoto et al., 1996) and proposed to be recruited by extracellular-leucine-rich repeat fibronectin type III domain containing 1, or ELFN1 (Tomioka et al., 2014). Sylwestrak and Ghosh demonstrated that ELFN1 knockdown in OLM interneurons decreases short-term facilitation and increases presynaptic release probability. Conversely, overexpression of ELFN1 in parvalbumin interneurons leads to short-term facilitation when these synapses typically undergo short-term depression (Sylwestrak and Ghosh, 2012). In slices from *Elfn1*$^{-/-}$ mice, presynaptic release probability, short term facilitation, and LTP are reduced in patch-clamp recordings from OLM interneurons (Tomioka et al., 2014). Although this evidence is indirect, it suggests that mGlu$_7$ may be involved in these forms of synaptic plasticity since mGlu$_7$ is likely to be a major regulator of presynaptic release probability at these synapses due to its recruitment by ELFN1.

In addition to its role as an autoreceptor on excitatory terminals, mGlu$_7$ is also located on the terminals of interneurons within the hippocampus and modulates the release of GABA (Somogyi et al., 2003; Summa et al., 2013). This function of mGlu$_7$ is required for LTP in wild-type animals at SC-CA1 synapses through a mechanism of disinhibition (Klar et al., 2015). Importantly, deficits in LTP at this particular synapse have been reported in several models of NDDs (Jiang et al., 1998; Moretti et al., 2006; von der Brelie et al., 2006). At SC-CA1 synapses, mGlu$_7$ is the only presynaptic mGlu receptor present in adult animals and activation of mGlu$_7$ has been repeatedly shown to reduce field potentials at SC-CA1 (Baskys and Malenka, 1991; Ayala et al., 2008; Jalan-Sakrikar et al., 2014). Klar et al. (2015) demonstrated that mGlu$_7$ activation by the agonist LSP4-2022 also reduces evoked inhibitory post-synaptic currents recorded from CA1 pyramidal cells. LTP induced by high-frequency stimulation was blocked by ADX71743, but only when GABAergic transmission was intact. Recently, we showed that a chemically distinct mGlu$_7$ NAM, VU6010608, also blocked LTP induced by high-frequency stimulation at SC-CA1 synapses (Reed et al., 2017). Interestingly, hippocampal slices from *Grm7*$^{-/-}$ mice have been reported to exhibit similar levels of LTP when compared to WT controls, but decreased short-term potentiation following high-frequency stimulation (Bushell et al., 2002). In these studies, slices from *Grm7*$^{-/-}$ mice showed reduced facilitation during the high-frequency train, an effect that was also seen with ADX71743 by Klar et al. (2015). The presence of LTP in *Grm7*$^{-/-}$ slices may be due to compensatory mechanisms during development, such as retained expression of mGlu$_8$, which is present at SC-CA1 synapses earlier in development (Ayala et al., 2008). Re-expression of mGlu$_8$ is not unprecedented as the selective mGlu$_8$ agonist (S)-3,4-DCPG was recently shown to reduce synaptic transmission at SC-CA1 in slices from pilocarpine-treated rats, but not in those of age-matched controls (Dammann et al., 2018). While further studies will be needed to explain the current discrepancy between genetic and pharmacological approaches, these data indicate that mGlu$_7$ regulates high-frequency transmission at SC-CA1 synapses. Recently, Martín et al. (2018) demonstrated that prolonged activation of mGlu$_7$ leads to potentiation of excitatory post-synaptic currents recorded by pyramidal cells in CA1. This potentiation of neurotransmitter release is dependent on PLC and the vesicle release proteins Munc13-2 and Rim1α. These studies indicate that, under conditions of high-frequency stimulation, mGlu$_7$ activation favors potentiation of excitatory transmission, which could be an additional mechanism by which mGlu$_7$ modulates long-term plasticity in the hippocampus.

Beyond the hippocampus, a role for mGlu$_7$ in LTP has also been established within the amygdala. Synaptic plasticity in the hippocampus is believed to underlie associative learning and working memory, whereas plasticity in the amygdala is associated with aversion and emotional learning (Brasted et al., 2003; Sigurdsson et al., 2007). The allosteric agonist AMN082 has been shown to block LTP at thalamo-amygdala synapses in slices from rats and mice (Fendt et al., 2008, 2013). This effect correlates with the ability of direct injection of AMN082 into the amygdala to block the acquisition of fear-potentiated startle behavior in rats (Fendt et al., 2008) and fear learning in mice (Fendt et al., 2008, 2013). Interestingly, *Grm7*$^{-/-}$ mice exhibit a general deficit in fear learning and decreased LTP at thalamo-amygdala synapses (Fendt et al., 2013). Reduction of LTP by both an agonist and gene ablation may be explained by AMN082's ability to cause rapid internalization of mGlu$_7$ receptors (Pelkey et al., 2007). This would suggest that AMN082 can act as a functional antagonist by decreasing surface expression and, therefore, receptor signaling. This hypothesis is further supported by the ability of the mGlu$_7$ antagonist XAP044 to block LTP within the amygdala, inhibit acquisition of conditioned fear, and reduce anxiety-like behavior (Gee et al., 2014). Together, these studies demonstrate that mGlu$_7$ promotes plasticity within the amygdala, which is in line with its involvement in behaviors of fear and anxiety.

ROLE OF mGlu$_7$ IN NDD-ASSOCIATED PHENOTYPES

Core symptoms and comorbidities of NDDs can include, but are not limited to: cognitive impairment, seizures, mood disorders, social deficits, and motor impairments (Mannion and Leader, 2013; Doshi-Velez et al., 2014). Many studies have demonstrated that modulation of mGlu$_7$ function via genetic

and/or pharmacologic techniques is able to mimic some of these phenotypes in animal models, and these studies will be reviewed here.

COGNITION

mGlu$_7$ knockout animals (*Grm7*$^{-/-}$) show deficits in tasks that test cognitive functioning. In a conditioned taste aversion task, which measures amygdala-dependent aversive learning, mice were given saccharin along with an intraperitoneal injection of the control, saline, or LiCl, which evokes malaise. In this task, *Grm7*$^{-/-}$ mice did not associate the adverse effects of LiCl to saccharin in comparison to wild-type littermates, exhibiting a deficit in fear learning (Masugi et al., 1999). In addition, Masugi et al. (1999) and Goddyn et al. (2008, 2015) demonstrated that *Grm7*$^{-/-}$ mice exhibit less freezing than wild-type animals in cued and contextual fear conditioning paradigms. Together, these results indicate a role for mGlu$_7$ in aversion learning, and also suggest that the loss of mGlu$_7$ causes impairments in these learning paradigms.

mGlu$_7$ has also been demonstrated to play a role in cognitive tasks that do not rely on fearful or aversive stimuli. Callaerts-Vegh et al. (2006) showed that *Grm7*$^{-/-}$ mice exhibit impaired short-term working memory in 4- and 8-arm radial maze tasks, committing more errors (visits to previously baited arms or un-baited arms) than their wild-type counterparts. Conversely, *Grm7*$^{-/-}$ mice performed similarly to wild-type animals in radial maze tasks when they were modified to assess long-term memory. Furthermore, both Callaerts-Vegh et al. (2006) and Goddyn et al. (2015) have reported that the loss of mGlu$_7$ causes increased latency to locate a platform in the Morris water maze task of spatial memory. Interestingly, *Grm7*$^{-/-}$ mice performed similarly to wild-type animals after increased training and in un-cued trials (Callaerts-Vegh et al., 2006). Together, these data demonstrate that mGlu$_7$ may play specific roles in tasks involving working and spatial memory.

Pharmacological studies have further confirmed a role for mGlu$_7$ in learning and memory. Hikichi et al. (2010) showed that administration of MMPIP, an mGlu$_7$ NAM, to wild-type mice reduced performance in object recognition and location tasks, suggesting that mGlu$_7$ is also involved in recognition memory. MMPIP also attenuates conditioned taste aversion learning in rats (Klakotskaia et al., 2013). Interestingly, MMPIP improved cognitive performance in Y-maze and object recognition assays in a mouse model of neuropathic pain with no effect on sham-treated animals (Palazzo et al., 2015). As discussed above, MMPIP exhibits cellular background-dependent differences *in vitro*, and also had no effect in an electrophysiological study of at SC-CA1 synapses in the hippocampus (Niswender et al., 2010), which may complicate interpretation of *in vivo* data. Inhibition of mGlu$_7$ with the antagonist XAP044 also resulted in reduced freezing in mice during a contextual fear conditioning task, further supporting a role for mGlu7 in amygdala function (Gee et al., 2014). Activation of mGlu$_7$ with an allosteric agonist, AMN082, has been shown to modulate both the acquisition and extinction of conditioned fear, though the results seem to contradict findings from studies performed with XAP044 and *Grm7*$^{-/-}$ animals (Fendt et al., 2008, 2013; Goddyn et al., 2008; Siegl et al., 2008; Dobi et al., 2013; Gee et al., 2014). Administration of AMN082 impairs the acquisition and enhances the extinction of fear learning (Fendt et al., 2008, 2013; Siegl et al., 2008; Dobi et al., 2013), but knockout animals exhibit similar phenotypes in conditioned fear paradigms (Goddyn et al., 2008; Fendt et al., 2013). AMN082 appears to exhibit a task-dependent phenotype, where mGlu$_7$ activation facilitates between-session extinction, but not within-session extinction in a fear conditioning model (Toth et al., 2012; Fendt et al., 2013). AMN082 was also shown to have effects in social fear; it impaired extinction and recall when administered prior to the social fear extinction task, but not when given before social fear conditioning (Slattery et al., 2017). However, Ahnaou et al. (2016) demonstrated that AMN082 produced similar sleep-wake and hypothermia phenotypes in *Grm7*$^{-/-}$ and wild-type mice, suggesting that there may be off-target effects elicited by the compound. Additionally, administration of VU6005649, an mGlu$_{7/8}$ PAM, to wild-type mice, increases freezing in contextual fear conditioning (Abe et al., 2017).

SEIZURES

Seizures are often present in patients with NDDs, and mGlu$_7$ and its interacting proteins have been implicated in seizure activity. Sansig et al. (2001) observed that *Grm7*$^{-/-}$ mice suffered from spontaneous sensory stimulus-seizures and were also more susceptible to subconvulsant doses of PTZ and bicuculline than their heterozygous or wild-type littermates. In addition, reduction of mGlu$_7$ activity with the NAM ADX71743 was sufficient to induce absence seizures (Tassin et al., 2016). Disruption of proteins that interact with mGlu$_7$ can also induce seizures in mice (Bertaso et al., 2008; Tomioka et al., 2014). For example, PICK1 is a PDZ-domain containing protein that interacts with the C-terminus of mGlu$_7$. The protein-protein interaction between PICK1 and mGlu$_7$ is important for stable mGlu$_7$ cell surface expression, proper trafficking of mGlu$_7$ to presynaptic active zones, and also for inhibition of P/Q-type calcium channels. Disruption of the interaction between PICK1 and mGlu$_7$ appears to interfere with mGlu$_7$'s inhibitory activity via decreased cell surface stability/expression or improper signaling and trafficking, resulting in a seizure phenotype in mice (Perroy et al., 2002; Bertaso et al., 2008; Zhang et al., 2008).

As mentioned previously, ELFN1 is a transmembrane protein that has been demonstrated to recruit mGlu$_7$ to distinct cell populations in the hippocampus and cortex (Tomioka et al., 2014). Most recently, ELFN1 was also shown to be a trans-synaptic allosteric modulator of Group III mGlu receptors; receptor modulation occurs through an ELFN1-mediated alteration of G-protein coupling efficiency to the Group III mGlu receptors (Dunn et al., 2018). Of note, ELFN1 mutations clustered in the region required for mGlu$_7$ recruitment have been found in patients with epilepsy and ADHD (Dolan and Mitchell, 2013; Tomioka et al., 2014), and ELFN1 knockout (*Elfn1*$^{-/-}$) animals exhibit a similar seizure phenotype to

Grm7$^{-/-}$ animals (Tomioka et al., 2014). Interestingly, *Elfn1*$^{-/-}$ mice also exhibit ADHD-like phenotypes such as hyperactivity and impulsivity. Dolan and Mitchell (2013) showed that *Elfn1*$^{-/-}$ animals display hyperlocomotion and increased activity in an open field. Administration of amphetamine to *Elfn1*$^{-/-}$ mice was able to attenuate hyperlocomotion, similar to the effects of stimulant therapies for ADHD patients. Tomioka et al. (2014) also demonstrated that *Elfn1*$^{-/-}$ mice displayed more spontaneous activity than wild-type animals and also exhibited decreased immobility in a forced swim test, which are behaviors suggestive of hyperactivity. *Elfn1*$^{-/-}$ mice spent more time in the open arms during an EPM task compared to wild-type littermates. These data are typically indicative of anxiolytic effects; however, *Elfn1*$^{-/-}$ mice showed no preferences between the light and dark boxes of the light-dark box transition task. Based on this finding, the authors hypothesized that the results of the EPM were indicative of impulsivity. Together, these data suggest a role for the ELFN1-mGlu$_7$ complex in seizures and in other disorders.

MOOD DISORDERS

mGlu$_7$ modulation has also been demonstrated to impact behavioral models of mood disorders such as anxiety or depression, which are common comorbidities seen in NDDs (Matson and Cervantes, 2014). The amygdala and hippocampus, areas of high mGlu$_7$ expression, are brain regions known for their importance in anti-anxiety and anti-depressive action (Shin and Liberzon, 2010). In comparison to cognitive tasks, where reductions in mGlu$_7$ cause deficits, the loss of mGlu$_7$ has been reported to result in anti-depressive and anxiolytic effects in these domains. For example, Cryan et al. (2003) showed that *Grm7*$^{-/-}$ animals spend more time in the open arms than their wild-type counterparts in an EPM paradigm, demonstrating that the loss of the receptor causes anxiolytic activity. In a light-dark box task, the knockout animals have a reduced latency to enter a covered, dark compartment as well as an increased number of transitions into an open, brightly lit compartment than wild-type mice (Cryan et al., 2003). Callaerts-Vegh et al. (2006) demonstrated that *Grm7*$^{-/-}$ mice bury fewer marbles than wild-type animals in a marble burying task, which also measures anxiety-like behavior in rodents. ADX71743, the mGlu$_7$-selective NAM, causes similar results in EPM, and reduces marble burying in wild-type mice (Kalinichev et al., 2013). Administration of the NAM MMPIP also reduces marble burying, consistent with the *Grm7*$^{-/-}$ phenotype (Palazzo et al., 2015). In tail suspension or forced swim tasks, where immobility is indicative of depression-like behavior, *Grm7*$^{-/-}$ mice are less immobile than wild-type animals (Cryan et al., 2003). In wild-type mice, the antagonist XAP044 also increases time in open arms in EPM and decreases immobility in tail suspension, recapitulating data from studies using knockout animals (Gee et al., 2014). In a mouse model of neuropathic pain, the NAM MMPIP also reduces immobility time during tail suspension (Palazzo et al., 2015). The mGlu$_7$ agonist AMN082 reduces immobility in tail suspension and forced swim tasks, and MMPIP can block the effect of AMN082 (O'Connor and Cryan, 2013; Pałucha-Poniewiera and Pilc, 2013). In summary, mGlu$_7$ has been implicated in a range of behaviors in rodent models, many of which mimic those reported in rodent models of NDDs.

GENETIC ASSOCIATIONS BETWEEN mGlu$_7$ AND NDDs

Genetic associations between NDDs and *GRM7*, the gene that encodes mGlu$_7$ in humans, provide a link between experiments in rodent models and the clinical population. ASD affects as much as 1% of the world's population (Lai et al., 2014), and family studies have suggested that the heritability of ASD is about 83% (Sandin et al., 2017), which indicates a strong genetic component. Heterozygous deletions in *GRM7* have been identified in three ASD patients by Gai et al. (2012), and in one patient by Liu et al. (2015). The latter patient exhibited language and cognitive impairments as well as hyperactivity, stereotyped behaviors, and deficits in social interaction (Liu et al., 2015). An additional ASD patient with a *de novo* point mutation in *GRM7*, resulting in a change from arginine to glutamate at amino acid 622, was reported by Sanders et al. (2012). This mutation affects the third transmembrane portion of the receptor. Yang and Pan (2013) identified the SNPs rs6782011 and rs779867, which encode a C to T change in intron 6 and a T to C or T to G change in intron 5 in *GRM7*, respectively. These two polymorphisms exhibited significant associations with ASD from a group of 22 ASD patients (Yang and Pan, 2013). In an Iranian cohort of 518 ASD patients, however, only rs779867 was identified as a SNP that associates *GRM7* with ASD (Noroozi et al., 2016). rs779867 is a T to C or T to G polymorphism in intron 5 hypothesized to have effects on a MRG protein binding motif. MRG motif-binding proteins are thought to bind chromatin and function in the regulation of gene transcription (Chen et al., 2010).

Attention deficit hyperactivity disorder is characterized by inattention, hyperactivity and impulsivity (American Psychiatric Association, 2013). Its estimated prevalence around the world is 7.2% in children and 3.4% in adults (Fayyad et al., 2007; Thomas et al., 2015). A genome wide copy-number variation study revealed that rs7623055, which encodes a G to C or G to T change, was significantly associated with ADHD, and also identified six different deletions in *GRM7* in patients with ADHD (Elia et al., 2011). Additionally, rs37952452 was found to have some association with ADHD in a study of 202 patients in Korea, though it was not significantly associated when using a case-control approach (Park et al., 2013). In contrast, neither rs37952452 nor rs7623055 were found to be significantly associated with ADHD in a later study (Akutagava-Martins et al., 2014). Interestingly, ADHD patients with the G/A genotype of rs37952452 showed an improved response to methylphenidate in comparison to those with the G/G genotype (Park et al., 2014).

Rare mutations in *GRM7* have also been implicated in undiagnosed NDDs. Whole-exome sequencing in 31 consanguineous Arab families with developmental delay and/or intellectual disability revealed two families with mutations in *GRM7*. Two brothers in the same family were homozygous for a 461T/C variant, which results in the missense mutation I154T in the ligand binding domain of mGlu$_7$. The same study also

identified two siblings (brother and sister) who are compound heterozygous for the mutations 1972C/T and 2024C/A, which lead to missense mutations, R658W and T675Y, respectively, in the third transmembrane domain. These four patients share symptoms that include developmental delay, ID, brain malformations and seizures (Charng et al., 2016). In a different set of consanguineous families, exome sequencing identified two female cousins with the homozygous mutation 1757G/A, which results in a premature truncation of $mGlu_7$ prior to its first transmembrane domain (W568*). These patients exhibit seizures, profound ID, microcephaly and leukodystrophy (Reuter et al., 2017). A search of the DECIPHER database (Firth et al., 2009) identified 69 patients with a deletion or duplication that included *GRM7*, although most of these also affected other genes. Three of these patients had a deletion or duplication restricted to the *GRM7* gene and their phenotypes are included in **Table 2**.

$mGlu_7$ IN *MECP2*-RELATED DISORDERS

Preclinical research in the NDD field has focused largely on mouse models of genetic syndromes due to their high construct validity. RTT is a monogenetic disorder in which $mGlu_7$ has recently gained particular interest as a potential therapeutic target (Gogliotti et al., 2017). RTT is a debilitating NDD affecting 1 in 20,000 births and is characterized by a period of normal development followed by sudden developmental regression and loss of acquired skills at 6 to 18 months of age. Following regression, RTT patients are burdened by life-long symptoms that include repetitive hand clasping, limited speech, intellectual disability, motor impairment, apneas, and epilepsy (Neul et al., 2010). The majority of RTT cases can be attributed to loss-of-function mutations in the X-linked gene *MECP2*, which encodes the transcriptional regulator methyl-CpG binding protein 2 (MeCP2) (Amir et al., 1999). Since this discovery, nearly two decades of research have yielded significant insight into the functions of MeCP2 within the brain. Of note, *MECP2* mutations have also been identified in patients with ASD and ID independent of a RTT diagnosis (Couvert et al., 2001; Carney et al., 2003), suggesting that pathways involving MeCP2 may underlie NDDs more broadly. MeCP2 is canonically thought to repress gene transcription through binding to methylated CpG dinucleotides and recruiting repressor complexes; however, MeCP2 has also been shown to activate gene transcription and play roles in long-range regulation of chromatin structure, mRNA splicing and micro-RNA processing (Guy et al., 2011). Although MeCP2 is involved in prenatal and postnatal development (Tate et al., 1996;

TABLE 2 | Summary of *GRM7* mutations identified in NDD patients.

Type	Chromosome 3 position	Nucleotide/ protein change NM_00844.3	Location in transcript NM_00844.3	Zygosity	Phenotype	Source
Duplication	6209671–6981117		5′ UTR and Exon 1	Heterozygous	Behavioral abnormality, ID	DECIPHER 289768
Point mutation	6861849	c.T461T > C p.I154T	Exon 1	Homozygous	Developmental delay, seizures, hypotonia, atrophy, thin corpus callosum	Charng et al., 2016
Deletion	7053179–7144453		Intron 1/2 and Exon 2	Heterozygous	ASD	Gai et al., 2012
Deletion	70664629–7172715		Exon 2	Heterozygous	ASD	Gai et al., 2012
Deletion	7065422–7172715		Exon 2	Heterozygous	ASD	Gai et al., 2012
Deletion	7257514–7442882		Exons 3–5	Heterozygous	Global developmental delay	DECIPHER 356330
Deletion	7221090–7524552		Exons 3–7	Heterozygous	ASD	Liu et al., 2015
Point mutation	7578663	c.1757 G > A p.W586*	Exon 8	Homozygous	Developmental delay, ID, microcephaly, seizures, leukodystrophy	Reuter et al., 2017
Point mutation	7578771	c.1865 G > A p.R622Q	Exon 8	Heterozygous	ASD	Sanders et al., 2012
Point mutation	7578878, 7578930	c.1972C > T p.R658W, c.2024C > A p.T675K	Exon 8	Compound Heterozygous	Developmental delay, ID, hypotonia, hypomyelination, brain atrophy, seizures	Charng et al., 2016
Duplication	7509664–7878406		Exons 8–10	Heterozygous	ID, microcephaly	DECIPHER 288108

Shahbazian et al., 2002; Bedogni et al., 2016), phenotypes of *Mecp2* knockout mice can be reversed if *Mecp2* expression is reintroduced in adult animals (Guy et al., 2007). Similarly, ablation of *Mecp2* expression in adult mice following normal development is sufficient to recapitulate the phenotype of constitutive *Mecp2* knockout mice (McGraw et al., 2011). MeCP2 is thus critical for proper neuronal function throughout life and there exists a therapeutic window to improve disease severity, even at adult stages. These proof-of-concept studies have fueled programs to develop *MECP2* replacement strategies, along with parallel efforts to identify targets downstream of MeCP2 dysfunction that may be amenable to pharmacological manipulation.

mGlu$_7$ is one of three mGlu receptors found to be decreased at the mRNA level in a RTT mouse model (Bedogni et al., 2016). These mGlu receptors represent a potential point of access to normalize synaptic function in RTT. Consistent with this initial report, we have shown that mGlu$_7$ protein expression is significantly decreased in motor cortex autopsy samples from RTT patients compared to those of controls matched for age, sex, and postmortem interval (Gogliotti et al., 2017). In global *Mecp2* knockout mice, mGlu$_7$ protein expression is decreased in a brain-region specific manner with a notable reduction in hippocampal synaptosomal fractions. This correlates with reduced depression of synaptic transmission at SC-CA1 synapses by LSP4-2022 in slices from RTT model mice, which can be restored by a PAM. Additionally, pre-application of two structurally distinct Group III mGlu receptor PAMs, VU0422288 and VU0155094, to slices was able to restore deficient LTP at SC-CA1 synapses in RTT model mice. Ablation of *Mecp2* selectively from GABAergic neurons is sufficient for LTP impairment (Chao et al., 2010); therefore, rescue of LTP by mGlu$_7$ potentiation is consistent with the proposed model by which mGlu$_7$-mediated inhibition of GABA release is required for LTP at SC-CA1 synapses (Klar et al., 2015).

At the behavioral level, mGlu$_7$ potentiation by intraperitoneal administration of the brain penetrant PAM, VU0422288, is able to improve performance in assays of cognition in RTT model mice (Gogliotti et al., 2017). While many studies in *Grm7*$^{-/-}$ mice have implicated a role for mGlu$_7$ in learning and memory (Hölscher et al., 2005; Callaerts-Vegh et al., 2006; Goddyn et al., 2008), this is the first report of mGlu$_7$ activity being modulated in a positive direction to reverse a deficit in cognition. VU0422288 is also able to increase performance in a social novelty task and reduce the number of apneas detected by whole body plethysmography (Gogliotti et al., 2017). These data suggest that mGlu$_7$ potentiation may be a valid approach to address multiple RTT-associated symptom domains. It is important to note that these experiments used mice with a global deletion of *Mecp2*. As RTT is most commonly caused by *MECP2* point mutations in humans, it will be important to elucidate the effect of various point mutations on mGlu$_7$ expression/function to identify patient subpopulations that would be predicted to benefit from an mGlu$_7$ PAM.

mGlu$_7$ has also been investigated for its therapeutic utility in a mouse of MDS. In contrast to RTT, MDS occurs when the region of the X chromosome containing *MECP2* is duplicated or triplicated, and is predicted to account for 1% of cases of unexplained X-linked intellectual disability (Lugtenberg et al., 2009). MDS patients present with infantile hypotonia, autism-associated symptoms, speech impairment, respiratory infections, and epilepsy (Ramocki et al., 2010). This disorder highlights the point that precise regulation of MeCP2 expression is required for normal brain function and that excess MeCP2 protein is detrimental. Fisher et al. (2017) tested whether mGlu$_7$ protein levels are affected in *MeCP2-Tg1* mice, a model for MDS. Contrary to a hypothesis of bidirectional regulation, mGlu$_7$ protein levels are unchanged in most brain regions in *MeCP2-Tg1* mice. Furthermore, neither genetic reduction of mGlu$_7$ protein levels or administration of the mGlu$_7$ NAM ADX71743 had any impact on anxiety and fear learning phenotypes in *MeCP2-Tg1* mice (Fisher et al., 2017). These findings suggest that that mGlu$_7$ expression/function may only be affected by MeCP2 hypofunction and not overexpression. More studies are warranted to understand the molecular interaction between MeCP2 and mGlu$_7$ expression. This information will inform future drug development of mGlu$_7$ PAMs for RTT and other NDDs in which *MECP2* mutations have been identified.

CONCLUSION

Neurodevelopmental disorders are a prevalent group of disorders with limited treatment options and mGlu$_7$ represents one potential access point for pharmacological intervention. *GRM7* gene disruptions identified in patients with NDDs provide clinical rationale for this approach. Pre-clinical studies in rodent models suggest that decreased mGlu$_7$ function is sufficient to mimic phenotypes that correlate to NDD symptom domains and that positive modulation of mGlu$_7$ activity can improve some deficits, specifically in a mouse model of RTT. However, NDDs are highly heterogeneous and are likely the result of unique molecular pathologies that converge to produce similar circuit and behavioral phenotypes. Therefore, further studies are needed to identify and understand which subpopulations may benefit from an mGlu$_7$-mediated therapy. In parallel, further development of improved tool compounds will facilitate studies focused on understanding mGlu$_7$ receptor function in brain circuits and behaviors associated with NDDs.

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CORRECTION
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Corrigendum: Metabotropic Glutamate Receptor 7: A New Therapeutic Target in Neurodevelopmental Disorders

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A Corrigendum on

Metabotropic Glutamate Receptor 7: A New Therapeutic Target in Neurodevelopmental Disorders

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In the original article, there was a mistake in **Figure 1** as published. The chirality of L-AP4 and LSP1-2111 was incorrect. pEC_{50} values have also been corrected for LSP1-2111 in **Table 1**. The authors apologize for these errors and state that they do not change the scientific conclusions of the article in any way. The original article has been updated.

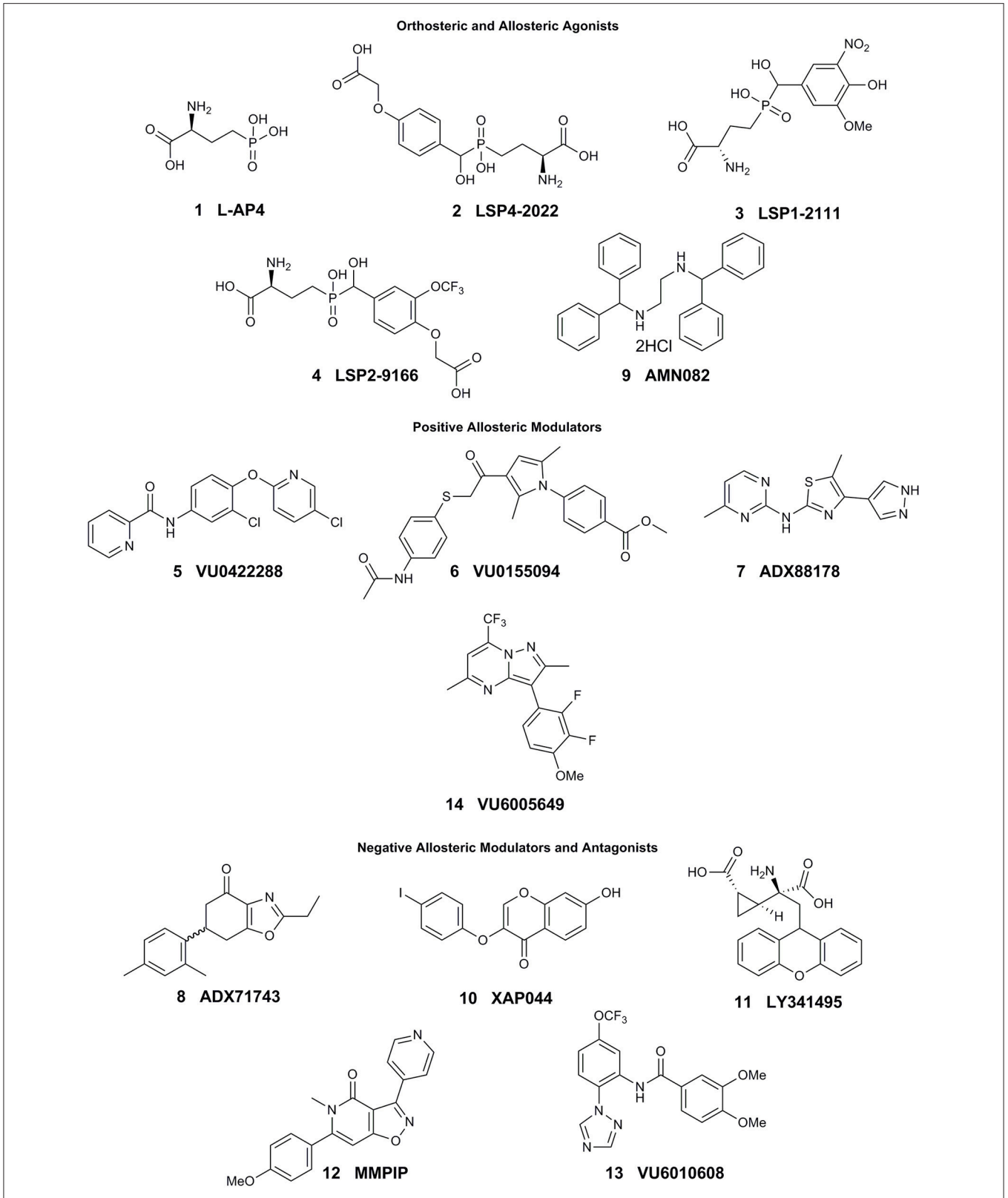

FIGURE 1 | Current tool compounds used to study $mGlu_7$.

TABLE 1 | Summary of current tool compounds used to study $mGlu_7$.

Name (#)	Type	$mGlu_7$ pEC_{50}/pIC_{50}	$mGlu_8$ pEC_{50}/pIC_{50}	$mGlu_4$ pEC_{50}/pIC_{50}	$mGlu_6$ pEC_{50}/pIC_{50}	Source
L-AP4 **(1)**	Orthosteric agonist	3.47 (PIH)	6.53 (PIH)	7.00 (PIH)	5.62 (PIH)	Acher et al., 2012; Selvam et al., 2018
		3.61 (Ca^{2+})	6.53 (Ca^{2+})	6.89 (Ca^{2+})	6.00 (Ca^{2+})	
LSP4-2022 **(2)**	Orthosteric agonist	4.34 (Ca^{2+})	4.54 (Ca^{2+})	6.96 (Ca^{2+})	5.36 (Ca^{2+})	Acher et al., 2012; Goudet et al., 2012; Selvam et al., 2018
LSP1-2111 **(3)**	Orthosteric agonist	4.28 (PIH)	4.18 (PIH)	5.66 (PIH)	5.77 (PIH)	Selvam et al., 2018
		4.00 (Ca^{2+})	4.71 (Ca^{2+})	6.05 (Ca^{2+})	5.49 (Ca^{2+})	
LSP2-9166 **(4)**	Orthosteric agonist	5.71 (Ca^{2+})	4.25 (Ca^{2+})	7.22 (Ca^{2+})	not reported	Acher et al., 2012
VU0422288 **(5)**	Group III PAM	6.85 (Ca^{2+})	6.93 (Ca^{2+})	6.98 (Ca^{2+})	not reported	Jalan-Sakrikar et al., 2014
VU0155094 **(6)**	Group III PAM	5.80 (Ca^{2+})	6.07 (Ca^{2+})	5.48 (Ca^{2+})	not reported	Jalan-Sakrikar et al., 2014
ADX88178 **(7)**	$mGlu_{4/8}$ PAM	>4.52 (Ca^{2+})	5.66 (Ca^{2+})	8.46 (Ca^{2+})	>5	Le Poul et al., 2012
ADX71743 **(8)**	$mGlu_7$ NAM	7.20 (human, Ca^{2+})	inactive	inactive	inactive	Kalinichev et al., 2014
		7.06 (rat, Ca^{2+})	inactive	inactive	inactive	
AMN082 **(9)**	Allosteric agonist	6.59 (GTPγS)	>5 (GTPγS)	>5 (GTPγS)	>5 (GTPγS)	Mitsukawa et al., 2005
XAP044 **(10)**	Antagonist	5.26 (cAMP)	4.48 (cAMP)	inactive	inactive	Gee et al., 2014
		5.55 to 5.46 (GTPγS)				
LY341495 **(11)**	Orthosteric antagonist	6.00 (cAMP)	6.76 (cAMP)	4.66 (cAMP)	not reported	Kingston et al., 1998
MMPIP **(12)**	$mGlu_7$ NAM	6.66 (cAMP)	>5 (cAMP)	>5 (cAMP)	not reported	Suzuki et al., 2007
		7.15 (Ca^{2+})				Niswender et al., 2010
		6.14 (Thallium)				Niswender et al., 2010
VU6010608 **(13)**	$mGlu_7$ NAM	6.12 (Ca^{2+})	>5 (Ca^{2+})	>5 (Ca^{2+})	inactive (>5)	Reed et al., 2017
VU6005649 **(14)**	$mGlu_{7/8}$ PAM	6.19 (Ca^{2+})	5.59 (Ca^{2+})	>5 (Ca^{2+})	inactive	Abe et al., 2017

NAM, negative allosteric modulator; PAM, positive allosteric modulator; EC_{50}, effective concentration 50; IC_{50}, inhibitory concentration 50. Assay type is indicated in parentheses: PIH, Phosphatidylinositol hydrolysis; cAMP, cAMP accumulation; Ca^{2+}, Calcium mobilization; GTPγS, GTPγS binding.

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REVIEW
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Emerging Trends in Pain Modulation by Metabotropic Glutamate Receptors

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Pain is an essential protective mechanism meant to prevent tissue damages in organisms. On the other hand, chronic or persistent pain caused, for example, by inflammation or nerve injury is long lasting and responsible for long-term disability in patients. Therefore, chronic pain and its management represents a major public health problem. Hence, it is critical to better understand chronic pain molecular mechanisms to develop innovative and efficient drugs. Over the past decades, accumulating evidence has demonstrated a pivotal role of glutamate in pain sensation and transmission, supporting glutamate receptors as promising potential targets for pain relieving drug development. Glutamate is the most abundant excitatory neurotransmitter in the brain. Once released into the synapse, glutamate acts through ionotropic glutamate receptors (iGluRs), which are ligand-gated ion channels triggering fast excitatory neurotransmission, and metabotropic glutamate receptors (mGluRs), which are G protein-coupled receptors modulating synaptic transmission. Eight mGluRs subtypes have been identified and are divided into three classes based on their sequence similarities and their pharmacological and biochemical properties. Of note, all mGluR subtypes (except mGlu6 receptor) are expressed within the nociceptive pathways where they modulate pain transmission. This review will address the role of mGluRs in acute and persistent pain processing and emerging pharmacotherapies for pain management.

Keywords: pain, GPCR (G-protein-coupled receptors), receptor, glutamate (Glu), neurotransmitter, chronic pain, pharmacology, neuromodulation

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INTRODUCTION

Acute pain is an important protective function, detecting harmful stimuli and preventing body damage. However, chronic pain persists for a long time after the initial affliction, losing its role as a warning signal and must be considered as a disease *per se*. Patients suffering from chronic pain not only experience exacerbated responses to both painful (hyperalgesia) and non-painful stimuli (allodynia) (Sandkühler, 2009) but also frequently express emotional and cognitive impairments often resulting in anxiety and depression (McWilliams et al., 2003; Moriarty et al., 2011; Bushnell et al., 2013).

Glutamate is the main excitatory neurotransmitter in the nervous system of adult mammals. Among the neurotransmitters involved in pain transmission from the periphery to the brain, glutamate has a leading role. Glutamate is also involved in central sensitization, which is

associated with chronic pain. Glutamate action is mediated through ionotropic and metabotropic receptors. Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels involved in the fast synaptic response to glutamate. Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors that are responsible for the slow neuromodulatory response to glutamate. Eight mGluRs have been identified so far. They are named mGlu1 to mGlu8 receptors by chronological order of discovery. Later, based on their sequence homology, signalization and pharmacology, they were subdivided in three groups. Group I mGluRs (mGlu1 and 5) are canonically coupled to Gαq/11 and lead to phospholipase C (PLC) activation that promotes neuronal excitability and are mostly expressed postsynaptically. In contrast, group II (mGlu2 and 3) and group III (mGlu4, 6, 7, and 8) mGluRs are predominantly coupled to Gαi/o triggering adenylate cyclase (AC) inhibition. Group II and III mGluRs also regulate neuronal excitability and synaptic transmission through Gβγ subunits, which notably inhibit voltage-sensitive calcium channels and activate potassium channels. Both group II and group III mGluRs are mainly localized on presynaptic terminals. Both iGluRs and mGluRs (except mGlu6 receptor) are expressed all along the pain neuraxis where they shape the transmission of pain information (**Figure 1**). They are also involved in the induction and the maintenance of central sensitization of the pain pathway (Latremoliere and Woolf, 2009). This phenomenon is associated with hyperexcitability of the glutamatergic system which leads to the development of the main sensory symptoms observed in persons suffering from chronic pain.

Acting on the molecular mechanisms of glutamatergic transmission may, therefore, be a way of developing future analgesics counteracting chronic pain. However, even if iGluR selective antagonists have proven efficacious in releasing several pain states, drastically inhibiting glutamatergic transmission via iGluR blocking inevitably induces numerous side effects, notably hallucinations, ataxia and sedation (Bleakman et al., 2006). Therefore, the strategy of pharmacological modulation of mGluRs for the treatment of pain has been favored and significant effort has been devoted to better understanding the expression, the function and the role of these receptors in pain processing. The present review will focus on the role of mGluRs in acute and chronic pain at different levels–from the periphery to higher brain center involved in the perception and modulation of pain–and report the recent advances in the pharmacological strategy used to achieve mGluRs modulation.

PHARMACOLOGY OF MGLURS

Both orthosteric and allosteric ligands are available for pharmacological manipulation of mGluRs. Given their different binding sites, orthosteric ligands and allosteric modulators have specific pharmacological properties.

Orthosteric ligands are binding in the same pocket than the natural ligand (the orthosteric pocket). They are also referred to as competitive ligands. In mGluRs, the glutamate-binding pocket is located in the extracellular domain of the receptor. Due to the high degree of conservation of the glutamate-binding pocket among the mGluRs, the identification of subtype selective ligands is highly challenging. Therefore, many orthosteric ligands are selective for a specific group but do not discriminate between receptors within the group. The typical specific group I, II or III mGluRs agonists are S-3, 5-DHPG, LY379268 and L-AP4, respectively, and have been used in many preclinical studies. Recently, selective orthosteric ligands have been generated, LY2794193 for mGlu3 receptor (Monn et al., 2015, 2018) and LSP4-2022 for mGlu4 receptor (Goudet et al., 2012). They bind to residues of the orthosteric site and to specific residues and pockets surrounding the glutamate-binding pocket. LSP4-2022 has notably been used in several pain studies.

Allosteric modulators regulate the activity of a receptor by binding at a site distinct from the orthosteric site of endogenous ligands. In mGluRs, the binding site for most synthetic allosteric modulators which has been identified so far is located in the seven transmembrane domain. Interestingly, this pocket is less well conserved between the different receptors of the family, allowing the discovery of subtype selective ligands. Allosteric modulators may inhibit (negatively modulate) or potentiate (positively modulate) the activity of a co-binding orthosteric ligand at a target receptor and so can act as negative or positive allosteric modulators, respectively. Moreover, neutral allosteric ligands capable of inhibiting the action of either positive or negative allosteric modulators but devoid of activity by themselves have also been described (also referred to as silent allosteric modulators, SAM). Negative allosteric modulators (NAM) act as non-competitive antagonists and can have inverse agonist properties, meaning that they can inhibit the constitutive activity of the receptor. Interestingly, due to their non-competitive mode of action, the action of NAMs is less dependent on the concentration of endogenous ligands. Positive allosteric modulators (PAM) can enhance either the potency or the efficacy, or both, of orthosteric agonists. Therefore, in contrast to agonists that maintain the receptor active, pure PAMs potentiate the cellular response resulting from the action of the endogenous ligand. Some PAMs can also directly activate the receptor, referred to as agoPAMs, although such activity is usually partial.

The first described allosteric modulators of mGluRs were CPCCOEt, BAY36-7620 and MPEP, which display inverse agonist activity on mGlu1 and mGlu5 receptors (Litschig et al., 1999; Pagano et al., 2000; Carroll et al., 2001). Shortly after, a series of PAMs of mGlu1 receptors were described (Knoflach et al., 2001). To date, PAMs and NAMs have been described for most mGluRs [see (Lindsley et al., 2016) for a review] and have proven to be useful in exploring the function of mGluRs in pain.

Photopharmacology is a recent advance in the field of mGluRs. It is based on freely diffusible, light-operated ligands to control the function of the ligand on its target by light. Contrary to optogenetics, neither genetic modification of the targeted receptor nor exogenous expression are required, enabling the photocontrol of endogenous receptors. Two types of drugs have been developed for photopharmacology: photoactivable and photoswitchable ligands (Goudet et al., 2018). It allows the pharmacological manipulation of mGluRs with high spatial and temporal precision and holds great promise for exploring their physiological and pathological functions, notably in pain (Font et al., 2017; Gómez-Santacana et al., 2017; Zussy et al., 2018).

TABLE 1 | Pain modulation following systemic administration of group I mGluRs ligands.

Receptor subtype	Drugs type	Name	Models Species		Effects Tests	References
Group I						
• mGlu1	NAM	FTIDC	Naïve Mice	∅	- No effect in thermal threshold - Tail immersion test	Satow et al., 2008
		EMQMCM	Naïve Rats	∅	- No effect in thermal threshold - Radiant heat source	Sevostianova and Danysz, 2006
		A-841720	CFA Rats	↘	- Dose dependent increase of withdrawal latencies - Radiant heat source	El-Kouhen et al., 2006
		LY456236	Formalin Mice	↘	- Dose dependent decrease of pain-related behavior - Licking and flinching	Varty et al., 2005
		EMQMCM	Formalin Rats	↘	- Reduced manifestation of both phases - No development of tolerance - Licking behavior	Sevostianova and Danysz, 2006
		FTIDC	Formalin Mice	↘	- Inhibit formalin-induced nociceptive behavior - Licking behavior	Satow et al., 2008
		A-841720	Skin incision Rats	↘	- Attenuation of spontaneous post-operative pain behavior - Significant motor side effects - Weight-bearing/Open field/Rotarod	Zhu et al., 2008
		A-794282	Skin incision Rats	↘	- Attenuation of spontaneous post-operative pain behavior - Significant motor side effects - Weight-bearing/Open field/Rotarod	Zhu et al., 2008
		A-841720	CCI Rats	↘	- Decrease mechanical allodynia - Motor and cognitive side effects at analgesic doses - Von frey	El-Kouhen et al., 2006
		LY456236	SNL Rats	↘	- Dose dependent increase of withdrawal threshold - Von Frey	Varty et al., 2005
		A-841720	SNL Rats	↘	- Decrease mechanical allodynia - Motor and cognitive side effects at analgesic doses - Von frey	El-Kouhen et al., 2006
• mGlu5	NAM	MPEP	Naïve Rats	∅	- No effect in thermal threshold - Radiant heat source	Sevostianova and Danysz, 2006
		MTEP	Naïve Rats	∅	- No effect in thermal threshold - Radiant heat source	Sevostianova and Danysz, 2006
		MPEP	AIW Mice	↘	- Dose-dependent reduction of writhing activity - Number of cramps	Zhu et al., 2004
		MPEP	Carrageenan Rats	↘	- Reversal of inflammatory hyperalgesia - Absence of locomotor side effects - Paw pressure/Rotarod assay	Walker et al., 2001a,b
		MPEP	Carrageenan Rats	↘	- Decrease thermal hyperalgesia without affecting paw oedema - Radiant heat source	Zhu et al., 2004
		MPEP	CFA Rats	↘	- Reversal of mechanical hyperalgesia - Paw pressure	Walker et al., 2001a,b
		MPEP	CFA Rats	↘	- Dose-dependent reversal of thermal and mechanical hyperalgesia - Paw pressure test/Radiant heat source	Zhu et al., 2004
		Fenobam	CFA Mice	↘	- Reduce thermal hypersensitivity - Increase in spontaneous locomotor activity, no effect in motor coordination - Radiant heat source/Open field/Rotarod	Montana et al., 2009
		MPEP	Formalin Rats	↘	- Reduce phase I and II - Paw flinches	Zhu et al., 2004
		MPEP	Formalin Mice	↘	- Dose dependent decrease of pain-related behavior - Licking and flinching	Varty et al., 2005

(Continued)

TABLE 1 | Continued

Receptor subtype	Drugs type	Name	Models Species	Effects Tests	References
		MTEP	Formalin Mice ↘	- Dose dependent decrease of pain-related behavior - Licking and flinching	Varty et al., 2005
		MPEP	Formalin Rats ↘	- Reduce the manifestation of both phases - Licking behavior	Sevostianova and Danysz, 2006
		MPEP	Formalin Rats ↘	- Reduce the manifestation of both phases - Development of tolerance - Licking behavior	Sevostianova and Danysz, 2006
		MPEP	Formalin Mice ↘	- Inhibit formalin-induced nociceptive behavior - Licking behavior	Satow et al., 2008
		Fenobam	Formalin Rats ↘	- Prevent formalin-induced spontaneous pain-related behavior - Licking, lifting, or flicking	Jacob et al., 2009
		Fenobam	Formalin Mice ↘	- Prevent formalin-induced spontaneous pain-related behavior - Licking, lifting, or flicking	Montana et al., 2009
		Fenobam	Formalin Mice ↘	- Both acute and chronic treatment reduce phase I and II - No tolerance, increase in exploratory behavior, no impact in motor coordination - Licking behavior, Open field, Elevated O maze	Montana et al., 2011
		MPEP	Skin incision Rats ↘	- Reduce post-operative pain - Von Frey/Radiant heat source	Zhu et al., 2004
		MPEP	CCI Rats ↘	- Dose-dependent reversal of mechanical allodynia - Von Frey	Zhu et al., 2004
		Fenobam	CCI Rats ↘	- No effect in mechanical allodynia - Electronic von Frey	Jacob et al., 2009
		MPEP	PSNS Rats ↘	- No effect - Von Frey/Paw pressure test/Radiant heat source	Hudson et al., 2002
		MPEP	SNL Rats ↘	- No effect - Paw pressure	Walker et al., 2001a,b
		MPEP	SNL Rats ↘	- Reverse thermal hyperalgesia - Fail to alter tactile allodynia or mechanical hyperalgesia - Von Frey/Paw pressure test/Radiant heat source	Hudson et al., 2002
		MPEP	SNL Rats ↘	- Dose-dependent reversal of mechanical allodynia - Von Frey	Zhu et al., 2004
		MPEP	SNL Rats ↘	- Anxiolytic effect in naïve animals, reduce locomotor activity and coordination - Vogel conflict test	Varty et al., 2005
		MTEP	SNL Rats ↘	- Anxiolytic effect in naïve animals, reduce locomotor activity and coordination - Vogel conflict test	Varty et al., 2005
		MPEP	CIPN Rats ↘	- Dose-dependent reversal of mechanical allodynia - Von Frey	Zhu et al., 2004

Symbols are used for model of pain induced by local injection, for inflammatory pain, for post-operative pain, for neuropathic pain and for chemotherapy-induced neuropathic pain models. ↘, Decrease pain; ↗, Increase pain; AIW, Acid-induced writhing; CCI, Chronic constriction injury; CFA, Complete Freund's Adjuvant; CIPN, Chemotherapy-induced peripheral neuropathy; PSNS, Partial sciatic nerve section; SNL, Spinal nerve ligation.

TABLE 2 | Pain modulation following systemic administration of group II mGluRs ligands.

Receptor subtype	Drugs type	Name	Models Species		Effects Tests	References
Group II						
• mGlu2/3-selective	**Agonist**	**LY379268**	Naïve Rats	⌀	- No effects acute thermal nociceptive function - Tail flick test on Radiant heat source	Simmons et al., 2002
		LY379268	Naïve Rats	⌀	- No effects on withdrawal latencies to either mechanical or thermal stimulation - Paw pressure/Radiant heat source	Sharpe et al., 2002
		LY2969822	CAP Rats	↘	- Prevent tactile hypersensitivity - Oral prodrug of LY2934747 - Von Frey	Johnson et al., 2017
		LY379268	Carrageenan Rats	↘	- Reduce inflammation induced hyperalgesia - Paw pressure/Radiant heat source	Sharpe et al., 2002
		LY2969822	CFA Rats	↘	- Reduce pain related behavior - Oral prodrug of LY2934747 - Paw pressure	Johnson et al., 2017
		LY354740	Formalin Rats	↘	- Reduce pain related behavior - Licking behavior	Simmons et al., 2002
		LY379268	Formalin Rats	↘	- Reduce pain related behavior - Reverse mGlu2/3 antagonist LY341495 - Licking behavior	Simmons et al., 2002
		LY389795	Formalin Rats	↘	- Reduce pain related behavior - Licking behavior	Simmons et al., 2002
		LY379268	Formalin Mice	⌀	- No effect - Licking behavior	Satow et al., 2008
		LY2934747	Formalin Rats	↘	- Reduce pain related behavior - Blocked by LY341495 - Licking behavior	Johnson et al., 2017
		LY379268	SNL Rats	↘	- Reverse mechanical allodynia - Von frey	Simmons et al., 2002
		LY2934747	SNL Rats	↘	- Prevent tactile hypersensitivity - Von Frey	Johnson et al., 2017

Symbols are used for model of pain induced by local injection, for inflammatory pain, for post-operative pain, for neuropathic pain and for chemotherapy-induced neuropathic pain models. ↘ Decrease pain; ↗, Increase pain; CAP, Capsaicin; CFA, Complete Freund's Adjuvant; SNL, Spinal nerve ligation.

TABLE 3 | Pain modulation following systemic administration of group III mGluRs ligands.

Receptor subtype	Drugs type	Name	Models Species	Effects Tests	References
Group III					
mGlu4	**Agonist**	**LSP4-2022**	Carrageenan Rats ↘	- Reduce mechanical hypersensitivity - Paw pressure	Vilar et al., 2013
mGlu7	**PAM**	**AMN082***	Carrageenan Rats ↘	- Prevent thermal hyperalgesia (before carrageenan) and inhibit thermal hyperalgesia and mechanical allodynia - Radiant heat source/Dynamic plantar aesthesiometer	Dolan et al., 2009
		AMN082*	Skin incision Rats ↘	- Pre surgical and postsurgical administration inhibits thermal hyperalgesia, but not mechanical allodynia - Radiant heat source/Dynamic plantar aesthesiometer	Dolan et al., 2009
	NAM	**MMPIP**	SNI Mice ↗	- Increase thermal and mechanical thresholds - Decrease anxiety-related behavior and improve cognitive performance - Radiant heat source/Dynamic plantar aesthesiometer/EPM/Tail suspension/Marble burying test.	Palazzo et al., 2015
		XAP044	SNI Mice ↗	- Increase thermal and mechanical thresholds - Decrease anxiety-related behavior - Radiant heat source/Dynamic plantar aesthesiometer/EPM/Tail suspension/Marble burying test.	Palazzo et al., 2015
mGlu8	**Agonist**	**DCPG**	Carrageenan Mice ↘	- Reduce carrageenan-induced thermal hyperalgesia and mechanical allodynia - Blocked by intra-PAG MSOP - Radiant heat source/Dynamic plantar aesthesiometer	Marabese et al., 2007
		DCPG	Formalin Mice ↘	- Decrease both early and delayed nociceptive responses - Blocked by intra-PAG MSOP - Licking, lifting, or flicking	Marabese et al., 2007
		DCPG	CCI Mice ↘	- Effective 3 days after surgery but ineffective in alleviating thermal hyperalgesia and mechanical allodynia 7 days after - Radiant heat source/Dynamic plantar aesthesiometer	Marabese et al., 2007

**Of note, in vivo actions of AMN082 should be interpreted with caution because they may involve other mechanisms in addition to mGlu7. Indeed, an AMN082 metabolite can inhibit monoamine transporters Sukoff Rizzo et al., 2011.*

symbols are used for model of pain induced by local injection, for inflammatory pain, for post-operative pain, for neuropathic pain and for chemotherapy-induced neuropathic pain models. ↘, Decrease pain; ↗, Increase pain; CCI, Chronic constriction injury; SNI, Spared nerve injury.

TABLE 4 | Pain modulation following local administration of group I mGluRs ligands.

Receptor subtype	Localization	Drugs type	Name	Models Species	Effects Tests		References
Group I							
• mGlu1/5 selective	Periphery	Agonist	RS-DHPG	Naïve Mice	↗	- Reduction of thermal withdrawal latency - Radiant heat source	Bhave et al., 2001
			RS-DHPG	Naïve Rats	↗	- Produce mechanical hyperalgesia - Paw pressure test	Walker et al., 2001a,b
			RS-DHPG	Naïve Rats	↗	- Decrease the mechanical threshold to noxious stimulation of the masseter muscle - Prevented by MPEP but not CPCCOEt - Von Frey	Lee and Ro, 2007
			S-DHPG	Naïve Rats	↗	- Reduction of thermal withdrawal latency - Radiant heat source	Jin et al., 2009
			RS-DHPG	Naïve Rats	↗	- Induce mechanical hyperalgesia in the masseter muscle - Attenuated by AMG9810, a specific TRPV1 antagonist - Von Frey	Chung et al., 2015
		NAM	CPCCOEt	CAP inj Rats	↘	- Dose dependent increase of withdrawal latencies - Radiant heat source	Jin et al., 2009
	Spinal cord	Agonist	RS-DHPG	Naïve Rats	↗	- Long lasting spontaneous nociceptive behaviors - Elevating, shaking, stamping of the hindpaw/elevating or whipping of the tail/liking or biting the tail	Fisher and Coderre, 1996
			RS-DHPG	Naïve Rats	↗	- Mechanical allodynia and hyperalgesia, thermal hyperalgesia - Von Frey/Tail clip/Hot plate	Fisher and Coderre, 1998
			S-DHPG	Naïve Sheep	↗	- Reduction of mechanical thresholds - Blocked by co-administration of the group I antagonist AIDA - Blunt pin	Dolan and Nolan, 2000
			RS-DHPG	Naïve Mice	↗	- Increase spontaneous nociceptive behavior - Licking of the flanks, tail, and hindpaws	Karim et al., 2001
			RS-DHPG	Naïve Rats	↗	- Increase spontaneous nociceptive behavior - Blocked by MPEP - Licking of tail and hindpaws	Lorrain et al., 2002
			RS-DHPG	Naïve Mice	↗	- Increase spontaneous nociceptive behavior - Blocked by MEK inhibitor U0126 - Licking, scratching and lifting behaviors	Adwanikar et al., 2004
			RS-DHPG	Naïve Rats	↗	- Spontaneous nociceptive behaviors induction - Licking of the flanks, tail, and hindpaw	Hu et al., 2007
			RS-DHPG	CCI Rats	↗	- Increase the hind paw frequency and duration of lifting - Blocked by MPEP - Cold plate	Hama, 2003
		Antagonist	LY393053	CFA Rats	↘	- Reduction of glutamate-induced spontaneous pain behaviors - Time spent licking	Vincent et al., 2017
			S-4CPG	CCI Rats	↘	- Attenuation of mechanical allodynia and cold hyperalgesia - Von Frey/1 cm deep 1°C water bath	Fisher et al., 1998
			LY393053	SNI Rats	↘	- Weak attenuation of glutamate-induced spontaneous pain behavior - Time spent licking the hind paws, lower legs or tail	Vincent et al., 2016
	Amygdala	Agonist	DHPG	Naive Mice	↗	- Side dependent increase of mechanical hypersensitivity - Von Frey	Kolber et al., 2010
			DHPG	Naïve Rats	↗	- Increased the duration of vocalizations - Decreased the hindlimb withdrawal threshold - Knee compression/colorectal distension	Li et al., 2011
	PAG	Agonist	S-DHPG	Naïve Mice	↘	- Increase the latency of the nociceptive reaction - Hot plate	Maione et al., 1998
			S-DHPG	Formalin Mice	↘	- Decrease phase II - Licking behavior	Maione et al., 2000
		Antagonist	RS-AIDA	Naïve Mice	↗	- Decrease the latency of the nociceptive reaction - Hot plate	Maione et al., 1998

Symbols are used for model of pain induced by local injection, for inflammatory pain, for post-operative pain, for neuropathic pain and for chemotherapy-induced neuropathic pain models. ↘ Decrease pain; ↗ Increase pain; CAP, Capsaicin; CCI, Chronic constriction injury; CFA, Complete Freund's Adjuvant; SNI, Spared nerve injury.

TABLE 5 | Pain modulation following local administration of selective mGlu1 or mGlu5 ligands.

Receptor subtype	Localization	Drugs type	Name	Models Species	Effects Tests		References
Group I							
• mGlu1	Periphery	Antagonist	LY367385	IL-1ß inj Rats	↘	- Decrease IL-1b-induced mechanical allodynia in orofacial area - Air puff	Ahn et al., 2005
		NAM	CPCCOEt	CAP inj Rats	↘	- Dose dependent increase of withdrawal latencies - Radiant heat source	Jin et al., 2009
			CPCCOEt	IL-1ß inj Rats	↘	- Decrease IL-1b-induced mechanical allodynia in orofacial area - Air puff	Ahn et al., 2005
	Spinal cord	Antagonist	RS-AIDA	CAP inj Rats	↘	- Reduction of mechanical hypersensitivity, no effect in thermal hyperalgesia - Von Frey/Paw immersion	Soliman et al., 2005
			RS-AIDA	CCI Rats	↘	- Pretreatment produced reductions in the development of mechanical and cold hypersensitivity - Von Frey/1 cm deep 1°C water bath	Fisher et al., 2002
		NAM	CPCCOEt	Formalin Mice	↘	- Decrease phase II - Licking behavior	Karim et al., 2001
	Amygdala	Antagonist	LY367385	Naïve Rats	⊘	- No effect - Knee compression/colorectal distension	Li et al., 2011
		NAM	CPCCOEt	Carrageenan Rats	↘	- Reduce mechanical hyperalgesia - Dynamic Plantar Aesthesiometer	Luongo et al., 2013
			CPCCOEt	MA Rats	↘	- Reduction of vocalizations induced by mechanical stimulation - Knee compression	Han and Neugebauer, 2005
	Striatum	NAM	CPCCOEt	MA Rats	⊘	- No effect - Knee compression	Han and Neugebauer, 2005
• mGlu5	Periphery	Agonist	CHPG	Naïve Rats	↗	- Produced mechanical hyperalgesia - Paw pressure test	Walker et al., 2001a,b
		NAM	MPEP	Naïve Rats	↘	- Inhibit the visceromotor responses - Colorectal distension	Lindström et al., 2008
			MPEP	CAP inj Rats	↘	- Dose dependent increase of withdrawal latencies - Radiant heat source	Jin et al., 2009
			MPEP	CFA Rats	↘	- Reduction of mechanical hyperalgesia - Paw pressure test	Walker et al., 2001a,b
			MPEP	IL-1ß inj Rats	↘	- Decrease IL-1b-induced mechanical allodynia in orofacial area - Air puff	Ahn et al., 2005
			SIB1893	IL-1ß inj Rats	↘	- Decrease IL-1b-induced mechanical allodynia in orofacial area - Air puff	Ahn et al., 2005
			JF-NP-26 Photoactivabl	Formalin Mice	↘	- Decrease both at phase I and phase II - Licking behavior	Font et al., 2017
			MPEP	Skin incision Rats	↘	- Dose-dependent reduction of non-evoked pain - Weight-bearing	Zhu et al., 2005
			SIB-1757	SNL Rats	↘	- No effect in acute pain - Reversal of thermal hyperalgesia - Von frey filaments/Radiant heat source	Dogrul et al., 2000
	Spinal cord	Agonist	trans-ADA	Naïve Rats	⊘	- No effect in spontaneous nociceptive behaviors - Elevating, shaking, stamping of the hindpaw/elevating or whipping of the tail/liking or biting the tail	Fisher and Coderre, 1996
		NAM	MPEP	CAP inj Rats	↘	- Reduction of mechanical hypersensitivity, no effect in thermal hyperalgesia - Von Frey/Paw immersion	Soliman et al., 2005
			Fenobam	CFA Rats	↘	- Reduction of glutamate-induced spontaneous pain behaviors and rewarding under pain conditions - Time spent licking/CPP	Vincent et al., 2017

(Continued)

TABLE 5 | Continued

Receptor subtype	Localization	Drugs type	Name	Models Species	Effects Tests		References
			MPEP	Formalin Mice	↘	- Decrease phase II - Licking behavior	Karim et al., 2001
			MPEP	Skin incision Rats	↘	- Dose-dependent reduction of non-evoked pain - Weight-bearing	Zhu et al., 2005
			MPEP	CCI Rats	↘	- Pretreatment produced reductions in the development of mechanical hypersensitivity (but not cold hypersensitivity) - Von Frey/1 cm deep 1°C water bath	Fisher et al., 2002
			MPEP	CCI Rats	⌀	- No effect in cold threshold - Cold plate	Hama, 2003
			Fenobam	SNI Rats	↘	- Reduction of glutamate-induced spontaneous pain behaviors and mechanical allodynia - Time spent licking the hind paws, lower legs or tail/Von frey	Vincent et al., 2016
			SIB-1757	SNL Rats	↘	- No effect in acute pain - Reversal of thermal hyperalgesia and partial reversal of tactile allodynia - Frey filaments/Radiant heat source	Dogrul et al., 2000
			SIB-1757	SNL Rats	↘	- No effect in acute pain - Reversal of thermal hyperalgesia and partial reversal of tactile allodynia - Frey filaments/Radiant heat source	Dogrul et al., 2000
			MPEP	CIPN Rats	↘	- Reversed pain hypersensitivity - Von Frey/Paw pressure test	Xie et al., 2017
	Amygdala	**NAM**	**MPEP**	Naïve Rats	⌀	- No effect - Knee compression/colorectal distension	Li et al., 2011
			MPEP	Carrageenan Rats	⌀	- No effect on mechanical hyperalgesia - Dynamic Plantar Aesthesiometer	Luongo et al., 2013
			MPEP	Formalin Mice	↘	- Side dependent mechanical hypersensitivity reduction - Von Frey	Kolber et al., 2010
			MPEP	MA Rats	↘	- Reduction of vocalizations induced by mechanical stimulation - Knee compression	Han and Neugebauer, 2005
			Alloswitch-1 Photoswitchable	CFA Mice	↘	- Restore mechanical sensitivity - Von Frey	Gómez-Santacana et al., 2017
	Thalamus	**NAM**	**JF-NP-26** Photoactivable	Formalin Mice	↘	- Decrease both at phase I and phase II - Licking behavior	Font et al., 2017
			JF-NP-26 Photoactivable	CCI Mice	↘	- Significantly increased pain thresholds - Von frey filaments	Font et al., 2017
	Striatum	**NAM**	**MPEP**	MA Rats	⌀	- No effect - Knee compression	Han and Neugebauer, 2005
	Prefrontal cortex	**NAM**	**MPEP**	SNL Rats	↘	- Decrease tactile hypersensitivity and depressive-like behavior - Von Frey/Forced swimming test/Open field/Conditioned place preference	Chung et al., 2017

Symbols are used for model of pain induced by local injection, for inflammatory pain, for post-operative pain, for neuropathic pain and for chemotherapy-induced neuropathic pain models. ↘ Decrease pain; ↗ Increase pain; CAP, Capsaicin; CCI, Chronic constriction injury; CFA, Complete Freund's Adjuvant; CIPN, Chemotherapy-induced peripheral neuropathy; MA, Mono arthritis; SNL, Spinal nerve ligation; SNI, Spared nerve injury.

TABLE 6 | Pain modulation following local administration of group II mGluRs ligands.

Receptor subtype	Localization	Drugs type	Name	Models Species	Effects Tests		References
Group II							
•mGlu2/3-selective	Periphery	Agonist	LY314582	Naïve Rats	↗	- Slight decrease of mechanical threshold - Paw pressure test	Walker et al., 2001a,b
			APDC	Naïve Rats	⌀	- No effect thermal withdrawal latency - Paw pressure test/Radiant heat source	Du et al., 2008
			L-CCG-1	Naïve Rats	⌀	- No effect thermal withdrawal latency - Radiant heat source	Jin et al., 2009
			APDC	CAP inj Rats	↘	- Attenuate capsaicin-induced nociceptive behaviors - Flinching and lifting/licking	Carlton et al., 2009
			APDC	Carrageenan Mice	↘	- Restore mechanical thresholds - Von Frey	Yang and Gereau, 2003
			APDC	Carrageenan Rats	↘	- Recovery of reduced weight load - Reduction of mechanical hyperalgesia - Von frey filaments/weight-bearing	Lee et al., 2013
			APDC	Formalin Rats	↘	- Reduce flinching and L/L - Blocked by LY341495 - Flinching and lifting/licking	Du et al., 2008
			APDC	IL-1ß inj Rats	↘	- Reduce IL-1b-induced mechanical allodynia - Inhibited by pretreatment with LY341495 - Air puff	Ahn et al., 2005
			DCG-IV	IL-1ß inj Rats	↘	- Reduce IL-1b-induced mechanical allodynia - Inhibited by pretreatment with LY341495 - Air puff	Ahn et al., 2005
			APDC	Inf soup Rats	↘	- Reduce heat and mechanical hyperalgesia - Radiant heat source/Von frey	Du et al., 2008
			APDC	PGE2 inj Mice	↘	- Restore mechanical thresholds - Blocked by LY341495 - Von Frey	Yang and Gereau, 2003
		Antagonist	MCCG	CAP inj Rats	⌀	- No significant changes in withdrawal latencies - Radiant heat source	Jin et al., 2009
			LY341495	Carrageenan Mice	↗	- Prolong mechanical allodynia - Von Frey	Yang and Gereau, 2003
			LY341495	PGE2 inj Mice	↗	- Prolong PGE2-induced mechanical allodynia - Von Frey	Yang and Gereau, 2003
	Spinal cord	Agonist	APDC	Naïve Rats	⌀	- No effect in spontaneous nociceptive behaviors - Elevating, shaking, stamping of the hindpaw/elevating or whipping of the tail/liking or biting the tail	Fisher and Coderre, 1996
			APDC	Naïve Rats	⌀	- No effect in spontaneous nociceptive behaviors - Elevating, shaking, stamping of the hindpaw/elevating or whipping of the tail/liking or biting the tail	Fisher and Coderre, 1996
			L-CCG-I	Naïve Sheep	↘	- Increase mechanical withdrawal thresholds - Blocked by EGLU - Blunt pin	Dolan and Nolan, 2000
			DCG-IV	Naïve Rats	↗	- Induce hyperalgesia - Paw pressure	Zhou et al., 2011
			APDC	CAP inj Rats	↘	- Reduction of mechanical hypersensitivity, no effect in thermal hyperalgesia - Von Frey/Paw immersion	Soliman et al., 2005
			APDC	CCI Rats	↘	- Pretreatment produced reductions in the development of mechanical and cold hypersensitivity - Von Frey/1 cm deep 1°C water bath	Fisher et al., 2002
			DCG-IV	SNL Rats	↘	- Dose-dependent attenuation of allodynia and hyperalgesia - Von Frey/Paw pressure	Zhou et al., 2011
	Thalamus	Antagonist	EGLU	CFA Rats	↘	- Decrease pain behavior - Ankle-bend test	Neto and Castro-Lopes, 2000
	PAG	Agonist	L-CCG-1	Naïve Mice	↗	- Decrease the latency of the nociceptive reaction - Hot plate	Maione et al., 1998
			L-CCG-1	Formalin Mice	↘	- Decrease phase II - Licking behavior	Maione et al., 2000
		Antagonist	EGLU	Naïve Mice	⌀	- No effect on nociceptive reaction - Hot plate	Maione et al., 1998

Symbols are used for model of pain induced by local injection, for inflammatory pain, for post-operative pain, for neuropathic pain and for chemotherapy-induced neuropathic pain models. ↘, Decrease pain; ↗, Increase pain; CAP, Capsaicin; CCI, Chronic constriction injury; CFA, Complete Freund's Adjuvant; Inf soup, Inflammatory soup; SNL, Spinal nerve ligation.

TABLE 7 | Pain modulation following local administration of group III mGluRs ligands.

Receptor subtype	Localization	Drugs type	Name		Models Species	Effects Tests		References
Group III								
•pan-group III selective	**Periphery**	**Agonist**	**L-AP4**		Naïve Rats	⊘	- No effect mechanical threshold - Paw pressure test	Walker et al., 2001a,b
			L-AP4		Naïve Rats	⊘	- No effect thermal withdrawal latency - Radiant heat source	Jin et al., 2009
			L-AP4		CAP injc Rats	↘	- No effect on paw withdrawal latency in acute pain - Attenuation of CAP-induced behavior - Flinching and lifting/licking/Radiant heat source	Govea et al., 2012
			L-AP4		Carrageenan Rats	↘	- Recovery of reduced weight load - Reduction of mechanical hyperalgesia - Von frey filaments/weight-bearing	Lee et al., 2013
		Antagonist	**MSOP**		CAP inj Rats	⊘	- No significant changes in withdrawal latencies - Radiant heat source	Jin et al., 2009
	Spinal cord	**Agonist**	**L-AP4**		Naïve Rats	⊘	- No effect in spontaneous nociceptive behaviors - Elevating, shaking, stamping of the hindpaw/elevating or whipping of the tail/liking or biting the tail	Fisher and Coderre, 1996
			L-AP4		CAP inj Rats	↘	- Reduction of mechanical hypersensitivity, weak effect in thermal hyperalgesia - Von Frey/Paw immersion	Soliman et al., 2005
			ACPT-I		Carrageenan Rats	↘	- Dose-dependent inhibition the nociceptive behavior - Paw pressure	Goudet et al., 2008
			ACPT-I		Formalin Rats	↘	- No effect in naive animals - Dose-dependent inhibition the nociceptive behavior - Licking behavior	Goudet et al., 2008
			ACPT-I		MA Rats	↘	- Dose-dependent inhibition the nociceptive behavior - Paw pressure	Goudet et al., 2008
			L-AP4		CCI Rats	↘	- Pretreatment produced reductions in the development of mechanical and cold hypersensitivity - Von Frey/1 cm deep 1°C water bath	Fisher et al., 2002
			ACPT-I		CCI Rats	↘	- Dose-dependent inhibition the nociceptive behavior - Paw pressure	Goudet et al., 2008
			L-AP4		SNL Rats	↘	- Reduction of mechanical hypersensitivity - Von Frey	Chen and Pan, 2005
			ACPT-I		CIPN Rats	↘	- Dose-dependent inhibition the nociceptive behavior - Paw pressure	Goudet et al., 2008
		Antagonist	**MAP4**		Naïve Rats	↗	- Increase mechanical and thermal hypersensitivity - Radiant heat/Paw pressure	Chen and Pan, 2005
	PAG	**Agonist**	**L-SOP**		Naïve Mice	↗	- Decrease the latency of the nociceptive reaction - Hot plate	Maione et al., 1998
			L-SOP		Formalin Mice	↗	- Increase of phase II - Licking behavior	Maione et al., 2000
		Antagonist	**MSOP**		Naïve Mice	↘	- Increase the latency of the nociceptive reaction - Hot plate	Maione et al., 1998

Symbols are used for model of pain induced by local injection, for inflammatory pain, for post-operative pain, for neuropathic pain and for chemotherapy-induced neuropathic pain models. ↘ Decrease pain; ↗ Increase pain; CAP, Capsaicin; CCI, Chronic constriction injury; CIPN, Chemotherapy-induced peripheral neuropathy; MA, Mono arthritis; SNL, Spinal nerve ligation.

PAIN MODULATION FOLLOWING SYSTEMIC ADMINISTRATION OF MGLURS LIGANDS

Since mGluRs are extensively expressed along the pain neuraxis (**Figure 1**), several preclinical studies have been performed to evaluate the impact of mGluRs ligands on pain following systemic administration (**Tables 1**–**3**). These preclinical studies outline the role of these different receptors on the regulation of pain. Additional studies have been performed to explore the role of these receptors at precise locations of the pain pathways and will be described in the following paragraphs.

Group I mGluRs

Systemic administration of mGlu1 receptor antagonists are inefficient at altering normal pain threshold in naive animals (Maione et al., 1998; Sevostianova and Danysz, 2006). However, mGlu1 receptor inhibition relieves both mechanical and thermal hypersensitivity in various models of both inflammatory and neuropathic pain (**Table 1**) (Varty et al., 2005; El-Kouhen et al., 2006; Sevostianova and Danysz, 2006; Satow et al., 2008; Zhu et al., 2008). Similarly, systemic administration of mGlu5 receptor antagonists fails to modify basal thermal threshold (Sevostianova and Danysz, 2006), whereas it prevents mechanical and thermal hyperalgesia in a broad range of pain conditions from sub-chronic inflammatory pain to long lasting neuropathic pain (**Table 1**) (Walker et al., 2001a,b; Hudson et al., 2002; Zhu et al., 2004; Varty et al., 2005; Sevostianova and Danysz, 2006; Satow et al., 2008; Jacob et al., 2009; Montana et al., 2009; Zammataro et al., 2011). Of note, mGlu1 receptor inhibition induces motor and cognitive side effects at analgesic doses that could limit its use in clinical trials (El-Kouhen et al., 2006; Zhu et al., 2008). Consequently, mGlu5 receptor seems to be a better target to develop analgesic drugs. Although mGlu5 antagonists have been reported to induce tolerance and some locomotor deficits (Varty et al., 2005; Sevostianova and Danysz, 2006), it is interesting to point out that mGlu5 receptor antagonists reduce anxiety in naïve animals, a comorbidity often associated with chronic pain states (Varty et al., 2005).

Group II mGluRs

Systematically administrated group II selective agonists have proven anti-hyperalgesic effects in both inflammatory and neuropathic pain without altering basal pain thresholds in healthy animals (**Table 2**) (Sharpe et al., 2002; Simmons et al., 2002; Satow et al., 2008; Johnson et al., 2017). Interestingly, selective group II mGluRs agonists have entered into clinical trials for the treatment of schizophrenia suggesting a safe profile of the drug in humans (Li et al., 2015; Muguruza et al., 2016).

Group III mGluRs

Only a few studies have investigated the effect of systemic administration of group III selective compounds in pain perception (**Table 3**). Systemic delivery of mGlu4 receptor agonist alleviates mechanical hypersensitivity provoked by carrageenan-induced inflammation (Vilar et al., 2013). AMN082, an mGlu7 receptor PAM prevents hyperalgesia in inflammatory models (Dolan et al., 2009). The same compound injected systematically reduces mechanical allodynia and thermal hyperalgesia induced by chronic constriction injury to the sciatic nerve and potentiates the effect of morphine (Osikowicz et al., 2008). This drug also exhibits antidepressant-like and anxiolytic-like effects (Bradley et al., 2012). In addition to the mGlu7 receptor, other mechanisms can contribute to these effects since the AMN082 compound is rapidly metabolized *in vivo* into a monoamine transporter inhibitor (Sukoff Rizzo et al., 2011). Surprisingly, systemically administrated mGlu7 receptor negative allosteric modulators (NAMs) also have anti-hyperalgesic effects in neuropathic pain models (Palazzo et al., 2015). As detailed further in this review, pharmacological activation of mGlu7 receptors can lead to opposite effects depending on the administration site. Neuropathic pain induces variation in mGlu7 receptor expression that could imbalance the pronociceptive and antinociceptive role of mGlu7 receptor (Osikowicz et al., 2009; Palazzo et al., 2013, 2015).

Systemic delivery of a mGlu8 receptor agonist also decreases nociceptive responses in inflammatory and neuropathic models, which is inhibited by blocking group III mGluRs in the PAG (Marabese et al., 2007).

ROLE OF METABOTROPIC GLUTAMATE RECEPTORS IN PERIPHERAL MECHANISMS OF SENSORY TRANSMISSION

Sensory transmission initiates with the detection by primary afferents in the periphery of a broad range of stimuli such as mechanical, thermal or chemical stimuli. Primary afferents are specialized neurons translating information detected at the periphery into electrical signals which are conveyed through their cell bodies located in the dorsal root ganglia (DRG) to their projections into the dorsal horn of the spinal cord. Spinal neurons then project to higher centers in the brain which process the sensory information. After nerve injury or inflammation, a number of dysregulations occur in sensory neurons affecting activity, properties or gene expression, driving an increased sensitivity to both non-noxious and noxious stimuli with or without ectopic activities. Because the primary afferents are the first relay of nociceptive transmission and can trigger the chronicization of pain, they represent an interesting target for the development of analgesic drugs.

Early evidence of a glutamate role in nociceptive transmission at the periphery derived from the observation of thermal and mechanical hypersensitivity following subcutaneous injection of glutamate into naive rat hind paw (Carlton et al., 1995; Jackson et al., 1995), first believed to be only triggered by iGluR activation (Zhou et al., 1996). Furthermore, in rodents, glutamate concentration rises in inflamed tissue (Omote et al., 1998) and after sciatic nerve stimulation (deGroot et al., 2000). Elevated levels of glutamate have also been measured in synovial fluid from knee joints of arthritis patients highlighting the clinical relevance of glutamate modulation as a peripheral mediator

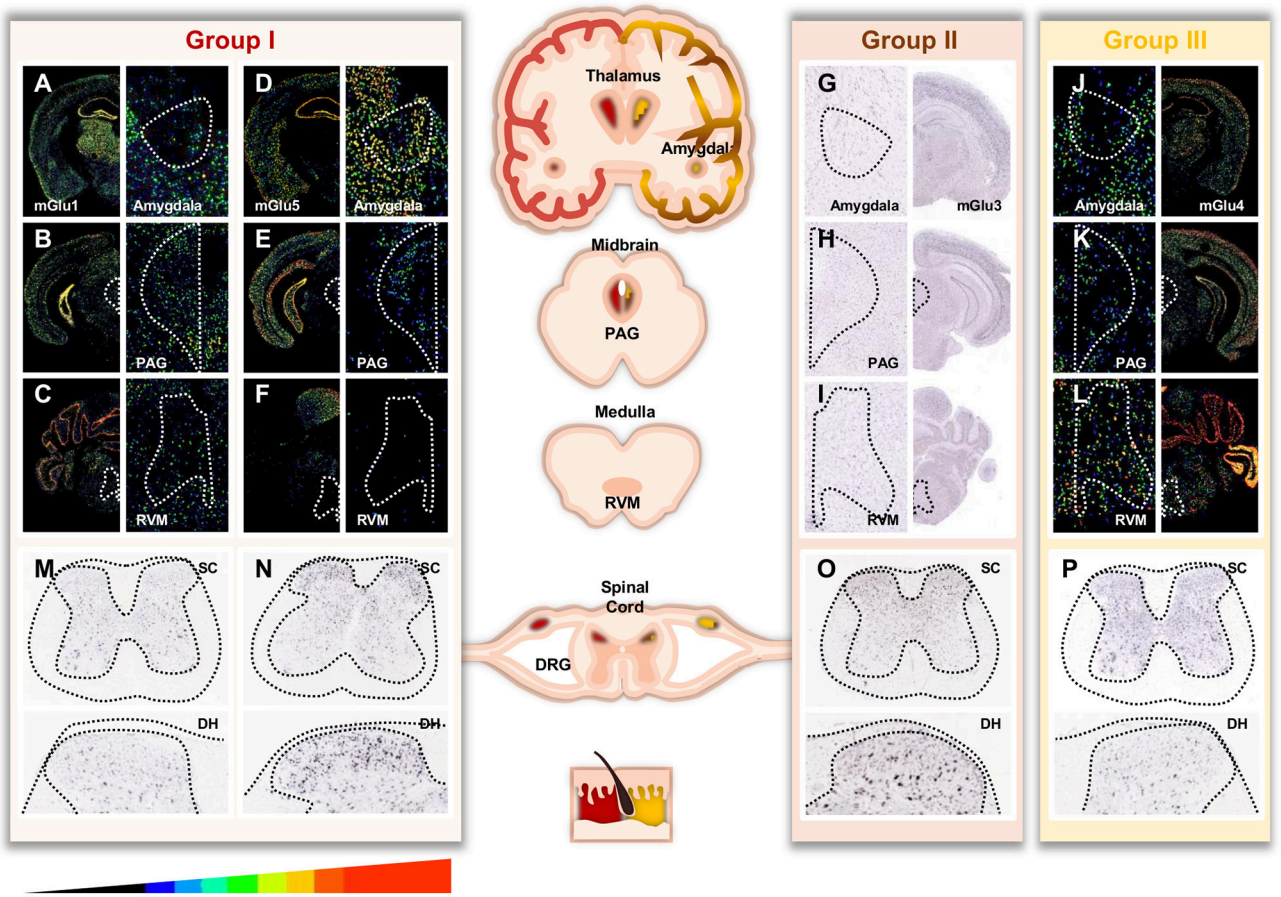

FIGURE 1 | Distribution of mGluRs throughout important areas involved in pain. For **(A-F, J-L)** pictures, masks with pseudo colors were used to color scale the relative expression level of mGluR transcripts across sections (scale displayed at the bottom of the figure). For **(G-I, M-P)**, no expression filter was applied to recolour the ISH pictures. Image credit: Allen Institute. Masked ISH images of mGlu1 **(A)** and mGlu5 **(B)** transcripts in mice coronal section, notably in Thalamus and Amygdala. CeA (central nucleus of the amygdala) is magnified in the right panels (white dotted line, drawn according to the Allen Brain Atlas). Distribution of mGlu1 **(B,C)** and mGlu5 **(E,F)** mRNA in mice midbrain and medulla sections involved in descending modulation of pain. Magnification of the periaqueductal gray (PAG) and rostro ventral medulla (RVM) areas are shown in the right panels (white dotted line, drawn according to the Allen Brain Atlas). ISH images of mGlu3 **(G)** transcript in mice coronal section, notably in Thalamus and Amygdala. CeA is magnified in the left panel (white dotted line). Distribution of mGlu3 **(H,I)** mRNA in mice midbrain and medulla. Magnification of the PAG and RVM nucleus are shown in the left panels (white dotted line). Masked ISH images of mGlu4 **(J)** transcript in mice coronal section, notably in Thalamus and Amygdala. CeA is magnified in the left panel (white dotted line). Distribution of mGlu4 **(K,L)** mRNA in mice midbrain and medulla. Magnification of the PAG and RVM nucleus are shown in the left panels (white dotted line). Images are available for mGlu1 receptor (GMR1 gene) at http://mouse.brain-map.org/experiment/show/79591723, for mGlu5 receptor (GRM5 gene) at http://mouse.brain-map.org/experiment/show/73512423, for mGlu3 receptor (GMR3 gene) at http://mouse.brain-map.org/experiment/show/539, and for mGlu4 receptor (GRM4 gene) at http://mouse.brain-map.org/experiment/show/71247631. Distribution of mGlu1 **(M)**, mGlu5 **(N)**, mGlu3 **(O)**, mGlu4 **(P)** transcripts in mice spinal cord. Bottom panels are magnification of the dorsal horn. Images are available for mGlu1 at http://mousespinal.brain-map.org/imageseries/show.html?id=100036413, for mGlu5 receptor at http://mousespinal.brain-map.org/imageseries/show.html?id=100033614, for mGlu3 receptor at http://mousespinal.brain-map.org/imageseries/show.html?id=100039062 and for mGlu4 receptor at http://mousespinal.brain-map.org/imageseries/show.html?id=100018200.

of pain perception (McNearney et al., 2000). Since then, an increasing number of studies have reported the involvement of mGluRs at the periphery.

Recently, a single-cell transcriptome analysis has reported the expression of mGluR transcripts in mice DRG. Among the most expressed are mGlu7, mGlu3, mGlu4, mGlu8, and mGlu5 receptors (Usoskin et al., 2015). Transcriptome analysis provides evidence for the expression of mGluRs in cell bodies but whether these receptors are expressed at the peripheral terminal, the spinal projection endings, or both, must be further investigated. mGluRs expression has also been reported in trigeminal ganglia, notably mGlu1, mGlu2/3, and mGlu8 receptors (Boye Larsen et al., 2014).

Group I mGluRs

Group I mGlu1 and mGlu5 receptors are expressed in nociceptive afferents (Bhave et al., 2001; Walker et al., 2001a,b). Together with iGluR, group I mGluRs are involved in capsaicin induced glutamate release, a process that could contribute to nociceptive responses evoked by the TRPV1 agonist (Jin et al.,

2009). Intraplantar injection of group I agonists in rodents enhances thermal sensitivity and reciprocally, peripherally applied group I antagonist reduced hyperalgesia in animal models of inflammatory or neuropathic pain (**Table 4**) (Dogrul et al., 2000; Bhave et al., 2001; Walker et al., 2001a,b) . Application of mGlu5 receptor antagonist at peripheral afferent endings also reduces visceral nociception (**Table 5**) (Lindström et al., 2008). More recently, the analgesic potential of peripheral mGlu5 receptor blockade has been highlighted using an mGlu5 selective photoactivable NAM. Photoactivable ligands, also called caged-ligands, are constituted of a ligand linked to a photo-labile protecting group that will be removed following illumination, enabling the precise control of the onset of drug activity at a specific location (Goudet et al., 2018). Following systemic injection of the inactive caged-mGlu5 NAM, analgesia in both phases of the formalin test can be induced by local illumination in the paw (**Table 5**) (Font et al., 2017).

Group II mGluRs

Primary sensory neurons express mGlu2 and mGlu3 receptors in both peripheral terminals and dorsal horn projection (Carlton et al., 2001; Carlton and Hargett, 2007). In DRG, mGlu2/3 receptors are largely co-localized with TRPV1 channel (Carlton et al., 2009). Consistent with this co-expression, group II mGluR antagonists increase hyperalgesia evoked by capsaicin, a TRPV1 agonist, and this effect is blocked by group II mGluR agonists (**Table 6**) (Carlton et al., 2011). However, a recent report has demonstrated that mGlu2/3 receptors activation abolishes TRPV1 sensitization in mouse sensory neurons, but not in humans (Sheahan et al., 2018).

In cultured DRG neurons, group II mGluRs also negatively regulate TTX resistant sodium channels (Yang and Gereau, 2004). Local administration of group II agonist in the knee joint both prevents and reduces carrageenan-induced arthritis (Lee et al., 2013). Due to the lack of selective compounds that can discriminate between mGlu2 and mGlu3 receptors, the individual contribution of those two receptors to pain modulation has remained unclear for a long time. However, the generation of mGlu2 and mGlu3 receptor knockout mice allowed the precise investigation of the role of each subtype in nociception and revealed a predominant role of the mGlu2 over mGlu3 receptor (Zammataro et al., 2011).

In line with the pharmacological evidence, mGlu2 receptor overexpression in DRG induces analgesia in models of inflammatory and neuropathic pain (Chiechio et al., 2002, 2009). L-acetylcarnitine, a drug known to enhance mGlu2 receptor expression in DRG through epigenetic mechanisms induces a long-lasting analgesia in both inflammatory and neuropathic pain models (Notartomaso et al., 2017). Strikingly, N-acetyl-cysteine, a drug enhancing mGlu2 receptor expression in rodents, reduces nociceptive transmission in humans (Truini et al., 2015). Moreover, in a recent report using cultured DRG neurons from both mice and humans, PGE2 evoked neuron hyperexcitability was blocked by group II mGluR activation (Davidson et al., 2016). This data suggests that activation of group II mGluRs leads to an analgesic effect in rodents and humans, making group II mGluRs an interesting target for development of peripherally active drugs for the treatment of chronic pain.

Group III mGluRs

Most group III mGluRs are expressed in the pain pathway, except the mGlu6 receptor which is expressed mainly in the retina (Vardi et al., 2000). The presence of mGlu4, mGlu7, and mGlu8 receptors have been detected in DRG and trigeminal ganglia (Li et al., 1996; Azkue et al., 2001; Carlton and Hargett, 2007). The mGlu8 receptor is expressed in DRG and peripheral terminals where it is widely co-expressed with TRPV1. Intraplantar injection of group III agonists significantly reduced capsaicin evoked pain behavior (**Table 7**; Govea et al., 2012). Similar to group II agonists, local administration in the knee joint of group III mGluRs agonist provokes analgesia in carrageenan-induced arthritic pain model (Lee et al., 2013). Specific contribution of each subtype to the antinociceptive effect of broad range group III mGluRs need to be further investigated.

ROLE OF MGLUR IN PAIN TRANSMISSION AT THE SPINAL CORD LEVEL

The spinal cord (SC) is the first relay in the transmission of sensory information from the periphery to the brain. It is submitted to control from peripheral inputs, interneurons within the SC and both inhibitory and excitatory descending pathways from supraspinal regions. This network makes the SC an important site for the modulation of signals generated at the periphery. Any alteration in neurons from the SC network can imbalance spinal relay and lead to chronic pain conditions.

The dorsal horn (DH) of the SC which receives nociceptive inputs is organized into different laminae, from the superficial laminae I to the deep laminae V. Most nociceptive fibers (Aδ- and C-fibers) superficially innervate laminae I-III and, to a lesser extent, laminae V, whereas low-threshold Aβ-fibers mainly project into laminae III–VI. Early studies have demonstrated that glutamate is released from primary afferent neurons into the DH in response to both acute and persistent painful stimuli, highlighting a role of the glutamatergic system in nociceptive transmission (Sluka and Westlund, 1992; Sorkin et al., 1992).

According to a recent single-cell RNA sequencing study of sensory neurons in the mouse DH, all mGluRs except mGlu6, are expressed within the spinal cord, the highest expression levels being measured for mGlu5 and 7 receptors (Häring et al., 2018). This high throughput data is in line with previous histological and pharmacological studies detailed below, and draw further attention to the relevance of targeting glutamate synapses for pain modulation in the dorsal horn of the spinal cord.

Group I mGluRs

Immunoreactive cell bodies for group I mGluRs are widely spread throughout the superficial laminae of DH (Jia et al., 1999; Tang and Sim, 1999; Hudson et al., 2002). Intrathecal administration of group I mGluR agonists provokes hyperalgesia whereas group I mGluR antagonists induces analgesia in inflammatory and neuropathic pain models (**Table 4**) (Fisher

and Coderre, 1996, 1998; Young et al., 1997; Fisher et al., 1998). Intrathecal injection of mGlu5 antagonist also reverses paclitaxel-induced neuropathic pain (**Table 5**; Xie et al., 2017). DH neuron excitability is increased after activation of spinal group I mGluRs in part due to due to inhibition of a voltage gated potassium channel (Hu et al., 2007). In line with this pharmacological evidence, knockdown or antibody approaches targeting mGlu1 receptor have demonstrated an antinociceptive effect in various pain models (Fundytus et al., 1998, 2001; Noda et al., 2003). Interestingly, recent studies have reported enhanced mGlu5 expression at the nuclear membrane in DH neurons after nerve injury. Using permeable mGlu5 antagonists reaching the cytoplasm, the authors have demonstrated that blocking intracellular mGlu5 had a greater antinociceptive effect than by blocking cell membrane expressed mGlu5 (Vincent et al., 2016). Pre-treatment with an excitatory amino acid transporter (EAAT) inhibitor, which is meant to decrease intracellular glutamate levels, decreases pain-related behavior in an inflammatory pain model (Vincent et al., 2017).

Group II mGluRs

Among group II mGluRs, mGlu3 receptor is the most expressed in the DH, and its transcript is restricted to laminae II (Valerio et al., 1997; Berthele et al., 1999; Jia et al., 1999). However, only mGlu2 receptor expression appears to be enhanced in the SC (and DRG neurons) after administration of L-acetylcarnitine and histone deacetylase inhibitors, two compounds with antinociceptive properties, suggesting a greater role of spinal mGlu2 receptors in pain modulation (Chiechio et al., 2002, 2009). This discrepancy could be explained by expression pattern differences. Indeed, mGlu2 receptor is mostly pre-synaptic, while mGlu3 receptor is both pre- and post-synaptic (Nicoletti et al., 2011). Moreover, mGlu2 is expressed in microglia while mGlu3 is expressed in both microglia and astrocytes (Spampinato et al., 2018).

Group III mGluRs

Transcripts of two group III members, mGlu4 and mGlu7 receptors, are detected in the spinal cord (Valerio et al., 1997). The expression of mGlu4 receptor is restricted to inner laminae II of the DH receiving nociceptive Aδ- and C-fibers inputs whereas mGlu7 receptor is expressed in both laminae I and II (Valerio et al., 1997; Vilar et al., 2013). In addition, the mGlu4 receptor may be expressed in spinal neurons, since its expression can still be observed after rhizotomy of the afferent fibers (Vilar et al., 2013). Activation of spinal group III mGluRs depletes glutamate release from primary afferents in nerve-injured rats (**Table 7**; Zhang et al., 2009). Furthermore, intrathecal administration of the group III broad-spectrum agonist L-AP4 reduces capsaicin-induced hypersensitivity and neuropathic pain symptoms (Fisher et al., 2002; Chen and Pan, 2005; Soliman et al., 2005). Intrathecal administration of the mGlu4 receptor PAM or agonist inhibits both inflammatory and neuropathic pain without altering acute pain thresholds in naive animals (**Table 8**; Goudet et al., 2008; Wang et al., 2011; Vilar et al., 2013). Conversely, the antiallodynic action of an mGlu4 agonist in inflammatory pain can be blocked by a photoswitchable mGlu4 NAM (Rovira et al., 2016). Positive allosteric modulation of spinal mGlu7 alleviates mechanical allodynia and thermal hyperalgesia induced by either carrageenan or skin incisions (Dolan et al., 2009). However, intrathecally administrated mGlu7 PAM has failed to relieve neuropathic pain (Wang et al., 2011). Both studies used the mGlu7 PAM named AMN082 (Mitsukawa et al., 2005). As mentioned earlier in the text, *in vivo*, AMN082 is rapidly metabolized and one of its metabolite inhibits several monoamine transporters (Sukoff Rizzo et al., 2011). Therefore, *in vivo* actions of AMN082 should be interpreted with caution since it may have multiple mode of action.

CONTRIBUTION OF MGLUR TO SUPRASPINAL MECHANISMS OF PAIN PERCEPTION

Integration of the nociceptive signal in the brain translates into a complex pain experience (Hunt and Mantyh, 2001). Pain processing in the supraspinal nervous system involves both ascending and descending pathways. Briefly, two main ascending pathways have been identified. The first one, the spinoparabrachial pathway, originates from the superficial dorsal horn and projects to areas of the brain concerned with affect: the parabrachial area (PB), the ventral medial nucleus (VMN) or the amygdala. The second one, the spinothalamic pathway, starts from the deep DH and projects to the thalamus and other areas of the cortex concerned with discrimination and affect. Different brain areas are involved in pain integration and processing. They are referred to as the pain matrix, a concept first described by Ronald Melzack in the late eighties (Melzack, 1990). It comprises several regions such as the primary and secondary sensorimotor cortex, insula, anterior cingulate cortex, thalamus, striatum, brainstem and cerebellum (Garcia-Larrea and Peyron, 2013). Descending pathways also involve high brain centers such as amygdala, hypothalamus and VMH, and nucleus in the midbrain and the brainstem, respectively, periaqueductal gray (PAG) and rostral ventromedial medulla (RVM).

mGluRs are widely express in neurons, astrocytes, oligodendrocytes, and microglia throughout the brain areas involved in pain processing. Consequently, there is an increasing interest in understanding the contribution of supraspinal mGluRs to pain modulation and many groups have investigated their potential for alleviating pain.

Group I mGluRs

Although it is clearly established that activation of group I mGluRs at both the periphery and the spinal cord promotes pain, group I activation at the supraspinal level can elicit both antinociceptive and pronociceptive effects depending on the region investigated (**Tables 4**, **5**). For instance, when applied in the amygdala, group I agonist promotes nociception (Li and Neugebauer, 2004; Kolber et al., 2010; Ren and Neugebauer, 2010; Tappe-Theodor et al., 2011). Reciprocally, stereotaxic injection of mGlu1 and mGlu5 receptor antagonists in the amygdala inhibits pain-related responses in a model of arthritic

TABLE 8 | Pain modulation following local administration of selective mGlu4, mGlu7 or mGlu8 ligands.

Receptor subtype	Localization	Drugs type	Name	Models Species	Effects Tests		References
Group III							
• mGlu4	Spinal cord	Agonist	LSP4-2022	Carrageenan Mice	↘	- Reduction of mechanical hypersensitivity - Von Frey	Vilar et al., 2013
			LSP4-2022	Carrageenan Rats	↘	- Reduction of mechanical hypersensitivity - Paw pressure	Vilar et al., 2013
			LSP4-2022	CCI Rats	↘	- No effect in naive animals - Reduction of mechanical hypersensitivity - Paw pressure	Vilar et al., 2013
		PAM	PHCCC	Carrageenan Rats	↘	- No effect in naive animals - Dose-dependent inhibition the nociceptive behavior - Paw pressure	Goudet et al., 2008
			PHCCC	CCI Rats	↘	- No effect in naive animals - Dose-dependent inhibition the nociceptive behavior - Paw pressure	Goudet et al., 2008
			VU0155041	SNL Rats	↘	- Dose dependent attenuation of hyperalgesia - Von Frey/Paw immersion	Wang et al., 2011
	Amygdala	Agonist	LSP4-2022	CFA Mice	↘	- Decrease mechanical allodynia and emotional components associated with chronic pain - Von Frey	Zussy et al., 2018
		PAM	Optogluram Photoswitchable	CFA Mice	↘	- Decrease mechanical allodynia and emotional components associated with chronic pain - Von Frey	Zussy et al., 2018
	Striatum	PAM	VU0155041	SNI Rats	↘	- No effect in both sham-operated and SNI rats - Tail flick	Rossi et al., 2013
• mGlu7	Spinal cord	PAM	AMN082*	SNL Rats	↘	- No effect - Von Frey/Paw immersion	Wang et al., 2011
	Amygdala	PAM	AMN082*	Naïve Rats	↘	- Decrease mechanical threshold and increase of vocalizations - Knee compression	Palazzo et al., 2008
			AMN082*	MA Rats	↘	- No effect in mechanical threshold and vocalization - Knee compression	Palazzo et al., 2008
	PAG	PAM	AMN082*	Naïve Mice	↘	- Decrease mechanical threshold - Radiant heat source	Marabese et al., 2007
	Striatum	PAM	AMN082*	Naïve Rats	↘	- Facilitation of pain - Dynamic Plantar Aesthesiometer/Tail Flick	Marabese et al., 2018
			AMN082*	SNI Rats	↘	- Decrease mechanical allodynia and thermal hypersensitivity - Dynamic Plantar Aesthesiometer/Tail Flick	Marabese et al., 2018
		NAM	ADX71743	SNI Rats	↘	- No effect in mechanical threshold - Dynamic Plantar Aesthesiometer	Marabese et al., 2018
• mGlu8	Amygdala	Agonist	DCPG	Naïve Rats	↘	- No effect in mechanical threshold nor vocalization - Knee compression	Palazzo et al., 2008
			DCPG	MA Rats	↘	- Increase mechanical threshold and reduce vocalization - Knee compression	Palazzo et al., 2008
	Striatum	Agonist	DCPG	SNI Rats		- Increase tail flick latency and mechanical threshold - No effect in sham animals - Tail flick/Von frey	Rossi et al., 2013
	Striatum	PAM	AZ12216052	SNI Rats	↘	- Increase the tail flick latency - No effect in sham animals - Tail flick	Rossi et al., 2013
	PAG	Agonist	DCPG	Carrageenan Mice	↘	- Reduce pain behavior - Dynamic Plantar Aesthesiometer/Radiant heat source	Marabese et al., 2007
			DCPG	Formalin Mice	↘	- Reduce pain behavior - Licking behavior	Marabese et al., 2007

**Of note, in vivo actions of AMN082 should be interpreted with caution because they may involve other mechanisms in addition to mGlu7. Indeed, an AMN082 metabolite can inhibit monoamine transporters Sukoff Rizzo et al., 2011.*

Symbols are used for model of pain induced by local injection, for inflammatory pain, for post-operative pain, for neuropathic pain and for chemotherapy-induced neuropathic pain models. ↘ Decrease pain; ↗ Increase pain; CCI, Chronic constriction injury; CFA, Complete Freund's Adjuvant; MA, Mono arthritis; SNL, Spinal nerve ligation; SNI, Spared nerve injury.

pain (Han and Neugebauer, 2005). Similarly, intra basolateral amygdala administration of group I mGluRs agonist alleviates inflammatory pain, an effect at least in part due to inhibition of prefrontal cortex neurons activity (Luongo et al., 2013). When applied to the thalamus, mGlu1 PAM potentiated nociceptive responses of thalamic neurons (Salt et al., 2014). Conversely, when administrated in the PAG, a region involved in modulation of the descending pain pathway, activation of group I mGluRs decreases the nociceptive response, likely through the inhibition of the GABAergic transmission (Maione et al., 2000; Drew and Vaughan, 2004). Moreover, PAG expressed mGlu5 contribute to the antinociceptive effect provoked by RVM cannabinoid receptor activation (de Novellis et al., 2005).

In an outstanding paper, authors used a selective photoactivable mGlu5 NAM enabling the precise spatiotemporal modulation of mGlu5 receptors to probe the involvement of thalamic mGlu5 receptors in pain processing. As expected, when injected systematically, the inactive caged compound has no effect on pain behavior of neuropathic animals. However, release of the active mGlu5 NAM by delivering light through implanted optical fibers in the ventrobasal thalamus, reduces neuropathic pain (Font et al., 2017).

An alternative photopharmacological strategy consists in using photoswitchable ligands that can be reversibly activated and inactivated by light (Goudet et al., 2018). This approach has been used to validate the role of amygdala-expressed mGlu5 in pain. A photoswitchable mGlu5 NAM has been injected locally in amygdala where it light-dependently reduced mechanical allodynia in a mice model of inflammatory pain (Gómez-Santacana et al., 2017), confirming previous preclinical studies (Han and Neugebauer, 2005).

Interestingly, global genetic disruption of mGlu5 in mice leads to increased basal mechanical withdrawal responses whereas conditional KO in the amygdala did not affect acute pain. However, both global and conditional KO prevent the establishment of mechanical hypersensitivity 180 min after formalin injection in the ipsi and contralateral paw (Kolber et al., 2010).

Group II mGluRs

Accumulating evidence demonstrates that stimulation of group II mGluRs in supraspinal areas mediates analgesia (**Table 6**). Administration into the amygdala by microdialysis of group II agonist diminishes the response to noxious stimulation in an arthritis model of chronic pain (Li and Neugebauer, 2006). In the PAG, group II mGluR activation reinforces antinociceptive descending pathway (Maione et al., 2000). Local inhibition in the PAG or the RVM of the degradation of an endogenous peptide acting as an mGlu3 receptor agonist relieves pain in rat models of inflammatory and neuropathic pain (Yamada et al., 2012). However, studies have also reported a pronociceptive effect of CNS expressed group II mGluRs. For instance, blockage in the thalamus elicits antinociceptive effects, possibly via an inhibition of GABAergic inhibitory neurones (Neto and Castro-Lopes, 2000). Furthermore, microinjection of a group II agonist in the PAG induces pronociceptive effects by inhibiting descending pathway (Maione et al., 1998).

Group III mGluRs

Broad range group III mGluR agonists were first used to elucidate the contribution of these receptors in pain processing in the CNS (**Table 7**). Early studies demonstrated that in the PAG a group III mGluR agonist facilitates pain related behavior (Maione et al., 1998, 2000), whereas in the amygdala group III agonist microinjection produces antinociceptive effects in an arthritis model (Li and Neugebauer, 2006). Development of more selective compounds for individual group III subtypes has allowed the more precise dissection of each members' contribution to nocifensive and affective pain responses within the CNS (**Table 8**). Of note, mGlu7 and mGlu8 have opposite effects in the PAG. Indeed, mGlu7 activation in PAG and amygdala is pronociceptive whereas mGlu8 activation is antinociceptive (Marabese et al., 2007; Palazzo et al., 2008). Similarly, in the nucleus tractus solitarius, mGlu7 activation has an antinociceptive effect on the cardiac-somatic reflex induced by pericardial capsaicin, while activation of mGlu8 receptors enhance cardiac nociception (Liu et al., 2012). Activation of mGlu7 in the nucleus accumbens by AMN082 has an antinociceptive effect and modulates relief learning (Kahl and Fendt, 2016). Blockade of mGlu7 in the PAG reduces the pain related behaviors in formalin and neuropathic pain models and differentially modulates RVM ON and OFF cell activity (Palazzo et al., 2013). Whereby, ON cells are neurons activated by noxious stimuli and inhibited by analgesics, and OFF cells are activated by analgesics and inhibited by painful stimuli (Palazzo et al., 2013).

Recently, dorsal striatum (DS) expressed mGlu7 receptors and their role in pain have been investigated. The DS is connected to the descending pain modulatory systems, including to the RVM. When locally administrated in the DS of sham animals, an mGlu7 PAM enhanced pain and simultaneously stimulates ON cells and inhibits OFF cells in the RVM. Whereas, in nerve-injured animals, the mGlu7 PAM has an anti-hyperalgesic effect in addition to increasing RVM OFF cell firing. This opposite effect of an mGluR7 PAM in acute or chronic pain conditions is assumed to be due to the recruitment of different pain pathways (Marabese et al., 2018). Interestingly, systemic administration of an mGluR7 PAM prevents the development of morphine tolerance (Gawel et al., 2018). A role of centrally expressed mGlu7 in epilepsy has also been reported (Sansig et al., 2001; Bertaso et al., 2008).

The first strong evidence of supraspinal mGlu4 involvement in pain processing is thanks to the recent development of an mGlu4 photoswitchable PAM allowing the time resolved control of endogenous receptors in freely behaving animals. Strikingly, dynamic modulation of mGlu4 receptor activation in the amygdala by the photoswitchable PAM reverses, in a light dependent manner, both inflammatory pain-related sensory and affective symptoms (Zussy et al., 2018). As compared to conventional compounds, this ligand enables precise temporal control of the mGlu4 receptor and, in contrast to optogenetics, allows endogenous receptor modulation, without the need of trangenesis. We expect that future development of photoswitchable ligands for other mGluRs

will greatly improve our understanding of mGluRs in the pain neuraxis and co-morbidities associated with chronic pain conditions.

ROLE OF GLIAL MGLUR IN PAIN

Beside neurons, mGluRs are also widely expressed in glial cells, noteworthy in microglia, astrocytes, and oligodendrocytes (for a recent review, see Spampinato et al., 2018). Astrocytes are the most abundant cell type in the brain, which are regulating neuronal function and remodeling synaptic structures. In addition to their physiological functions, astrocytes are involved in numerous diseases, such as chronic pain. Microglia act as resident macrophages, which function as sentinels of the CNS surveying potential damage. Following nerve injury, activated microglia surround the injured peripheral nerve terminals in the dorsal horn where they release different factors, such as brain-derived neurotrophic factor (BDNF), cytokines (TNFα, IL-1β, IL-6…) and glutamate, that will contribute to neuroinflammation, excitotoxicity and central sensitization. Numerous studies have shown that glial cells play a critical role in the development of neuropathic and inflammatory pain (Ji et al., 2013). For instance, microglia and astrocytes contribute to the central sensitization process that occurs in the setting of injury (Basbaum et al., 2009). Interestingly, all three groups of mGluRs are expressed in microglia and play a critical role in regulating microglial activity (Taylor et al., 2002, 2003; Byrnes et al., 2009; McMullan et al., 2012). *In vitro*, neuroinflammatory factors trigger an opposite regulation in the gene expression of the two predominant mGluR subtypes found in astrocytes and microglia, namely an upregulation of mGlu3 and a downregulation mGlu5 (Berger et al., 2012). Concerning group I mGluRs, activation of mGlu5 receptors inhibits microglial-associated inflammation and neurotoxicity (Byrnes et al., 2009), while little is known about mGlu1 receptors. Activation of group II mGluRs *in vitro* yields two opposite effects in cultured microglia, mGlu2 activation enhancing neurotoxicity whilst mGlu3 activation promotes neuroprotection (Taylor et al., 2002, 2005; Pinteaux-Jones et al., 2008). However, further studies are needed to understand the particular roles of these receptors, since activation of both mGlu2 and mGlu3 receptors have been reported to be neuroprotective *in vivo* (Fazio et al., 2018). Activation of group III mGluRs, notably mGlu4 receptors, reduces microglial reactivity (Taylor et al., 2003; Pinteaux-Jones et al., 2008; Ponnazhagan et al., 2016). Glial mGluRs modulate neuronal excitability and glutamate concentration in the synaptic and extrasynaptic regions (Pál, 2018). Of note, activation of group II and III, but not group I, attenuates export of glutamate from activated microglia through a cAMP-dependent mechanism (McMullan et al., 2012). Taken together, these results suggest that although less well studied than their neuronal counterparts, glial mGluRs may represent novel targets for the treatment of chronic pain.

CONCLUSION

The growing number of selective compounds for the different mGluRs has significantly improved our understanding of the specific role of each subtype in nociception. Numerous evidences tend to suggest these receptors are promising targets for the treatment of chronic pain. However, at doses proven to be analgesic, mGlu1 antagonists are associated with motor and cognitive impairment (El-Kouhen et al., 2006; Zhu et al., 2008). Similarly, deficits in motor coordination phenotype has also been observed in mGlu1 conditional knockouts in the cerebellum (Nakao et al., 2007). Although mGlu5 antagonists may have psychoactive properties (Swedberg et al., 2014), mGlu5 blockade seems to elicit less side effects than mGlu1, suggesting that targeting mGlu5 may be more promising for the development of new analgesics. Regarding group II agonists, which have proven antinociceptive effects, a major concern for the treatment of persistent pain is the development of tolerance after repeated systematic injections (Jones et al., 2005; Zammataro et al., 2011). Nevertheless, epigenetic upregulation of endogenous mGlu2 receptor expression could counteract the drawback of tolerance. Group III metabotropic receptors are of a particular interest in drug development because their targeting may also decrease affective and cognitive disorders associated with chronic pain such as anxiety, depression, or fear (Zussy et al., 2018).

Given the analgesic effects observed after targeting peripheral mGluRs, peripherally restricted molecules may have satisfying analgesic effectiveness while decreasing the central-associated side effects. Furthermore, the use of new pharmacological tools such as photoswitchable or caged ligands, which allow the spatiotemporal tuning of mGluRs, could reduce off-target effects related to the modulation of the glutamatergic system outside the pain neuraxis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Metabotropic Glutamate Receptors in Glial Cells: A New Potential Target for Neuroprotection?

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Neurodegenerative disorders are characterized by excitotoxicity and neuroinflammation that finally lead to slow neuronal degeneration and death. Although neurons are the principal target, glial cells are important players as they contribute by either exacerbating or dampening the events that lead to neuroinflammation and neuronal damage. A dysfunction of the glutamatergic system is a common event in the pathophysiology of these diseases. Metabotropic glutamate (mGlu) receptors belong to a large family of G protein-coupled receptors largely expressed in neurons as well as in glial cells. They often appear overexpressed in areas involved in neurodegeneration, where they can modulate glutamatergic transmission. Of note, mGlu receptor upregulation may involve microglia or, even more frequently, astrocytes, where their activation causes release of factors potentially able to influence neuronal death. The expression of mGlu receptors has been also reported on oligodendrocytes, a glial cell type specifically involved in the development of multiple sclerosis. Here we will provide a general overview on the possible involvement of mGlu receptors expressed on glial cells in the pathogenesis of different neurodegenerative disorders and the potential use of subtype-selective mGlu receptor ligands as candidate drugs for the treatment of neurodegenerative disorders. Negative allosteric modulators (NAM) of mGlu5 receptors might represent a relevant pharmacological tool to develop new neuroprotective strategies in these diseases. Recent evidence suggests that targeting astrocytes and microglia with positive allosteric modulators (PAM) of mGlu3 receptor or oligodendrocytes with mGlu4 PAMS might represent novel pharmacological approaches for the treatment of neurodegenerative disorders.

Keywords: neurodegeneration, metabotropic glutamate receptor, transforming growth factor-β1, apoptosis, neuroprotection

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INTRODUCTION

Neurodegenerative disorders, among the most prevalent, devastating and yet poorly treated illnesses are progressive diseases characterized by slow neuronal death. Dysfunction of glutamatergic transmission plays a central role in the pathogenesis of neurodegenerative diseases (Nguyen et al., 2011). Malfunctioning or aberrant expression of glutamate transporters leads in fact

to the accumulation of this neurotransmitter followed by over-activation of ionotropic glutamate receptors, mainly NMDA receptors, a primary event in the pathophysiology of neuronal damage. Activation of NMDA and/or AMPA receptor lacking the GluR2 subunit (Dugan and Choi, 1994; Zipfel et al., 2000), leads to an excessive influx of extracellular Ca^{++} that triggers a cascade of events leading to apoptotic and necrotic death. This occurs both in acute and chronic neurodegenerative conditions such as AD, ischemia, ALS (Doble, 1999; Hardingham and Bading, 2003).

The underlying context is a condition of neuroinflammation, defined as an innate immunological response of the nervous system, involving glial cells, microglia, astrocytes, and cytokines, chemokines, ROS, and other factors they release (Kim and de Vellis, 2005; Block and Hong, 2007; Benatti et al., 2016). Excitotoxicity and neuroinflammation are strictly interconnected since increased extracellular levels of glutamate critically favor activation of glial cells and promotion of neuroinflammatory phenomena in the brain (Olmos and Llado, 2014). In this scenario, glial cells (astrocytes, microglia, and oligodendrocytes) reciprocally interact to contribute to the pathophysiology of neurodegeneration. Under physiological conditions, astrocytes play a key role in the homeostatic control of CNS environment, by removing glutamate from the extracellular space through specific transporters, GLAST and GLT1 (Oliet et al., 2001), as well as by controlling formation (Ullian et al., 2001) and pruning of synapses in response to changes of neuronal activity (Stevens et al., 2007). Dysfunction of astrocytes causes glutamate accumulation with ensuing excitotoxicity (Werner et al., 2001). Reactive astrocytes can further precipitate neuroinflammation (Verite et al., 2018) through the release of pro-inflammatory cytokines and chemokines, including CCL2, which recruits peripheral monocytes into the CNS. Accordingly, apoptotic astrocytes and reactive astrogliosis critically contribute to neurodegenerative processes in different forms of dementia (Heneka et al., 2010) including AD (Kobayashi et al., 2002), vascular (Tomimoto et al., 1997), and frontotemporal dementia (Martin, 2000).

Microglial cells are professional phagocytes (Gomez-Nicola and Perry, 2015) that regulate synapses pruning (Schafer et al., 2012) and phagocytosis of cells undergoing programmed death, both during development and in the mature healthy brain. They also support immune surveillance in the CNS (Zabel and Kirsch, 2013). In response to a prolonged inflammatory stimulus or to the accumulation of misfolded proteins, such as aggregated Aβ, α-synuclein, mutant huntingtin, SOD1, hyperactivated microglia can amplify neurodegeneration, by releasing pro-inflammatory cytokines (Block and Hong, 2007; Mosher and Wyss-Coray, 2014; Streit and Xue, 2014) and ROS (Wilkinson and Landreth, 2006; Dewapriya et al., 2013). Microglia also strongly influence glutamatergic transmission by regulating the expression of glutamate receptors and transporters in neighbor cells (Aronica et al., 2005a; Pickering et al., 2005; Tilleux et al., 2007). Increased extracellular levels of glutamate under pathological conditions, induce microglia chemotaxis to the injury site, through activation of both ionotropic and mGlu receptors expressed in microglia cells (Liu et al., 2009).

In addition to astrocytes and microglia, oligodendrocytes have an essential role in maintaining CNS homeostasis by supporting neuronal myelination and protecting axonal membrane (Rosenbluth, 2009; Bakiri et al., 2011; Harris and Attwell, 2012). Oligodendrocyte dysfunction is mainly involved in the pathogenesis of classical demyelinating diseases (MS and NMO) and leukodystrophies (Fellner and Stefanova, 2013; Ettle et al., 2016). Recent studies suggest that ischemic insults, trauma, and accumulation of abnormal protein aggregates (i.e., α-synuclein, tau, PrP) also cause oligodendrocytes malfunction, leading to myelin disruption and thus neuronal conduction impairment, as reviewed in Ferrer (2018).

mGlu RECEPTORS IN GLIAL CELLS: DISTRIBUTION AND FUNCTION

As stated above, glutamate, through the activation of ionotropic receptors, plays a central role in the onset of excitotoxicity. Glutamate activates also a class of G-protein coupled receptors, mGlu receptors, that form a family of eight subtypes (mGlu1 to mGlu8) subdivided into three groups on the basis of their amino acid sequence, G-protein coupling, and pharmacological profile. Group I includes mGlu1 and mGlu5 receptors, which are coupled to G_q/G_{11} and are functionally linked to polyphosphoinositide hydrolysis and negatively coupled with K^+ channels (Abdul-Ghani et al., 1996; Nicoletti et al., 2011). Group II (mGlu2, mGlu3) and group III (mGlu4, mGlu6, mGlu7, mGlu8) subtypes are coupled to G_i/G_o, negatively regulate adenylate cyclase, but can also activate MAP kinase and PI-3-kinase pathways (Iacovelli et al., 2002; Niswender and Conn, 2010; Nicoletti et al., 2011).

mGlu receptors are widely distributed in the CNS, where they are localized at synaptic and extra synaptic levels in neurons and glia. Group I mGlu receptors are generally localized postsynaptically, surrounding ionotropic receptors, and they modulate depolarization and synaptic excitability. Group II and III are mostly expressed at presynaptic level and control the release of neurotransmitters as reviewed in Niswender and Conn (2010), Ribeiro et al. (2017). mGlu receptor subtypes form homo- and heterodimers (Kammermeier, 2012; Yin et al., 2014; Vafabakhsh et al., 2015). In addition, G_i-coupled mGlu receptors dimerize with other receptors coupled to G_q such as 5-HT_{2A}, β_1-adrenergic, and $GABA_B$ receptors (Pin and Bettler, 2016).

Abbreviations: 6-OHDA, 6-hydroxydopamine; AD, Alzheimer disease; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor; ASL, amyotrophic lateral sclerosis; Aβ, beta amyloid; BBB, blood brain barrier; BDNF, brain derived neurotrophic factor; CHPG, (RS)-2-chloro-5-hydroxyphenylglycine; CCL2, monocyte chemoattractant protein-1; CNS, central nervous system; DHPG, (S)-3,5-dihydroxyphenylglycine; EGF, epidermal growth factor; FGF, fibroblast growth factor; GalC+, galactocerebroside; GLAST, L-glutamate/L-aspartate transporter; GLT-1, glutamate transporter-1; IL-6, interleukin 6; JNK, c-Jun N-terminal kinase; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; LPS, lipopolysaccharide; LTP, long term potentiation; MBP, myelin basic protein; mGlu, metabotropic glutamate receptor; MPEP, 2-methyl-6-(phenylethynyl)pyridine; MS, Multiple sclerosis; NAM, negative allosteric modulator; NMDA, *N*-methyl-D-aspartate receptor; NMO, neuromyelitis optica; OGD, oxygen glucose deprivation; OPC, oligodendrocytes progenitor cell; PAM, positive allosteric modulator; PD, Parkinson disease; PI3K, phosphatidylinositol-3-kinase; ROS, reactive oxygen species; sAPPα, soluble amyloid precursor protein; SCI, spinal cord injury; SOD-1, superoxide dismutase; TBI, traumatic brain injury; TGF-β1, transforming growth factor β1; TNFα, tumor necrosis factor α; TrkB, tyrosin receptor kinase B.

Evidence of functional interactions between mGlu receptors and estrogen receptors in neurons also exists (Spampinato et al., 2012b).

Intracellular signaling triggered by mGlu receptors has been mainly studied in neuronal cells, whereas less is known in glial cells (Gerber et al., 2007). Group I mGlu receptors activate MAP kinase playing a key role in protein synthesis-dependent neuronal plasticity (Gerber et al., 2007; Hellyer et al., 2017). Translation and transcription factors targeted by MAPK cascades following mGlu receptors activation have been well characterized (Gerber et al., 2007). Group I mGlu receptors dependent phosphorylation of JNKs increases transcription mediated by activator protein-1 (Yang et al., 2006), whereas activation of p38 regulates NF-κB (O'Riordan et al., 2006). More detailed analysis has been carried out in glial cells, and specifically in astrocytes, where stimulation of MAPK and PI3K pathways *via* mGlu3 receptor increases the production of neurotrophic factors (Bruno et al., 1998; Caraci et al., 2011; Durand et al., 2017) promoting neuroprotection against different toxic insults (Ribeiro et al., 2017). When moving to group III mGlu receptors, mGlu4 receptor activation in cultured rat neural stem cells results in inhibition of JNK and p38 mitogen-activated protein kinase, which downregulates the expression of procaspase-8/9/3 and reverses the Bcl-2/Bax balance, finally preventing H_2O_2-mediated cell death (Zhang et al., 2015). A protective role for mGlu7 receptor has also been recently found in glial cells and it involves the activation of PI3K/Akt and MAPK/ERK1/2 pathways (Jantas et al., 2018).

According to the principles of "ligand bias" and "functional selectivity," a G-protein coupled receptor can signal *via* a canonical pathway mediated by the Gα subunit and *via* non-canonical pathways (e.g., MAPK activation) mediated by scaffolding proteins such as β-arrestin (Iacovelli et al., 2014; Hathaway et al., 2015). Recent evidence suggests that mGlu receptors associate with β-arrestin in the initiation of intracellular cascades affecting cellular responses (Hathaway et al., 2015; Hellyer et al., 2017). The recruitment of β-arrestin-dependent signaling pathways occurs in response to G-protein coupled Receptor Kinase (GRK)-dependent phosphorylation and it is strictly ligand-dependent (Hellyer et al., 2017). Future studies are needed in astrocytes and microglial cells to assess whether specific ligands with a functional selectivity can exert different effects on intracellular signaling pathways (e.g., MAPK and PI3K) in neuronal and glial cells.

Of note, the expression of mGlu receptors is developmentally regulated. mRNA levels for mGlu1, mGlu2, and mGlu4 receptors are low at birth and increase during postnatal development (Lujan et al., 2005). In addition, the expression of the shorter mGlu5a receptor isoform is higher in prenatal stages, and mainly detected in cortex, hippocampus and subventricular zone, where it colocalizes with neural progenitors (Boer et al., 2010), astrocytes and microglia. In contrast, in mature brain, mGlu5b receptor is the main isoform expressed (Romano et al., 2002; Lujan et al., 2005).

In glial cells, mGlu1, mGlu3, and mGlu5 receptors are found in astrocytes whereas mGlu2, mGlu3, and mGlu5 receptors are expressed in microglial cells. In oligodendrocytes, mGlu1 and mGlu4 are highly expressed (Ribeiro et al., 2017), whereas mGlu5a and mGlu2/3 receptors are present in early developmental stages and downregulated in mature MBP+ oligodendrocytes (Luyt et al., 2003; Deng et al., 2004; Spampinato et al., 2014).

Glial mGlu receptors regulate glial cell proliferation (Ciccarelli et al., 1997), the release of growth factors, cytokines (Ciccarelli et al., 1999; Aronica et al., 2005b), and neurotransmitters including glutamate, ATP and adenosine, which propagate Ca^{++} signaling between astrocytes and other glial cells (Hamilton et al., 2010). Glial mGlu receptors modulate also the activity and the expression of glutamate transporters, thus participating in the regulation of synaptic function (Aronica et al., 2003b; Vermeiren et al., 2005). Glutamatergic system plays a key role in the pathophysiology of chronic pain and in particular in central sensitization (Guida et al., 2015; Hossain et al., 2017) and neurodegenerative processes leading to cognitive deficits (Giordano et al., 2012). Microglial activation significantly contributes to central sensitization and neurodegeneration promoting the transition from acute to chronic pain (Ji et al., 2014; Hossain et al., 2017). According to this scenario mGlu receptors expressed on glial cells (microglia and astrocytes) might exert a key role in the pathogenesis of chronic pain by modulating both glutamate release and neuroinflammatory phenomena (Chiechio, 2016; Palazzo et al., 2017).

GROUP I mGlu RECEPTORS

In physiological conditions, the expression of mGlu1 receptor is very low in astrocytes as well as in cultured cortical astrocytes grown in conventional media. In contrast, the expression is higher in reactive astrocytes of ALS spinal cord (Agrawal et al., 1998; Aronica et al., 2001; Anneser et al., 2004).

Expression of mGlu5 in astrocytes is high prenatally, but decreases after birth (Cai et al., 2000; Yang et al., 2012; Iyer et al., 2014). In physiological conditions, the activity of mGlu5 receptor in cortical astrocytes defines the frequency of Ca^{++} oscillations (Bradley and Challiss, 2011) and the release of gliotransmitters (Agulhon et al., 2008; Fiacco et al., 2009). mGlu5 overexpression has been reported in different neurodegenerative disorders (Ribeiro et al., 2017), in particular in reactive astrocytes surroundings Aβ plaques (Shrivastava et al., 2013), spinal cord lesions (Gwak and Hulsebosch, 2005), MS lesion (Geurts et al., 2003), ALS (Aronica et al., 2001), PD (Tison et al., 2016), and in hippocampal astrocytes from Down syndrome patients (Iyer et al., 2014).

Accordingly, *in vitro*, mGlu5 receptor expression occurs as a reactive response: both mRNA and protein levels are induced in astrocytes grown in media enriched with growth factors (FGF, EGF, TGF-β1) (Miller et al., 1995; Balazs et al., 1997), or exposed to Aβ oligomers (Casley et al., 2009; Lim et al., 2013).

mGlu5 receptor actively regulates glutamate transmission, acting as a sensor of extracellular glutamate concentrations and inducing activation of the glial glutamate transporter GLT-1 (Vermeiren et al., 2005). In contrast, after sustained mGlu5 stimulation, both GLAST and GLT-1 activity are reduced (Aronica et al., 2003a). In astrocytes derived from

hSOD1-G93A rats, an established model of ALS, increased expression of mGlu5 receptor mRNA is accompanied by reduced GLT-1 activity and enhanced glutamate-induced excitotoxicity (Vermeiren et al., 2006). Similarly, the accumulation of the glial glutamate and the consequent excitotoxicity described in a mouse model of epilepsy have been related to mGlu5 receptor overexpression in hippocampal astrocytes. Accordingly, the mGlu5 receptor antagonist MPEP, attenuates gliotransmission, preventing neuronal death, with no change of synaptic transmission (Ding et al., 2007).

In the AD APPswe/PS1 transgenic mouse model, high expression of mGlu5 receptor has been described in astrocytes surroundings Aβ plaques, associated to Ca^{++} signaling dysregulation and ATP abnormal release (Shrivastava et al., 2013). As previously described for mGlu1 receptor in neurons exposed to an excitotoxic insult (Spampinato et al., 2012a), astrocytic mGlu5 receptor may activate two opposite pathways: on one side, stimulation of phospholipase C, with ensuing increased intracellular Ca^{++} concentrations, may lead to cell death; on the other hand, however, this effect could be counteracted by alternative activation of the ERK1/2 pathway, through a Homer-dependent mechanism (Paquet et al., 2013). Interestingly, in cultured cortical astrocytes, inflammatory cytokines reduce the expression of mGlu5 receptor (Aronica et al., 2005c; Berger et al., 2012), suggesting a protective adaptation to prevent excitotoxicity (Berger et al., 2012). Furthermore, pharmacological blockade of mGlu5 in astroglial cells prevents motor neurons excitotoxicity (D'Antoni et al., 2011). The inhibition of mGlu5 receptor activity on astrocytes may contribute to the reduction of an inflammatory state in the CNS. Treatment with the mGlu5 receptor antagonist MPEP prevented in fact astrocytic secretion of the inflammatory cytokines IL-6 and IL-8 (Shah et al., 2012).

In cultured microglia, the expression of mGlu1 receptor is barely detectable (Byrnes et al., 2009), but it has been reported *in vivo* in selected microglia cell populations in MS (Klaver et al., 2013). Similarly, the expression of mGlu5 receptor mRNA is low in cultured microglia compared to astrocytes. However, PET imaging studies in animal models exposed to inflammatory stimuli have shown that mGlu5 receptor activation reduced the inflammatory response (Drouin-Ouellet et al., 2011).

In vitro, administration of the non-selective group I agonist DHPG, reduced the number of activated microglia (Farso et al., 2009), while the selective mGlu5 receptor agonist CHPG prevented microglial proliferation induced by LPS (Huang et al., 2018), microglial death induced by OGD (Ye et al., 2017), and the expression of several inflammatory cytokines (Byrnes et al., 2009; Loane et al., 2009; Beneventano et al., 2017). The potential of mGlu5 receptor as a new pharmacological target appears also very interesting in traumatic conditions, such as spinal cord lesions or other traumatic events, where reactive microglia, surroundings the area of the lesion, overexpress mGlu5 receptor. In both TBI models and spinal cord lesions, the delayed CHPG administration, also one month after the traumatic event, reduced the number of reactive microglia and the chronic post-injury inflammation (Byrnes et al., 2012; Wang et al., 2013). In TBI and spinal cord lesion, BBB damage may further activate microglia, due to the access in the CNS of blood-borne proteins such as fibrinogen, that induces microglial phagocytic phenotype and the release of inflammatory cytokines, leading to neurotoxicity (Piers et al., 2011). The BBB in normal conditions prevents the access of fibrinogen and other proteins and immune cells that are present in the blood, but its damage is a common event in traumatic injuries (TBI, SCI), ischemic events and neurodegenerative disorders (Zhao et al., 2015), such as AD, where increased barrier permeability is observed (Spampinato et al., 2017). *In vitro*, exposure of microglia to fibrinogen in the presence of the Glu5 receptor PAM (CDPPB) prevented microglia activation and neuronal toxicity (Piers et al., 2011), further underlying the neuroprotective potential of mGlu5 agonists in reducing neuroinflammation.

(RS)-2-chloro-5-hydroxyphenylglycine may prevent microglial activation by releasing BDNF and inducing expression of its receptor Trkb, as observed in BV2 microglia cells (Ye et al., 2017). Recently it has been demonstrated that microglia, as many other cell types, communicate with the neighbor cells through shedding of microvesicles that may represent a cargo for neuromodulators, cytokines, and microRNA (Verderio, 2013). In BV2 microglia cells, CHPG induced an increased release of microvesicles carrying the inflammatory miRNA146a (Beneventano et al., 2017), suggesting a pro-inflammatory role of mGlu5 receptor. It has also been suggested that LPS binds directly to mGlu5 receptor inducing Ca^{++} oscillations and NF-κB activity, while attenuating TNFα production (Liu et al., 2014). All these data suggest that microglial mGlu5 receptor exerts an ambivalent role in inflammation.

The neuroprotective potential of mGlu5 receptor agonist CHPG in reducing microglia-induced neuroinflammation may be limited by the fact that the drug has only partial selectivity, poor BBB penetration, and induces a rapid receptor desensitization (Homayoun and Moghaddam, 2010). mGlu5 receptor PAMs have been investigated as potential therapeutic agents in neurological disorders (Xue et al., 2014). *In vitro*, exposure of microglia to mGlu5 receptor PAMs has demonstrated a better control in comparison to CHPG in preventing microglia activation after inflammatory insults (Xue et al., 2014). *In vivo* administration of the mGlu5 receptor PAM, VU0360172, prevented neuronal loss in a TBI model in mice by reducing microglia-induced inflammation (Loane et al., 2014). An open question for future drug discovery processes in neurodegenerative disorders remains how to reconcile the protective effects observed with mGlu5 receptor antagonists on astrocytes, in different experimental models of neurodegeneration, with the anti-inflammatory action of mGlu5 receptor PAMs on microglia, as reported in TBI (Xue et al., 2014). Furthermore we cannot forget that, in neurons, mGlu5 receptors physically interact with NMDA receptors playing a permissive role in mechanisms of excitotoxic neuronal death (Bruno et al., 2017). Accordingly, selective NAMs of mGlu5 receptors are consistently neuroprotective in models of PD and AD (Bruno et al., 2017).

As already stated, the expression of group I mGlu receptors in oligodendrocytes is stage dependent. mGlu1 receptor is expressed in the somas of GalC+ oligodendrocytes in prenatal ages and

during the first two postnatal weeks (P3–P14), while later on mGlu1 receptor is localized exclusively at cell processes. mGlu5 receptor shows a similar distribution, although its expression is lower than mGlu1 and it peaks earlier, at P3–P6. A similar pattern is described in human white matter (Jantzie et al., 2010). Both oligodendrocytes and OPC are very sensitive to glutamate mediated toxicity after hypoxia-ischemia (Deng et al., 2003; Fern et al., 2014) and in MS (Macrez et al., 2016). Activation of mGlu1 receptor by DHPG prevented OPC death induced by kainate (Kelland and Toms, 2001; Deng et al., 2004) and non-excitotoxic agents by maintaining the intracellular levels of glutathione and thus reducing oxidative stress (Deng et al., 2004). mGlu5 receptor activation prevented also staurosporine-induced OPC death (Luyt et al., 2006). Starting from this evidence, selective group I mGlu receptor agonists have been studied in periventricular leukomalacia, a condition characterized by OPC damage, that affects the white matter in premature infants after hypoxia-ischemia (Jantzie et al., 2010). Butt et al. (2017) demonstrated that group I receptor agonists can prevent hypoxia-ischemia-induced oligodendrocyte death at all stages of differentiation. Further studies are needed to establish the role of mGlu1 receptor as a new pharmacological target to prevent oligodendrocyte loss in neurodegenerative disorders such as MS, where OPCs are highly vulnerable to excitotoxic damage (Newcombe et al., 2008).

GROUP II mGlu RECEPTORS

Group II includes mGlu2 and mGlu3 receptors, which are coupled to G_i/G_o proteins and have been recently studied as a relevant pharmacological target in neurodegenerative disorders (Bruno et al., 2017). Both mGlu2 and mGlu3 receptors are preferentially localized in the pre-terminal region of axon terminals, where they negatively regulate neurotransmitter release. Only mGlu3 receptor is expressed in astrocytes and is present at all developmental stages (Sun et al., 2013), whereas microglial cells express both mGlu2 and mGlu3 receptors (Geurts et al., 2003). mGlu2/3 receptors levels increase in astrocytes in response to FGF and EGF (Aronica et al., 2003a) and after exposure to pro-inflammatory cytokines (TNFα and IL-1β) (Berger et al., 2012). mGlu3 receptor actively participates in the control of extracellular glutamate by increasing the expression of GLAST and GLT-1 (Gegelashvili et al., 2000; Aronica et al., 2003a; Yao et al., 2005; Zhou et al., 2006). Hence, the use of mGlu3 receptor agonists and/or PAMs has been proposed in the treatment of ALS in which a defect of GLT-1 has been well described (Rothstein et al., 1995; Battaglia et al., 2015). In addition, astrocytic mGlu3 receptors, through activation of MAPK and PI3K pathways, lead to neuroprotection by increasing synthesis and secretion of neurotrophic factors (Bruno et al., 2017), among others, TGF-β1, that prevents both NMDA- and Aβ-induced toxicity on neurons (Bruno et al., 1998; Corti et al., 2007; Caraci et al., 2011) and GDNF. The latter is an established neurotrophic agent for nigral dopaminergic neurons, and has shown neuroprotective and restorative activity in a variety of preclinical models of parkinsonism (Ibanez and Andressoo, 2017). It also protects cultured spinal motor neurons from excitotoxicity (Battaglia et al., 2015). Pharmacological activation of mGlu3 receptor in mice increases GDNF mRNA and protein levels in striatal neurons (Battaglia et al., 2009). Hence, selective mGlu3 receptor enhancers may be effective in slowing neuronal degeneration in different conditions such as ALS (Battaglia et al., 2015) and PD (Bruno et al., 2017).

In this regard, a glial-neuronal interaction mediated by astrocytic mGlu3 receptors seems to play a critical role. Early studies have shown that mGlu2/3 receptors agonists protect cortical neurons against excitotoxic death only in the presence of astrocytes (Caraci et al., 2012; Bruno et al., 2017). Studies carried out in cultured astrocytes from mGlu3(−/−) mice (Corti et al., 2007; Caraci et al., 2011; Battaglia et al., 2015) have clearly demonstrated the key role of astrocytic mGlu3 receptor in mediating the neuroprotective effects of mGlu2/3 receptor agonists. Activation of mGlu3 receptor activity also protects astrocytes from OGD (Ciccarelli et al., 2007) and nitric oxide damage, due to the reduction of cAMP content and consequent activation of PI3K/Akt pathway (Durand et al., 2010, 2013).

mGlu3 receptor might represent a relevant pharmacological target to develop disease-modifying drugs in AD (Caraci et al., 2018a). Although no clear data are available in human AD brains, mGlu3 receptor expression appears reduced in several animal models of AD (Dewar et al., 1991; Cha et al., 2001; Durand et al., 2014; Knezevic and Mizrahi, 2018). When treated with the mGlu2/3 receptor agonist LY379268, astrocytes *in vitro* reduced neuronal Aβ toxicity through the release of neuroprotective factors such as TGF-β1 (Caraci et al., 2011) and BDNF (Durand et al., 2017). TGF-β1 is known to exert anti-inflammatory and neuroprotective effects in experimental models of AD (Chen et al., 2015), and stimulates Aβ clearance by microglia (Tichauer and von Bernhardi, 2012). It also exerts a key role in synaptic plasticity and memory formation promoting the transition from early to late LTP (Caraci et al., 2015). A selective deficit of TGF-β1 signaling has been found in an early phase of AD and appears to critically contribute to neuroinflammation and cognitive decline in AD (Caraci et al., 2018b). Rescue of TGF-β1 signaling represents therefore a new pharmacological strategy to yield neuroprotection in AD. Activation of mGlu3 receptor can positively interfere also with other relevant steps of AD pathogenesis by reducing Aβ production (Durand et al., 2014) or increasing Aβ clearance (Durand et al., 2017). Astroglial mGlu3 receptors stimulate the activity of α-secretase, the enzyme that cleaves APP downstream of the N-terminus domain of $A\beta_{(1-42)}$ (Durand et al., 2014). When exposed to LY379268, astrocytes reduce the levels of β-secretase, while increasing the expression of sAPPα, thereby reducing neurotoxic Aβ. Recently, it has been demonstrated that LY379268 can increase Aβ uptake in astrocytes and microglia, finally promoting Aβ removal from the extracellular space (Durand et al., 2017). The contribution of mGlu3 receptor seems equivocal because Aβ phagocytosis was not prevented by LY2389575, a selective mGlu3 receptor NAM, suggesting that the effects observed after LY379268 stimulation can also involve mGlu2 receptor activation (Durand et al., 2017).

Microglia respond to Aβ with increased glutamate release (Barger and Basile, 2001). Exposure of microglial cells to the active fragment $A\beta_{(25-35)}$ induces also mGlu2 receptor

activation, that can lead to increased neurotoxicity (Taylor et al., 2002, 2005). Activation of mGlu2, but not mGlu3 receptors, promotes in fact a pro-inflammatory and neurotoxic phenotype that releases TNF-α and FAS-L, and enhanced microglial reactivity in response to chromogranin-A, up-regulated in AD (Taylor et al., 2002, 2005).

An open question remains whether activation of microglial mGlu3 receptor can promote the release of TGF-β1, then contributing to the overall neuroprotective activity of LY379268 observed in Aβ-treated mixed neuronal cultures (Caraci et al., 2011).

It is well known that microglial activation plays a central role in the pathogenesis of MS (Strachan-Whaley et al., 2014). Exposure to myelin fragments induces microglia activation *in vitro*, promoting the release of glutamate and TNF-α, followed by neuronal death. Interestingly, activation of microglial mGlu2 receptor exacerbates myelin-evoked neurotoxicity, whilst activation of mGlu3 receptor is protective (Pinteaux-Jones et al., 2008).

Suboptimal neuroprotective effects of orthosteric mGlu2/3 receptor agonists have been observed in animal models of global and focal brain ischemia (Bond et al., 1998; Bond et al., 2000), probably due to the involvement of mGlu2 receptors expressed in neurons (Corti et al., 2007; Motolese et al., 2015; Mastroiacovo et al., 2017). However, the role of microglial mGlu2 receptor in stroke ischemia has not been fully elucidated. mGlu2 and mGlu3 receptors are expressed by microglia in the ischemic penumbra, where apoptotic neuronal death develops slowly, making this area more amenable to therapeutic intervention. Microglial cells mediate neurotoxicity in the stroke penumbra (Kaushal and Schlichter, 2008) and in experimental models of ischemia, it has been demonstrated that glutamate, released by "ischemic" neurons, activates microglia through group II mGlu receptors with the following activation of NF-κB, induction of TNF-α, and subsequent neuronal death (Kaushal and Schlichter, 2008). New studies should be conducted in cultured microglia from mGlu2(−/−) mice to better understand the role of microglial mGlu2 receptor in the pathophysiology of stroke ischemia.

GROUP III mGlu RECEPTORS

The function of group III mGlu receptors in astrocytes has not been fully explored. They are almost undetectable in gray matter of normal human brains (Blumcke et al., 1996; Tang and Lee, 2001), although the expression of mGlu4 receptor, and occasionally of mGlu8 receptor, was described in reactive astrocytes surrounding MS lesions (Geurts et al., 2005) as well as in other pathological conditions (Tang and Lee, 2001; Aronica et al., 2003b). The expression of mGlu4 receptor in astrocytes cultured *in vitro* is still debated. Some studies, but not others (Ciccarelli et al., 1997), reported the expression in primary cortical cultures (**?**), and induction after exposure to LPS (Spampinato et al., 2014). In contrast, mGlu7 receptor subtype is not expressed in glial cells (Ciccarelli et al., 1997; Aronica et al., 2001; Taylor et al., 2003). Of note, stimulation of mGlu7 and mGlu8 receptors may have a role in the differentiation of progenitor cells in the ventral midbrain (Vernon et al., 2011). Stimulation with the group III mGlu receptor agonist L-AP4 reduces in fact the proliferation of fetal mouse neocortical progenitor, and promotes their differentiation toward an oligodendrocytic and astrocytic phenotype (Nakamichi et al., 2008).

One of the principal effects exerted by mGlu4 receptor agonists is the reduction of the inflammatory response. The expression of the chemoattractant chemokine Rantes (CCL5), whose role in neuroinflammation has been well documented (Sorensen et al., 1999), was significantly downregulated when astrocytes were exposed to inflammatory cytokines in the presence of L-AP4 (**?**). This *in vitro* evidence was supported by reduction of the disability score in mice with experimental autoimmune encephalomyelitis treated with l-AP4 (Besong et al., 2002). In astrocyte and oligodendrocyte co-cultures, L-AP4 prompted astrocytic release of TGF-β1, preventing kainate-induced cell death in oligodendrocytes (Spampinato et al., 2014). In contrast, L-AP4 direct treatment on oligodendrocytes was not able to prevent kainate-induced toxicity, but accelerated the differentiation of OPC into mature MBP+ and fully branched oligodendrocytes (Spampinato et al., 2014).

Acting on astrocytes, group III mGlu receptors may also improve glutamate uptake, modulating the expression of both GLT-1 and GLAST. Zhou et al. (2006) reported that L-AP4 prevented neurotoxicity of LPS-treated astrocytes, an effect likely mediated by the increased expression of glutamate transporters. Similar effects were reported in astrocytes exposed to MPTP in the presence of mGlu4 receptor agonists (Yao et al., 2005). In conditions of energy failure, e.g., ischemia, GLT-1 may act paradoxically, running in a reverse mode and thus aggravating the load of glutamate (Rossi et al., 2000; Bonde et al., 2003). Under these conditions, stimulation of mGlu4 receptor may prevent GLT-1 upregulation in reactive astrocytes, thus reducing the aberrant glutamate transport and contributing to neuroprotection (Rodriguez-Kern et al., 2003).

In cultured microglia, the expression of mGlu4, 6 and 8 receptors has been clearly reported (Taylor et al., 2003). In MS patients, mGlu8 receptor was described in the microglial/macrophage line, in particular in the parenchyma and perivascular cuff (Geurts et al., 2003). The overexpression of the receptor in these areas may be induced by the presence of specific cytokines and growth factors released by the environment surrounding the lesions.

As reported (Taylor et al., 2003), agonists acting on group III mGlu receptors prevent microglia activation *in vitro*. The mechanisms involved in these processes were not clarified, but the release of trophic factors from microglia (Conn and Pin, 1997), or reduced glutamate discharge (Taylor et al., 2003) could be claimed. Glutamate may in fact act in a negative feedback loop reducing its own release in inflammatory states (McMullan et al., 2012). Further, *in vitro* exposure of microglia to the mGlu4 receptor PAM, ADX88178, reduced the LPS-induced expression of MHCII and iNOS, while reducing the release of TNFα (Ponnazhagan et al., 2016).

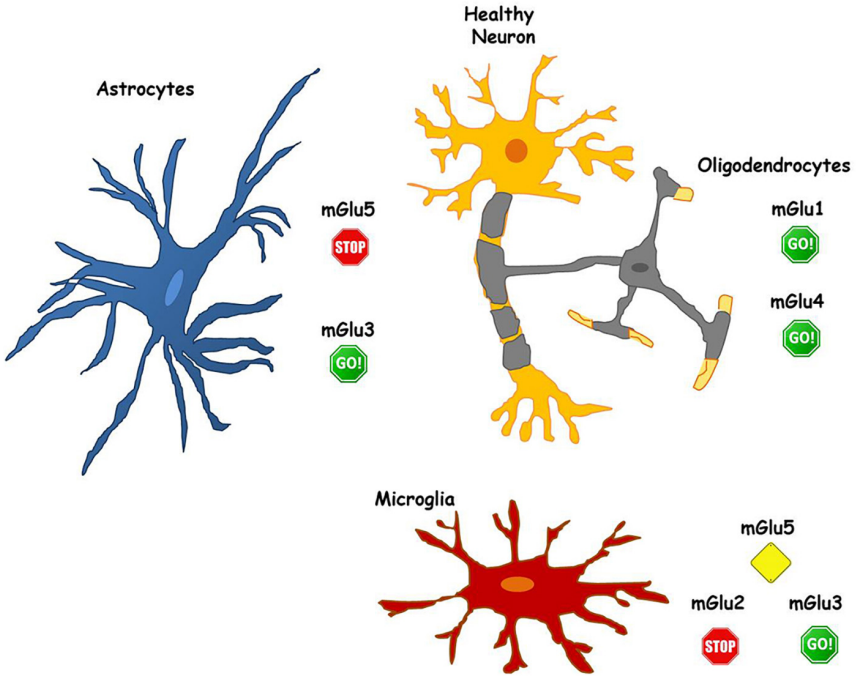

FIGURE 1 | The role of mGlu receptors in different glial cell types. Astrocytes express both mGlu3 and mGlu5 receptors. mGlu3 receptor stimulation initiates mechanisms that induce neuroprotection, while mGlu5 receptor activity promotes neuronal damage. Hence, allowing pharmacological activation of mGlu 3 receptor (GO!) and blocking mGlu5 receptor activity (STOP) in astrocytes could be valuable for the maintenance of neuronal health. Similarly, in microglia, mGlu3 receptor stimulation plays beneficial effects on neurons (GO!), while blockade of mGlu2 (STOP) appears necessary to prevent neurotoxicity. Less defined is the function of glial mGlu5 receptor that, playing a dual role, may be a more complex target for pharmacological intervention (Alert yellow sign). Pharmacological activation of both mGlu1 and mGlu4 receptors, expressed in oligodendrocytes, appear to be neuroprotective (GO! sign).

mGluR4, for its anatomical distribution and function, seems to be an interesting pharmacological target for the treatment of PD. mGluR4 orthosteric agonists have been tested in neurotoxin-based rat models of PD, where they reduced signs of inflammation and the consequent dopamine neuronal loss (Battaglia et al., 2006; Zhou et al., 2006; Betts et al., 2012). These effects were also observed using more potent, selective and orally bioavailable mGlu4 receptor PAMs, such as ADX71743 (Le Poul et al., 2012). The increasing importance of the potential use of mGlu4 receptor agonists in PD relies in their capability to modulate directly neuronal circuits, and as additive effects, to attenuate pro-inflammatory immune mechanisms associated with PD. Accordingly, VU0155041, a mGlu4 receptor PAM, reduces microglia activation in the substantia nigra pars compacta of 6-OHDA-treated rats (Betts et al., 2012).

CONCLUSION

Metabotropic glutamate receptors are highly and diffusely expressed in glial cells. This, on one side, increases the options for therapeutic interventions, but, on the other side, makes even more difficult the possibility to target selectively single receptors to yield neuroprotection. As mentioned above, different mGlu receptors may give rise to contrasting outcomes when activated in neurons or in glial cells or even in different types of glial cells (see **Figure 1**).

mGlu5 receptor agonists for instance, might be detrimental for neuroprotection. On neurons, mGlu5 receptor stimulation has been linked to increased synaptotoxicity in AD and PD models (Bruno et al., 2017). A similar potentiation of neurotoxicity is also observed following activation of mGlu5 receptor in astrocytes. Therefore, the anti-inflammatory effects mediated by the activation of mGlu5 receptor on microglia may be vanished by the effects that mGlu5 receptor agonists could exert acting directly on neurons and/or on astrocytes. However, when considering as a whole the different role of mGlu5 receptor in astrocytes and microglia in neurodegenerative disorders, NAMs of mGlu5 receptor should continue to represent a relevant pharmacological tool to develop new neuroprotective strategies in these diseases, with astrocytes as the main target (see **Figure 1**).

mGlu3 receptor represents a validated pharmacological target to develop disease-modifying drugs in neurodegenerative disorders such as AD, where the development of mGlu3 receptor PAMs might be successful (**Figure 1**). These drugs acting on receptors expressed in glial cells exert a relevant neuroprotective activity in AD models through multiple mechanisms such as the release of neurotrophic factors (TGF-β1, BDNF) and the reduction of Aβ production (Bruno et al., 2017). More specifically, drugs with mGlu2 NAM/mGlu3 PAM activities might be considered excellent candidates for the treatment of

AD. The potential disease-modifying activity of pure mGlu2/3 receptors agonists may be vanished by the detrimental effects of mGlu2 receptor in neurons. Drugs endowed with mGlu2 NAM activity may limit this effect and also cater the potential to restrain microglia-induced neuroinflammation that is consistently found in different neurodegenerative disorders such as AD and PD.

Finally, the effects mediated by mGlu4 receptor expressed either in astrocytes, microglia and oligodendrocytes appear promising for the development of mGlu4 receptor modulators in the treatment of neurodegenerative disorders (**Figure 1**). In this regard, the possibility to prevent neuroinflammatory phenomena with mGlu4 PAMs seems particularly intriguing since the effect exerted on glial cells may be synergized by the modulatory activity shown by mGlu4 receptor agonists on the peripheral immune system (Fallarino et al., 2010; Fazio et al., 2014, 2018).

Moving from the evidence discussed in the present review, we believe that targeting astrocytes and microglia with mGlu3 PAM or oligodendrocytes with mGlu4 PAMs might actually represent a novel pharmacological approach for the treatment of neurodegenerative disorders.

AUTHOR CONTRIBUTIONS

SFS, MAS, and FC wrote the paper. AC and FN contributed to write the paper and revised it critically for important intellectual content.

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REVIEW
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Role of Metabotropic Glutamate Receptors in Neurological Disorders

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Glutamate is a fundamental excitatory neurotransmitter in the mammalian central nervous system (CNS), playing key roles in memory, neuronal development, and synaptic plasticity. Moreover, excessive glutamate release has been implicated in neuronal cell death. There are both ionotropic and metabotropic glutamate receptors (mGluRs), the latter of which can be divided into eight subtypes and three subgroups based on homology sequence and their effects on cell signaling. Indeed, mGluRs exert fine control over glutamate activity by stimulating several cell-signaling pathways *via* the activation of G protein-coupled (GPC) or G protein-independent cell signaling. The involvement of specific mGluRs in different forms of synaptic plasticity suggests that modulation of mGluRs may aid in the treatment of cognitive impairments related to several neurodevelopmental/psychiatric disorders and neurodegenerative diseases, which are associated with a high economic and social burden. Preclinical and clinical data have shown that, in the CNS, mGluRs are able to modulate presynaptic neurotransmission by fine-tuning neuronal firing and neurotransmitter release in a dynamic, activity-dependent manner. Current studies on drugs that target mGluRs have identified promising, innovative pharmacological tools for the treatment of neurodegenerative and neuropsychiatric conditions, including chronic pain.

Keywords: glutamate, metabotropic glutamate receptors, neurodegeneration, neuroinflammation, pain

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INTRODUCTION

Metabotropic Glutamate Receptors (mGluRs): Brain Distribution and Role in Neuroinflammatory and Neurodegenerative Diseases

Glutamate, a non-essential amino acid, is the main excitatory neurotransmitter of the central and peripheral nervous systems (CNS and PNS, respectively; Ferraguti et al., 2008). There are two major types of GluRs: ionotropic and metabotropic. Ionotropic glutamate receptors (iGluRs), such as N-methyl-d-aspartate (NMDA), amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors, are ligand-gated ion channels that stimulate fast excitatory neurotransmission (Dingledine et al., 1999). In contrast, metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors (GPCRs) that have been categorized into three groups based on their signal transduction pathways and pharmacological profiles.

Group I metabotropic receptors, which include mGluR1 and mGluR5, are normally stimulatory and associated with phospholipase C activation and second messengers such as inositol and diacylglycerol production. **Group II** metabotropic receptors include mGluR2 and mGluR3, while **Group III** metabotropic receptors include mGluR4, mGluR6, mGluR7, and mGluR8.

Both Group II and Group III receptors share a major sequence homology (∼70%), and they normally inhibit glutamatergic neurotransmission (Conn and Pin, 1997). In addition, Group II and Group III metabotropic receptors are both negatively coupled to adenylyl cyclase. Elevated levels of mGluR1 have been reported in the neurons of the olfactory bulb, cerebellar cortex, ventral pallidum, globus pallidus, entopeduncular nucleus, lateral septum, magnocellular preoptic nucleus, and thalamic nuclei (Hubert et al., 2001). Their presence is also widespread in cerebellar Purkinje cells and in the mitral/tufted cells of the olfactory bulb. Moreover, notable Group I mGluR expression has been observed in the substantia nigra pars compacta (SNc), globus pallidus, lateral septum, and thalamic relay nuclei (Martin et al., 1992). In addition, the hypothalamus contains more mGlu1β receptors than mGlu1α receptors (Mateos et al., 1998). Previous studies have demonstrated that mGluR1 is associated with the postsynaptic specialization of excitatory synapses due to its subcellular localization, where it seems to be concentrated in the perisynaptic and extrasynaptic areas. Therefore, when concentrations of glutamate are elevated, excess glutamate leaks into the synaptic cleft, leading to activation of mGluRs. Several studies have investigated the role of mGluR1 in the cerebellar cortex, revealing that activation of such receptors is necessary for the stimulation of long-term depression (LTD) of excitatory neurotransmission at parallel Purkinje-fiber cellular synapses. Diacylglycerol is formed following activation of mGluR1; it is then split into 2-arachidonylglycerol (2-AG), the main endocannabinoid species of the CNS, by diacylglycerol lipase (Yoshida et al., 2006). Elevated mGluR5 expression has been observed in the telencephalon, particularly in the cerebral cortex, hippocampus, subiculum, nucleus accumbens, striatum, olfactory bulb, and lateral septal nucleus (Shigemoto et al., 1993; Romano et al., 1995). High expression of mGluR5 has also been observed in the superficial dorsal horn of the spinal cord (Berthele et al., 1999; Jia et al., 1999).

Among the Group II receptors, mGluR2 has been identified in only a few brain regions, such as the olfactory bulb and cerebellar cortex. In addition, mGluR2 is exclusively concentrated in neurons, primarily in the pre-terminal region of axons, far from the sites of neurotransmitter release (Tamaru et al., 2001). Presynaptic mGluR2/mGluR3 can be activated either by a surplus of synaptic glutamate or by glutamate released from astrocytes *via* the cystine–glutamate membrane antiporter (Kalivas, 2009). Modifications to the expression and activity of the cysteine–glutamate antiporter may influence the function of mGluR2 and mGluR3 in brain areas involved in drug dependence.

A key function of presynaptic mGluR2/mGluR3 is to reduce the release of neurotransmitters. Both receptor types are known to play a role in the modulation of synaptic plasticity, particularly in stimulating LTD of excitatory synaptic transmission (Grueter and Winder, 2005; Nicholls et al., 2006; Altinbilek and Manahan-Vaughan, 2009). A specific arrangement of synaptic plasticity has been observed in the mouse olfactory bulb, where stimulation of mGluR2 reduces gamma-aminobutyric acid (GABA)-ergic inhibition of mitral cells. Such inhibition enables the realization of a particular olfactory memory that closely reproduces the memory of the male pheromones that are produced during mating (Hayashi et al., 1993; Kaba et al., 1994). In the CNS, mGluR3 is extensively expressed in the olfactory tubercle, dentate gyrus, cerebral cortex, nucleus accumbens, lateral septal nucleus, striatum, amygdaloid nuclei, cerebellar cortex, and substantia nigra pars reticulata (Tanabe et al., 1993; Petralia et al., 1996; Tamaru et al., 2001). Expression of mGluR3 is observed presynaptically, postsynaptically, and on glial cells (Ohishi et al., 1993; Ferraguti and Shigemoto, 2006).

Group III mGluRs are expressed in the olfactory bulb, lateral reticular nucleus of the medulla oblongata, and pontine nuclei (Duvoisin et al., 1995; Saugstad et al., 1997; Corti et al., 1998). Several signaling pathways containing mitogen-activated protein kinases (MAPK) and PI3-kinase are coupled to the Group III mGluRs, allowing for control of synaptic transmission (Iacovelli et al., 2002, 2004). While mGluR7 is widely expressed in the brain, mGluR6 is not, instead exhibiting limited expression in the retina (Nakajima et al., 1993; Kinoshita et al., 1998). High mGluR7 expression has also been observed in the hippocampus, thalamus, neocortex, amygdala, hypothalamus, and locus coeruleus (Ngomba et al., 2011). Peripherally, mGluR7 is found in the adrenal glands, colon, and stomach, among other regions (Scaccianoce et al., 2003; Julio-Pieper et al., 2010).

Two other Group III receptors, mGlu4 and mGlu8, exhibit restricted expression in the brain (Pilc et al., 2008; Julio-Pieper et al., 2011). Although mGluR4 is primarily found in the cerebellum (Kinoshita et al., 1996; Shigemoto et al., 1997), it has also been observed in other areas, including the cerebral cortex, olfactory bulb, hippocampus, lateral septum, septofimbrial nucleus, striatum, thalamic nuclei, lateral mammillary nucleus, pontine nuclei, and dorsal horn (Fotuhi et al., 1994; Azkue et al., 2001; Corti et al., 2002). Moreover, previous studies have revealed that mGluR4 exhibits widespread peripheral expression in the gastrointestinal tract, pancreas, and adrenal glands (Chang et al., 2005; Sarría et al., 2006), and that such expression is highly concentrated around the active presynaptic area. While mGluR4, mGluR7, and mGluR8 are expressed in neurons, they are also expressed in oligodendrocyte precursor cells and recently formed oligodendrocytes.

Expression of mGluR8 in the CNS has been observed at the presynaptic level in the cerebellum, olfactory bulb, hippocampus, and cortical areas (Ferraguti and Shigemoto, 2006). However, mGluR8 expression has also been observed in peripheral tissues, such as the pancreas and testes (Julio-Pieper et al., 2011). Remarkably, previous studies have suggested that levels of mGluR8 are typically lower than those of mGluR4 and mGluR7 (Niswender and Conn, 2010).

Today, combined treatment approaches are the most attractive therapeutic strategies for numerous disorders, and several recent studies have highlighted the potential of multifunctional drug approaches (Kaiser and Nisenbaum, 2003). Because trauma and neurodegeneration in the CNS are influenced by several factors, multiple therapeutic approaches will likely be more effective than those directed at a single target. Neurons, astrocytes, microglia, oligodendrocytes, endothelial cells, and other circulating immune cells act in response to both

acute and subacute injury and in chronic neurodegeneration. One multifunctional treatment strategy involves targeting mGluRs, which are expressed in several cell types commonly distributed throughout the CNS (Ferraguti and Shigemoto, 2006). Glial cells express both, ionotropic and mGluRs, as well as glutamate transporters. The different and heterogeneous locations of mGluRs in the CNS provide a promising opportunity to investigate drugs that selectively target different receptor subtypes. Several studies have demonstrated that mGluRs are expressed in lymphocytes as well as antigen-presenting cells, such as dendritic cells, microglia, and macrophages (Pacheco et al., 2006; Fallarino et al., 2010). In addition, mGlu5 and mGlu3 receptor activation can independently or cooperatively control several astrocyte functions, such as glutamate transporter activity, including astrocyte–arteriolar and astrocyte–neuronal interactions (Bradley and Challiss, 2012). In astrocytes, mGlu5 is the predominant or exclusive group I mGlu receptor subtype. Both mGluR3 and mGluR5 exert positive and negative influences on cell proliferation, and they are both highly expressed in cultured oligodendrocyte progenitor cells (Aronica et al., 2003). Moreover, mGluRs regulate cell migration, glutamate release, and the induction of the inflammatory phenotype in microglia (Barker-Haliski and White, 2015). Researchers have focused heavily on characterizing the involvement of mGluRs in various immune pathologies, including neuroinflammatory processes, in order to exploit them as novel targets for therapeutic strategies. Inflammatory events occur at different levels in the CNS, relative to those observed in other tissues. First, resident dendritic cells are absent in the CNS parenchyma, along with perivascular macrophages and vascular pericytes, which may shed light on the function of mature dendritic cells in the CNS. Second, stimulation of innate immune cells in the CNS parenchyma (e.g., astrocytes, microglia, and, in some regions, mast cells) may be reduced even under physiological conditions (Skaper et al., 2012). Moreover, the extravasation of immune cells and molecules towards the inflamed area—a process that is necessary for the activation of complement cascades and sustaining the immune response—is critical for the inflammatory response of the whole organism. Nevertheless, the blood–CNS barrier reduces the permeability of CNS microvessels, decreasing the magnitude of the inflammatory reaction. Only activated T-cells can penetrate the blood–CNS barrier, but they do not elicit an efficient (Patel et al., 2005) reaction to inflammation similar to that observed in peripheral tissues, where dendritic cells play a role in the adaptive immune response (Melchior et al., 2006). Consequently, the CNS reacts to inflammatory events when these events exert a direct effect on the CNS (i.e., in the case of pathogens and tissue damage, and when the inflammatory events are so severe that infiltrating T-cells are involved). In this way, neuroinflammation differs from inflammatory reactions that occur in other tissues, and can be thought to reflect the response of the CNS to altered homeostasis. This response is primarily mediated by the contribution of one or two cell systems: the glia of the CNS and the lymphocytes, monocytes, and macrophages of the hematopoietic system (Stoll and Jander, 1999). Neuroinflammation can be elicited by infection, autoimmunity, and toxins, but also by neurogenic factors such as noxious stimuli or psychological stress. However, extended neuroinflammation exceeds the limits of physiological control, resulting in harmful outcomes such as the stimulation of pro-inflammatory signaling pathways, increase doxidative stress, and the death of neighboring neurons. Neuroinflammation typically affects the severity and progression of neurodegenerative and psychiatric disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS), stroke, and others (Lyman et al., 2014). Several cytokines such as interleukin-23 (IL-23), IL-12 (Bennett, 2013), IL-1b, and IL-6 affect neurodegenerative processes by attacking leukocytes, thus resolving neuroinflammation. Previous studies have demonstrated that different cell types such as oligodendrocytes, microglia, astrocytes (Domingues et al., 2016), both local and circulating lymphocytes, different dendritic cell subsets (Colton, 2013), and endothelial cells (Combes et al., 2012) are involved in neuroinflammation. Remarkably, several mGluR subtypes are expressed in these subtypes, both under stable conditions and during immune activation (Pacheco et al., 2007), suggesting that mGluRs play a role in regulating dissimilar immune responses in the CNS. The mechanisms by which mGluRs modulate immune responses are dependent on the specific subtype of mGluR that is implicated, and on the subset of targeted immune cells that bind the receptor. Normally, mGluR stimulation plays similar roles in the nervous and immune systems by theoretically responding to the negative effects of glutamate (Boldyrev et al., 2005). Indeed, mGluRs trigger widespread activation of various cell-signaling pathways in the CNS, suggesting that they are involved in some physiological and pathological processes associated with neurodegenerative disorders. Due to its potential roles in the pathophysiology of acute and chronic neurodegenerative diseases, glutamate has received much attention. Excess of glutamate is knowingly associated with excitotoxicity. Thus, the elevation in glutamate released from neuronal cells may induce acute neurodegeneration in both traumatic brain injury and cerebral ischemia (Ishikawa, 2013). Stimulation of AMPA, N-methyl-d-aspartate (NMDA), kainate, and Group I metabotropic receptors is involved in the neurotoxic processes underlying neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), motor neuron disease (MND), HD, AD, and PD. In status epilepticus, neuronal death is strictly correlated with NMDA receptor activation; whereas both, NMDA and AMPA receptors, are linked to the degeneration of neuronal tissue in cerebral ischemia.

mGluRs IN AD

AD is a progressive neurodegenerative disease that represents the main cause of dementia. Patients with AD experience memory loss associated with cognitive decline and motor fluctuations (Goedert and Spillantini, 2006). Due to increases in the number of older adults in the population, approximately 36 million people have been diagnosed with AD worldwide—a situation that is expected to double by 2050. Characterized by massive loss of synapses and neuronal death, AD affects approximately 10% of individuals over the age of 65, and approximately

40% of people over the age of 80 (Palop et al., 2006). AD progression disturbs brain areas involved in cognitive functions, such as the hippocampus, entorhinal and cerebral cortices, and ventral striatum. To date, treatments for AD provide provisional symptomatic relief only, and there is currently no cure or method for slowing disease progression. AD is associated with extracellular plaques that largely consist of beta-amyloid peptide (Aβ) aggregates (Glenner and Wong, 1984) and aberrantly phosphorylated tau, a microtubule-associated protein (Grundke-Iqbal et al., 1986). The protein Aβ, derived from amyloid precursor protein (APP) is the main constituent of the amyloid plaques. The need for drugs that can slow the progression of the pathological events that lead to synaptic dysfunction and neurodegeneration in AD is urgent. To date, the European Union (EU) has officially approved only four drugs for the treatment of AD (three cholinesterase inhibitors and memantine); however, none of these drugs has been shown to significantly modify disease activity. However, Group I mGluR agonists have demonstrated both neuroprotective and neurotoxic effects in *in vitro* and *in vivo* models of neurodegeneration (Nicoletti et al., 1999). Both Group II and Group III mGluRs are principally located at the levels of the presynaptic terminal in GABAergic and glutamatergic neuronal cells. Thus, activation of these receptors may decrease glutamate release (Cartmell and Schoepp, 2000). *In vivo* and *in vitro* studies have revealed that activation of Group III mGluRs exerts neuroprotective effects (Bruno et al., 2000). One *in vivo* study reported that treatment with low doses of the Group III mGluR agonist (+)-4-phosphonophenylglycine (PPG) exerts neuroprotective effects in wild-type mice, but not in mGluR4-knockout mice (Bruno et al., 2000). These results suggest that activation of mGluR4 is essential for neuroprotection. In addition, several studies have reported that Group III mGluR orthotropic agonists, such as L-AP4 and L-SOP, play neuroprotective roles in models of Aβ toxicity or excitotoxicity (Winkler et al., 1995; Bruno et al., 1996). In fact, numerous reports have indicated that discriminatory damage to cholinergic neurons in the basal forebrain (BF) is one of the most reliable modifications linked with AD at the initial stage of the disease (Winkler et al., 1995). Because it lowers NMDA levels, activation of mGluR7 protects BF neurons against such damage, thereby diminishing excitotoxicity (Gu et al., 2014). Furthermore, there is evidence that Aβ oligomers cause synaptotoxic effects at NMDA receptors (Malinow, 2012), which may represent the cause of cognitive dysfunction in AD. Despite such progress, precisely how mGluR signaling contributes to AD remains to be elucidated. Further *in vivo* studies using mGluR agonists and antagonists are required in order to determine whether targeting mGluRs is an effective pharmacological strategy for the treatment of AD. Such studies should aim to determine the role of mGluRs in various brain functions and neurological disorders in an effort to identify suitable treatment options.

mGluRs IN PD

PD represents the second most common neurodegenerative disease worldwide. The key feature of PD is selective loss of dopaminergic neurons in the SNc, leading to decreased dopamine levels in the striatum (Frisina et al., 2009). When degeneration surpasses 50%, diminished dopamine triggers the usual symptoms of the disease: postural instability, resting tremor, and hypokinesia (Lee and Liu, 2008). Moreover, degeneration of dopamine neurons in the SNc leads to increased glutamatergic activity in the subthalamic nucleus (STN), aggravating the motor symptoms of PD (Delong and Wichmann, 2015). Conventional PD therapy consists of the administration of 3, 4-dihydroxyphenylalanine (L-DOPA), which aims to enhance motor function by increasing dopamine levels in the striatum (Schapira et al., 2006). While L-DOPA management is the gold-standard therapy for PD, chronic L-DOPA use is associated with a harmful "on-off" syndrome, a clinical state known as L-DOPA-provoked dyskinesia (LID; Lundblad et al., 2004). Recent studies have indicated that LID is caused by dysfunctional neuronal plasticity in the striatum due to the imbalance between glutamate and dopamine signaling (Picconi et al., 2012). Thus, targeting glutamate receptors may aid in the treatment of LID symptoms.

The first attempts to pharmacologically oppose glutamate hyperactivity involved the use of iGluR antagonists. While iGluRs exert adequate antiparkinsonian activity in preclinical models, they have been associated with debilitating side effects in humans, decreasing their applicability in clinical settings. Because of their modulatory role on glutamatergic transmission, mGluRs provide an alternative pathway for regulating increased glutamatergic transmission in the basal ganglia (Paoletti, 2011). Positive allosteric modulators (PAMs) of GluR4 have been proposed for the symptomatic management of PD. Stimulation of mGluR4 inhibits GABAergic discharge at synapses between striatal projection neurons and neurons of the globus pallidus (GPext), thus limiting the activity of the indirect pathway (Conn et al., 2005). Furthermore, mGlu4 PAMs have been shown to reduce motor symptoms in animal models of PD (Niswender and Conn, 2010). Interestingly, additional studies have demonstrated that mGlu4 PAMs exert protective effects against nigrostriatal damage induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice and 6-hydrxydopamine in rats (Betts et al., 2012). Therefore, mGluR4 PAMs may play a role as neuroprotective agents in PD. However, unlike mGluR5 PAMs, they are not believed to exert important therapeutic activity on LID (Ribeiro et al., 2014). Although selective mGlu5 receptor PAMs have been associated with antipsychotic activity, they may induce neurotoxicity in brain regions with high mGlu5 expression, such as the auditory cortex and hippocampus.

Recent research has indicated that the mGluR4 PAMVU0364770 enriches the motor response to a subthreshold dose of L-DOPA, but does not exert anti-dyskinetic activity (Iderberg et al., 2015). Another mGluR4 PAM, Lu AF21934, has been shown to reduce the incidence of LID, but not the severity. Using specific pharmacological tools, previous authors (Conn et al., 2005) reported that mGlu4 homodimers are presynaptically concentrated in the GPext, whereas mGlu2/mGlu4 heterodimers are expressed in the corticostriatal terminals. However, it remains to be determined whether the neuroprotective effects

of mGluR4 PAMs, which are selective for homodimers, vary from those of PAMs that are selective for heterodimers. Adverse effects including dizziness and hallucinations have been reported, necessitating further clarification before clinical strategies can be developed. Additional research has indicated that mGlu2 and mGlu3 receptors do not exert beneficial effects on motor symptoms in animal models of PD: while the selective mGlu2/3 receptor agonist LY379268 improves rotarod performance in animal models of the disease, it does not modify akinesia in 6-OHDA-lesioned rats and may even worsen motor symptoms (Johnson et al., 2009). Group II mGluR agonists may be more appropriate for the treatment of neuropsychiatric symptoms associated with PD (Han et al., 2006).

Group III mGluRs (i.e., mGluR 4, mGluR7, and mGluR8) are expressed in GABAergic and glutamatergic terminals in the basal ganglia. Because these three mGluRs are expressed at the presynaptic level and are coupled to Gi/o, agonists or PAMs for such receptors can inhibit the release of both glutamate and GABA in PD. Moreover, Betts et al. (2012) revealed that allosteric potentiation of mGluR4 in the SNc diminished levels of inflammatory markers, improved motor deficits, and attenuated loss of dopaminergic neurons in a 6-OHDA rat model of PD. Several other studies have demonstrated that PAMs for this receptor can reverse both akinesia and catalytic disease caused by haloperidol, in addition to enhancing motor stimulation by L-DOPA in 6-OHDA-lesioned rats (Le Poul et al., 2012). Along with mGluR4, mGluR7 is particularly expressed at the level of the basal ganglia, where presynaptic actions at these receptors inhibit the release of glutamate. Thus, these receptors may represent targets for reducing excessive synaptic activation in PD. However, due to the dearth of selective ligands, the precise role of mGluR7 in PD remains unknown. To date, the first selective PAM for mGluR7 is AMN082, which exhibits modest antiparkinsonian effects (Mitsukawa et al., 2005). As with mGluR7, the lack of selective agonists for mGluR8 limits our ability to study its potential benefits in PD (Broadstock et al., 2012).Recent studies have demonstrated that the orthosteric agonist (S)-3, 4-dicarboxyphenylglycine (DCPG) exerts no influence in rodent models of PD (Broadstock et al., 2012). However, Johnson et al. (2013) reported that DCPG decreases haloperidol-induced catalepsy. The authors further reported that DCPG decreases reserpine-induced akinesia in a protracted, but not acute, 6-OHDA rodent model.

In summary, accumulating evidence suggests that targeting mGluRs can aid in managing motor symptoms and LID in PD. Thus, further preclinical and clinical studies that demonstrate the efficacy of agonists, antagonists, PAMs, and negative allosteric modulators (NAMs) for all mGluR types are critical in advancing therapeutic strategies for PD.

mGluRs IN HD

HD is an autosomal dominant neurodegenerative disease associated with the presence of polyglutamine, which is localized in the amino-terminal region of the huntingtin protein (htt). Specifically, this pathology is represented by a single genetic mutation that promotes the development of the disease in animal models with genetic modifications that summarize the traits of HD (Pouladi et al., 2013). HD is associated with several symptoms, including the loss of cognitive function, involuntary body movements and chorea, psychiatric disturbances, and death (Li and Li, 2004). Among the possible mechanisms, many studies have focused on the mutation of htt as the cause of gradual neuron loss in the neocortical regions and caudate-putamen in patients with HD.

Previous studies have indicated that inhibition of presynaptic glutamate release *via* activation of both Group II and Group III mGluRs may attenuate the processes associated with excitotoxicity in patients with HD. Group II and Group III mGluRs, particularly those positioned at corticostriatal presynaptic terminals, can mediate negative feedback control for glutamate release (Calabresi et al., 1999). Treatment with LY379268 (1.2 mg/kg VO) has been reported to increase survival time and decrease early pathological hyperactivity in a transgenic mouse model of HD (e.g., R6/2 mouse); however, such treatment does not improve rotarod performance or htt intranuclear inclusions (Schiefer et al., 2004). However, subcutaneous administration of LY379268 at 20 mg/kg was associated with positive effects in R6/2 mice, including increased survival time, improved rotarod performance, normalization of locomotor performance, and a 20% decrease in neuronal loss in both the cortex and striatum. Nevertheless, LY379268 was unable to modify the frequency or size of htt aggregates (Schiefer et al., 2004). Another study reported that brain-derived neurotrophic factor (BDNF) expression increases in layer 5 of the motor cortex following administration of LY379268, indicating that activation of mGluR2/3 may counteract neuronal cell death by increasing or diminishing levels of BDNF.

In summary, activation of mGluR2/mGluR3 may counteract the release of glutamate and diminish excitotoxicity. However, the roles of mGluR1/mGluR5 in regulating neuronal death remain to be clarified. Partial activation of these receptors, as well as activation of mGluR2/mGluR3, excites neuroprotective cell-signaling pathways, stimulating increases in BDNF expression. Such effects may be associated with improvements in the symptoms of HD (Li and Li, 2004).

mGluRs IN CHRONIC STRESS-RELATED DISORDERS

Depression and anxiety are psychiatric conditions related to chronic stress, and they are classified as important public health issues. The etiologies of these disorders are complex, and psychosocial stressors are among the most debated risk factors. Considering the significance of glutamate in the brain, pharmacological interventions for these disorders should target excesses in glutamate transmission while leaving normal glutamatergic transmission unaltered. Pharmacological modulation of mGluR subtypes may allow for such modification (Bergink et al., 2004). The functional diversity and distribution of the different mGluR subtypes may allow for selective

targeting of individual receptor subtypes, which may in turn lead to the development of novel strategies for the treatment of emotional disorders. Preclinical data have suggested that ligands for mGluR subtypes can aid in the management of mood disorders such as depression and anxiety. Furthermore, selective mGluR ligands have begun to show promise in clinical trials, with some compounds exhibiting outstanding clinical efficacy.

Among the stress-related psychiatric conditions, major depressive disorder (MDD), anxiety, and drug abuse are significant health concerns worldwide (Cryan and Holmes, 2005). These pathologies are very complex, and chronic psychosocial stressors have been recognized as the greatest risk factors associated with these conditions (Cryan and Holmes, 2005). Research has revealed strong comorbidity between depression/mood disorders and anxiety (Cortese and Phan, 2005): approximately half of patients with anxiety also meet the criteria for MDD. Both disorders are characterized by disproportionate excitability within crucial brain circuits. The L-glutamate system, considered the primary excitatory neurotransmitter system within the circuits of emotion and cognition, plays a prominent role in the etiopathology and persistence of disorders related to mental health. One study using human data has linked dysfunction in the L-glutamate system to the pathogenesis of psychiatric conditions (Cortese and Phan, 2005). In fact, alterations in glutamate levels have been found in the cerebrospinal fluid (CSF), plasma, and brains of patients with mood and anxiety disorders. Postmortem findings have confirmed these data, revealing that patients with depression and bipolar disorder exhibit substantial increases in glutamate levels in both the frontal and dorsolateral prefrontal cortex, respectively (Lan et al., 2009). Moreover, clinical neuroimaging data have consistently reported volumetric alterations in brain areas where glutamatergic neurons predominate, such as the amygdala, hippocampus, and numerous cortical regions (Lorenzetti et al., 2009). In addition, expression of mGlu2Rs in the hippocampus has been strongly correlated with the mechanisms underlying resilience (or non-resilience) to stress, which lie at the core of the pathophysiology of MDD and other stress-related disorders (McEwen et al., 2015).

mGluRs IN THE PHYSIOLOGY OF STRESS

Group III mGluRs have received less attention than Group I and Group II mGluRs, likely due to the lack of selective and brain-penetrant pharmacological tools (Semyanov and Kullmann, 2000). However, Group III mGluRs are thought to be implicated in several psychological disorders and physiological conditions due to their role in regulating glutamatergic and GABAergic neurotransmission (Semyanov and Kullmann, 2000). Domin et al. (2014) demonstrated that intraventricular injection of the Group III mGluR agonist (1S,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-I) can induce antidepressant- and anxiolytic-like effects. In particular, such anxiolytic effects have been observed in stress-induced hyperthermia (SIH), elevated plus maze (EPM) and Vogel conflict tests. Moreover, the antidepressant-like effects of ACPT-I have been observed in mice subjected to the forced swim test (FST; Domin et al., 2014). Klak et al. (2007) reported that combined treatment with the mGlu4-selective PAM 7-hydroxyimino-N-phenyl-1,7 adihydrocyclopropa[b]chromene-1a-carboxamide (PHCCC) and a non-effective dose of ACPT-1 exerts antidepressant-like effects. Subsequent studies have demonstrated that local ministration of PHCCC to the basolateral amygdala exerts dose-dependent anti-conflict effects in rats subjected to the Vogel conflict test. These results indicate that positive allosteric modulation of mGlu4Rs may represent an alternative therapeutic strategy for the treatment of state anxiety (Stachowicz et al., 2004).

Recent data have further revealed that (1R,2S)-2-[(3, 5-dichlorophenyl)carbamoyl]cyclohexane-1-carboxylic acid (VU0155041), an mGlu4 PAM, exerts anxiolytic effects in animals subjected to the elevated-zero maze. Furthermore, the novel mGlu4 PAMs (1S,2R)-2-[(aminooxy)methyl]-N-(3, 4-dichlorophenyl)cyclohexane-1-carboxamide (Lu AF21934) and 4-methyl-N-[5-methyl-4-(1H-pyrazol-4-yl)-1,3-thiazol-2-yl]pyrimidin-2-amine (ADX88178) have been shown to promote anxiolytic effects in acute rodent models [e.g., stress-induced hyperthermia (SIH), four-plate test (FPT) and marble-burying test (MBT)], and to be effective in several PD models (Kalinichev et al., 2014). Remarkably and reciprocally, mGlu4-deficient mice exhibit increased anxiety in acute models (i.e., open-field text and elevated-zero maze), as well as decreased sensorimotor function in the rotarod test. Such data suggest that mice exhibited improvements in amygdala-dependent cued-fear conditioning (Davis et al., 2013). In accordance with these results, additional studies have reported that mice lacking mGluR8 exhibit higher levels of anxiety than control animals. Moreover, mice exposed to new, aversive environments exhibited greater neuronal activation in stress-related brain areas (Linden et al., 2003). These data suggest that mice deficient in mGluR4 or mGluR8 exhibit improved reactivity to stressors.

The stimulation of mGluR8 with the selective agonist (S)-3, 4-DCPG diminishes innate anxiety levels in the open-field and EPM tests, as well as the expression of contextual fear, without disturbing processes associated with cued fear (Fendt et al., 2013). An mGlu8 receptor-preferring agonist, 2-amino-2-(4-phosphonophenyl)acetic acid (RS-PPG), provokes dose-dependent antidepressant-like effects in the FST following central administration, while the mGlu8-selective PAM 2-[(4-bromophenyl)methyl-sulfanyl]-N-(4-butan-2-ylphenyl) acetamide (AZ12216052) decreases levels of anxiety in both the EPM and open-field tests (Duvoisin et al., 2010). Over the last several decades, preclinical and clinical studies have provided encouraging data, revealing that the glutamatergic system of the brain plays a key role in the physiology of psychiatric disorders. Unfortunately, very few studies have investigated the contribution of the glutamatergic system to the pathophysiology of chronic psychosocial stress (Hammen, 2005). For these reasons, current drug discovery efforts targeting mGluRs have focused on identifying pharmacological agents that can effectively treat psychiatric disorders (Mercier and Lodge, 2014).

mGluRs AND PAIN

Due to the concurrence of different types of pain, chronic pain is a complex syndrome that remains difficult to treat. Nevertheless, mGluRs may represent suitable targets for counteracting the nociceptive and persistent forms of pain. Previous studies have established the role of mGluRs, expressed at peripheral and brain area associated with pain modulation, in efficiently reducing pain hypersensitivity. Such studies have demonstrated that mGluRs control the perception of physiological pain, and that they are associated with the development of peripheral and central pain (Chiechio and Nicoletti, 2012).

Over the last several decades, research has demonstrated that mGluRs represent promising targets in the treatment of chronic pain. For example, these studies have revealed that pain hypersensitivity is efficiently controlled be either blocking Group I mGluRs or activating both Group II and III mGluRs. These effects can be achieved using orthotropic ligands that can block or activate specific mGluR subtypes, or using allosteric ligands that positively (PAM) or negatively (NAM) regulate mGluR functions (Govea et al., 2012). In this review article, we focus on the role of Group III mGluRs in the treatment of chronic pain.

Group III mGluRs are expressed throughout the pain neuraxis, from the peripheral nerves to the CNS. Research has indicated that mGluR8 is expressed in unmyelinated fibers of the digital nerves, where they adversely modify the activity of transient receptor potential vanilloid 1 (TRPV1) receptors on nociceptors by inhibiting the activity of adenylyl cyclase (Govea et al., 2012). Several studies have also demonstrated that intraplantar injection of a Group III mGluRs agonists, such as L-AP4, diminishes the hyperalgesia triggered by the TRPV1 agonist capsaicin (Govea et al., 2012). Similar to Group II mGluRs, peripheral Group III mGluRs have been implicated in the management of hyperalgesia after inflammatory states. In one study, *in situ* treatment with L-AP4, a Group III mGluR agonist, reduced hyperalgesia in a carrageenan-induced model of arthritic pain in the knee joint (Lee et al., 2013). Moreover, stimulation of Group III mGluRs in the dorsal horn of the spinal cord reduces the shooting pain sensation generated by second-order neurons by monitoring excess glutamatergic transmission in models of both neuropathic and inflammatory pain (Zhang et al., 2009).

Then GluR4 subtype is expressed on the presynaptic terminals of C-fibers and spinal neuron terminals in inner laminae II of the dorsal horn. Vilar et al. (2013) reported that stimulation of mGluR4 in the dorsal horn inhibits the development of both neuropathic and inflammatory pain by decreasing glutamatergic transmission. The mGluR7 subtype has also been discovered in the presynaptic terminals of sensory neurons in laminae I and laminae II of the dorsal horn. Interestingly, mGluR7 does not appear to play a relevant role in chronic pain, as intrathecal administration of mGluR7 PAMs does not diminish hyperalgesia in a model of neuropathic pain (Wang et al., 2011). Moreover, previous studies have revealed that Group III mGluRs are significantly expressed in the supraspinal region in conditions associated with pain. Nevertheless, mGluR7 and mGluR8 appear to exert opposing effects in the periaqueductal gray (PAG), amygdala, and rostral ventromedial medulla (RVM). Many authors have reported that systemic activation of both mGluR7 and mGluR8 is efficient in decreasing neuropathic and inflammatory pain; moreover, local stimulation of mGluR7 within the PAG and amygdala intensifies pain, while mGluR7 blockade reduces both inflammatory and neuropathic pain. Stimulation of mGluR8 in the amygdala, PAG, dorsal striatum, and RVM has also been reported to reduce inflammatory and neuropathic pain (Marabese et al., 2007b). Taken together, these findings indicate that Group III mGluR ligands may inhibit pain hypersensitivity in patients with chronic pain. These results are particularly significant for mGluR4, since activation of such receptors in the spinal cord decreases the perception of chronic pain without affecting normal pain perception.

Few studies have investigated the impact of systemic administration of Group III mGluR agonists or antagonists on pain management. Systemic administration of the mGlu8 receptor agonist (S)-3,4-DCPG results in formalin-induced nocifensive behaviors in models of carrageenan-induced mechanical allodynia and thermal hyperalgesia, and in the first stage of neuropathic pain (Marabese et al., 2007a). Research regarding selective mGluR7 NAMs has revealed that mGluR7 plays a key role in physiological and pathological pain conditions. *In vivo* studies of the mGluR7 NAM 6-(4-methoxyphenyl)-5-methyl-3-pyridinyl-4-isoxazolo[4,5-c]pyridin-4(5H)-one (MMPIP) have reported that negative allosteric alteration of the mGluR7 worsens cognitive performance in both radial arm maze tasks and object recognition tests, in addition to reducing social interaction. Additional studies have indicated that MMPIP does not affect depression- or anxiety-like behaviors, motor coordination, sensorimotor gating, seizure threshold, or nociception in healthy rats and mice (Hikichi et al., 2010). Subcutaneous administration of MMPIP has been found to trigger sensory and affective/cognitive symptoms of neuropathic pain in a spared nerve injury model of neuropathic pain, although such treatment exerted no effect in control mice. Palazzo et al. (2015) demonstrated that alterations in receptor expression in supraspinal areas such as the dorsal raphe, basolateral amygdala, PAG, hippocampus, and prelimbic cortex—which are observed in neuropathic pain—may be required for MMPIP efficacy. Among the selective mGluR7 NAMs, 7-hydroxy-3-(4-iodophenoxy)-4H-chromen-4-one (XAP044) inhibits long-term potentiation in brain tissues enclosing the lateral amygdala in wild-type mice, but not in mGluR7-knockout mice, suggestive of XAP044-specific functions for mGluR7 (Gee et al., 2014). Palazzo et al. (2015) further demonstrated that subcutaneous treatment with XAP044 can modify both mechanical allodynia and anxiety- and depression-like behaviors in mice with neuropathic pain. Subcutaneous administration of another selective mGluR7 NAM, (+)-6-(2,4-dimethylphenyl)-2-ethyl-6,7-dihydrobenzo [d]oxazol-4(5H)-one (ADX71743), in rodents leads to anxiolytic-like effects in the EPM and MBTs. This selective mGluR7 NAM has been reported to decrease amphetamine-provoked hyperactivity without altering locomotor activity under physiological conditions (Kalinichev et al., 2013).

CONCLUSION

Knowledge regarding mGluRs has improved exponentially over the last several years. Research has uncovered new mechanisms of action and ligands for these receptors, which may allow for the development of novel therapeutic strategies for neuroinflammatory diseases associated with brain excitability. The discovery of new compounds has encouraged the development of selective tools, some of which can be implemented in clinical practice. Moreover, the diversity and heterogeneous distribution of mGluR subtypes in the brain may allow for the targeting of specific mGluR subtypes implicated in different functions of the CNS, which may in turn aid in the development of novel treatment strategies for psychiatric and neurological disorders, including depression, anxiety, chronic pain, AD, and PD.

Treatments targeting iGluRs in the CNS have failed due to multiple side effects, including cognitive and motor impairment. In general, targeting glutamatergic neurotransmission *via* the modulation of mGluRs holds great promise for the management of several CNS diseases, with the potential for fewer side effects. Drugs targeting mGluRs achieve their therapeutic effects by reducing excitatory drive, either *via* antagonism of Group I mGluRs or activation of Group II and III mGluRs (one exception is represented by the use of mGluR5 PAMs for the management of cognitive deficits linked with schizophrenia).

In clinical practice, the results of targeting mGluRs will depend on whether the targeted glutamatergic pathways are directly or indirectly linked to the pathological condition of interest. Thus, clinical efficacy may depend on indirect potentiation of GABAergic, dopaminergic, or other neurotransmitter systems. Novel therapeutic approaches may benefit from selective targeting of multiple mGluRs. For example, mixed Group I antagonism/Group II or III agonism may be the key to developing effective treatment strategies for some disorders. However, few clinical studies have supported the therapeutic benefit of mGluR modulation in the management of psychiatric and neurological disorders, with the exception of a positive clinical trial for the mGluR2/3 agonist LY2140023 in the treatment of schizophrenia. Further clinical studies are required to determine whether targeting of multiple mGluRs can be effective in human patients with neuropsychiatric and neurological disorders.

AUTHOR CONTRIBUTIONS

RC and DI wrote this review article. SC edited and revised it.

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