January 2016

mGluR2/3 agonist LY379268 rescues NMDA and GABAA receptor level deficits induced in a two-hit mouse model of schizophrenia

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Abstract

Rationale An imbalance of excitatory and inhibitory neurotransmission underlies the glutamate hypothesis of schizophrenia. Agonists of group II metabotropic glutamate receptors, mGluR2/3, have been proposed as novel therapeutic agents to correct this imbalance. However, the influence of mGluR2/3 activity on excitatory and inhibitory neurotransmitter receptors has not been explored. Objectives We aimed to investigate the ability of a novel mGluR2/3 agonist, LY379268, to modulate the availability of the excitatory N-methyl-d-aspartate receptor (NMDA-R) and the inhibitory gamma-aminobutyrate-A receptor (GABAA-R), in a two-hit mouse model of schizophrenia. Methods Wild type (WT) and heterozygous neuregulin 1 transmembrane domain mutant mice (NRG1 HET) were treated daily with phencyclidine (10 mg/kg ip) or saline for 14 days. After a 14-day washout, an acute dose of the mGluR2/3 agonist LY379268 (3 mg/kg), olanzapine (antipsychotic drug comparison, 1.5 mg/kg), or saline was administered. NMDA-R and GABAA-R binding densities were examined by receptor autoradiography in several schizophrenia-relevant brain regions. Results In both WT and NRG1 HET mice, phencyclidine treatment significantly reduced NMDA-R and GABAA-R binding density in the prefrontal cortex, hippocampus, and nucleus accumbens. Acute treatment with LY379268 restored NMDA-R and GABAA-R levels in the two-hit mouse model comparable to olanzapine. Conclusions We demonstrate that the mGluR2/3 agonist LY379268 restores excitatory and inhibitory deficits with similar efficiency as olanzapine in our two-hit schizophrenia mouse model. This study significantly contributes to our understanding of the mechanisms underlying the therapeutic effects of LY379268 and supports the use of agents aimed at mGluR2/3.

Disciplines

Medicine and Health Sciences

Publication Details

Engel, M., Snikeris, P., Matosin, N., Newell, K. Anne., Huang, X. & Frank, E. (2016). mGluR2/3 agonist LY379268 rescues NMDA and GABAA receptor level deficits induced in a two-hit mouse model of schizophrenia. Psychopharmacology, 233 (8), 1349-1359.

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Original investigation

mGluR2/3 agonist LY379268 rescues NMDA and GABAA receptor level deficits induced in a two-hit mouse model of schizophrenia

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Acknowledgments
This work was supported by the Schizophrenia Research Institute, utilising infrastructure funding from the NSW Ministry of Health. LY379268 was kindly gifted by Eli Lilly & Co (Indianapolis, USA). The funding bodies had no role in the study design, data collection and publication decisions.

Abstract

Rationale An imbalance of excitatory and inhibitory neurotransmission underlies the glutamate hypothesis of schizophrenia. Agonists of Group II metabotropic glutamate receptors, mGluR2/3, have been proposed as novel therapeutic agents to correct this imbalance. However, the influence of mGluR2/3 activity on excitatory and inhibitory neurotransmitter receptors has not been explored.

Objectives We aimed to investigate the ability of a novel mGluR2/3 agonist, LY379268, to modulate the availability of the excitatory *N*-methyl-D-aspartate receptor (NMDA-R) and the inhibitory gamma-aminobutyrate-A receptor (GABAA-R), in a two-hit mouse model of schizophrenia.

Methods Wild type (WT) and heterozygous neuregulin 1 transmembrane domain mutant mice (Nrg1 HET) were treated daily with phencyclidine (10mg/kg ip) or saline for 14 days. After a 14 day washout, an acute dose of the mGluR2/3 agonist LY379268 (3mg/kg), olanzapine (antipsychotic drug comparison, 1.5mg/kg) or saline was administered. NMDA-R and GABAA-R binding densities were examined by receptor autoradiography in several schizophrenia-relevant brain regions.

Results In both WT and NRG1 HET mice, phencyclidine treatment significantly reduced NMDA-R and GABAA-R binding density in the prefrontal cortex, hippocampus and nucleus accumbens. Acute treatment with LY379268 restored NMDA-R and GABAA-R levels in the two-hit mouse model comparable to olanzapine.

Conclusions We demonstrate that the mGluR2/3 agonist LY379268 restores excitatory and inhibitory deficits with similar efficiency as olanzapine in our two-hit schizophrenia mouse model. This study significantly contributes to our understanding of the mechanisms underlying the therapeutic effects of LY379268, and supports the use of agents aimed at mGluR2/3.

Keywords mGluR2/3, LY379268, agonist, NMDA receptor, GABAA receptor, Schizophrenia, Neuregulin 1; Phencyclidine; two hit; antipsychotic.

1. Introduction

Schizophrenia is a severe neuropsychiatric disorder caused by an interaction of genetic and environmental factors. Substantial evidence from human and animal studies links an imbalance of excitatory glutamate and inhibitory gamma-aminobutyric acid (GABA) neurotransmission to the pathophysiology of schizophrenia (Javitt, 2010; Nakazawa et al., 2012). Recent studies report reduced expression of glutamatergic *N*-methyl-D-aspartate receptors (NMDA-R) and suggest an increase in glutamatergic state via activation of non-NMDA glutamate receptors in individuals with schizophrenia (Geddes et al., 2011; Hu et al., 2014; Moghaddam and Javitt, 2012; Nakazawa et al., 2012; Weickert et al., 2013). Furthermore, reduced expression levels of GABA synthesizing enzymes and transporters together with increased expression of GABAA receptors (GABAA-R) have been identified in several disease-relevant brain regions in individuals with schizophrenia (recently reviewed by Inan et al., 2013). Consequently, the hyperglutamatergic hypothesis suggests that an overstimulation of glutamatergic neurons and thus reduced GABA signaling in schizophrenia contributes to the severe symptoms (Moghaddam and Javitt, 2012). A promising strategy for antipsychotic drug development might therefore be to reduce hyperglutamatergia in order to re-balance GABAergic neurotransmission.

Group II metabotropic glutamate receptors, mGluR2 and mGluR3, have been reported to inhibit the release of glutamate and GABA in response to neurotransmitter levels in the synaptic cleft (Newell et al., 2014). mGluR2/3 are G-protein coupled glutamate receptors primarily expressed on the pre-terminal region of the presynapse. They are negatively coupled to adenylate cyclase and cyclic adenosine monophosphate via $G_{\alpha q}$ activation, and largely function as auto- and hetero-receptors. Notably, mGluR2/3 have the ability to regulate glutamatergic tone and are distributed in regions implicated in schizophrenia pathology (Lu et al., 1997; Tamaru et al., 2001), highlighting their potential as a therapeutic target to correct the hyperglutamatergia in schizophrenia. Agonist activation of mGluR2/3 reduces release of glutamate and GABA, as well as reversing the effects of psychomimetics in both rodents and humans (Hashimoto et al., 2013; Imre, 2007; Krystal et al., 2005; Spooren et al., 2003). Several clinical, preclinical and *in vitro* studies have explored the consequences of mGluR2/3 endogenous and exogenous activation; however, the therapeutic suitability of mGluR2/3 agonists remains unclear. Recently, the mGluR2/3 agonist LY2140023 has been tested in clinical trials against symptoms of schizophrenia, with initially promising results (Patil et al., 2007), but was later stopped due to limited benefits in phase III (Adams et al., 2013). A follow up study reported a positive correlation between treatment outcome and a mutation in the neuregulin 1 (*NRG1)* and serotonin receptor genes, suggesting treatment benefits for specific populations of individuals with schizophrenia (Liu et al., 2012).

Genome wide association studies and population studies indicate that schizophrenia is a multi-factorial disorder, with both environmental and genetic factors contributing to its onset (Brown, 2010). Animal models for treatment development are thus most advantageous and relevant when they incorporate both environmental and genetic aspects. For example, several single nucleotide polymorphisms within the *NRG1* gene have been associated with an increased risk for developing schizophrenia in different patient populations (Agim et al., 2013; Buonanno, 2010; Petryshen et al., 2005; Stefansson et al., 2002). Furthermore, mild schizophrenia-like behavior and neurochemical impairments have been reported to result from different modifications of the *NRG1* gene in animals, supporting the contribution of this gene to the development of schizophrenia (Desbonnet et al., 2009). Additionally, stressors that contribute to the development of schizophrenia symptoms include the consumption of illicit substances such as cannabis (Wilkinson et al., 2014), methamphetamine (Callaghan et al., 2012) and phencyclidine (PCP) during adolescence (Swartz et al., 2014). Pre-clinical animal models that combine genetic risk factors with late developmental stressors result in a wide range of behavioral and neurochemical impairments, strongly supporting the "two-hit" hypothesis of schizophrenia (Karl and Arnold, 2014).

We therefore aimed to investigate the treatment effect of the mGluR2/3 agonist LY379268, with tenfold higher mGluR2/3 affinity than LY2140023(Mezler et al., 2010), on glutamatergic and GABAergic neurotransmitter systems relevant to schizophrenia. We chose the *NRG1* transmembrane heterozygous mouse (NRG1 HET) as a relevant genetic predisposition model (Karl et al., 2007; Long et al., 2013; Newell et al., 2013; O'Tuathaigh et al., 2010) combined with PCP treatment as a subsequent second hit insult during adolescence. Chronic PCP treatment of rodents has been reported to result in several schizophrenia-like behavioral impairments as well as differences in the glutamatergic and GABAergic neurotransmitter systems (reviewed in Mouri et al., 2007). The specific aim of this two-hit strategy was to induce a dysregulation of glutamatergic and GABAergic neurotransmission, similar to neurochemical alterations observed in patients with schizophrenia (Moghaddam and Javitt, 2012). Due to the role of mGluR2/3 as modulator of glutamate and GABA presynaptic release, we hypothesized that mGluR2/3 agonists would reduce the impact of the two-hit model on NMDA-R and GABAA-R expression in schizophrenia-relevant brain areas. To evaluate the therapeutic benefit of LY379268 compared to existing treatment options, we selected the commonly used antipsychotic olanzapine as comparison treatment.

2. Material and methods

2.1 Animals

Heterozygous *NRG1*$^{+/-}$ transmembrane domain knockout mice (NRG1 HET; allele tm2Zhou, backcrossed over 10 generations to C57BL/6JArc backgrounds) and C57BL/6JArc wild type (WT) littermates, were provided from a colony maintained at the Garvan Institute of Medical Research (Sydney, Australia), as previously described (Karl et al., 2007). Animals were subsequently bred and pair-housed under standard conditions (20 ºC±2; 12 hour light/dark cycle, light on at 6 AM) with food and water available ad libitum, in the animal facility of the University of Wollongong. Details regarding the generation, genotyping and characterization of NRG1 HET mice have been described elsewhere (Stefansson et al., 2002). The Animal Ethics Committee of the University of Wollongong and the Australian Code of Practice approved all animal and research procedures in this study. Every effort was made to minimize suffering and the number of animals used in this study.

2.2 Animal Treatment

At 7 weeks of age, 36 male NRG1 HET mice and 36 male WT littermates were given a daily subcutaneous injection of either saline (3 ml/kg; pH 7.4; n=18/group) or 10 mg/kg PCP (diluted in saline, Sigma; n=18/group) for 14 days, which has shown to cause several relevant behavioral impairments in mice (Barzilay et al., 2011; Corbett et al., 1999; Nagai et al., 2009; Wang et al., 2007). Thereafter, all animals remained untreated for a period of 14 days. Two hours before euthanasia, the mice were given an intraperitoneal injection of olanzapine (Eli lily, USA), which at 1.5 mg/kg has shown to achieve clinically relevant D2 occupancy (Kapur et al., 2003), the mGluR2/3 agonist LY379268 (Eli Lilly, USA), which at 3 mg/kg has shown to prevent PCP-induced hyperlocomotion (Cartmell et al., 1999), or vehicle (saline). Final groups consisted of 6 animals/treatment/genotype. Immediately after euthanasia, brains were rapidly removed, snap frozen and stored at -80 ºC until sectioning.

2.3 Animal tissue dissection and preparation

Brains were sectioned at -17 ºC into 14 µm coronal sections using a cryostat (Leica CM1950, Germany), at the levels of the prefrontal cortex (cingulate/prelimbic area, PFC, Bregma +2.1 mm), striatum (caudate putamen, CPu; Nucleus accumbens, NAcb, Bregma +0.98 mm), lateral septum (LS, Bregma +0.5 mm), whole hippocampus and subregions (CA1 and dentate gyrus, DG, Bregma -1.7 mm) and amygdala regions (central amygdala, CeA; basolateral amygdala, BLA; medial amygdala,

MeA, Bregma -1.7 mm). Identification of regions was based on a standard mouse brain atlas (Paxinos and Franklin, 2001). Sections were thaw-mounted onto Polysine™ slides and stored at -20 ºC until use.

2.4 Receptor autoradiography

NMDA-R and GABAA-R binding was based on the protocols previously described by Du Bois et al. (2009).

2.4.1 NMDA-R [^{3}H]MK-801 binding

Sections were incubated in 30 mM HEPES buffer (pH 7.5), 100 µM glycine, 100 µM glutamate, 1 mM EDTA and 20 nM [^{3}H]MK-801 (specific activity 27.5 Ci/mmol; PerkinElmer, USA) for 2.5 h at room temperature and washed 2x20 min in 30 mM HEPES containing 1 mM EDTA at 4 °C.

2.4.2 GABAA-R [^{3}H]Muscimol binding

Sections were pre-incubated in a 50 mM Tris buffer (pH 7.0; citrate adjusted), 3x5 min at 4 °C. Incubation was performed with a 12 nM [^{3}H]Muscimol solution (specific activity 29.5 Ci/mmol; PerkinElmer, USA) in Tris buffer for 45 min at 4°C, followed by 4x2 sec washes in Tris buffer at 4 °C.

2.4.3 Analyses

All slides were rinsed in cold distilled water, air-dried, and were exposed to Kodak BioMax MR film and analyzed using the Multi-Analyst imaging system (Bio-Rad, USA). The PFC, CPu, NAcb, LSW, CA1, DG, CeA, BLA, MeA regions were identified using a standard brain atlas (Paxinos and Franklin, 2001) and the average density of left and right hemispheres of two separate sections per animal were analyzed in these regions. Optical density values were compared to co-exposed standard [3H]microscales (Amersham, UK) and transformed into radiographic densities using the Multi-Analyst imaging system (Bio-Rad, USA).

2.5 Statistical Analysis

Statistical analysis was performed using SPSS 17.0. Normal distribution for all data was confirmed using the Kolmogorov-Smirnov test. Two-way ANOVAs were used to analyze effects and interactions of the two-hit options (WT SAL, WT PCP, NRG1 HET SAL or NRG1 HET PCP) with the acute treatment options (vehicle, olanzapine or LY379268), followed by post-hoc Bonferroni

multiple comparisons. Spearman's correlations were used to determine whether NMDA-R binding levels were associated with GABAA-R levels following the different modelling strategies and acute treatment options. Significance was accepted at $p<0.05$. Data are presented as means±standard error of the means (SEM).

3. Results

3.1 PCP treatment reduces NMDA-R binding in several brain regions of NRG1 transgenic mice

To confirm the two-hit mouse model for evaluating the role of the mGluR2/3 agonist in altering glutamatergic neurotransmission via the NMDA-R, we measured the binding density of NMDA-R antagonist MK801 in several schizophrenia relevant brain regions. Two-way ANOVA revealed an overall effect of the model options on NMDA-R binding density in the PFC, Nacb, Hipp and LS ($F_{3, 60}$>5.31, P<0.01; Table 1A). Analyzing the vehicle groups of each two-hit model option (WT or NRG1 HET with chronic PCP or saline) revealed that NRG1 HET PCP mice had reduced NMDA-R binding in the PFC (52.86%, P<0.001), Nacb (61.15%, P<0.001), Hipp (50.81%, P<0.001), CA1 (87.78%, P<0.01), DG (81.76%, P<0.001) and LS (74.67%, P<0.05) when compared to saline treated WT littermates (**Error! Reference source not found.**C-D). The NRG1 HET mice showed no difference in NMDA-R density in the regions examined when treated with saline only. WT animals had reduced NMDA-R density following PCP treatment in the PFC (73.95%, P<0.01) and Hipp (74.91%, P<0.05; **Error! Reference source not found.**B) when compared to saline treated WT animals. There were no differences in NMDA-R density values in the amygdala regions (CeA, BLA, MeA) between saline treated WT mice and the two-hit models (data not shown).

Table 1: *Two-way analysis of variance values for NMDA-R binding densities across several brain regions with factor (a) two-hit models (wild type or neuregulin 1 heterozygous transmembrane domain knockout mice with sub-chronic saline or phencyclidine treatment) and factor (b) acute treatment options (vehicle, olanzapine, LY379268) on NMDA-R binding.*

Brain region	(a) Two-hit models		(b) Acute treatments		Interaction	
	$F_{(3, 60)}$	P-value	$F_{(2, 60)}$	P-value	$F_{(6, 60)}$	P-value
PFC	8.73	**<0.001**	47.26	**<0.001**	6.611	**<0.001**
CPU	1.33	>0.05	9.67	**<0.001**	1.315	>0.05
Nacb	5.31	**<0.01**	37.28	**<0.001**	6.085	**<0.001**
Hipp	5.55	**<0.01**	49.46	**<0.001**	5.783	**<0.001**
CA1	2.54	>0.05	22.54	**<0.001**	2.946	**<0.01**
DG	1.49	>0.05	27.99	**<0.001**	2.196	>0.05
CeA	1.10	>0.05	11.09	**<0.001**	0.319	>0.05
BLA	0.72	>0.05	3.58	**<0.05**	0.222	>0.05
MeA	0.44	>0.05	6.77	**<0.01**	0.531	>0.05
LS	6.76	**<0.001**	9.43	**<0.001**	0.917	>0.05

Abbreviations: N-methyl-D-aspartate receptor (NMDA-R), prefrontal cortex (PFC), caudate putamen (CPu), Nucleus accumbens (NAcb), whole hippocampus (Hipp) and subregions (CA1) dentate gyrus (DG), central amygdala (CeA), basolateral amygdala (BLA), medial amygdala (MeA), lateral septum (LS).

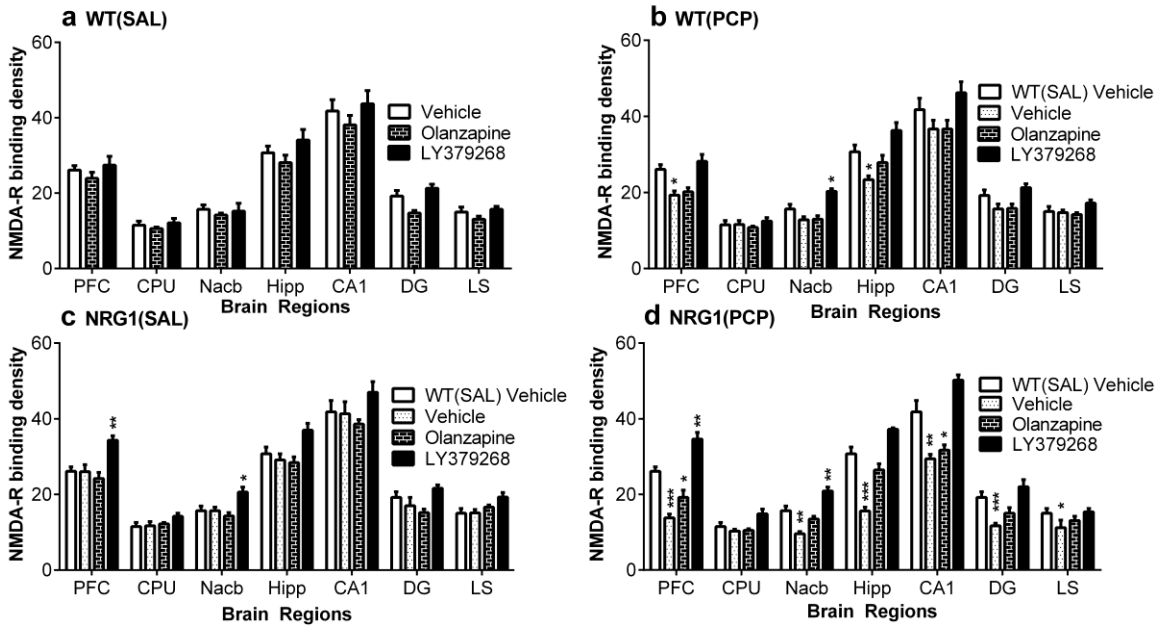

Fig. 1 Acute LY379268 and olanzapine treatment restore NMDA-R binding density levels in several brain regions of two-hit NRG1 HET (PCP) mice.

Wild type mice (WT) and neuregulin 1 heterozygous transmembrane domain knockout mice (HET) received chronic phencyclidine (PCP) or saline (SAL) treatment for 14 days and an acute treatment of LY379268 or olanzapine after 14 days washout. N-methyl-D-aspartate receptors (NMDA-R) binding was quantified in the prefrontal cortex (PFC), caudate putamen (CPu), Nucleus accumbens (NAcb), whole hippocampus (Hipp) and subregions (CA1) dentate gyrus (DG), lateral septum (LS). Data presented as mean binding density nCi/mg tissue± standard error of the mean (n=6), Statistical significance:*P<0.05, **P<0.01, ***P<0.001 as compared to WT(SAL) Vehicle

3.2 Deficits in NMDA-R binding are restored following acute treatment with LY379268 and olanzapine

Two weeks after the chronic PCP or saline treatment, animals received an acute single injection of the mGluR2/3 agonist LY379268, olanzapine or vehicle. Two-way ANOVA revealed an overall effect of acute treatments on NMDA-R binding density in all assessed brain regions ($F_{2, 60}$>3.58, P<0.03; Table 1B). In WT saline animals, neither LY379268 nor olanzapine acute treatment affected NMDA-R density (**Error! Reference source not found.**A). In WT PCP mice, LY379268 and olanzapine increased NMDA-R binding deficits in the PFC above PCP levels, with LY379268 treatment also increasing NMDA-R binding density in the Nacb (167.77%, P<0.001; **Error! Reference source not found.**B). Treating NRG1 HET saline mice with LY379268 increased NMDA-R density in the Nacb (170.25%, P<0.001) and PFC (125.17%, P<0.01), while olanzapine had no effect (**Error! Reference source not found.**C). In the combined two-hit NRG1 HET PCP mice, LY379268 treatment restored NMDA-R density to WT saline vehicle levels in the Hipp, CA1 and DG, while increasing the density in the PFC (126.28%, P<0.01) and Nacb (172.72%, P<0.001) (**Error! Reference source not found.**D). Olanzapine reduced binding levels in the PFC (76.56%, P<0.05) and CA1 (75.83%,

P<0.05) of NRG1 HET PCP animals, while restoring binding levels in the Nacb, Hipp and DG (**Error! Reference source not found.**D).

3.3 PCP treatment reduces GABAA-R binding in several brain regions of NRG1 transgenic mice

GABAA-R density, as assessed through the binding potential of muscimol, was significantly altered in the PFC, Nacb and DG ($F_{(3, 60)}$>3.59, P<0.05; Table 2A) following the different modelling strategies. Comparing the vehicle group of each two-hit option revealed that WT PCP animals had reduced GABAA-R density in the Hipp and DG (66.66%, P<0.001 and 64.57%, P<0.01 compared to WT saline animals; **Error! Reference source not found.**B). NRG1 HET saline mice showed no difference in GABAA-R density in any of the quantified brain regions (**Error! Reference source not found.**C). NRG1 HET PCP mice displayed reduced GABAA-R binding in the PFC (52.59%, P<0.001), Hipp (60.41%, P<0.001), CA1 (56.07%, P<0.01) and DG (53.54%, P<0.001) when compared to WT saline littermates (**Error! Reference source not found.**B). There were no differences in GABAA-R density values in the amygdala regions (CeA, BLA, MeA) between saline treated WT mice and the two-hit models (data not shown).

Table 2. *Two-way analysis of variance values for GABAA-R binding densities across several brain with factor (A) two-hit models (wild type or neuregulin 1 heterozygous transmembrane domain knockout mice with sub-chronic saline or phencyclidine treatment) and factor (B) acute treatment options (vehicle, olanzapine, LY379268) on GABAA-R binding.*

Brain region	(A) Two-hit models		(B) Acute treatments		Interaction	
	$F_{(3, 60)}$	P-value	$F_{(2, 60)}$	P-value	$F_{(6, 60)}$	P-value
PFC	6.71	**<0.001**	6.47	**<0.01**	2.157	>0.05
CPU	1.35	>0.05	9.63	**<0.001**	0.841	>0.05
Nacb	8.02	**<0.001**	5.37	**<0.01**	0.547	>0.05
Hipp	2.02	>0.05	34.63	**<0.001**	4.180	**<0.001**
CA1	1.14	>0.05	10.99	**<0.001**	3.707	**<0.01**
DG	3.59	**<0.05**	10.54	**<0.001**	2.790	**<0.05**
CeA	1.41	>0.05	10.35	**<0.001**	0.675	>0.05
BLA	0.82	>0.05	5.67	**<0.01**	0.642	>0.05
MeA	1.042	>0.05	11.53	**<0.001**	0.679	>0.05
LS	1.78	>0.05	7.85	**<0.001**	1.102	>0.05

Abbreviations: the inhibitory gamma-aminobutyrate-A receptor (GABAA-R), prefrontal cortex (PFC), caudate putamen (CPu), Nucleus accumbens (NAcb), whole hippocampus (Hipp) and subregions (CA1) dentate gyrus (DG), central amygdala (CeA), basolateral amygdala (BLA), medial amygdala (MeA), lateral septum (LS).

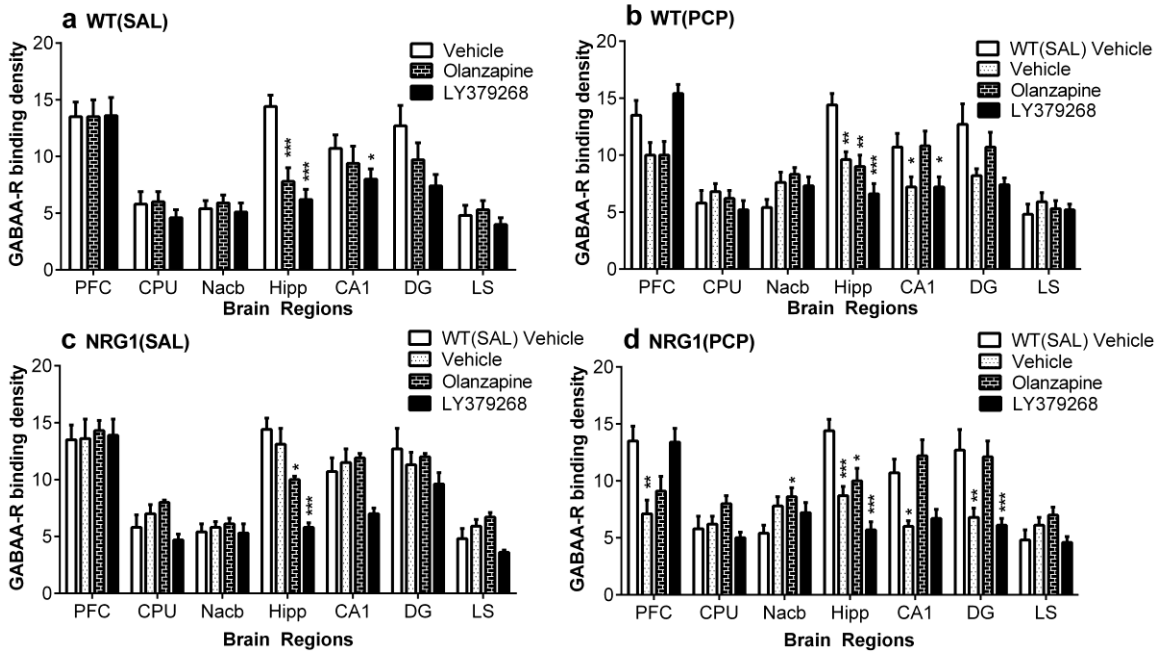

Fig. 2 Acute LY379268 and olanzapine treatment restore GABAA-R binding density levels in several brain regions of two-hit NRG1 HET (PCP) mice.

Wild type mice (WT) and neuregulin 1 heterozygous transmembrane domain knockout mice (HET) received chronic phencyclidine (PCP) or saline (SAL) treatment for 14 days and an acute treatment of LY379268 or olanzapine after 14 days washout. Gamma-aminobutyric acid receptor A (GABAA-R) binding was quantified in the prefrontal cortex (PFC), caudate putamen (CPu), Nucleus accumbens (NAcb), whole hippocampus (Hipp) and subregions (CA1) dentate gyrus (DG), lateral septum (LS). Data presented as mean binding density nCi/mg tissue ± standard error of the mean (n=6), Statistical significance:*P<0.05, **P<0.01, ***P<0.001 as compared to WT(SAL) Vehicle.

3.4 Deficits in GABAA-R binding are restored following acute treatment with LY379268

The acute treatments significantly affected GABAA-R density in all assessed brain regions ($F_{2,60}$>5.37, P<0.01; Table 2B). In WT saline animals, LY379268 reduced GABAA-R density in the Hipp and DG (43.05%, P<0.001 and 58.25%, P<0.01 respectively) compared to vehicle treatment (**Error! Reference source not found.**), while acute olanzapine reduced binding levels in the Hipp (54.16%, P<0.001) (**Error! Reference source not found.**A). In WT PCP animals, GABAA-R binding levels remained below WT saline animals following LY379268 (Hipp: 45.83%, P<0.001; DG: 58.26%, P<0.01) and olanzapine (Hipp: 62.5%, P<0.001) treatment (**Error! Reference source not found.**B). NRG1 HET saline mice also showed reduced GABAA-R binding levels in the Hipp following either LY379268 (40.27%, P<0.001) or olanzapine (69.44%, P<0.01) treatment compared to WT saline vehicle animals (**Error! Reference source not found.**C). In the combined two-hit NRG1 HET PCP mice, LY379268 treatment restored binding density in the PFC and CA1, while Hipp and DG levels were below WT saline vehicle (Hipp: 39.57%, P<0.001; DG: 48.03%, P<0.001)

(**Error! Reference source not found.**D). GABAA-R density following olanzapine treatment in NRG1 HET PCP mice showed increased GABAA-R binding in the Nacb (159.26%, P<0.05) and reduced in the Hipp (69.44%, P<0.01) compared to WT saline vehicle animals (**Error! Reference source not found.**D).

3.5 LY379268 treatment restored correlation between NMDA-R and GABAA-R binding

Spearman's correlations for NMDA-R and GABAA-R binding levels are presented in **Error! Reference source not found.**. Wild type mice expressed a strong positive correlation between NMDA-R and GABAA-R binding levels across all brain regions. This correlation was severely impaired following chronic PCP treatments in both WT and NRG1 HET mice. Acute treatment with LY379268 restored the correlation between NMDA-R and GABAA-R binding in PCP treated NRG1 HET mice to control levels, without altering the binding correlation in WT animals. NMDA-R and GABAA-R levels did not correlate in any group following acute olanzapine treatment (**Error! Reference source not found.**).

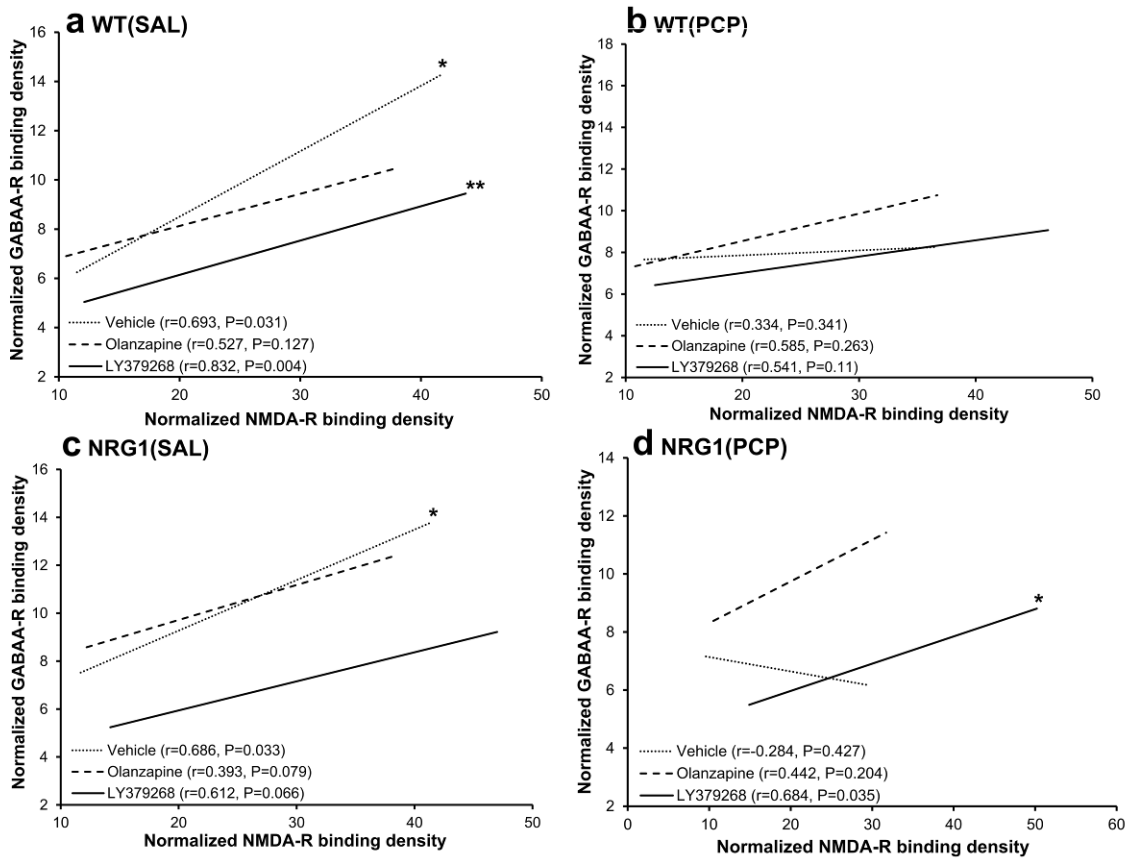

Fig. 3 Acute LY379268 treatment restores positive correlation between NMDA-R and GABAA-R binding levels across brain regions in two-hit NRG1 HET(PCP) mice.

Spearman’s correlation plots depicting the relationship between NMDA-R and GABAA-R binding levels across all brain regions, following the different modelling strategies and acute treatment options. **Abbreviations:** wild type mice (WT), neuregulin 1 heterozygous transmembrane domain knockout mice (NRG1), chronic phencyclidine treatment (PCP), chronic saline treatment (SAL) *P>0.05, **P>0.01

4. Discussion

We have for the first time explored the ability of the mGluR2/3 agonist LY379268 to restore NMDA-R and GABAA-Rs, as measured by binding densities, in a schizophrenia-relevant animal model. The combination of a *NRG1* mutation with adolescent chronic PCP treatment resulted in a robust reduction in NMDA-R and GABAA-R in several schizophrenia-relevant brain regions, particularly in the PFC and hippocampus. Subsequently, we showed that a single treatment with the mGluR2/3 agonist restored NMDA-R and GABAA-R levels as efficient as olanzapine. These findings suggest that mGluR2/3 activity moderates alterations in excitatory-inhibitory neurotransmitter receptors, and thus mGluR2/3 might be a valuable therapeutic target to restore the imbalance between excitatory and inhibitory neurotransmission in schizophrenia.

4.1 The two-hit NRG1-PCP model shows reduced NMDA-R and GABAA-R expression

NMDA-R and GABAA-R receptor binding were reduced in several brain regions, including in the schizophrenia-relevant prefrontal cortex and hippocampus in PCP-treated NRG1 HET mice. Reduced NMDA-R expression in several brain regions, including the frontal cortex and hippocampus, has been found in individuals with schizophrenia (Errico et al., 2013; Geddes et al., 2014, 2011; Weickert et al., 2013). GABAA-R expression level differences vary between receptor subtypes and brain regions in individuals with schizophrenia (Benes et al., 1996a, 1996b; Duncan et al., 2010). The reduction in NMDA-R and GABAA-R binding supports our two-hit modelling strategy as neither NRG1 HET mice nor chronic PCP treatment alone showed such robust impairments in the present study or in previously published experiments (Beninger et al., 2010; Bullock et al., 2009; Dean et al., 2008; Hanania et al., 1999; Lindahl and Keifer, 2004; Long et al., 2013, 2012; Newell et al., 2013; Stefansson et al., 2002; Wang et al., 2005). Cognitive and memory functions rely on balanced glutamatergic and GABAergic neurotransmission (Klausberger and Somogyi, 2008). The NRG1 HET genetic predisposition would likely result in impaired cognitive and memory abilities following the PCP insult by extending the impact onto glutamatergic and GABAergic neurotransmission in the PFC and hippocampus. While differences in amygdala-related behavior and glutamatergic neurotransmission have previously been reported following PCP treatment (Bustillo et al., 2012; Katayama et al., 2009; Lee et al., 2005; Zavitsanou et al., 2008), our data suggests that NMDA-R and GABAA-R are not contributing to these symptoms.

The treatment potential of LY379268 has previously been assessed in single-hit rodent models, including acute and chronic PCP treatment and shown to improve behavioral impairments (Amitai and Markou, 2010; Cartmell et al., 2000, 1999; Clark et al., 2002). To increase the relevance of the

treatment outcomes for the clinical setting, we developed a two-hit mouse model and assessed the treatment potential against neurochemical impairments. The combination of a mutation in the known susceptibility gene *NRG1,* with the pharmacological insult of chronic PCP during adolescence, caused robust glutamatergic and GABAergic receptor differences with strong relevance to schizophrenia pathophysiology in our two-hit model.

4.2 The mGluR2/3 agonist LY379268 restores NMDA-R and GABAA-R expression levels

As mGluR2/3 are located on the presynapse where they function as endogenous modulators of glutamatergic tone, it has been hypothesized that these receptors have the potential to correct imbalances of excitatory and inhibitory neurotransmission (Gu et al., 2008). The expression of mGluR2/3 in cortical regions appears to vary between populations of patients, with reduced (Corti et al., 2007; Ghose et al., 2009; González-Maeso et al., 2008; Gupta et al., 2005) and unchanged levels being reported (Frank et al., 2011; Matosin et al., 2014). In accordance, increasing the activity of mGluR2/3 receptors might therefore be a viable treatment strategy. The upregulation of mGluR2/3 signaling using agonists or modulators of mGluR2/3 has been shown to have therapeutic potential in schizophrenia relevant behavioral paradigms (Li et al., 2015). However, none of these studies have assessed the potential of these agents to correct core NMDA-R and GABAA-R deficits and/or imbalances.

We thus set out to explore the consequence of increased mGluR2/3 signaling on schizophrenia-relevant neurochemical impairments. Compellingly, a single injection with LY379268 restored the NMDA-R density levels in all brain regions that were previously impaired by the two-hit strategy. Furthermore, LY379268 treatment restored reduced GABAA-R binding levels in the PFC and hippocampus, specifically CA1 and DG. Finally, acute LY379268 treatment restored the correlation between NMDA-R and GABAA-R binding in NRG1 HET PCP mice to a similar level as in saline WT animals. These findings provide the first evidence for the ability of mGluR2/3 agonists to restore NMDA-R and GABAA-R levels and ratios, indicative of rebalancing excitatory:inhibitory neurotransmission.

As aforementioned, existing animal studies have shown that mGluR2/3 agonists can counteract the psychotic behavioral effects induced by PCP, ketamine and MK-801 (potent NMDA-R antagonists) treatment in rodents (Cartmell et al., 2000, 1999; Harich et al., 2007; Hikichi et al., 2013, 2010; Imre et al., 2006; Moghaddam and Adams, 1998; Patil et al., 2007; Pitsikas and Markou, 2014; Rorick-Kehn et al., 2007; Spooren et al., 2000). The therapeutic potential has however been shown to vary between different mGluR2/3 agonists, as some agonists failed to improve PCP-induced behavioral

impairments (Schlumberger et al., 2009). Treatment specifically with the mGluR2/3 agonist LY379268 used in this study has been shown to reduce MK-801-induced hippocampal-PFC synaptic transmission and gamma band oscillation; this supports the utility of LY379268 to restore the balance between glutamatergic and GABAergic neurotransmission in cortical and subcortical regions (Blot et al., 2013; Hiyoshi et al., 2014). Together with the present results, these findings suggest activating mGluR2/3 receptors could attenuate hypoglutamatergia by increasing the number of active NMDA-R, possibly via the NMDA-R regulating SRC kinase (Trepanier et al., 2013). mGluR2/3 activation has also been shown to increase non-NMDA glutamate receptor levels (Wang et al., 2013), which could contribute to the restoration of GABAA-R binding levels through reduced GABA release (Drew and Vaughan, 2004; Satake et al., 2000). Finally, Gorrie and colleagues have reported that more than 6 h are required for the synthesis of GABAA-R and its integration into the cell membrane (Gorrie et al., 1997), with GABAA-receptors more likely being recycled back into the membrane than replaced with new receptors (Thomas et al., 2005). The rapid increase in GABAA-R binding within 2 h after acute treatment thus suggests that LY379268 triggered the integration of existing, stored GABAA-R into the cell membrane over the production of new receptors. While our experiment cannot fully elucidate the neurochemical events following LY379268 administration, by restoring the NMDA-R and GABAA-R binding in the two-hit schizophrenia model, LY379268 shows a promising antipsychotic potential.

4.3 The mGluR2/3 agonist LY379268 shows stronger therapeutic potential than olanzapine in restoring NMDA-R and GABAA-R expression levels in this study

To evaluate the treatment potential of LY379268, we administered olanzapine as a benchmark comparison for currently used antipsychotics. A single injection with olanzapine restored the NMDA-R binding density in all areas previously impaired by the two-hit strategy. GABAA-R levels remained below WT levels in the Hipp following olanzapine treatment, while NAcb GABAA-R levels were increased and CA1 and DG levels restored to WT levels. Furthermore, NMDA-R and GABAA-R binding did not correlate in WT, NRG1 HET and NRG1 HET + PCP mice following acute olanzapine. LY379268 was similarly effective as olanzapine in restoring NMDA-R and GABAA-R binding levels in the two-hit model. Research by Elsworth and colleagues has shown that chronic PCP-induced dendritic spine loss can be restored by a single olanzapine injection within 90 min of treatment (Elsworth et al., 2011), a mechanism possibly contributing to the recovery of NMDA-R and GABAA-R binding levels. Olanzapine, however, did not restore the positive correlation between NMDA-R and GABAA-R binding levels, while LY379268 did. The LY379268 dose used in the present study (3 mg/kg) has been shown to have no effect on rodent behavior, while olanzapine at therapeutically comparable concentrations has been reported to cause behavior impairments in some

studies (Cartmell et al., 2000; Gleason and Shannon, 1997). Combined with the present finding, LY379268 clearly appears as a valuable treatment option against schizophrenia-relevant molecular and behavioral impairments.

With their role in modulating glutamate, GABA, dopamine and serotonin release, mGluR2/3 receptors at their pre-synaptic location in schizophrenia-relevant brain regions present themselves as critical treatment targets. While the mGluR2/3 agonist LY2140023 has been discontinued during the clinical trial phase III due to a lack of significant improvement above placebo, more selective agonists, including LY379268, have been hypothesized to exert a stronger therapeutic action (Li et al., 2015). In the present study, we have shown that acute treatment with the mGluR2/3 agonist LY379268 restores NMDA-R and GABAA-R binding levels in several brain regions of a robust schizophrenia-relevant two-hit mouse model. The findings from the present study thus support the therapeutic potential of mGluR2/3 agonist LY379268, specifically to correct an imbalance in excitatory:inhibitory neurotransmission in individuals with schizophrenia. However, considering that genetic variations influenced the impact on the patient treatment response to LY2140023 (Liu et al., 2012), future studies should aim to determine the patient subtype that will benefit from mGluR2/3 agonist treatments.

5. References

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