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ARTICLE

Pharmacological insights into the role of $P2X_4$ receptors in behavioural regulation: lessons from ivermectin

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Abstract

Purinergic ionotropic P2X receptors are a family of cation-permeable channels that bind extracellular adenosine 5′-triphosphate. In particular, convergent lines of evidence have recently highlighted $P2X_4$ receptors as a potentially critical target in the regulation of multiple nervous and behavioural functions, including pain, neuroendocrine regulation and hippocampal plasticity. Nevertheless, the role of the $P2X_4$ receptor in behavioural organization remains poorly investigated. To study the effects of $P2X_4$ activation, we tested the acute effects of its potent positive allosteric modulator ivermectin (IVM, 2.5–10 mg/kg i.p.) on a broad set of paradigms capturing complementary aspects of perceptual, emotional and cognitive regulation in mice. In a novel open field, IVM did not induce significant changes in locomotor activity, but increased the time spent in the peripheral zone. In contrast, IVM produced anxiolytic-like effects in the elevated plus maze and marble burying tasks, as well as depression-like behaviours in the tail-suspension and forced swim tests. The agent induced no significant behavioural changes in the conditioned place preference test and in the novel object recognition task. Finally, the drug induced a dose-dependent decrease in sensorimotor gating, as assessed by pre-pulse inhibition (PPI) of the acoustic startle reflex. In $P2X_4$ knockout mice, the effects of IVM in the open field and elevated plus maze were similar to those observed in wild type mice; conversely, the drug significantly increased startle amplitude and failed to reduce PPI. Taken together, these results suggest that $P2X_4$ receptors may play a role in the regulation of sensorimotor gating.

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Introduction

Purinergic ionotropic P2X receptors are a family of hetero- and homotrimeric cation-permeable channels that bind extracellular adenosine 5′-triphosphate (North, 2002). The biophysical and pharmacological properties of P2X receptors are defined by their subunit composition (Khakh & North, 2006). In particular, of the seven P2X subunits characterized to date (termed P2X1 to P2X7), recent evidence has highlighted $P2X_4$ subunits as a potentially interesting target in the regulation of nervous functions. $P2X_4$ subunits form homotrimeric receptors with distinct functional properties (Bo *et al.* 1995; Soto *et al.* 1996), as well as heteromers with other P2X subunits (Guo *et al.* 2007; Nicke *et al.* 2005; Ormond *et al.* 2006; for contrasting results, see Antonio *et al.* 2011). $P2X_4$ receptors are abundant in the central nervous system (Rubio & Soto, 2001; Tsuda *et al.* 2003) and involved in the regulation of neuropathic pain (Tsuda *et al.* 2003; Ulmann *et al.* 2008; Zhang *et al.* 2006), neuroendocrine regulation (Zemkova *et al.* 2010) and hippocampal plasticity (Baxter *et al.* 2011; Lorca *et al.* 2011; Sim *et al.* 2006). Furthermore, $P2X_4$ receptors have been recently shown to modulate the activity of γ-amino-butyric acid $(GABA)_A$ (Jo *et al.* 2011) and *N*-methyl-D-aspartate glutamate (Baxter *et al.* 2011) receptors. Recent studies have documented that some antidepressants may inhibit $P2X_4$ receptors (Nagata *et al.* 2009), although this evidence remains controversial in view of contrasting findings (Sim & North, 2010; Toulme *et al.* 2010*a*). Furthermore, converging lines of *in vitro* and *in vivo* evidence suggest that $P2X_4$ receptors may be an important therapeutic target for alcohol use disorders (Asatryan *et al.* 2010, 2011; Kimpel *et al.* 2007; Popova *et al.* 2010; Tabakoff *et al.* 2009).

In contrast with this background, the pharmacological armamentarium to probe $P2X_4$ receptors is very limited.

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$P2X_4$ receptors are poorly sensitive to P2X receptor antagonists, such as suramin and pyridoxal phosphate-6-azo-(benzene-2,4-disulfonic acid) (Buell *et al.* 1996). Conversely, research has shown that the anti-parasitic ivermectin (IVM) acts as a potent positive allosteric regulator of $P2X_4$ in either its homomeric or heteromeric configurations, but does not interact with other P2X receptors (Khakh *et al.* 1999; Priel & Silberberg, 2004). IVM is a mixture of semi-synthetic macrocyclic lactone disaccharides derived by hydroxylation of avermectins B_{1a} and B_{1b}, natural fermentation products of the actinomycete *Streptomyces avermitilis*. IVM is widely adopted in human and veterinarian medicine as a broad-spectrum anthelmintic and insecticide agent (Geary, 2005; Richard-Lenoble *et al.* 2003), with high therapeutic index and limited side-effects. The anti-parasitic mechanism of IVM is based on the activation of glutamate-gated chloride channels specific to invertebrates (Cully *et al.* 1994; Vassilatis *et al.* 1997).

In mammalians, in addition to its action on $P2X_4$ receptors, IVM has been shown to activate $GABA_A$ receptors and exert weak effects on the positive modulation of other ionotropic channels, including glycine, and acetylcholine nicotinic receptors (Collins & Millar, 2010; Dawson *et al.* 2000; Krause *et al.* 1998; Sattelle *et al.* 2009; Shan *et al.* 2001; Sung *et al.* 2009).

Previous studies have revealed that long-term administration of IVM has subtle behavioural effects on locomotor and sensory reactivity of mice (Davis *et al.* 1999); furthermore, this substance has been shown to exert anxiolytic-like properties in rats (de Spinosa *et al.* 2002). Capitalizing on our discovery that IVM antagonizes ethanol-mediated inhibition of $P2X_4$ receptors (Asatryan *et al.* 2010), we recently found that this compound reduces alcohol consumption (Yardley *et al.* 2012). Nevertheless, to the best of our knowledge, the acute behavioural effects of IVM have not been the object of systematic investigations. Thus, in consideration of the potential role of $P2X_4$ receptors in the regulation of brain functions, we addressed the present study to determine the perceptual, emotional and cognitive impact of IVM in C57BL/6 mice, using a broad spectrum of well-validated paradigms targeting complementary aspects of behavioural regulation.

Materials and method

Animals

Studies were performed on drug-naive, C57BL/6J (Jackson Laboratory, USA) wild-type (WT) and $P2X_4$ knockout (KO) male mice, aged 6–10 wk at the time of testing. $P2X_4$ KO mice were generated and genotyped according to previous protocols (Sim *et al.* 2006). Animals were acclimatized to the housing facility for a minimum of 1 wk and group-housed (four mice per cage) in polycarbonate cages, with *ad libitum* access to food and water. The holding room was maintained at approximately 22 °C with a 12:12 h light:dark cycle (lights on 06:00 hours). All procedures were in compliance with the National Institute of Health guidelines and the protocols were approved by the University of Southern California Institutional Animal Care and Use Committee.

Generation and identification of KO $P2X_4^{-/-}$ mice

The current $P2X_4$ KO colony in our laboratory was started by rederivation (USC Transgenic Core, USA) of frozen heterozygous $P2X_4^{+/-}$ embryos (via surrogate female C57BL/6 mice (Jackson Laboratory). Presently, we are maintaining the $P2X_4R$ KO colony using sixth generation heterozygous $P2X_4^{+/-}$ backcrossed onto C57BL/6J background. Upon weaning, offspring are genotyped, separated by sex and maintained in groups of four in individually ventilated cages with free access to food and water under a 12:12 h light/dark cycle at 26 ± 1 °C.

Drugs

IVM (Norbrook Inc., USA) and its vehicle propylene glycol (Alfa Aesar, USA) were diluted in a 0.9% saline solution, to a concentration that would allow for an injection volume of 0.01 ml/g body weight. Throughout all experiments, IVM was injected 8 h before behavioural testing, as our previous studies documented that the maximal efficacy and the T_{max} of the drug in brain and plasma was reached at this time (Yardley *et al.* 2012).

Preliminary physical assessment

The impact of IVM in mice was tested on posture, gait, heart rate, breathing frequency and neurological reflexes (righting reflex, postural reflex, eye-blink reflex and whisker-orienting reflex; Davis *et al.* 1999).

Sticky tape test

Mice were briefly restrained and a circular piece of tape was placed on the bottom of each forepaw. Mice were then released and the latency to remove the first piece of tape was recorded.

Hot plate

Mice were individually exposed to a hot plate (IITC Life Science, USA) at 47.5 and 50 °C and the latency to lick their paws was measured. We used a cut-off time of 40 s to eliminate any potential tissue damage.

Novel open field

The open field was a Plexiglas square grey arena (40×40 cm) surrounded by four black walls (40 cm high). On the floor, two concentric zones of equivalent areas were defined: a central square quadrant and a peripheral frame directly adjacent to the walls. Mice were placed in the centre and their behaviour was monitored for 5 min.

Analysis of locomotor activity was performed using Ethovision (Noldus Instruments, The Netherlands). Behavioural measures included the distance travelled, duration in the central zone, percent activity in the centre (defined as the distance travelled in the centre divided by the total distance travelled), meandering (defined as the ratio of the turn-angle degrees over total distance; Kalueff *et al.* 2007), number of rearing episodes and percent time moving (calculated as the time spent moving over the total time).

Elevated plus maze

Behaviour in the elevated plus maze was monitored as described elsewhere (Bortolato *et al.* 2009). The apparatus was black Plexiglas with a light grey floor and consisted of two open (25×5 cm) and two closed ($25 \times 5 \times 5$ cm) arms, which extended from a central platform (5×5 cm) at 60 cm from the ground. Mice were individually placed on the central platform facing an open arm and their behaviour was observed for 5 min by an experimenter unaware of the genotype. An arm entry was counted when all four paws were inside the arm. Behavioural measures included time spent and entries into each partition of the elevated plus maze, number of head dips, stretch-attend postures (both defined as in Rodgers *et al.* 1992) and rears.

Marble burying

Marble burying was tested as previously described (Bortolato *et al.* 2009). Briefly, mice were acclimatized for 10 min to novel cages (35×28 cm) filled with sawdust. At the end of this phase, they were briefly removed and 20 glass marbles were placed on the surface of the cage at even distances. Animals were then reintroduced into the cages and their behaviour was monitored for the following 10 min. The number of marbles buried, as well as the frequency and overall duration of digging was scored. A marble was considered buried if at least two-thirds of its surface area was covered in sawdust.

Forced swim test

The forced swim test was performed as previously described (Gobbi *et al.* 2005). Briefly, mice were habituated to clear Plexiglas cylinders (40 cm $\times$ 19 cm in diameter) filled to 15 cm with water for 1 min. The water temperature was maintained at 30 °C. On the following day, mice were re-exposed to the cylinder using the above-mentioned conditions for 5 min. Environmental light was kept at 300 lux. Animals were video-recorded and the duration of immobility (s) and the latency to immobility (s) were measured.

Tail suspension test

The tail suspension test was performed as described elsewhere (Scott *et al.* 2008). Mice were individually suspended by the tail using medical tape affixed to a hook, at 30 cm from the floor. Environmental light was kept at 300 lux. Animals were video-recorded for 6 min and the duration of immobility (s), the latency to immobility (s) and number of faecal boli were measured.

Conditioned place preference (CPP)

CPP was evaluated as previously described (Bortolato *et al.* 2006; Maldonado *et al.* 1997). The apparatus consisted of two different compartments ($15 \times 15 \times 15$ cm) separated by a central neutral area (start compartment), through two guillotine doors. The two compartments featured different visual cues (striped or triangular patterns) on the walls and tactile cues (square or chequered grid) on the floor. The combinations of visual and tactile cues were present in the compartments in a counterbalanced order. The experiment was conducted with a biased design and consisted of three consecutive phases. In phase I (pre-conditioning), the animals were habituated to the apparatus for 2 d and their initial side preferences were recorded in a 15-min trial. Phase II (training) lasted 10 d. On odd days, mice received IVM or vehicle and were placed in the non-preferred (NP) compartment (with the guillotine door closed) for 15 min; conversely, on even days, mice received vehicle and were placed in the preferred compartment for 15 min. On the test day (post-conditioning, phase III), the animals were given no treatment and were placed in the start compartment, with free access to both sides for 15 min. IVM preference was measured as the difference between the time spent in the drug-paired compartment (NP) during the post-conditioning and pre-conditioning phases.

Acoustic startle and pre-pulse inhibition (PPI) of the startle reflex

Acoustic startle reflex and PPI were tested as previously described (Bortolato *et al.* 2005). The apparatus used for detection of startle reflex (San Diego Instruments, USA) consisted of one standard cage placed in sound-attenuated chambers with fan ventilation. Each cage consisted of a Plexiglas cylinder of 3 cm diameter, mounted on a piezoelectric accelerometric platform connected to an analogue–digital converter. Background noise and acoustic bursts were conveyed by two separate speakers, each one properly placed so as to produce a variation of sound within 1 dB across the startle cage. Both speakers and startle cages were connected to a main PC, which detected and analysed all chamber variables with specific software. Before each testing session, acoustic stimuli were calibrated via specific devices supplied by San Diego Instruments. Mice were placed in a cage for a 5-min acclimatization period with a 70 dB white noise background, which continued for the remainder of the session. Each session consisted of three consecutive sequences of trials (periods). Unlike the first and the third period – during which mice were presented

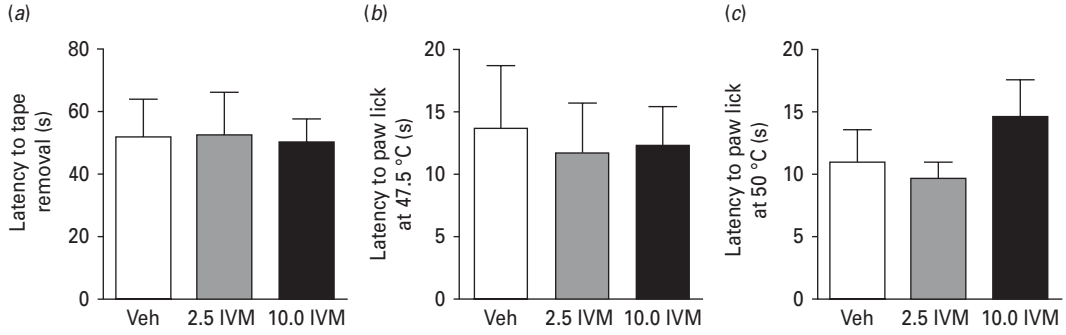

Fig. 1. Effects of ivermectin (IVM) and its vehicle (Veh) on haptic stimulation and sensorimotor coordination. IVM did not alter the latencies to remove a sticky tape (*a*) or to lick the paw in a hot plate at 47.5 °C (*b*) and 50 °C (*c*). IVM doses are expressed in mg/kg (i.p.). Values represented as mean ± S.E.M., $n = 6$–8 per treatment group for sticky tape and hot plate tests.

with only five pulse-alone trials of 115 dB – the second period consisted of a pseudorandom sequence of 40 trials, including 12 pulse-alone trials, 30 trials of pulse preceded by 73, 76 and 82 dB pre-pulses (respectively defined as PP3, PP6 and PP12; 10 for each level of pre-pulse loudness) and eight no stimulus trials, where only the background noise was delivered. The duration of pulses and pre-pulses was 80 and 40 ms, respectively. Pre-pulse–pulse delay amounted to 100 ms. Inter-trial intervals were selected randomly between 10 and 15 s. Percent PPI was calculated using the following formula: 100-[(mean startle amplitude for pre-pulse pulse trials/mean startle amplitude for pulse alone trials) × 100].

Novel object exploration and recognition

We used a modified version of the protocol described in Bortolato *et al.* (2010). Mice were individually acclimatized to Makrolon cages for 15 min each. The day after, animals were exposed to two novel black plastic cylinders (8 cm tall × 3.5 cm in diameter), affixed to the floor and symmetrically placed at 6 cm from the two nearest walls. Mice were placed in a corner, facing the centre and at equal distance from the two objects. Their start position was rotated and counterbalanced for each treatment throughout the test. Twenty-four hours later, mice were placed in the same cage for long-term memory testing. One of the cylinders was replaced by a novel plastic rectangular block (6 cm tall × 3 × 3 cm), which was placed in a counterbalanced fashion to avoid experimental bias. Behaviours for both sessions were videotaped for 15 min. Analysis included the number and total duration of exploratory approaches between novel and familiar objects. Exploration was defined as sniffing or touching either of the two objects with the snout; sitting on the object was not considered exploration. In the second exploration trial, an object exploration index was calculated as the ratio of the duration of the exploratory approaches targeting the novel objects over the time of exploration of both objects. The locomotor activity was defined as the number of crossings on a grid superimposed onto the image of each cage in a video monitor.

Statistical analyses

Normality and homoscedasticity of data distribution were verified using the Kolmogorov–Smirnov and Bartlett's test. Parametric analyses were performed with one-way analyses of variance (for repeated measures or independent factors, as appropriate), followed by Tukey's test with Spjøtvoll–Stoline correction for *post-hoc* comparisons. Non-parametric comparisons were carried out by Kruskal–Wallis test, followed by Nemenyi's test for *post-hoc* comparisons. Significance threshold was set at $p = 0.05$.

Results

Sensorimotor functions, tactile sensitivity and thermal nociception

In an initial set of experiments, we ascertained that IVM did not exert any significant effect on reflex and home-cage activity (data not shown). We then studied the ability of IVM to modify haptic perception and somatosensory coordination in the sticky tape test. The $P2X_4$ positive allosteric modulator did not significantly affect the latency to remove the tape from the forepaws of the mice ($F_{2,15} = 0.02$, n.s.; Fig. 1*a*). Furthermore, in consideration of the role of P2X in pain modulation (Toulme *et al.* 2010*b*), we tested its effects on acute thermal nociception in the hot-plate test. However, IVM did not modify the latency to the first paw-licking episode in mice, at either 47.5 °C ($F_{2,17} = 1.51$, n.s.; Fig. 1*b*) or 50 °C ($F_{2,17} = 1.69$, n.s.; Fig. 1*c*).

Novel open field

We evaluated the effects of IVM in the regulation of the locomotor and exploratory responses within a novel open field. While IVM did not induce significant variations of locomotor activity ($F_{2,15} = 2.12$, n.s.; Fig. 2*a*), it dose-dependently reduced the time spent in the centre ($F_{2,15} = 8.29$, $p < 0.01$; Fig. 2*b*), as well as the percent locomotor activity in the centre ($F_{2,15} = 6.46$, $p < 0.01$; Fig. 2*c*). In contrast, no differences were detected between

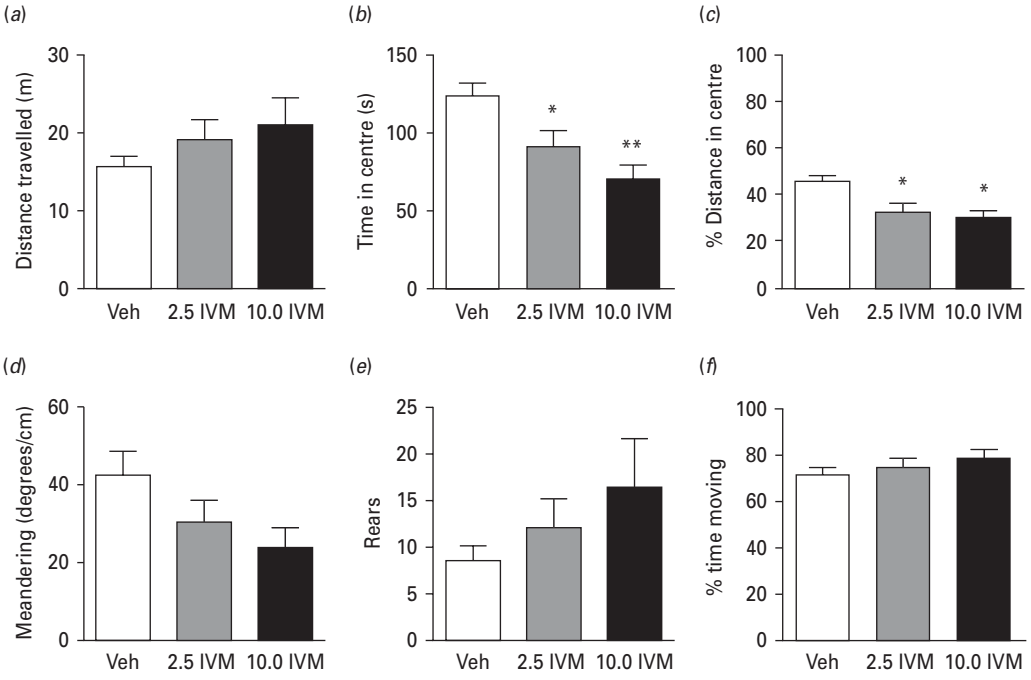

Fig. 2. Ivermectin (IVM) administration increases thigmotactic behaviour in the open field. While IVM did not affect the overall locomotor activity (*a*), the time spent (*b*) and percent activity (*c*) in the central zone were significantly reduced following IVM treatment. Conversely, drug administration did not produce any changes in meandering (*d*), number of rears (*e*) or percent time moving (*f*) between groups. IVM doses are expressed in mg/kg (i.p.). Values represented as mean ± S.E.M. for $n = 10$ per treatment group. *$p < 0.05$ and **$p < 0.01$ compared to vehicle (Veh)-treated mice.

treatment groups in meandering ($F_{2,15} = 2.50$, n.s.; Fig. 2*d*), number of rears ($F_{2,15} = 1.80$, n.s.; Fig. 2*e*) or the percentage of time spent moving ($F_{2,15} = 2.04$, n.s.; Fig. 2*f*). Collectively, these data indicate that IVM enhanced the thigmotactic responses evoked in mice by a novel open arena in mice, without modifying their exploratory behaviour.

Elevated plus maze

To further qualify the role of IVM in the regulation of anxiety-like responses, we tested a separate group of mice in the elevated plus maze paradigm. The highest IVM dose significantly increased the time spent in open arms ($F_{2,20} = 5.73$, $p < 0.05$; Fig. 3*a*). This effect was accompanied by a significant decrease in the closed arm duration ($F_{2,20} = 8.00$, $p < 0.01$; Fig. 3*b*) and an increase in the time spent on the central platform ($F_{2,20} = 10.17$, $p < 0.01$; Fig. 3*c*). Administration of the highest dosage of IVM also significantly elevated the number of total entries ($F_{2,20} = 10.84$, $p < 0.001$; Fig. 3*d*). Additionally, treatment with the 10 mg/kg dose of IVM resulted in increments of head dips ($F_{2,20} = 5.54$, $p < 0.05$; Fig. 3*e*) and rears ($F_{2,20} = 6.10$, $p < 0.01$; Fig. 3*f*). Conversely, the 2.5 mg/kg dose of IVM did not elicit any significant effect in any of the aforementioned parameters. No significant differences were found in stretch-attend postures (data not shown). Taken together, these data show that the 10 mg/kg dose of IVM increased the exploratory activity and reduced anxiety-like reactions in the elevated plus maze, without exerting significant effects on risk assessment.

Marble burying

In line with the effects of IVM in the elevated plus maze, the highest dose of this agent (10 mg/kg i.p.) elicited a significant decrease in marble-burying activity ($F_{2,26} = 16.04$, $p < 0.001$; Fig. 4*a*). The 10 mg/kg dose also induced a decrease in digging duration ($F_{2,25} = 4.34$, $p < 0.05$; Fig. 4*b*), but not number of digging bouts ($F_{2,25} = 2.35$, n.s.; Fig. 4*c*).

Tail suspension

We proceeded with the assessment of IVM's effects on depression-related responses in mice. In the tail-suspension paradigm, the 2.5 mg/kg dose of the drug significantly increased immobility time in comparison to both the vehicle and the 10 mg/kg dose ($F_{2,18} = 11.17$; Fig. 5*a*). No significant difference, however, was found between animals treated with the 10 mg/kg dose and vehicle. IVM did not induce significant changes in latency to immobility ($H2, 21 = 2.14$, n.s.; data not shown).

Forced swim test

In the forced swim paradigm, IVM treatment induced a dose-dependent increase in the duration of immobility ($F_{2,24} = 3.54$, $p < 0.05$; Fig. 5*b*). *Post-hoc* analyses revealed that this effect was due to the difference between the

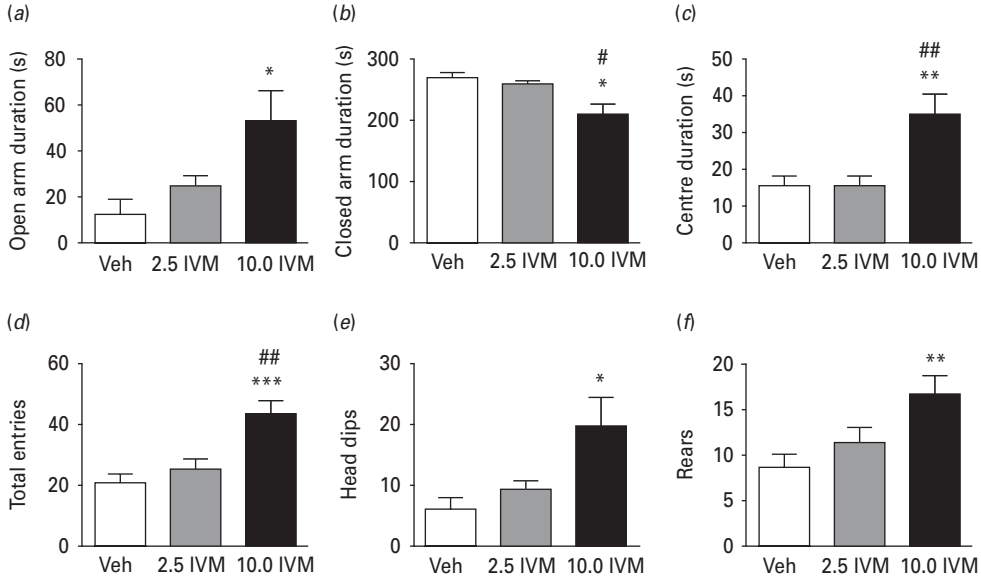

Fig. 3. Behavioural effects of ivermectin (IVM) on the elevated plus maze. (*a–c*) IVM increased the time spent in open arms and on central platform, but reduced in the time spent in the closed arms. (*d–f*) IVM treatment at high doses also enhanced the total number of entries, as well as exploratory head dips and rearing behaviour. IVM doses are expressed in mg/kg (i.p.). Values are indicated as mean ± S.E.M. for $n = 7$–8 per treatment group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ in comparison with vehicle (Veh)-treated animals; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ in comparison with IVM at 2.5 mg/kg.

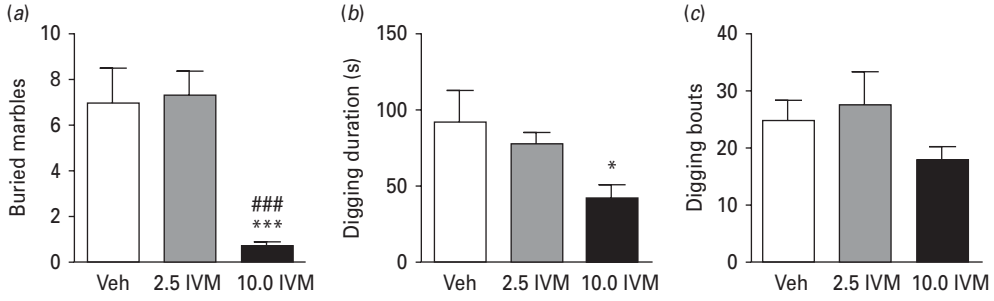

Fig. 4. Ivermectin (IVM) treatment significantly reduces marble-burying activity. (*a–c*) IVM significantly decreased marble-burying behaviour and the duration of digging time, but not digging frequency. IVM doses are expressed in mg/kg (i.p.). Values represented as mean ± S.E.M. for $n = 10$ per treatment group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to vehicle (Veh)-treated mice; $^{\#\#\#}p < 0.001$ compared to IVM at 2.5 mg/kg.

10 mg/kg dose and the vehicle ($p < 0.05$). A statistical trend was also detected between the 2.5 mg/kg dose and the vehicle ($p < 0.10$). No significant difference was found between the groups treated with the two IVM doses. Similar to the results in the tail-suspension tests, IVM failed to affect the latency to immobility ($F_{2,25} = 0.10$, n.s.).

Conditioned place preference

In the CPP test, IVM did not elicit any overt rewarding or aversive effect, as indicated by the absence of significant changes in preference for the drug-paired compartments ($F_{2,19} = 0.24$, n.s.; Fig. 5*c*). No significant differences were found in either locomotor activity during the pre- and post-conditioning phases or in the differences of time spent in the other compartment of the apparatus (data not shown).

PPI of the acoustic startle

Treatment with IVM did not induce significant alterations of acoustic startle magnitude ($F_{2,12} = 2.82$, n.s.; Fig. 6*a*). The drug, however, elicited a significant PPI reduction (treatment: $F_{2,12} = 9.65$, $p < 0.01$; Fig. 6*b*). *Post-hoc* analysis revealed that mice treated with 10 mg/kg IVM showed a significantly lower PPI than both the groups treated with vehicle ($p < 0.01$) and 2.5 mg/kg IVM ($p < 0.05$).

Novel object exploration/recognition

As shown in Fig. 7*a*, IVM decreased the duration of novel object exploration ($F_{2,18} = 3.49$, $p = 0.05$). This effect was due to a significant difference between the IVM doses of 2.5 and 10 mg/kg ($p < 0.05$). While the drug did not have any effect on the number of exploratory approaches

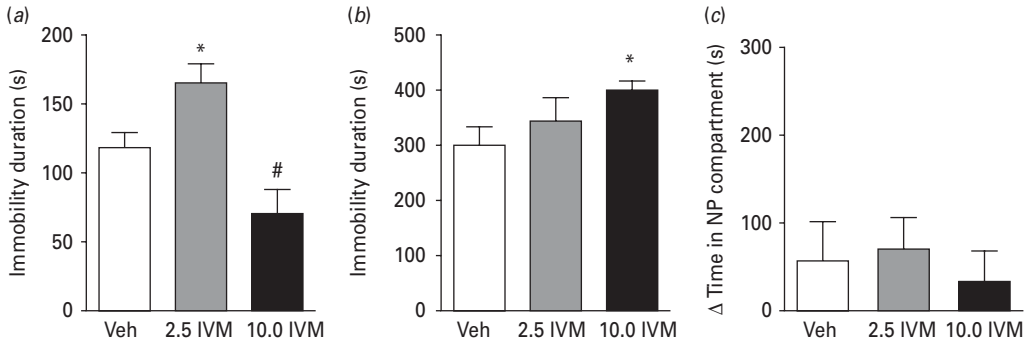

Fig. 5. Ivermectin (IVM) increases depressive-related behaviours. (*a*) In the tail-suspension test, IVM significantly increased immobility time at 2.5 mg/kg (i.p.), but not at 10.0 mg/kg (i.p.). (*b*) Conversely, high, but not low dose administration of IVM increased immobility in the forced swim test. (*c*) IVM treatment did not produce any alterations in conditioned place preference [non-preferred (NP)] compared to vehicle (Veh) treatment. IVM doses are expressed in mg/kg (i.p.). Values represented as mean ± S.E.M. $n = 7$–10 per treatment group for tail suspension, forced swim, and conditioned place preference. $^{*}p < 0.05$ compared to Veh-treated mice; $^{\#}p < 0.05$ compared to IVM at 2.5 mg/kg.

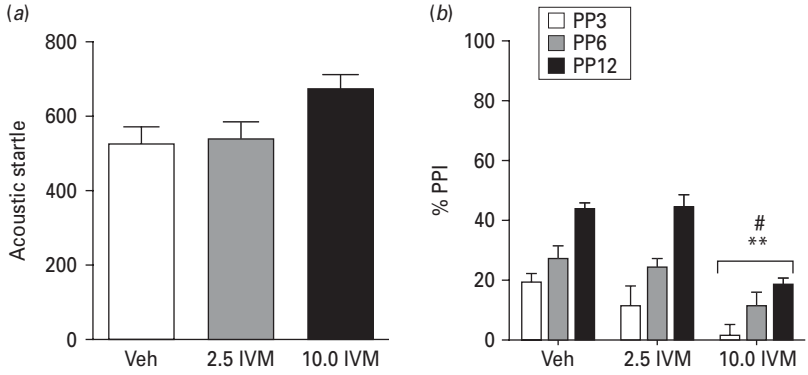

Fig. 6. Ivermectin (IVM) treatment disrupts sensorimotor gating. IVM did not produce any alterations in the acoustic startle response (*a*), but impaired pre-pulse inhibition (PPI) compared to the vehicle (Veh) in a dose-dependent fashion (*b*). Pre-pulses (PP) are indicated by the intensity corresponding to decibels above background noise. IVM doses are expressed in mg/kg (i.p.). Values represented as mean ± S.E.M. for $n = 9$–10 per treatment group. $^{**}p < 0.01$ compared to Veh-treated mice; $^{\#}p < 0.05$ compared to IVM at 2.5 mg/kg.

directed towards the objects ($F_{2,18} = 0.58$, n.s.; Fig. 7*b*), it did affect the mean duration of each sniffing episode ($F_{2,19} = 3.75$, $p < 0.05$; data not shown). Further scrutiny of this latter effect depended on a significant difference between the two doses of IVM ($p < 0.05$), as well as by a statistical trend ($p < 0.10$) between vehicle and 2.5 mg/kg IVM (Tukey). No difference in preference between the two objects was detected in any of the treatment groups.

The $P2X_4$ receptor activator failed to affect the mnemonic encoding of novel objects, as revealed by the equivalence of the novel exploration index at 24 h after the first exposure ($F_{2,18} = 0.82$, n.s.; Fig. 7*c*). In line with the results from the open field, no significant differences were observed in locomotor activity between treatment groups (data not shown).

Behavioural effects of IVM in $P2X_4$ KO mice

To verify whether the observed behavioural effects of IVM were mediated by $P2X_4$ receptors, we tested the high dose (10 mg/kg i.p.) of this compound in $P2X_4$ KO mice across the main paradigms significantly affected by it. In the open field, IVM evoked a spectrum of responses similar to those observed in WT mice, including the lack of changes in overall distance ($F_{1,12} = 0.04$, n.s.; Fig. 8*a*), as well as significant reductions in time ($F_{1,13} = 22.61$, $p < 0.001$; Fig. 8*b*) and percent distance travelled in the centre quadrant ($F_{1,12} = 54.76$, $p < 0.001$; Fig. 8*c*).

In the elevated plus maze, IVM significantly increased the duration of time spent in the open arms ($F_{1,13} = 6.04$, $p < 0.05$; Fig. 8*d*) and reduced the time spent in the closed arms by $P2X_4$ KO mice ($F_{1,13} = 14.49$, $p < 0.01$; Fig. 8*e*). In addition, IVM-treated $P2X_4$ KO mice exhibited significant enhancements in the total number of arm entries ($F_{1,13} = 11.43$, $p < 0.01$; Fig. 8*f*)] and head dips ($F_{1,13} = 11.75$, $p < 0.01$; Fig. 8*g*).

In contrast with the effects of IVM in WT mice, this drug significantly increased startle amplitude ($F_{1,11} = 6.25$, $p < 0.05$; Fig. 8*e*) but did not reduce %PPI ($F_{3,9} = 1.30$, n.s.; Fig. 8*f*).

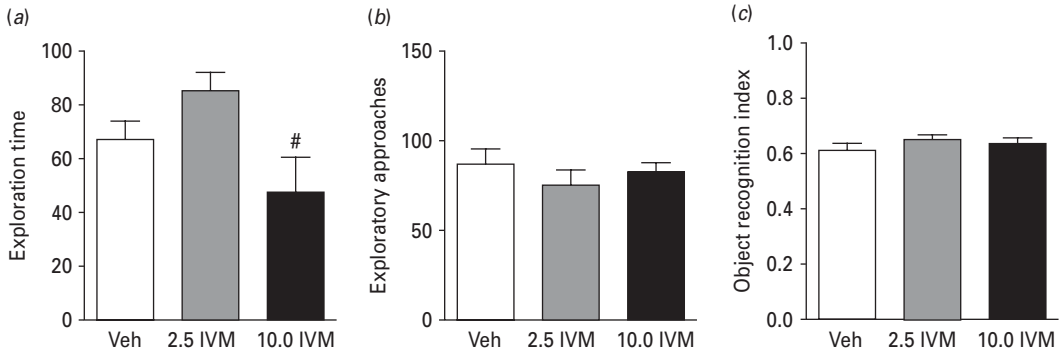

Fig. 7. Ivermectin (IVM) administration had no effect on mnemonic parameters in the object interaction and recognition test. (*a*) IVM treatment significantly decreased exploration duration as compared to vehicle (Veh)-treated mice. (*b*, *c*) Conversely, IVM did not affect the total number of exploratory approaches or the object recognition index. IVM doses are expressed in mg/kg (i.p.). Values represented as mean ± S.E.M. for $n = 7$–8 per treatment group. $^{\#}p < 0.05$ compared to IVM at 2.5 mg/kg.

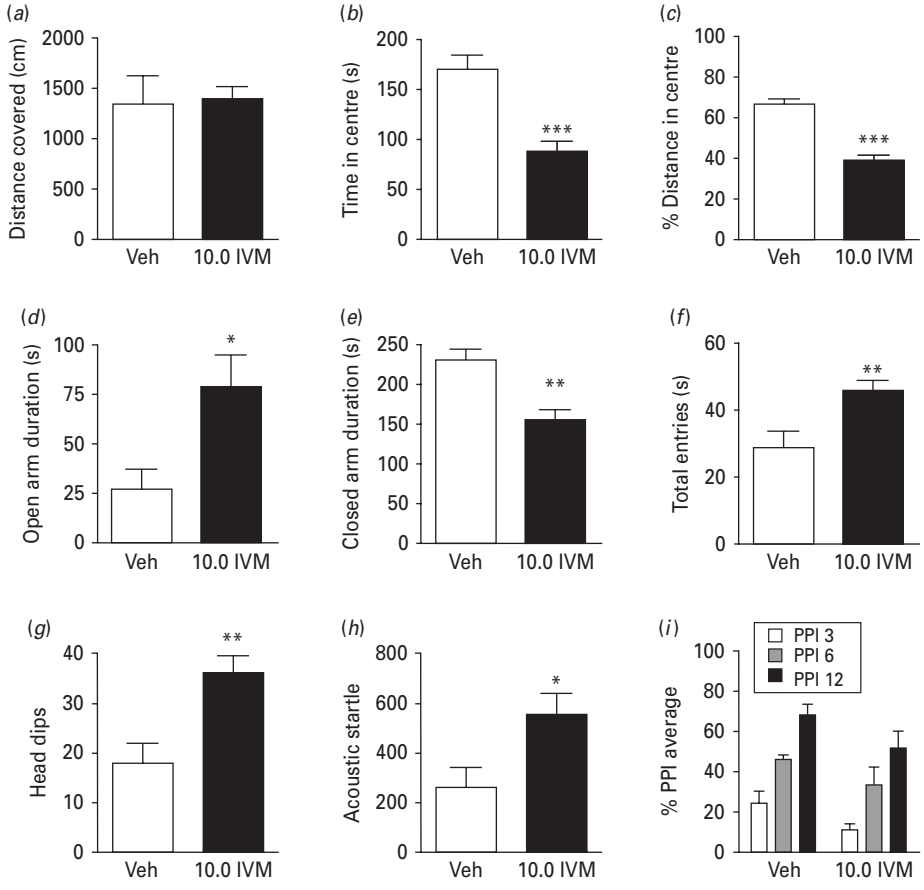

Fig. 8. Behavioural effects of ivermectin (IVM, 10 mg/kg i.p.) administration in $P2X_4$ receptor knockout mice. In the open field (*a*–*c*), IVM did not affect the overall locomotor activity (*a*), but reduced the time spent (*b*) and percent activity in the central quadrant (*c*). In the elevated plus maze (*d*–*g*), IVM significantly increased the time spent in open arms (*d*), reduced the time spent in the closed arms (*e*), increased the total numbers of entries (*f*) and head dips (*g*). IVM significantly increased startle amplitude (*h*), but did not significantly affect the pre-pulse inhibition (PPI) of the startle (*i*). Pre-pulses (PP) are indicated by the intensity corresponding to decibels above background noise. The IVM dose is expressed in mg/kg. Values represented as mean ± S.E.M. for $n = 8$ per treatment group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle (Veh)-treated mice.

Discussion

The results of the present study document that acute administration of IVM resulted in a complex set of effects on the regulation of emotional, cognitive and perceptual functions. In particular, the drug exhibited a multi-faceted, complex profile with regard to complementary aspects of anxiety-like responsiveness. IVM induced a

highly heterogeneous array of behavioural responses across different anxiety-related tasks. In a novel open field, mice treated with this drug displayed a dose-dependent increase in thigmotaxis, which was not accompanied by any other significant variation in locomotor and exploratory parameters. While these results are generally associated with anxiogenic-like effects (Prut & Belzung, 2003), this interpretation is in stark contrast to the finding that the 10 mg/kg (but not 2.5 mg/kg) dose of IVM exerted a marked reduction of anxiety-like behaviours in the elevated plus maze and marble-burying assays.

The discrepancies among IVM-induced effects on anxiety-like responses are likely to reflect the differential impact of this drug on diverse aspects of anxiety-related and exploratory responses. Although the ethological construct underlying most paradigms for anxiety testing in rodents is based on the conflict between exploration and neophobia in response to different environmental cues, several studies have documented that each test captures different dimensions of anxiety-related responsiveness (Belzung & Le Pape, 1994; Carola *et al.* 2002; Ramos *et al.* 1997).

Additionally, IVM increased depression-associated indices in both the tail suspension and forced swim tests and reduced sensorimotor gating in the PPI assay. These results suggest that IVM induced depression-like reactions (albeit at different doses) in response to acute, inescapable stressors. Both assays carry high predictive validity for depression and treatments that increase immobility duration are generally interpreted to worsen mood, particularly if, like IVM, they elicit no intrinsic hypolocomotion or sedation. Although previous reports indicated that very high doses of IVM induced signs of depression in animals (Basudde, 1989), the increased immobility in these studies is unlikely to reflect the same phenomena, since they were elicited by the low IVM dose in the tail suspension and not associated by any sign of acute toxicity in this or other studies (Yardley *et al.* 2012). Notably, we found divergent dose–response relations of IVM-induced depressive-like effects in the tail-suspension test (in which immobility was observed in response to the 2.5 mg/kg, but not 10 mg/kg, dose) and in the forced swim test (which resulted in immobility only at the dose of 10 mg/kg). The differences between these two results are currently unknown, but they may reflect different sensitivities of these two paradigms to the various neurotransmitter systems that regulate stress reactivity. Indeed, although both paradigms are based on the same ethological construct, based on the immobility caused by inescapable situations (Castagné *et al.* 2011), they have been shown to respond differently to several pharmacological agents (Chatterjee *et al.* 2012).

Another interesting finding of our studies was that, in WT mice, IVM induced a dose-dependent reduction in PPI, without affecting acoustic startle magnitude, signifying that this drug has an acute impact on sensorimotor gating and information processing. This finding is in line with previous reports (Davis *et al.* 1999), which documented PPI deficits following long-term administration of IVM in drinking water to mice. These impairments are connected to a broad set of neuropsychiatric deficits, ranging from psychotic disorders to obsessive–compulsive disorder and attention-deficit hyperactivity disorder. In addition, pharmacological disruptors of PPI in rodents have been shown to elicit psychotomimetic effects in humans. Irrespective of these considerations, it is interesting to note that IVM-induced gating impairments did not overtly affect declarative mnemonic encoding, as revealed by the novel object recognition test. This observation tempers the possibility that the observed changes in PPI may have a repercussion on cognition and learning.

It is relevant to note that IVM did not elicit overt rewarding and/or aversive actions in the CPP test. As the rewarding effects of a drug are regarded as a necessary premise for abuse and dependence, these data strongly suggest that IVM does not significantly modify incentive behaviour. Given the biased design of our protocol (which was used to enhance the sensitivity of the test to the potential rewarding effects of IVM), the apparent lack of aversive effects remains inconclusive, as it may be masked by potential floor effects. Irrespective of this issue, the lack of inherent addictive potential of IVM is in line with the absence of clinical notes on its recreational consumption in humans and is particularly important in view of the recent finding that this drug reduces alcohol intake in mice, as measured in several models (Yardley *et al.* 2012).

To our knowledge, few studies have been published on the behavioural effects of IVM in rodents. In line with our findings, previous work showed the lack of effects of this drug on general parameters of health and motor coordination in mice and rats (Davis *et al.* 1999; Nafstad *et al.* 1991). Davis *et al.* (1999) found that prolonged administration of IVM in tap water elicited significant psychotropic effects in several murine strains, including an enhancement in motor and acoustic reactivity as well as an attenuation of gating, but that it did not alter spatial learning. Furthermore, i.p. injections of low IVM doses were found to exert rapid anxiolytic-like properties in the elevated plus maze and Vogel test in rats (de Spinosa *et al.* 2002). Although these findings cannot be directly compared to our results, in view of differences in regimens of administration and species (or strains) tested, our study confirmed that IVM exerts psychotropic properties and induces a complex array of effects on emotional regulation.

The multi-faceted behavioural profile of IVM across the tests in this study is likely to reflect the involvement of multiple, divergent neurochemical targets of this drug, also in relation to the different sensitivity of each paradigm to different systems. In mammals, high-affinity

binding sites for IVM have been characterized on $GABA_A$ (Huang & Casida, 1997) and $P2X_4$ receptors (Priel & Silberberg, 2004).

Given the lack of available effective $P2X_4$ receptor antagonists, we tested the effects of $P2X_4$ KO mice to identify the specific contributions of this target to IVM-induced behavioural effects. The effects of IVM remained substantially unaltered in the open field and elevated plus maze, suggesting that $P2X_4$ receptors do not play a critical role in the modulation of anxiety-related responses in paradigms based on the conflict between approach and avoidance with regard to unprotected environments. Thus, it is likely that the apparently antithetical effects of IVM in the open field and elevated plus maze may reflect the impingement of other receptors. For example, the behavioural changes in the elevated plus maze are in line with the anxiolytic-like properties of $GABA_A$ receptor agonists (Davis *et al.* 1999).

In contrast, IVM elicited significantly divergent effects in WT and $P2X_4$ KO mice with regard to startle and PPI. In particular, IVM enhanced the startle amplitude of $P2X_4$ KO mice, suggesting a role of these receptors in the regulation of acoustic startle response. Further research will be needed to fully understand the specific neurobiological underpinnings of the role of these targets in the modulation of startle.

In addition, we found that IVM did not induce any impairments of PPI in $P2X_4$ KO mice, suggesting that the deficits in sensorimotor gating caused by this drug were in fact mediated by $P2X_4$ receptor activation.

Although the tail-suspension effects of IVM were not tested in $P2X_4$ KO mice, the enhancement of tail-suspension immobility by IVM is also unlikely to strictly reflect the outcomes of $GABA_A$ activation, as this effect is dose-dependent and reflects a reduction in overall locomotor activity (Steru *et al.* 1987). Given that tail-suspension and forced swim behaviours are poorly sensitive to the outcomes of $GABA_A$ receptor activation (Borsini & Meli, 1988; Steru *et al.* 1987), it is possible that IVM-induced responses may reflect a more prominent influence by $P2X_4$ receptors. These results indicate that positive allosteric modulation of $P2X_4$ receptors may directly modulate emotional reactivity in response to acute stressors, possibly inducing anxiety- and depression-like responses. Complementary to this idea, previous studies suggest that the behavioural effects of some antidepressant agents may in fact be mediated by the blockade of $P2X_4$ receptors, (Sim & North, 2010; Toulme *et al.* 2010*b*; for contrasting results see Nagata *et al.* 2009).

Recent evidence suggests that $P2X_4$ receptors may negatively interact with the effects of $GABA_A$ receptor activation (Jo *et al.* 2011), providing a possible explanation for the contrasting outcomes observed across different tests. In particular, it is possible that $P2X_4$ receptor activation may induce aversive effects, which may contrast the rewarding properties of $GABA_A$ receptor activation. Thus, the simultaneous stimulation of both targets may reduce the addictive potential associated with $GABA_A$ receptor agonists.

The identification of novel pharmacological targets for behavioural regulation is a task of paramount importance, in consideration of the largely inadequate therapeutic armamentarium for neuropsychiatric conditions. The translational implications of our data afford a broader perspective on the behavioural effects of IVM. Further studies are necessary to qualify the nature of the potentially untoward effects documented by this study, such as the increase in depression-like responses and the sensorimotor gating deficits. Our results point to a role of $P2X_4$ receptors in the modulation of startle responsiveness, as well as sensorimotor gating. Future studies with selective antagonists or small interfering RNAs for $P2X_4$ receptors will be critical to ascertain the implication of the activation of these targets. Irrespective of these aspects, the lack of rewarding properties of IVM in the CPP paradigm, its anxiolytic-like properties in two complementary paradigms for anxiety-related behaviours and its ability to attenuate alcohol intake (Yardley *et al.* 2002) strongly suggest that IVM and its derivatives may be of great interest for the development of novel therapeutic avenues in neuropsychiatric therapy.

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Statement of Interest

None.

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