RESEARCH PAPER

Cerebellar α₆-subunit-containing GABA_A receptors: a novel therapeutic target for disrupted prepulse inhibition in neuropsychiatric disorders

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BACKGROUND AND PURPOSE

The pathophysiological role of α₆-subunit-containing GABA_A receptors, which are mainly expressed in cerebellar granule cells, remains unclear. Recently, we demonstrated that hispidulin, a flavonoid isolated from a local herb that remitted a patient's intractable motor tics, attenuated methamphetamine-induced hyperlocomotion in mice as a positive allosteric modulator (PAM) of cerebellar α₆GABA_A receptors. Here, using hispidulin and a selective α₆GABA_A receptor PAM, the pyrazoloquinolinone Compound **6**, we revealed an unprecedented role of cerebellar α₆GABA_A receptors in disrupted prepulse inhibition of the startle response (PPI), which reflects sensorimotor gating deficits manifested in several neuropsychiatric disorders.

EXPERIMENTAL APPROACH

PPI disruptions were induced by methamphetamine and NMDA receptor antagonists in mice. Effects of the tested compounds were measured in *Xenopus* oocytes expressing recombinant α₆β₃γ₂ₛGABA_A receptors.

KEY RESULTS

Hispidulin given i.p. or by bilateral intracerebellar (i.cb.) injection rescued PPI disruptions induced by methamphetamine, ketamine, MK-801 and phencyclidine. Intracerebellar effects of hispidulin were mimicked by Ro15-4513 and loreclezole (two α₆GABA_A receptor PAMs), but not by diazepam (an α₆GABA_A receptor-inactive benzodiazepine) and were antagonized by furosemide (i.cb.), an α₆GABA_A receptor antagonist. Importantly, Compound **6** (i.p.) also rescued methamphetamine-induced PPI disruption, an effect prevented by furosemide (i.cb.). Both hispidulin and Compound **6** potentiated α₆β₃γ₂ₛGABA_A receptor-mediated GABA currents.

CONCLUSIONS AND IMPLICATIONS

Positive allosteric modulation of cerebellar α_6GABA$_A$ receptors rescued disrupted PPI by attenuating granule cell activity. α_6GABA$_A$ receptor-selective PAMs are potential medicines for treating sensorimotor gating deficits in neuropsychiatric disorders. A mechanistic hypothesis is based on evidence for cerebellar contributions to cognitive functioning including sensorimotor gating.

Abbreviations

α_6GABA$_A$ receptors, α_6-subunit-containing GABA$_A$ receptors; DCN, deep cerebellar nuclei; MIH, methamphetamine-induced hyperlocomotion; PAM, positive allosteric modulator; PCP, phencyclidine; PFC, prefrontal cortex; PPI, prepulse inhibition of the startle response; TS, Tourette syndrome

Introduction

Some years ago, we identified a patient with intractable motor tic disorders who responded well to a herbal remedy consisting of the leaf juice of *Clerodendrum inerme* (L.) Gaertn (*C. inerme*), without apparent haemotoxicity, hepatic toxicity or renal toxicity (Fan *et al*., 2009). Tic disorders and Tourette syndrome (TS), an idiopathic spectrum of motor tic disorders, are attributed to an over-reactive dopaminergic system in the cortico–thalamic–striatal circuit (Singer, 2005). Therefore, using a hyper-dopaminergic behaviour model, **methamphetamine**-induced hyperlocomotion (MIH), we identified the flavonoid hispidulin (6-methoxy-4′,5,7-trihydoxyflavonoid) (Figure 1A) as an active constituent of the ethanol extract of *C. inerme* leaves (Huang *et al*., 2015).

Hispidulin has micromolar affinity for the benzodiazepine site of human **GABA$_A$ receptors** (Kavvadias *et al*., 2003) and is a positive allosteric modulator (PAM) of oocyte-expressed recombinant GABA$_A$ receptors comprising the $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ subunits (Kavvadias *et al*., 2004). It is noteworthy that hispidulin is a PAM of **α_6-subunit**-containing GABA$_A$ receptors (α_6GABA$_A$ receptors) that are mainly expressed in the granule cells of the cerebellum (Gutierrez *et al*., 1996; Pirker *et al*., 2000). Among 92 neurotransmitter receptors, enzymes and transporters, we found that hispidulin displayed micromolar affinity only at GABA$_A$ receptors and **COMT** (Liao *et al*., 2016). Further, hispidulin alleviated MIH in mice by acting as a PAM at cerebellar α_6GABA$_A$ receptors, but not as a COMT inhibitor, at doses without benzodiazepine-like effects, such as sedation, anxiolysis and motor impairment (Liao *et al*., 2016).

In addition to motor tics, TS patients also manifest a sensorimotor gating deficit, which may contribute to their premonitory urges (Leckman *et al*., 1993) and can be measured by a disruption in the prepulse inhibition of the startle response (PPI) (Swerdlow *et al*., 2001). In addition to tic disorders/TS, PPI disruptions are also manifested in patients with several neuropsychiatric disorders, such as obsessive–compulsive disorder (Swerdlow *et al*., 1993), attention deficit disorder (Ornitz *et al*., 1992), panic disorder (Ludewig *et al*., 2002), nocturnal enuresis (Freitag *et al*., 2006), Huntington's disease (Swerdlow *et al*., 1995), premenstrual dysphoric disorder (Kask *et al*., 2008), mania in bipolar disorder (Giakoumaki *et al*., 2007), antisocial personality disorder (Kumari *et al*., 2005) and schizophrenia (Braff *et al*., 1978). Especially in patients with schizophrenia, PPI disruption is a well-known endophenotype manifestation (Braff *et al*., 1978).

In PPI disruption mouse models induced by methamphetamine and **NMDA channel** blockers, based on hyper-dopaminergic and hypo-glutamatergic hypotheses of schizophrenia, respectively (Geyer and Moghaddam, 2002), the ethanolic extract of *C. inerme* was also effective (Chen *et al*., 2012). Here, we have investigated whether the effects of *C. inerme* extracts on PPI disruptions could also be attributed to hispidulin and whether PAM effects on α_6GABA$_A$ receptors and/or inhibitory effects on COMT were involved. To substantiate whether PAM action at cerebellar α_6GABA$_A$ receptors can rescue PPI disruptions, we also investigated the effects of the pyrazoloquinolinone Compound **6** (Figure 1B), which, in contrast to hispidulin, is a highly selective PAM at $\alpha_6\beta_2\gamma_2$ and $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors (Varagic *et al*., 2013). Finally, we examined whether effects of hispidulin and Compound **6** could be prevented by an intracerebellar (i.cb.) microinjection of **furosemide**, a selective α_6GABA$_A$ receptor antagonist.

Methods

Animal experiments

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of National Taiwan University, College of Medicine (NTUMC) and Meijo University, Japan. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al*., 2010; McGrath and Lilley, 2015). All animal procedures were performed in male ICR mice (6-8 week-old; BioLASCO Taiwan Co., Ltd. Ilan, Taiwan) as reported in our previous studies conducted in NTUMC (Chen *et al*., 2012; Huang *et al*., 2015) except the experiments with **phencyclidine (PCP),** which were conducted in male 6 week-old ddY mice (Nihon SLC,
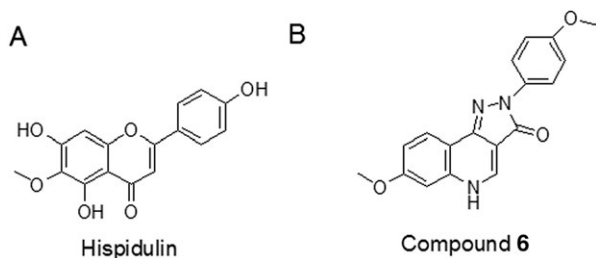

Figure 1

Chemical structures of hispidulin and Compound **6**.

Shizuoka, Japan) at Meijo University. Animals were randomly divided into several groups as needed. The experimenters were blind to the treatment(s) in each group.

The PPI test

Except for the experiments with the PPI disruption model induced by PCP (PCP-PPI), all PPI tests were performed with two acoustic stimulation protocols (71–115 and 77–115 dB) as reported previously with a PPI apparatus (SR-LAB; San Diego Instruments, San Diego, CA, USA) consisting of a startle chamber equipped with various programming acoustic stimulations (Chen *et al.*, 2012). Briefly, the mouse was placed in the startle chamber for a 4 min acclimation period with a 65 dB background noise. There were four types of startle trials: PULSEALONE (115 dB, 20 ms), two types of PREPULSE + PULSE (71 + 115 and 77 + 115 dB, interval: 120 ms) and NOSTIM (no stimulation with background 65 dB only). In each session, the mouse received four NOSTIM trials first, followed by four types of trials randomly given 14 times (56 trials in total) and finally four PULSEALONE trials. The magnitude of PPI (PPI%) was determined by the summarized startle responses in PULSEALONE and PREPULSE + PULSE trials, according to the equation: (PULSEALONE − PREPULSE + PULSE) PULSEALONE × 100%. To induce PPI disruptions, mice were injected i.p. (pretreatment) with 2 mg·kg^{-1} methamphetamine (METH-PPI) or 30 mg·kg^{-1} **ketamine** (Ketamine-PPI) for 10 min, or with 0.3 mg·kg^{-1} **MK-801 (dizocilpine;** MK-PPI), 15 min before receiving the PPI test, unless stated otherwise.

In the PCP-PPI disruption model, three PPI protocols, 69–120, 73–120 and 81–120 dB, were used, and PCP (1 mg·kg^{-1}) was injected s.c. into ddY mice 10 min before the PPI test (Zou *et al.*, 2008).

Bilateral intracerebellar microinjection

Bilateral i.cb. microinjection was performed as described previously (Liao *et al.*, 2016). Briefly, the mouse was, under anaesthesia, implanted with two 24-gauge stainless-steel guide cannulas, respectively, directed towards the right and left lateral cerebella (−6.4 mm caudal, ±1.5 mm lateral and −1.0 mm ventral from bregma) (Paxinos and Franklin, 2001). Seven days after the cannulation, mice received bilateral i.cb. microinjections, which were performed by slowly infusing 0.5 μL (each side) drug solution for 30 s with a 60 s hold time through a microinfusion pump (KDS311; KD Scientific Inc., Holliston, MA, USA). The microinjection site was confirmed by the positive staining with Trypan blue.

Electrophysiological measurements at recombinant $\alpha_6\beta_3\gamma_2$GABA$_A$ and $\alpha_6\beta_3\delta$GABA$_A$ receptors

Two-electrode voltage-clamp recordings were performed in *Xenopus laevis* oocytes expressing recombinant $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors as described previously (Varagic *et al.*, 2013). To obtain $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors with efficient incorporation of the γ_2 subunit and thus consistent PAM responses of tested compounds, we utilized concatenated subunits as described previously (Minier and Sigel, 2004), and the constructs were a kind gift from E. Sigel (University of Bern, Institute for Biochemistry and Molecular Medicine. Bern, Switzerland).

Specifically, cRNA coding for one triple concatemer γ_2-β_3-α_6 was co-injected in oocytes with cRNA coding for one double concatemer β_3-α_6 in a 1:1 ratio, instead of non-concatenated α_6, β_3 and γ_2 subunits. The PAM effect of the tested compound on $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors was measured by the increment of the Cl$^-$ current induced by GABA at EC$_{3-5}$, that is, the concentration needed to elicit 3–5% of the maximum GABA current in each oocyte.

Because the arrangement of the δ-subunit-containing GABA$_A$ receptors is still not clear, subunit concatenation for $\alpha_6\beta_3\delta$GABA$_A$ receptors is not a viable option. To determine whether drugs also modulate these receptors, mRNA of the individual subunits was mixed and injected as described previously (Mirheydari *et al.*, 2014). We collected data only from cells that displayed at least 500% enhancement of the GABA EC$_{10}$ currents in response to the subunit-selective compound, **DS2** (4-chloro-N-[2-(thiophen-2-yl)imidazo[1,2-a]pyridin-3-yl]benzamide), which is an established method to test incorporation of the δ subunit.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Data are expressed as the mean ± SEM. Statistical comparisons among groups were analysed by ANOVA with Tukey's *post hoc* test, and differences between two groups were analysed by Student's *t*-test. Differences were considered significant if $P < 0.05$.

Materials

Hispidulin was purchased from Tocris Bioscience (Bristol, UK) or isolated from the ethanol extract of *C. inerme* leaves as reported previously (Chen *et al.*, 2012; Huang *et al.*, 2015) with the structure identified by NMR and mass spectroscopy. Isolated hispidulin displayed the same efficacy in alleviating MIH as commercial hispidulin purchased from Tocris Bioscience (Huang *et al.*, 2015). Compound **6** was synthesized as reported previously (Zhang *et al.*, 1995). **Loreclezole**, **Ro15-4513**, **diazepam** and furosemide were purchased from Tocris Bioscience, (+)MK-801 hydrogen maleate, methamphetamine and OR-486 from Sigma-Aldrich (St. Louis, MO, USA) and ketamine from Parke-Davis (Taoyuan, Taiwan). Diazepam, methamphetamine and ketamine were purchased under the approval from Food and Drug Administration, the Ministry of Health and Welfare, Taiwan. PCP hydrochloride was synthesized according to the method of Maddox *et al.* (1965), and its purity has been confirmed by the melting point and UV spectrum.

Methamphetamine, ketamine, MK-801 and PCP were dissolved in normal saline. Hispidulin, Compound **6**, OR-486 and diazepam, when given by i.p. injection, were dissolved in a vehicle containing 20% DMSO, 20% Cremophor® EL (polyoxyethylene castor; Sigma-Aldrich) and 60% normal saline. Hispidulin, Ro15-4513, loreclezole, diazepam and furosemide, when administered by i.cb. microinjection, were dissolved in DMSO as reported previously (Liao *et al.*, 2016). All drugs were administered 10 min before the test session unless stated otherwise. The i.p. or s. c. injection volume was 10 mL·kg^{-1}. For oocyte experiments, hispidulin, Compound **6** and OR-486 were dissolved in DMSO as stock solutions. From the stock solutions, the

compounds were diluted to final concentrations in the *Xenopus* Ringer solution, where the final concentration of DMSO was less than 0.1% (Varagic *et al*., 2013).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharma-cology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al*., 2017), and are permanently archived in the Concise Guide to PHARMA-COLOGY 2017/18 (Alexander *et al*., 2017a,b,c)

Results

Hispidulin rescued METH-PPI disruption at doses not eliciting benzodiazepine-like effects

In the METH-PPI disruption model, a hyper-dopaminergic model that was responsive to antipsychotic drugs and the *C. inerme* ethanol extract (Chen *et al*., 2012), hispidulin was tested at 1, 4, 10 and 50 mg·kg^{-1} (i.p.). In the METH-PPI group, PPI magnitudes elicited by 71–115 and 77–115 dB were significantly lower than in control mice (Figure 2A, E, F), suggesting that METH disrupts PPI. Hispidulin at 4 mg·kg^{-1} (i.p.) and above significantly reduced METH-PPI disruption. At 10 mg·kg^{-1}, it restored PPI magnitudes elicited by 71–115 and 77–115 dB, respectively, to control levels (Figure 2A). Increasing the dose to 50 mg·kg^{-1}, hispidulin also completely reversed the disruption in METH-PPI (Figure 2A). Note that hispidulin at doses below 100 mg·kg^{-1} did not exert benzodiazepine-like effects (Liao *et al*., 2016),

Hispidulin rescued PPI disruptions induced by NMDA channel blockers

Hispidulin also rescued PPI disruptions induced by NMDA channel blockers. In the ketamine disruption model (Figure 2B), PPI magnitudes elicited by 71–115 and 77–115 dB were reduced by ketamine (30 mg·kg^{-1}, i.p.). Hispidulin, at 10 mg·kg^{-1} (i.p.), completely inhibited ketamine-induced PPI disruption (Figure 2B). In the MK-PPI disruption model (Figure 2C), PPI magnitudes elicited by 71–115 and 77–115 dB were reduced by MK-801 (0.3 mg·kg^{-1}, i.p.) and hispidulin at 50 mg·kg^{-1} (i.p.) restored PPI magnitudes elicited by 71–115 and 77–115 dB to control levels (Figure 2C).

Hispidulin also significantly reversed the PPI disruption induced by PCP (Figure 2D). As shown in Figure 2D, the PPI magnitude elicited by 81–120 dB in ddY mice was reduced by PCP (1 mg·kg^{-1}, s.c.) and was restored by hispidulin (10 mg·kg^{-1}, i.p.) to control levels. Notably, hispidulin (10 mg·kg^{-1}, i.p.) *per se* did not affect PPI in control mice (without PCP treatment) (Figure 2D).

Diazepam did not rescue METH-PPI disruption at motor-impairing doses

In addition to α$_6$GABA$_A$ receptors, hispidulin is also a PAM of other benzodiazepine-sensitive GABA$_A$ receptors (Kavvadias *et al*., 2004). We, therefore, examined whether diazepam, a typical benzodiazepine ineffective at α$_6$GABA$_A$ receptors,

would affect METH-PPI disruption. Diazepam, at doses up to 10 mg·kg^{-1} (i.p.) that displayed significant sedative, anxiolytic and motor-impairing activities in ICR mice (Liao *et al*., 2016), did not rescue METH PPI disruption (Figure 2E). Thus, it is unlikely that hispidulin reversed PPI disruptions *via* acting at diazepam-sensitive GABA$_A$ receptors.

OR-486 rescued METH-PPI disruption

Hispidulin displayed micromolar affinity only at GABA$_A$ receptors and COMT in a binding affinity screening of 92 different neurotransmitter receptors, enzymes and transporters (Liao *et al*., 2016). To investigate the contribution of COMT inhibition to the effect of hispidulin on PPI disruptions, we examined the effect of OR-486, a selective COMT inhibitor (Nissinen *et al*., 1988), on METH-PPI disruption. OR-486 was 2.75 times more potent than hispidulin in inhibiting COMT activity in a concurrent assay (Liao *et al*., 2016). OR-486, at the same dose (10 mg·kg^{-1}, i.p.) as hispidulin, also partly reversed METH-PPI disruption in mice (Figure 2F). Therefore, inhibition of COMT by hispidulin may also contribute to its ability to restore PPI disruption.

Intracerebellar hispidulin rescued METH-PPI, Ketamine-PPI and MK-PPI disruptions

We further investigated whether α$_6$GABA$_A$ receptors, which are mainly expressed in cerebellar granule cells (Gutierrez *et al*., 1996; Pirker *et al*., 2000), could be the target of hispidulin. For this, hispidulin (10 nmol) was administered by bilateral microinjections into the cerebellum (i.cb.) of mice and, as shown in Figure 3, it restored the PPI disruption induced by methamphetamine (Figure 3A) or ketamine (Figure 3B). In the MK-PPI model, i.cb. hispidulin was also effective, although a higher dose (50 nmol) was needed to completely reverse the PPI disruption (Figure 3C).

Hispidulin (i.cb.)-induced PPI restoration was mimicked by α$_6$GABA$_A$ receptor PAMs and prevented by an α$_6$GABA$_A$ receptor antagonist

In a second set of experiments, we used a pharmacological approach with diazepam, Ro15-4513, loreclezole and furosemide to confirm that hispidulin was acting as a PAM at α$_6$GABA$_A$ receptors in the cerebellum. Diazepam is a classical benzodiazepine that is ineffective at α$_6$GABA$_A$ receptors (Derry *et al*., 2004). Ro15-4513, although being a negative allosteric modulator at the benzodiazepine site of diazepam-sensitive GABA$_A$ receptors, is a PAM at diazepam-insensitive GABA$_A$ receptors, including the α$_6$GABA$_A$ receptors (Hadingham *et al*., 1996; Knoflach *et al*., 1996). Loreclezole is a PAM at the majority of β$_{2/3}$-containing GABA$_A$ receptors independent of the α-subunit type (Wingrove *et al*., 1994). Furosemide is a non-competitive selective antagonist of α$_6$GABA$_A$ receptors (Korpi *et al*., 1995).

Bilateral i.cb. microinjections of Ro15-4513 (10 nmol) and loreclezole (10 nmol), but not diazepam (10 nmol), significantly reversed METH-PPI disruption (Figure 4A). These effects of Ro15-4513, loreclezole and hispidulin were prevented by i.cb. co-microinjection of furosemide (10 nmol) (Figure 4B). However, i.cb. furosemide *per se*
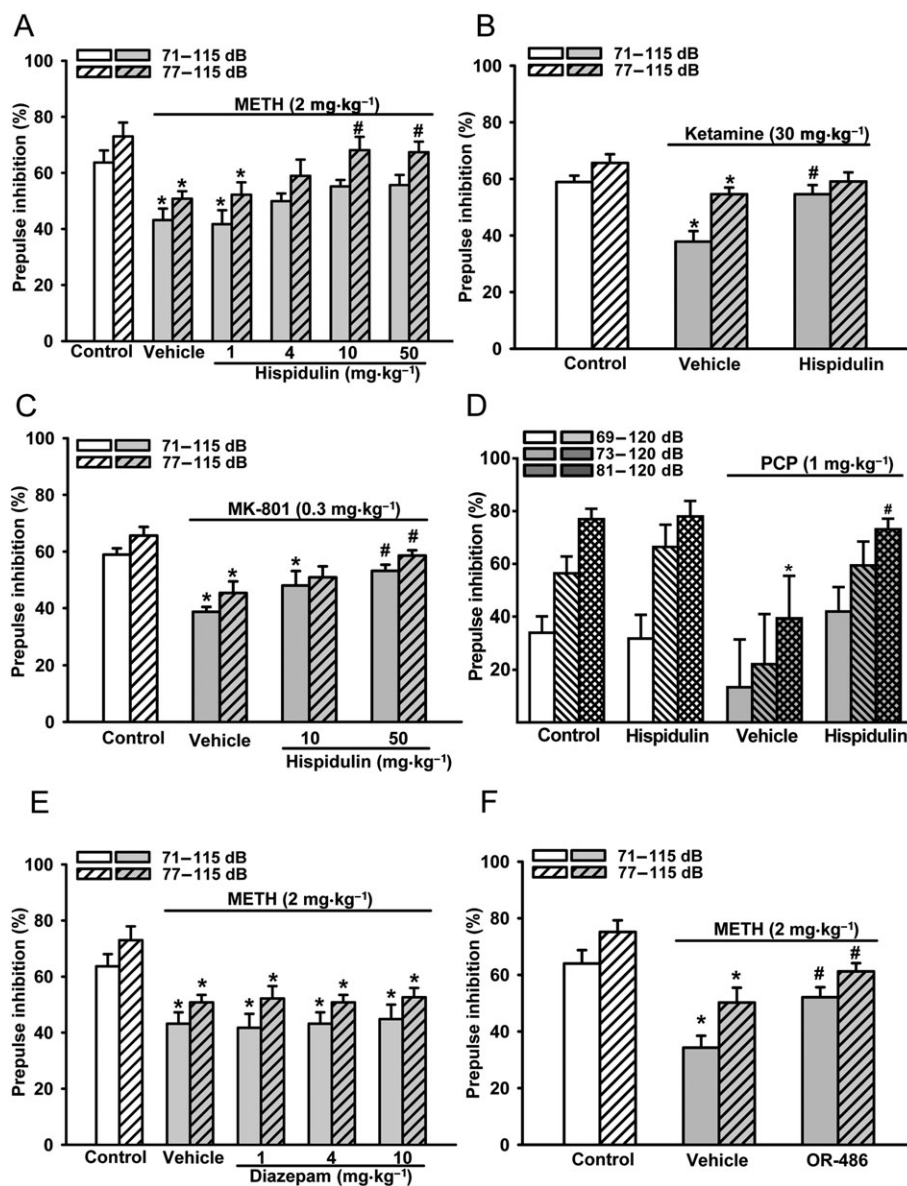
Figure 2

Effects of hispidulin, diazepam and OR-486 on disruptions of prepulse inhibition of acoustic startle response (PPI) in mice. PPI disruptions were induced by methamphetamine (METH-PPI), ketamine (KI-PPI), MK-801 (MK-PPI) or PCP (PCP-PPI). (A) Effects of hispidulin (1, 4, 10 and 50 mg·kg^{-1}, i.p.) on METH-PPI. (B) The effect of hispidulin (10 mg·kg^{-1}, i.p.) on Ketamine-PPI. (C) Effects of hispidulin (10 and 50 mg·kg^{-1}, i.p.) on MK-PPI. (D) The effect of hispidulin (10 mg·kg^{-1}, i.p.) on PCP-PPI. (E) Effects of diazepam (1, 4 and 10 mg·kg^{-1}, i.p.), an α_6GABA$_A$ receptor-inactive classical benzodiazepine, on METH-PPI. (F) The effect of OR-486 (10 mg·kg^{-1}, i.p.), a selective COMT inhibitor, on METH-PPI. The magnitude of PPI was measured by the inhibition ratio of the startle response in mice receiving a 115 dB acoustic stimulation with and without a prepulse of 71 dB (71–115 dB) or 77 dB (77–115 dB) 120 ms ahead, except in the PCP-PPI experiment where three startle protocols, 69–120, 73–120 and 81–120 dB, were used. Mice were i.p. pretreated with the tested drug or vehicle for 10 min, followed by methamphetamine (2 mg·kg^{-1}, i.p.), ketamine (30 mg·kg^{-1}, i.p.) or PCP (1 mg·kg^{-1}, s.c.) for 10 min, or MK-801 (0.3 mg·kg^{-1}, i.p.), 15 min, before the PPI test. The control group of mice was treated with normal saline 10 min before the PPI test. Data shown are means ± SEM; $n = 8$. *$P < 0.05$, significantly different from the control group; #$P < 0.05$, significantly different from the vehicle group; one-way ANOVA with Tukey's *post hoc* test.

affected neither METH-PPI disruption (Figure 4A) nor the PPI in control mice without disruption (Supporting Information Figure S1). Similarly, i.cb. Ro15-4513 (10 nmol) and hispidulin (10 nmol), but not diazepam (10 nmol), were also effective (Figure 4C) in the Ketamine-PPI disruption model.

Effects of Ro15-4513 and hispidulin were also prevented by i.cb. furosemide (10 nmol), in this model, (Figure 4D). These results suggest that hispidulin rescues METH-PPI and Ketamine-PPI disruptions by acting as a PAM of α_6GABA$_A$ receptors in the cerebellum.
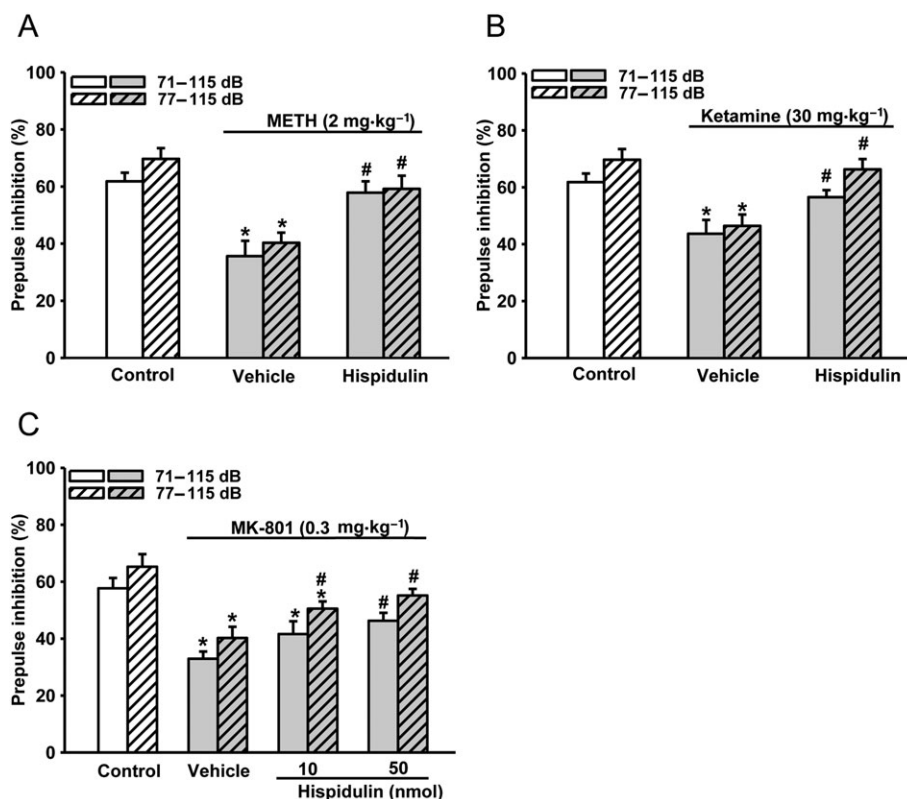
Figure 3

Effects of i.cb. microinjection of hispidulin on disruptions of METH-PPI, Ketamine-PPI and MK-PPI. Hispidulin (10 or 50 nmol) or vehicle was administered by microinjection at the lateral cerebellum (i.cb.) bilaterally 10 min before injection of (A) methamphetamine (METH; 2 mg·kg^{-1}, i.p.), (B) ketamine (30 mg·kg^{-1}, i.p.) or (C) MK-801 (0.3 mg·kg^{-1}, i.p.). PPI was measured and analysed as in Figure 2. Data shown are means ± SEM; $n = 8$. $^*P < 0.05$, significantly different from the control group; $^#P < 0.05$, significantly different from the vehicle group.

Compound 6 reversed METH-PPI disruption and this reversal was prevented by i.cb. furosemide

To directly support the conclusion that PAM action at cerebellar α_6GABA$_A$ receptors can rescue PPI disruption, we examined whether Compound 6, a highly selective α_6GABA$_A$ receptor PAM identified recently (Varagic *et al.*, 2013), can rescue METH-PPI disruption. Indeed, Compound 6, at 3 and 10 mg·kg^{-1} (i.p.), significantly reversed METH-PPI disruption in mice (Figure 5A). Effects of 3 and 10 mg·kg^{-1} were not significantly different, suggesting that the maximal effective dose of Compound 6 is 3 mg·kg^{-1} or lower. Importantly, these effects of Compound 6 (10 mg·kg^{-1}) given systemically, was prevented by i.cb. furosemide (10 nmol) (Figure 5B), suggesting that the cerebellar α_6GABA$_A$ receptors were the target of Compound 6 administered systemically.

Hispidulin and Compound 6 effectively rescued already established METH-PPI disruption

The results described above suggested that hispidulin and Compound 6 could prevent PPI disruptions as both compounds were administered before mice were treated with METH or NMDA channel blockers. To further examine whether they are effective after PPI disruptions had been established, a clinically more relevant condition, we treated mice with Compound 6 or hispidulin 10 min after methamphetamine (2 mg·kg^{-1} i.p), when hyperlocomotion was clearly present in these mice (Liao *et al.*, 2016). As shown in Figure 6, both hispidulin and Compound 6, applied after methamphetamine, effectively reversed the PPI disruptions, as they did when they were applied before methamphetamine. In contrast, neither hispidulin nor Compound 6 *per se* affected PPI after injections of saline (the vehicle of methamphetamine).

Compound 6 had no significant effect on COMT or psychoactive neurotransmitter receptors/transporters

Because inhibition of COMT can also contribute to the restoration of PPI by hispidulin (Figure 2F), we further examined whether Compound 6 could inhibit COMT activity. In an assay of the COMT activity (PT #: 1203067) conducted by Eurofins Panlabs (Beitou, Taipei, Taiwan). (https://www.eurofinspanlabs.com), as used for hispidulin (Liao *et al.*, 2016), Compound 6 at concentrations up to 10 μM displayed only 4% inhibition of COMT activity (Study No. AB61114; Eurofins Panlabs).

We also conducted a receptor binding screen for Compound 6 on a panel of assays of 46 receptors, transporters and channels, including the hERG channel, by the
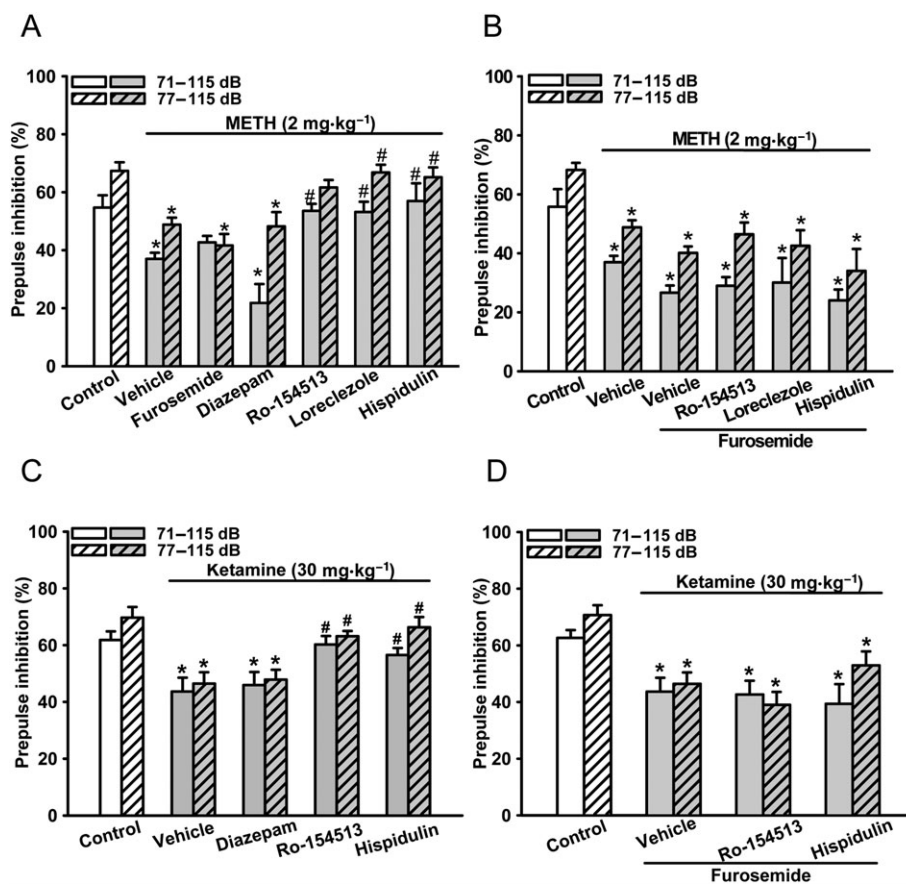
Figure 4

Effects of i.cb. microinjection of hispidulin, Ro15-4513, loreclezole or diazepam on METH-PPI and Ketamine-PPI disruptions and their interactions with i.cb. furosemide. Hispidulin, Ro15-4513 (an α_6 GABA$_A$ receptor-active benzodiazepine), loreclezole (an anticonvulsant triazole derivative effective at most GABA$_A$ receptors), diazepam (an α_6GABA$_A$ receptor-inactive classical benzodiazepine) and furosemide (an α_6GABA$_A$ receptor-selective allosteric antagonist) were i.cb. administered at 10 nmol (A, C) without or (B, D) with i.cb. co-administration of furosemide 10 min before administration of (A, B) methamphetamine (2 mg·kg^{-1}, i.p.) or (C, D) ketamine (30 mg·kg^{-1}, i.p.). PPI was measured and analysed as in Figure 2. Data shown are means ± SEM; $n = 8$. $^*P < 0.05$, significantly different from the control group; $^\#P < 0.05$, significantly different from the vehicle group.

Psychoactive Drug Screening Program of the National Institute of Mental Health. Compound **6** at 10 μM did not display significant (>50%) inhibition of the radioligand binding on almost all of the tested targets (Supporting Information Table S1).

Hispidulin, but not OR-486, positively modulated $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors where Compound 6 is a potent and selective PAM

Hispidulin has previously been reported as a PAM at $\alpha_6\beta_2\gamma_2$GABA$_A$ receptors (Kavvadias *et al.*, 2004), and we demonstrated that Compound **6** is a highly selective PAM at GABA$_A$ receptors consisting of $\alpha_6\beta_2\gamma_2$ or $\alpha_6\beta_3\gamma_2$ subunits (Varagic *et al.*, 2013). To investigate a possible effect of OR-486 on α_6GABA$_A$ receptors, under the same conditions as hispidulin and Compound **6**, here we compared the effects of all three compounds at $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors expressed in *Xenopus* oocytes with concatenated subunits. Compound **6** (10 μM) increased GABA currents (at EC$_{3-5}$) by more than eight-fold (Figure 7), which was identical to its PAM effect

on $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors in our previous study, using non-concatenated subunit expression (Varagic *et al.*, 2013) (diamond symbols in Figure 7), supporting the validity of data obtained from concatenated $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors. At these concatenated $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors, hispidulin (0.3–10 μM) increased GABA currents in a concentration-dependent manner, but its efficacy was lower than that of Compound **6** at 10 μM (Figure 7). In contrast, OR-486 did not elicit significant modulatory effects on these receptors (Figure 7), even at 10 μM.

Hispidulin, in contrast to Compound 6, did not modulate $\alpha_6\beta_3\delta$GABA$_A$ receptors

The γ_2-subunit-containing and δ-subunit-containing α_6GABA$_A$ receptors mediate phasic and tonic inhibition of cerebellar granule cells respectively (Nusser *et al.*, 1998). Although Compound **6** is a highly selective PAM at $\alpha_6\beta_{2/3}\gamma_2$GABA$_A$ receptors (Varagic *et al.*, 2013), it may also modulate the δ-subunit-containing α_6GABA$_A$ receptors. We therefore examined whether Compound **6** and hispidulin modulated $\alpha_6\beta_3\delta$GABA$_A$ receptors. In
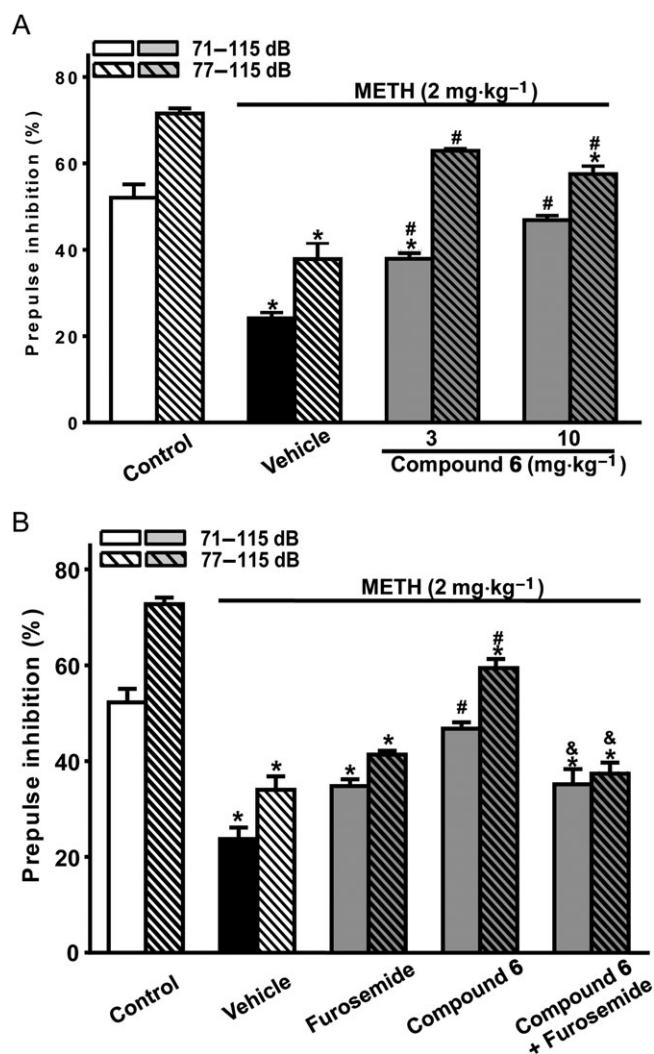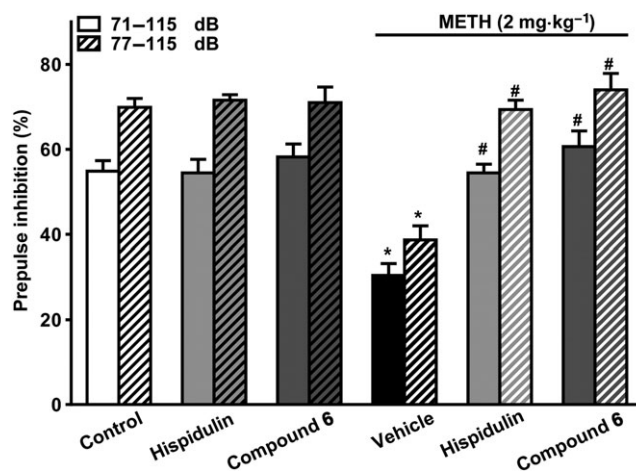
Figure 6

Effects of hispidulin and Compound **6** on PPI in mice without and with methamphetamine pretreatment. Mice without or with methamphetamine (2 mg·kg^{-1}, i.p.) pretreatment for 10 min were i.p. injected with hispidulin (10 mg·kg^{-1}), Compound **6** (10 mg·kg^{-1}) or vehicle 10 min before performing the PPI test. PPI was measured and analysed as in Figure 2. Data shown are means ± SEM; $n = 6$. $^{*}P < 0.05$, significantly different from the control group; $^{\#}P < 0.05$, significantly different from the vehicle group.

in the presence of 3 μM hispidulin and were 100% and 130% with 10 μM hispidulin. This indicates that hispidulin at concentrations up to 10 μM did not significantly affect $\alpha_6\beta_3\delta$GABA$_A$ receptors and, therefore, modulation of only the $\alpha_6\beta_{2/3}\gamma_2$GABA$_A$ receptors is necessary and sufficient to account for the restoration of PPI by hispidulin and Compound **6**.

Discussion

Hispidulin and Compound 6 rescued PPI disruptions as PAMs at cerebellar $\alpha_6\beta_{2/3}\gamma_2$GABA$_A$ receptors

Here, we demonstrated that hispidulin, a flavonoid and benzodiazepine site ligand, significantly restored PPI disruptions in several mouse models based on the hyper-dopaminergic and hypo-glutamatergic hypotheses of schizophrenia. Several lines of evidence indicate that hispidulin restored disrupted PPI by acting as a PAM at cerebellar $\alpha_6\beta_{2/3}\gamma_2$GABA$_A$ receptors. First, hispidulin restored the disrupted PPI after systemic administration of a dose which had no benzodiazepine-like effects (Liao *et al.*, 2016). Second, diazepam, a typical benzodiazepine ineffective at α_6GABA$_A$ receptors, did not affect PPI disruption. Third, direct microinjection of hispidulin into the cerebellum restored PPI disruptions, and this effect was mimicked by i.cb. α_6GABA$_A$ receptor PAMs, such as Ro15-4513 or loreclezole, or systemic administration of the α_6GABA$_A$ receptor-selective PAM, Compound **6**, but not by i.cb. diazepam. Fourth, effects of all α_6GABA$_A$ receptor PAMs on PPI disruption were prevented by i.cb. furosemide, an allosteric α_6GABA$_A$ receptor antagonist. Fifth, hispidulin seemed to restore PPI disruptions *via* $\alpha_6\beta_{2/3}\gamma_2$GABA$_A$ receptors, as it did not

Figure 5

Effects of Compound **6** (i.p.) on METH-PPI disruption in mice with and without i.cb. pretreatment with furosemide. Compound **6** (3 or 10 mg·kg^{-1}) or vehicle was administered by i.p. injection 10 min before injection of methamphetamine (2 mg·kg^{-1}, i.p.). Furosemide was administered by i.cb. bilaterally alone or 5 min before Compound **6** (10 mg·kg^{-1}, i.p.) was given. PPI was measured and analysed as in Figure 2. Data shown are means ± SEM; $n = 8$. $^{*}P < 0.05$, significantly different from the control group, $^{\#}P < 0.05$, significantly different from the vehicle group, $^{\&}P < 0.05$, significantly different from the Compound **6** alone group.

recombinant $\alpha_6\beta_3\delta$GABA$_A$ receptors, the GABA current levels were too low to measure at the EC$_{3-5}$ of GABA. Even at the EC$_{10}$, GABA currents in most of oocytes were too low to be detectable. Nevertheless, in two independent experiments with successful recordings of EC$_{10}$ GABA currents, Compound **6** at 3 μM increased GABA currents to 270 and 270% of control and at 10 μM to 440 and 500%. As compared with $\alpha_6\beta_{2/3}\gamma_2$GABA$_A$ receptors where 10 μM Compound **6** increased GABA currents to 800 ± 70% of control (Varagic *et al.*, 2013), Compound **6** is a weaker PAM in $\alpha_6\beta_3\delta$GABA$_A$ receptors. In a concurrent assay with the same oocytes expressing $\alpha_6\beta_3\delta$GABA$_A$ receptors, GABA currents were 95% and 105%
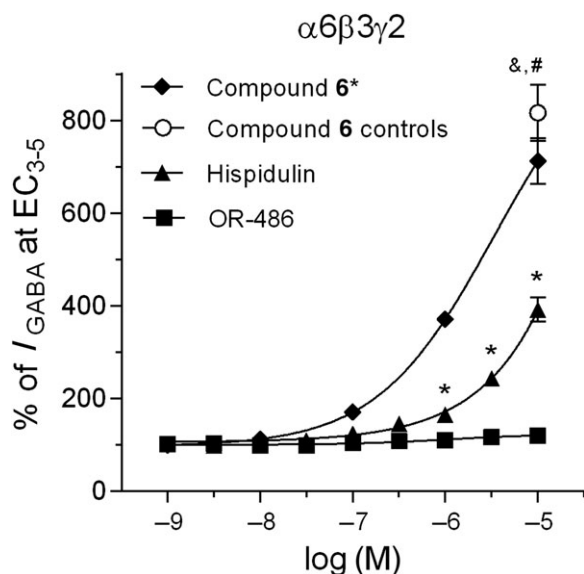
α6β3γ2

Figure 7

Effects of hispidulin and OR-486, in comparison with Compound **6**, on GABA currents of recombinant $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors expressed in *Xenopus* oocytes. Concatenated recombinant $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors were expressed in *Xenopus laevis* oocytes. The ordinate indicates the modulatory effect of hispidulin, OR-486 or Compound **6** on the GABA current elicited by GABA at EC$_{3-5}$ (the effective concentration that induced 3–5% of maximum GABA currents), expressed as % of the control GABA current ($I_{GABA, EC3-5}$). Hispidulin (30 nM–10 μM) and OR-486 (1 nM–10 μM) were tested. Compound **6**, serving as the positive control of the tested oocytes, was tested at 10 μM. The concentration–response curve of Compound **6** (Compound **6***) was taken from Varagic *et al.* (2013). Data are means ± SEM for the Compound **6**, hispidulin and OR-486 groups obtained from seven, five and six cells, respectively, which were prepared from three separate cell batches. *$P < 0.05$, hispidulin significantly different from solvent; one-way ANOVA with Dunnett's *post hoc* test, &$P < 0.05$, Compound **6** significantly different from solvent; paired *t*-test, #$P < 0.05$, Compound **6** significantly different from hispidulin; unpaired *t*-test.

modulate $\alpha_6\beta_3\delta$GABA$_A$ receptors. Sixth, combining the target screening results of hispidulin (Liao *et al.*, 2016) and Compound **6** (Supporting Information Table S1) and their effects on COMT activity, the only common target of hispidulin and Compound **6** in restoring PPI seems to be the $\alpha_6\beta_{2/3}\gamma_2$GABA$_A$ receptor.

COMT inhibition contributes to hispidulin-induced, but not Compound 6-induced, PPI restoration

In addition to modulating α_6GABA$_A$ receptors, hispidulin is also a COMT inhibitor (Liao *et al.*, 2016). The selective COMT inhibitor OR-486 also restored the METH-PPI disruption model. This effect does not involve α_6GABA$_A$ receptors as OR-486 did not modulate $\alpha_6\beta_3\gamma_2$GABA$_A$ receptors. Therefore, COMT inhibition may also contribute to hispidulin-induced PPI restoration. In contrast, Compound **6** did not inhibit COMT, excluding the contribution of COMT inhibition in its restoration of PPI.

Hispidulin is a constituent of C. inerme, effective in attenuating both MIH and PPI disruptions

Hispidulin alleviated MIH by acting as a PAM at cerebellar α_6GABA$_A$ receptors (Liao *et al.*, 2016). Here, we demonstrate that hispidulin also rescued PPI disruptions *via* cerebellar α_6GABA$_A$ receptors at a similar dose. The extract of *C. inerme* from which we isolated hispidulin (Huang *et al.*, 2015) also alleviated MIH and restored PPI (Chen *et al.*, 2012), suggesting hispidulin is the active constituent of the extract, in both models, by modulation of cerebellar α_6GABA$_A$ receptors.

In contrast, COMT inhibition only contributes to hispidulin-induced PPI restoration (this study) but not MIH alleviation (Liao *et al.*, 2016). Although both PPI deficit and MIH are hyper-dopaminergic behavioural models, their underlying circuitries and mechanisms are different. In the striatum, which is involved in hyperlocomotion (Nelson and Kreitzer, 2014), COMT plays a minor role in dopamine clearance compared with synaptic uptake by the **dopamine transporter**. In contrast, dopamine transporters are sparse in the prefrontal cortex (PFC), which is involved in PPI processing and, therefore, COMT activity is crucial in regulating dopamine levels in the PFC (Tunbridge *et al.*, 2006; Sagud *et al.*, 2010). In addition, reduced dopaminergic activity in the PFC leads to impaired PPI (Koch and Bubser, 1994; Ellenbroek *et al.*, 1996), and there is an inverted-U relationship between the dopamine level and cognitive functions in PFC (Farrell, 2012): PPI and cognitive functions are improved at low but inhibited at high dopamine levels. However, increased dopaminergic activity in the nucleus accumbens (a part of the ventral striatum) also impairs PPI (Swerdlow *et al.*, 2007; Swerdlow *et al.*, 2013). This may explain why methamphetamine, by inducing massive release of dopamine in both PFC and nucleus accumbens, causes PPI disruption whereas COMT inhibitors, by increasing dopamine levels in the PFC only, can restore PPI.

How can a cerebellar α6GABA_A receptor PAM restore PPI disruptions?

To the best of our knowledge, this is the first study demonstrating that positive allosteric modulation of cerebellar α_6GABA$_A$ receptors can rescue disrupted PPI. Several reports in the literature shed some light on a possible explanation (Figure 8).

α_6GABA$_A$ receptors are mainly located at cerebellar Golgi cell–granule cell synapses and extrasynaptic sites (Gutierrez *et al.*, 1996; Pirker *et al.*, 2000), mediating phasic and tonic inhibition of granule cells *via* the γ2-subunit-containing and δ-subunit-containing ones respectively (Nusser *et al.*, 1998). Granule cells are important excitatory interneurons in the cerebellar cortex, providing a direct excitatory input onto Purkinje cells (PCs). PCs are the major output neurons of the cerebellar cortex, providing an inhibitory control on the downstream deep cerebellar nuclei (DCN) (Sacchetti *et al.*, 2005). By enhancing GABAergic inhibition of granule cells, α_6GABA$_A$ receptor PAMs may reduce the activity of PCs, ultimately enhancing the activity of DCN and downstream DCN-innervated brain regions (Figure 8).

The diagram shown in Figure 8, however, does not take into account the dynamics of the system. Both PCs and
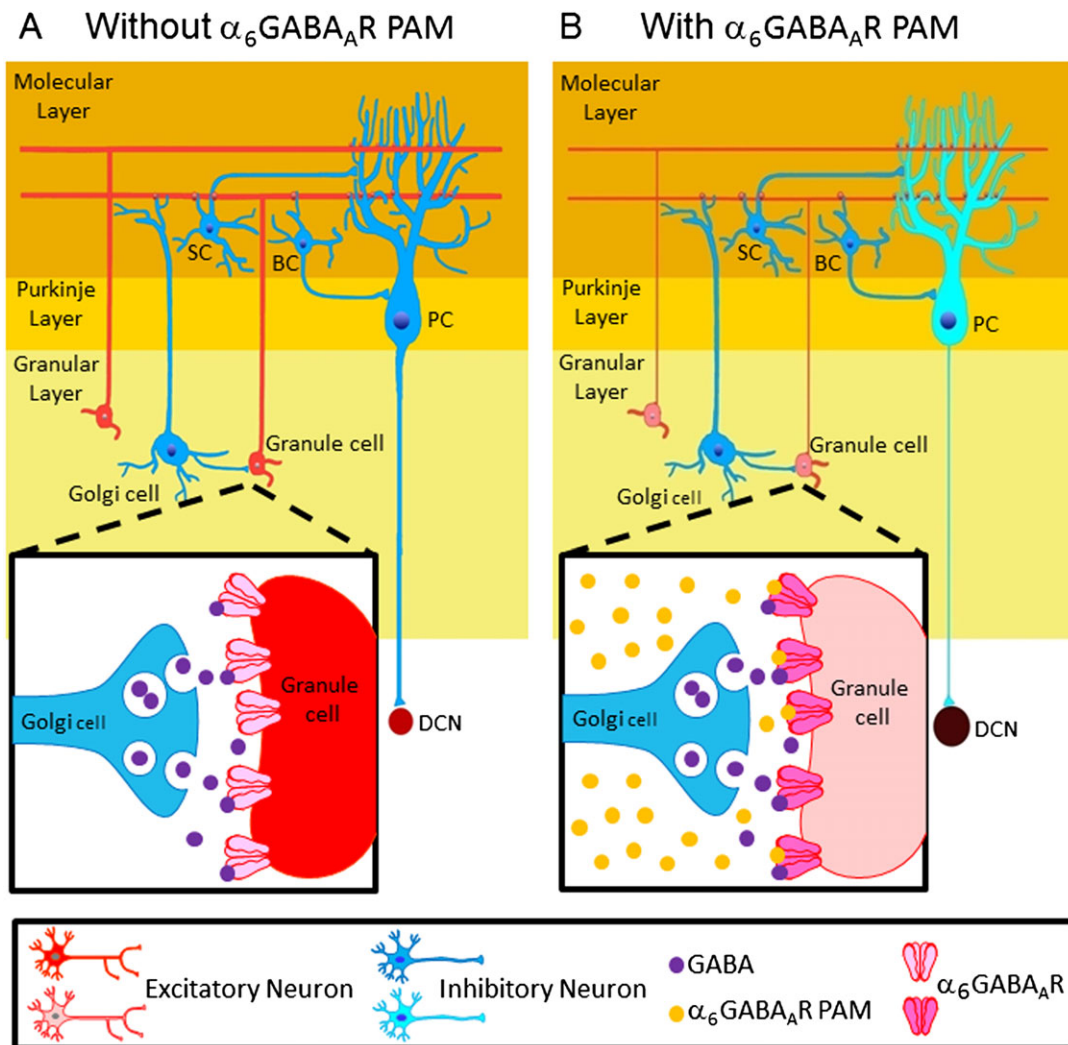
Figure 8

A proposed model for how an α_6GABA$_A$ receptor PAM affects canonical cerebellar circuits and ultimately leads to the restoration of prepulse inhibition. Schemes of the basic circuit of the cerebellar cortex, which consists of molecular, Purkinje and granule layers, (A) before and (B) after treatment with an α_6GABA$_A$ receptor PAM, such as hispidulin or Compound **6**. The excitatory (red) and inhibitory (blue) neurons in the cerebellar cortex (the yellow part) that may be involved in the action of α_6GABA$_A$ receptor PAMs, including Golgi cells that form GABAergic synapses onto granule cells, where the α_6GABA$_A$ receptors are located synaptically and extrasynaptically (enlarged squares). Granule cells form excitatory synapses on dendrites of PCs and also send feedforward inhibition onto PCs indirectly through GABAergic interneurons such as basket cells (BCs) and stellate cells (SCs). PCs are GABAergic output neurons in the cerebellar cortex, providing an inhibitory control onto the downstream DCN. Thinner lines and lighter colours represent reduced neurotransmission and neuronal activity, respectively, after α_6GABA$_A$ receptor PAM treatment. α_6GABA$_A$ receptor PAMs act by enhancing Golgi-GABAergic transmission to granule cells, which then decreases PC activity as a result of the attenuated excitatory inputs from granule cells and this sequence of events ultimately decreases the inhibitory output from the cerebellar cortex. This will activate the DCN, to increase dopamine levels in the PFC, leading to the restoration of prepulse inhibition.

DCN neurons spontaneously fire tens of action potentials per second *in vivo* when animals are not engaged in cerebellar behaviours and firing rates can be increased by synaptic excitation and decreased by synaptic inhibition (Pugh and Raman, 2009). Due to their specific ion channels, even a short excitation can drive DCN neurons into a depolarization block. Their firing usually resumes only after an active hyperpolarization, likely to be due to the inhibitory GABAergic input from PCs (Pugh and Raman, 2009).

Granule cells, in addition to activating PCs, also increase feedforward and lateral inhibition of PCs. Feedforward inhibition limits the excitation of directly activated PCs, whereas lateral inhibition reduces the activity of other PCs, leading to increased spike precision. In addition, phasic, but not tonic, inhibition also plays an important role in shaping the timing and precision of granule cell firing (Nieus *et al.*, 2014). Therefore, $\alpha_6\beta_{2/3}\gamma_2$GABA$_A$ receptor-selective PAMs would only modulate those granule cells that are activated and thus increase spike precision and synchronization of

associated PCs, which is important for coupling to a specific DCN neuronal output. DCN neurons preferentially relay the spike timing of synchronized PCs to downstream premotor and other brain areas (Pugh and Raman, 2009; Person and Raman, 2012; Najac and Raman, 2015).

Increasing evidence suggests that the cerebellum, aside from its role in motor coordination, contributes to cognitive functioning. This probably involves the connections of PCs in the posterior lateral cerebellum to DCN neurons distinct from those controlling motor activity that convey the information from the cerebellar cortex to the PFC *via* the thalamus (Caligiore *et al.*, 2016). Therefore, the cerebellum has been proposed to be involved in the pathophysiology of neuropsychiatric disorders (Phillips *et al.*, 2015; Caligiore *et al.*, 2016). For example, patients with schizophrenia had lower emotion-induced cerebellar activity (Mothersill *et al.*, 2015) and reduced glucose consumption in the dorsal PFC than normal subjects (Andreasen *et al.*, 1996) when performing a memory task, suggesting a low activity of this connection. At the cellular level, reduced GABA-synthesizing enzyme has been reported in *post mortem* schizophrenia cerebellar tissues, as well as at cerebellar Golgi–granule synapses, of rats chronically treated with PCP (Bullock *et al.*, 2008), an animal model for schizophrenia (Grayson *et al.*, 2016). These results suggest that patients with schizophrenia have impaired Golgi-inhibitory control and overactive granule cells and thus overactive PCs in the cerebellar cortex.

The circuits involved in PPI are complex, especially the role of the cerebellum in PPI regulation is far from clear (Takeuchi *et al.*, 2001). Nevertheless, PPI was enhanced in mice with impaired excitatory transmission at PCs, elicited by a targeted deletion of PC-specific glutamate receptors (Takeuchi *et al.*, 2001). If a reduced excitatory input at PCs *via* granule cells causes an enhanced PPI (Takeuchi *et al.*, 2001), then overactive granule cells, possibly driven by a multitude of unfiltered information contributing to cognitive impairments in patients with schizophrenia (Lewis *et al.*, 2004; Bullock *et al.*, 2008), may contribute to PPI disruptions.

Electrical stimulation at DCN can evoke dopamine efflux in the PFC (Mittleman *et al.*, 2008) and decreased PFC dopaminergic activity leads to impaired PPI (Koch and Bubser, 1994; Ellenbroek *et al.*, 1996). Therefore, α_6GABA$_A$ receptor PAMs may restore disrupted PPI by reducing granule cell activity and enhancing synchronization of PCs, which is important for activating appropriate DCN neurons, and then increase dopamine levels in the PFC, leading to PPI restoration. Further experiments will have to investigate this tentative scheme providing a possible explanation for the present observations. However, our findings that hispidulin and Compound **6** only restored disrupted PPI but did not affect PPI in control animals (Figure 2D, Figure 6) are consistent with the synchronizing activity of α_6GABA$_A$ receptor PAMs: they will not change synchronization of adequately synchronized PCs in control animals.

In conclusion, using hispidulin and a structurally unrelated pyrazoloquinolinone Compound **6**, we demonstrated that positive allosteric modulation of cerebellar α_6GABA$_A$ receptors, for which so far no function was known, could prevent or treat PPI disruptions. Moreover, we provided a tentative explanation for this finding. PPI disruptions are considered sensorimotor gating deficits that are usually manifested in patients with several neuropsychiatric disorders, including but not limited to tic disorders and schizophrenia. Therefore, this study may pave the way for the development of α_6GABA$_A$ receptor-selective PAMs as a novel treatment for sensorimotor gating deficits in these neuropsychiatric disorders. Compound **6**, which was systemically effective and thus blood–brain barrier permeable and is devoid of HERG channel activity (and thus lacking cardiotoxicity, see Supporting Information Table S1), may serve as a lead compound for the future drug development.

Author contributions

L.-C.C., H.-J.L., M.E., H.-L.C., J.-F.C., A.M. and T.N. designed the study; H.-J.L., H.-L.C., J.-F.C., M.T. and A.M. acquired and analysed the data; L.-C.C., W.-J.H., D.E.K., C.W. and J.C. provided the compounds; M.E., P.-C.F., A.M. and T.M. participated in discussing the data and writing the manuscript; and L.-C.C., M.E., W.S. and T.N. wrote and finalized the article, which all authors reviewed and approved for publication.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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Figure S1 Intra-cerebellar (*i.cb.*) microinjection of furosemide alone did not affect PPI. Furosemide (10 nmol) or vehicle was administered by bilateral *i.cb.* microinjection. PPI was measured and analysed as in Figure . Note that there is no significant difference in the magnitude of PPI elicited by either 70–115 dB or 77–115 dB. $N = 6$.

Table S1 Radioligand displacement studies conducted by the Psychoactive Drugs Screening Program, National Institute of Medical Health to determine the binding affinity of Compound 6 over a panel of 46 receptors, channels and transporters.