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Role of Purinergic P2X4 Receptors in Regulating Striatal Dopamine Homeostasis and Dependent Behaviors

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Abstract

Purinergic P2X4 receptors (P2X4Rs) belong to the P2X superfamily of ion channels regulated by ATP. We recently demonstrated that P2X4R knockout (KO) mice exhibit deficits in sensorimotor gating, social interaction and ethanol drinking behavior. Dopamine (DA) dysfunction may underlie these behavioral changes, but there is no direct evidence for P2X4Rs' role in DA neurotransmission. To test this hypothesis, we measured markers of DA function and dependent behaviors in P2X4R KO mice. P2X4R KO mice exhibited altered density of presynaptic markers including tyrosine hydroxylase, dopamine transporter; postsynaptic markers including dopamine receptors and phosphorylation of downstream targets including dopamine and cyclic-AMP regulated phosphoprotein of 32kDa (DARPP-32) and cyclic-AMP response element binding protein (CREB) in different parts of the striatum. Ivermectin, an allosteric modulator of P2X4Rs, significantly affected DARPP-32 and ERK 1/2 phosphorylation in the striatum. Sensorimotor gating deficits in P2X4R KO mice were rescued by DA antagonists. Using the 6-Hydroxydopamine (6-OHDA) model of DA depletion, P2X4R KO mice exhibited an attenuated levodopa (L-DOPA) induced motor behavior, whereas IVM enhanced this behavior. Collectively, these findings identified an important role for P2X4Rs in maintaining DA homeostasis and illustrate how this association is important for CNS functions including motor control and sensorimotor gating.

Keywords

P2X4 receptors; ivermectin; dopamine receptors; 6-OHDA; schizophrenia; Parkinson's disease

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Introduction

Purinergic P2X receptors are cation permeable ion channels gated by ATP. They form homo- and heterotrimeric channels from seven subunits namely, P2X1-P2X7 (North 2002, Khakh & North 2012). Amongst them, P2X4 receptors (P2X4Rs) are abundantly expressed on neurons and glial (microglia and astrocytes) cells across the CNS as well as the PNS (Le *et al.* 1998). Gene knockout and pharmacological strategies have implicated P2X4R mediated transmission in hippocampal synaptic plasticity, inflammatory processes in the spinal cord and neuroendocrine functions (Sim *et al.* 2006, Ulmann *et al.* 2008, Zemkova *et al.* 2010). Despite this growing body of evidence, there remains a paucity of information regarding the functional significance of P2X4Rs in the CNS.

We recently reported that mice deficient in the *p2rx4 gene* [i.e. P2X4R knockout (KO)] exhibited deficits in sensorimotor gating, social behavior and ethanol drinking behavior (Wyatt *et al.* 2013, Wyatt *et al.* 2014). However, we did not identify any molecular mechanism that could explain these behavioral deficits. One plausible mechanism could be a result of P2X4Rs modulating major neurotransmitter systems including the glutamate and GABA systems. For instance, P2X4Rs are suggested to regulate postsynaptic currents mediated by NMDA receptors, AMPA receptors and GABA_A receptors as well as presynaptic release of glutamate and GABA (Baxter *et al.* 2011, Andries *et al.* 2007, Jo *et al.* 2011, Gu & MacDermott 1997, Hugel & Schlichter 2002). Moreover, P2X4R KO mice exhibited altered subunit expression of multiple glutamatergic and GABA_A receptors across multiple brain regions. This latter finding suggests that P2X4R deficiency disrupts homeostasis of postsynaptic ionotropic receptors (Wyatt *et al.* 2013, Wyatt *et al.* 2014). Notably, disruption of glutamatergic and GABAergic function has been linked to deficits in sensorimotor gating, social interaction and ethanol drinking behavior (Duncan *et al.* 2004, Du *et al.* 2012, Blednov *et al.* 2003). Together, these findings support the hypothesis that P2X4Rs can interact with other ionotropic receptors in regulation of multiple CNS functions.

In contrast to the building evidence supporting a role for P2X4Rs in glutamatergic and GABAergic function, little is known regarding the interaction of P2X4Rs with dopamine (DA) neurotransmission. Early evidence suggests that P2X4Rs are indirectly involved in DA neurotransmission (Krugel *et al.* 2001, Krügel *et al.* 2003, Xiao *et al.* 2008), but the direct role for P2X4Rs in regulating DA homeostasis has not been demonstrated. Considering that P2X4Rs are expressed on DA neurons and GABAergic medium spiny neurons (MSNs) of the basal ganglia (Heine *et al.* 2007, Amadio *et al.* 2007) and the behavioral deficits exhibited by P2X4R KO mice may represent DA dysfunction (Gendreau *et al.* 2000, Rodriguiz *et al.* 2004, Zhou *et al.* 1995, Ralph *et al.* 2001), we hypothesized that P2X4Rs control DA signaling with a relevant impact on DA associated behaviors.

In the present study, we utilized a P2X4R KO mouse model as a genetic approach and ivermectin (IVM), a positive allosteric modulator of P2X4Rs (Priel & Silberberg 2004, Khakh *et al.* 1999, Jelinkova *et al.* 2008, Jelinkova *et al.* 2006, Hattori & Gouaux 2012), as a pharmacological approach to test the aforementioned hypothesis. We measured protein densities of different markers of DA neurotransmission including tyrosine hydroxylase (TH),

dopamine transporter (DAT), dopamine D1 and D2 receptors (D1Rs and D2Rs) and downstream targets integral to DA signaling including dopamine and cyclic-AMP regulated phosphoprotein of 32 kDa (DARPP-32), extracellular regulated kinase-1/2 (ERK 1/2) and cyclic-AMP response element binding protein (CREB) in different regions of the striatum of P2X4R KO and wildtype (WT) male mice. We also measured the degree of phosphorylation of DARPP-32, ERK 1/2 and CREB isolated from different striatal regions of WT and P2X4R KO mice in the presence and/or absence of IVM. The interaction between P2X4Rs and DA system in the regulation of CNS functions was addressed by employing behavioral pharmacology paradigms. The 6-Hydroxydopamine model (6-OHDA) of DA depletion was used to link P2X4R function with DA neurotransmission in modulation of motor control. Finally, using the prepulse inhibition (PPI) of acoustic startle reflex coupled with DA antagonists, we evaluated the effects of DA dysregulation as it is pertained to sensorimotor gating deficits. Overall, the findings support the hypothesis that P2X4R function plays a role in maintaining DA signaling with an impact on DA associated behaviors such as motor control and sensorimotor gating.

Experimental procedures

Animals

Experimentally naïve male WT and P2X4R KO mice were obtained from our breeding colony at the University of Southern California. The breeding colony was established from a previous P2X4R KO colony that was maintained on a C57BL/6 background (Sim et al. 2006). Our overall breeding scheme for generation of P2X4R KO mice and genotyping has been described previously (Wyatt et al. 2013, Wyatt et al. 2014). Mice were housed in groups of 5 per cage in rooms maintained at 22°C with 12 h/12 h light: dark cycle and *ad libitum* access to food and water. All experiments were undertaken in compliance to guidelines established by National Institute of Health (NIH) and approved by the Institutional Animal Care and Use Committee (IACUC) of University of Southern California.

2-3 month old P2X4R KO and WT mice were used for biochemical assays. 2-4 month old P2X4R KO and WT mice were used for the motor behavior studies. 4-6 month old mice were used for the PPI of acoustic startle reflex study with DA antagonists.

Materials

Levodopa (L-DOPA) (Sinemet; 100 mg L-DOPA, 25 mg Carbidopa per pill) was diluted in 0.9% saline solution to achieve a concentration of 0.75 mg/ml. IVM (Norbrook, Lenexa, KS) was diluted in 0.9% saline solution, to achieve a concentration of 0.5 mg/ml and injected at a volume of 0.01 ml/g of body weight. Propylene glycol (Sigma Aldrich, St. Louis, MO) was used as the vehicle control for IVM. SCH-23390 HCl and raclopride tartrate (Sigma-Aldrich, St. Louis, MO) were dissolved in 0.9% saline at concentrations of 0.2 mg/ml and 0.6 mg/ml respectively. Both drugs were injected at a volume of 0.005 ml/g of body weight.

Western immunoblotting

Tissue preparation—The dorsal and ventral striata were dissected from P2X4R KO and WT mice following euthanasia with CO₂ asphyxiation. For the experiment that tested the effects of IVM (5 mg/kg, i.p.) on dopaminergic signaling, the dorsal and ventral striatal regions were dissected out 8 h post drug administration. The dorsal and ventral striata were dissected out as per the neuroanatomical landmarks described in the mouse brain atlas (Franklin & Paxinos 2007). The brain tissues were homogenized in a buffer containing 50 mM Tris-HCl pH (8.0), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1/100 dilution proteinases inhibitor cocktail (Millipore, Temecula, CA) and protein content was determined using BCA assay kit (Thermo Scientific, Rockford, IL). Homogenates were treated with a cocktail of phosphatase inhibitors (1 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.5 mM sodium orthovanadate, 10 mM β-glycerol phosphate, 1 μM microcysteine LR) (Sigma-Aldrich, St. Louis, MO) for detection of phosphoproteins.

Immunoblotting procedure—Protein samples of 50 μg ran on 10% SDS PAGE gels and transferred onto polyvinylidene difluoride membranes using semi-dry transfer method (Trans turbo blot; BioRad Laboratories, Hercules, CA). Striatal samples from WT and P2X4R KO mice were made to run on the same gel and transferred onto the same membrane. Non specific binding was blocked by incubation in 5% non fat dry milk, followed by incubation with primary antibodies overnight at 4°C. The antibodies used were rabbit anti- TH (1:5000), mouse anti-DAT (1:1000), rabbit anti-D2 receptor (1:1000), rabbit anti-DARPP-32 (1:1000) (Millipore, Temecula, CA), rabbit anti -D1 receptor (1:500; SantaCruz biotechnology, Santacruz, CA), mouse anti-total ERK 1/2 (1:1000), rabbit anti- total CREB (1:1000) (Cell Signaling Technology, Beverly, MA), mouse anti-β-actin (1:20,000; Sigma Aldrich, St. Louis, MO), mouse anti-α-tubulin (1:10,000; Millipore, Temecula, CA). The antibodies for phospho proteins included rabbit anti-phospho-Thr34- DARPP-32 (1:400; Millipore, Temecula, CA), rabbit anti-phospho-Ser133- CREB (1:500), rabbit anti-diphospho- Thr202/Tyr204- ERK 1/2 (1:500) (Cell Signaling Technology, Beverly, MA). Secondary antibodies included goat anti-mouse and goat anti-rabbit antibodies (1:10,000; BioRad Laboratories, Hercules, CA). Bands were visualized using chemiluminescence method (Clarity western plus ECL substrate; BioRad Laboratories, Hercules, CA) followed by exposure to HyBlot autoradiography films (Denville Scientific, Metuchen, NJ). Protein quantification was carried out by optical densitometry using ImageJ software (NIH, Bethesda, MD). Protein densities were normalized to β-actin or α-tubulin levels.

HPLC assay

Brain tissue was homogenized with 0.5 M perchloric acid, centrifuged at $16,873 \times g$ for 12 mins at 4°C and protein was resuspended in 0.5 M NaOH. Protein content was detected by BCA assay. DA concentrations were determined using electrochemical detection method consisting of a ESA model Coularray 5600A coupled with a four channel analytical cell at -175, 50, 220, 300 mV. Samples were injected with ESA autosampler (Chelmsford, MA) and DA was separated by a 150 × 3.2 mm reverse phase 3 μm diameter C-18 column regulated at 28°C. The mobile phase MD-TM (ESA) consisted of acetonitrile in phosphate buffer and a non ion-pairing reagent delivered at a rate of 0.6 ml/min. The HPLC was integrated with a

Dell GX 280 computer with analytical programs including ESA Coullarray for Windows software.

Acoustic Startle Reflex and PPI of Startle

Apparatus—Acoustic startle reflex and PPI were tested as previously described (Wyatt et al. 2013). The apparatus used for detection of startle reflex (San Diego Instruments, San Diego, CA) consisted of a standard cage placed in sound attenuated chambers with fan ventilation. Each cage consisted of a Plexiglass cylinder of 3 cm diameter, mounted on piezoelectric accelerometric platform connected to an analog-digital converter. Background noise and acoustic bursts were conveyed by two separate speakers, each one oriented appropriately so as to produce a variation of sound within 1 dB across the startle cage. Both speakers and startle cages were connected to the main PC, which detected and analyzed all chamber variables with Startle software (San Diego Instruments, San Diego, CA). Before each testing session, acoustic stimuli were calibrated via a digital sound level meter.

Startle and PPI session—During the baseline session, mice were exposed to background noise of 70 dB and after an acclimatization period of 5 mins, were presented with 12 40 ms trials of 115 dB interposed with 3 trials of a 82 dB prestimulus preceding the 115 dB by 100 ms. Subsequently, treatment groups were established so that the average startle response and %PPI were equivalent within the WT and P2X4R KO groups. On the testing day, each mouse was placed in the cage and exposed to a 5 mins acclimatization period with a 70 dB white noise background, which continued for remainder of the session. Each session consisted of three consecutive sequences of trials. Unlike the first and third session, wherein the mice were exposed to five alone pulse trials of 115 dB, the second period consisted of a pseudorandom sequence of 50 trials, including 12 pulse alone trials, 30 trials of pulse preceded by 73 dB, 76 dB or 82 dB prepulse (respectively, defined as PPI 3, PPI 6 and PPI 12; 10 for each level of prepulse loudness) and 8 no stimulus trials, wherein the mice were presented with background noise without any prepulse or pulse stimuli. Inter trial intervals were randomly chosen between 10 and 15 seconds. Delta PPI (PPI) was calculated as mean startle amplitude for pulse alone trials – (mean startle amplitude for prepulse trial). DA antagonists, SCH-23390 (1 mg/kg, i.p) and raclopride (3 mg/kg, i.p) were administered 10 mins prior to testing session.

6-OHDA lesioning in mice and motor behavior testing

6-OHDA lesioning surgery—Mice were treated with desipramine hydrochloride (25 mg/kg, i.p.) (Sigma-Aldrich, St. Louis, MO) 30 mins prior to surgery to prevent concurrent damage of noradrenergic pathways by 6-OHDA. Mice were anesthetized with avertin (25 mg/kg, i.p.) and placed in the stereotaxic apparatus. 2 µl of freshly prepared 6-OHDA bromide salt (4 mg/ml in 0.2% ascorbic acid and 0.9% saline; Sigma-Aldrich, St. Louis, MO) was unilaterally infused into left median forebrain bundle (from Bregma point: 1.2 mm posterior, 1.1 mm lateral, 5 mm ventral) (Franklin & Paxinos 2007) at a rate of 0.5 µl/min using a 10 µl Hamilton syringe and a microlitre syringe pump. The injection cannula was left in place for 3-5 mins to prevent reflux and ensure complete absorption. Postoperative procedures involved daily subcutaneous (s.c.) injections with sucrose (5% w/v in saline) and warming on a heating pad for 2 weeks.

Motor behavior testing—Mice were subjected to behavioral testing 3 weeks after 6-OHDA lesioning surgery. Mice were placed in a plastic cylinder (318 mm in diameter) with a video camera mounted above it. Baseline rotational behavior was established by saline injections which causes the mouse to rotate towards the lesioned side with lesser DA activity (ipsilateral rotations). L-DOPA (5 mg/kg, s.c.) was administered 5 mins prior to behavioral testing and the number of contralateral and ipsilateral rotations were counted in every ten minute interval for a total period of 90 mins. After monitoring the behavioral activity with L-DOPA alone, the same cohort of WT and P2X4R KO mice received a combination of IVM (5 mg/kg, i.p.) and L-DOPA (5 mg/kg, s.c.) to study the modulatory effect of IVM on L-DOPA induced motor behavior. IVM and L-DOPA were administered 8 h and 5 mins respectively prior to behavioral testing. To monitor effect of IVM alone on rotational behavior, a separate cohort of 6-OHDA lesioned WT mice received IVM (5 mg/kg, i.p) 8 h prior to behavioral testing and motor activity was monitored for 2 h. The 8 h time point was selected since IVM has been shown to achieve maximal concentration in brain and plasma post 8 h (Yardley *et al.* 2012).

Peroxidase based immunohistochemistry

Collection and processing of brain tissues—Transcardial perfusion was performed using 0.9 % NaCl/ 4% phosphate buffered paraformaldehyde. Perfused brains were post fixed overnight followed by storage in 20% sucrose for 48 h and quickly frozen with 4-Methylbutane on dry ice. Perfused brains were cut coronally at 25 μ m thickness in a cryostat and stored in a cryoprotective solution containing 30% sucrose in PBS at 4°C until further use.

TH staining using peroxidase method of immunohistochemistry in ventral mesencephelon—Brain sections were heated in 10 mM sodium citrate buffer (pH 8.5) for 30 mins at 80°C followed by permeabilization in TBS + 0.2% Triton-X for 30 mins. Slices were then quenched in solution containing 10% methanol and 3% H₂O₂ in TBS for 10 mins and subsequently blocked in 5% non fat dry milk and in 5% normal goat serum (each step for 30 mins). Sections were incubated overnight with rabbit anti-TH (1:5000) (Millipore, Temecula, CA) diluted in TBS +0.2% Triton-X containing 1% normal goat serum. The avidin-biotin complex method of detection was used, wherein slices were incubated with biotinylated goat anti-rabbit antibody (1:500) for 1 h and then with 1% avidin linked peroxidase complex for 45 mins (Vector Laboratories, Burlingame, CA). This was followed by treatment with solution containing 0.05% 3,3-diaminobenzidine and 0.015% H₂O₂ in PBS for 5 mins, dehydration of slides in a dilution series of ethanol, clearance in xylene and evaluation under light microscope.

Statistical analyses

For the Western blotting analysis, the average of densitometry values of WT striatal samples was used to arbitrarily normalize WT samples to 1 and the P2X4R KO striatal samples were normalized by dividing each densitometry value by the average of WT samples. Normalization of the two genotypes was done within the same membrane and presented as fold change of P2X4R KO versus WT in that membrane. For D1, D2, DARPP-32, ERK1/2 and CREB immunoblotting; WT and P2X4R KO mice were generated in separate cohorts.

The normalization of P2X4R KO to WT samples was done within the same cohort followed by combining data from two cohorts for analyses. The same method of normalization was used for the study that tested the effects of IVM on dopaminergic signaling in the dorsal and ventral striatum of WT and P2X4R KO mice. The average of vehicle treated WT mice was used to arbitrarily normalize the WT control samples to 1 and the IVM treated WT, vehicle and IVM treated P2X4R KO mice were normalized by dividing each value by the average of vehicle treated WT mice. Normalized density of proteins were expressed as mean \pm SEM. Phosphorylation was calculated as ratio of normalized values of phosphorylated form to total form of protein. DA levels were expressed as ng/mg of protein content. Unpaired Student t-test was used for analyzing the differences in protein densities and DA levels between WT and P2X4R KO groups. Two-way ANOVA with Bonferroni post hoc test was used to evaluate the effect of IVM on phosphorylation of various signaling molecules between WT and P2X4R KO. Pharmacological studies for motor behavior and sensorimotor gating were analyzed by two way repeated measures ANOVA with time/PPI intensity and genotype/treatment as within and between subjects variability respectively followed by Bonferroni post hoc test for multiple comparisons. Significance was set at $P < 0.05$. All data was analyzed using GraphPad Prism software (San Diego, CA).

Results

P2X4R KO mice exhibit alterations in expression of presynaptic DA markers in striatum

To investigate changes in presynaptic markers of DA neurotransmission, we compared TH and DAT protein density between P2X4R KO and WT mice using Western immunoblotting. P2X4R KO mice exhibited a significant increase in TH protein density in the dorsal striatum by 64% ($p < 0.01$) but no change in protein density in the ventral striatum [Fig 1A & 1B (i)]. P2X4R KO mice exhibited significant increases in DAT protein density by 106% ($p < 0.05$) and by 98% ($p < 0.01$) in the dorsal and ventral striatum, respectively [Fig 1A & 1B (ii)].

The significant increase in TH protein density in the dorsal striatum did not appear to be associated with an increase in DA levels as there were no significant changes in DA levels in the dorsal striatum (Fig S1A). Moreover, there were no changes in DA levels in the ventral striatum (Fig S1B) between P2X4R KO and WT mice.

P2X4R KO mice exhibit significant alterations in expression of DA receptors and downstream targets

Based on the significant changes in TH and DAT protein densities, we posit that changes within presynaptic DA environment could impact the densities of DA receptors and downstream targets. Among the DA receptor subtypes, D1Rs belong to excitatory $G_{\alpha_s/olf}$ family of G-coupled protein receptors (GPCRs), whereas D2Rs belong to inhibitory $G_{\alpha_i/olf}$ family of GPCRs (Girault 2012). To test this hypothesis, we measured protein densities of D1Rs, D2Rs and phosphorylation states of major downstream targets in striatum. P2X4R KO mice exhibited significant increases in D1R protein density by 159% [$p < 0.05$; Fig 1A & 1B (iii)] and in D2R protein density by 42% [$p < 0.05$; Fig 1A & 1B (iv)] in the ventral striatum as compared to WT mice. There were no changes in either of protein densities for DA receptors in the dorsal striatum of P2X4R KO mice.

To investigate downstream pathways regulated by DA receptors, we measured total and phosphorylated form of DARPP-32, ERK 1/2 and CREB in the dorsal and ventral striatum using Western immunoblotting. P2X4R KO mice exhibited a significant increase in DARPP-32 phosphorylation (by 164%, $p < 0.05$) without any changes in total DARPP-32 protein density in the dorsal striatum as compared to WT counterparts [Fig 2A & 2B (i)]. In the ventral striatum, P2X4R KO mice exhibited a significant decrease in DARPP-32 phosphorylation (by 48%, $p < 0.05$) without any changes in protein density of total DARPP-32 [Fig 2A & 2B (i)]. P2X4R KO mice did not exhibit any significant changes in total ERK 1/2 protein density or ERK 1/2 phosphorylation in the dorsal and ventral striatum compared to WT mice [Fig 2A & 2B (ii)]. There were no significant changes in protein density of total CREB or CREB phosphorylation in the dorsal striatum of P2X4R KO mice [Fig 2A & 2B (iii)]. On the other hand, P2X4R KO mice did exhibit a significant decrease in total CREB density (by 36%, $p < 0.01$) and a corresponding increase in CREB phosphorylation (by 141%, $p < 0.001$) in the ventral striatum compared to WT mice [Fig 2A & 2B (iii)].

IVM significantly affected DARPP-32 and ERK 1/2 phosphorylation, but not CREB phosphorylation in the dorsal striatum

IVM was used to gain pharmacological insights into the role of P2X4Rs in dopaminergic signaling in the dorsal striatum. There was no significant effect of IVM treatment or genotype on total DARPP-32 levels or phosphorylation but the drug treatment \times genotype interaction was significant for both the total DARPP-32 levels [$F(1,18)=5.61$, $p < 0.05$] and DARPP-32 phosphorylation [$F(1,18)=5.48$, $p < 0.05$]. Bonferroni post hoc test confirmed significant increase in DARPP-32 phosphorylation upon IVM treatment in WT ($t=2.834$, $p < 0.05$), but not in P2X4R KO mice ($t=0.5829$, $p > 0.05$) [Fig 3A & 3B (i)]. There was no significant effect of IVM treatment or genotype on total ERK 1/2 levels, but the drug treatment \times genotype interaction was significant [$F(1,18)=10.00$, $p < 0.01$]. There was a significant effect of IVM treatment [$F(1,18)=$, $p < 0.05$] but not genotype or drug treatment \times genotype interaction for ERK 1/2 phosphorylation. There was no significant effect of IVM treatment, genotype or drug treatment \times genotype interaction for total CREB levels. There was no significant effect of IVM treatment or drug treatment \times genotype interaction, but the effect of genotype trended towards significance for CREB phosphorylation [$F(1,16)=4.37$, $p=0.0529$] [Fig 3A & 3B (iii)].

IVM significantly affected DARPP-32 phosphorylation, but not ERK 1/2 or CREB phosphorylation in the ventral striatum

Similar to dorsal striatum, we investigated the effect of IVM on phosphorylation of the same signaling molecules in the ventral striatum. There was no significant effect of IVM treatment or genotype on total DARPP-32 levels or DARPP-32 phosphorylation but the drug treatment \times genotype interaction was significant for both total DARPP-32 levels [$F(1,16)=6.40$, $p < 0.05$] and DARPP-32 phosphorylation [$F(1,16)=6.20$, $p < 0.05$] [Fig 4A & 4B (i)]. There was no significant effect of IVM treatment, genotype or drug treatment \times genotype interaction for total ERK 1/2 levels or ERK 1/2 phosphorylation [Fig 4A & 4B (ii)]. There was no significant effect of IVM treatment, genotype or drug treatment \times genotype interaction for total CREB levels. Similarly, there was no significant effect of IVM treatment

or genotype on CREB phosphorylation. However, the drug treatment \times genotype interaction trended towards significance for CREB phosphorylation [F(1,15)=4.43, $p=0.0527$] [Fig 4A& 4B (iii)].

Pharmacological inhibition of D1Rs and D2Rs significantly enhanced prepulse inhibition (PPI) of acoustic startle reflex in P2X4R KO mice

We investigated the response of P2X4R KO mice to selective antagonists for D1Rs (SCH-23390; 1 mg/kg) and D2Rs (raclopride; 3 mg/kg) on PPI startle response, to link imbalances in DA homeostasis to PPI deficits in P2X4R KO mice. The doses tested were chosen based on previous studies that investigated PPI function in C57BL/6 mice (Doherty *et al.* 2008, Ralph-Williams *et al.* 2003, Ralph *et al.* 2001). We found a significant effect of genotype on PPI function [F(1,26)= 5.50, $p<0.05$] (Fig 4A) which supports our previous findings (Wyatt *et al.* 2013). SCH-23390 significantly increased PPI in P2X4R KO mice [F(1,28)=9.33, $p<0.01$] with Bonferroni post hoc test identifying a significant increase at PPI 6 ($t=3.294$, $p<0.01$) and PPI 12 ($t=3.240$, $p<0.01$) (Fig 4A). Similarly, raclopride significantly enhanced PPI function in P2X4R KO mice [F(1,28)=11.98, $p<0.01$] as compared to saline treated P2X4R KO mice with Bonferroni post hoc test identifying a significant increase at PPI 6 ($t=3.748$, $p<0.001$) and PPI 12 ($t=3.507$, $p<0.01$) (Fig 4A). There were no significant changes in PPI function in WT mice upon treatment with SCH-23390 or raclopride (Fig 4A). Moreover, neither SCH- 23390 nor raclopride induced any significant change in startle amplitude in P2X4R KO mice (Fig 4B).

Pharmacological or genetic manipulation of P2X4R function significantly influenced L-DOPA induced motor behavior in the 6-OHDA model of DA depletion

Using the 6-OHDA model of DA depletion, we investigated the role of P2X4Rs in regulation of motor behavior (Schwartz & Huston 1996). As presented, ablation of DA neurons in ventral mesencephalon in both genotypes (Fig S2) resulted in reduced TH expression by 95.7% and 96.3% in the striatum of WT ($p<0.001$) and P2X4R KO mice ($p<0.01$) respectively (Fig S2). In the presence of L-DOPA treatment (5 mg/kg), both genotypes exhibited contralateral rotations, since L-DOPA metabolizes into DA in the synapses, followed by activation of the supersensitive postsynaptic DA receptors on lesioned striatum (Ungerstedt 1971) (Fig 5A). In the presence of L-DOPA, we found that P2X4R KO mice exhibited significantly fewer contralateral turns as compared to WT mice (Fig 5A). There was a significant effect of genotype [F(1,20)=4.39, $p<0.05$] and time [F(8,160)=58.46, $p<0.001$] on L-DOPA induced rotational behavior in P2X4R KO. There was no significant time \times genotype interaction for L-DOPA induced motor behavior. Bonferroni post hoc test confirmed significant reduction in L-DOPA induced motor behavior in P2X4R KO mice during the 45-55 mins interval ($t=3.154$, $p<0.05$) (Fig 5A).

We reasoned that if genetic disruption of P2X4Rs reduces L-DOPA response on motor behavior, then pharmacological potentiation of P2X4Rs should enhance motor behavior. In support of this hypothesis, we found that IVM (5 mg/kg) significantly increased the number of L-DOPA induced rotations in WT mice. There was a significant effect of time [F(8,208)=95.32, $p<0.001$] and IVM treatment [F(1,26)=33.80, $p<0.001$] on L-DOPA induced motor behavior. There was also a significant time \times drug treatment interaction

[F(8,208)=10.99, $p<0.001$] with Bonferroni post hoc test identifying a significant increase in contralateral rotations upon IVM treatment during 5-15 mins ($t=5.509$, $p<0.001$), 15-25 min ($t=4.161$, $p<0.001$), 25-35 min ($t=5.491$, $p<0.001$), 35-45 min ($t=6.254$, $p<0.001$), 45-55 min ($t=6.484$, $p<0.001$) and 55-65 min ($t=4.878$, $p<0.001$) intervals (Fig 5A). IVM alone induced an average of 12 rotations \pm 2 rotations (SEM) for period of 2 hours post drug treatment, although it was still statistically significant compared to sham controls [F(1,9)=26.64, $p<0.001$] (Fig S3).

There was a significant effect of time [F(8,112)=36.93, $p<0.001$] and IVM treatment [F(1,14)=4.80, $p<0.05$] on L-DOPA induced motor behavior in P2X4R KO mice (Fig 5A). There was also a significant time \times drug treatment interaction [F(8,112)=2.88, $p<0.01$] with Bonferroni post hoc test confirming a significant increase in L-DOPA induced motor behavior during the 5-15 mins interval ($t=3.481$, $p<0.01$). Taking into consideration the lower L-DOPA baseline in P2X4R KO mice, we analyzed the difference in the number of contralateral rotations between L-DOPA alone and IVM + L-DOPA in WT and P2X4R KO mice. There was a significant effect of time [F(8,160)=11.33, $p<0.001$] and a non-significant trend of effect of genotype [F(1,20)=3.17, $p=0.0903$] on contralateral rotations between L-DOPA and L-DOPA + IVM (Fig 5B). There was a significant time \times genotype interaction [F(8,160)=2.07, $p<0.05$], suggesting that IVM's ability to increase L-DOPA induced motor behavior differed between WT and P2X4R KO mice. Bonferroni post hoc test identified significant differences in L-DOPA induced motor behavior upon IVM treatment between WT and P2X4R KO mice at 55-65 mins ($t=2.948$, $p<0.05$) (Fig 5B).

Discussion

Our current study investigated the role of P2X4Rs in the dopaminergic system and its impact on DA dependent behaviors. Impairments in DA neurotransmission were observed with respect to changes in protein densities of pre- and postsynaptic markers. There was increased TH protein density in the dorsal, but not the ventral, striatum of P2X4R KO mice. DAT was significantly increased in both parts of the striatum in P2X4R KO mice, which is indicative of higher presynaptic reuptake of DA. In addition to changes in presynaptic markers of DA activity, we also identified significant changes in protein densities of DA receptors in the ventral, but not the dorsal, striatum of P2X4R KO mice. The increased DA receptors' density in the ventral striatum could be an adaptive response to the altered synaptic DA availability due to increased DAT expression in the same brain region of P2X4R KO mice. This interpretation is based on previous studies that have postulated a positive correlation between DAT and DA receptor density (Fauchey *et al.* 2000, Ghisi *et al.* 2009). The increased DAT and DA receptor density levels suggest that P2X4R KO mice may have alterations in DA neurotransmission. However, there were no significant changes in DA levels in both parts of the striatum of P2X4R KO mice despite significant alterations in TH density levels in the dorsal striatum of P2X4R KO mice. There are several other factors that control DA levels such as storage, release, reuptake, and catabolism (Eells 2003). Based on our finding of increased density of DAT and DA receptors, future studies will be necessary using *in vivo* microdialysis or fast scan cyclic voltammetry to help to identify any differences in the extracellular DA levels between WT and P2X4R KO mice and elucidate

the dopaminergic tone in P2X4R KO mice. Overall, the findings suggest that *p2rx4* deficiency affects DA synthesis and transport that could impact normal DA neuron function.

In addition to DA receptors, we identified dysregulation of signaling molecules (i.e. DARPP-32 and CREB) that can be regulated through DA receptors in different striatal regions of P2X4R KO mice. D1R stimulation on striatonigral MSNs phosphorylates DARPP-32 at Thr34 via protein kinase A (PKA) that inhibits PP-1 activity and allows phosphorylation of ERK1/2 and CREB. D2R stimulation produces the opposite effects in the striatopallidal MSNs (Girault 2012, Bertran-Gonzalez *et al.* 2008). Despite the lack of significant changes in D1Rs protein density, P2X4R KO mice exhibited a significant increase in DARPP-32 phosphorylation in the dorsal striatum, which is typically indicative of upregulated D1R mediated signaling function. On the other hand, we saw a significant decrease in DARPP-32 phosphorylation in the ventral striatum of P2X4R KO mice that correlates well with the increased D2R protein density in the same brain region. But, we saw significant increases in D1R density and CREB phosphorylation in the same brain region of P2X4R KO mice that did not corroborate with the decreased DARPP-32 phosphorylation. One possible explanation for these neurochemical differences identified in the P2X4R KO mice is that there are multiple interactions or involvement of various neurotransmitter systems besides DA in regulating DARPP-32 phosphorylation such as glutamate, GABA and serotonin (Svenningsson *et al.* 2004). Thus, the alterations in signaling molecules including DARPP-32 and CREB in P2X4R KO mice suggest a complex compensatory change other than that of DA receptors. Taken together; the increased density of pre- and postsynaptic markers suggests dysregulation of DA system in P2X4R KO mice which may partially underlie the behavioral deficits previously reported in P2X4R KO mice.

In addition to the genetic approach, we used a pharmacological approach to explore a role for P2X4Rs in regulating DA receptor associated signaling pathways. Since, there are limited specific antagonists to test P2X4R related signaling *in vivo*, we used the P2X4R allosteric modulator, IVM, to investigate a link between P2X4R function and DA receptor associated signaling molecules in the dorsal and ventral striatum. As presented above, there was a significant increase in DARPP-32 phosphorylation upon IVM treatment in the WT, but not in P2X4R KO mice, suggesting a role for P2X4Rs in regulating DARPP-32 phosphorylation in the dorsal striatum. The changes in DARPP-32 phosphorylation upon P2X4R potentiation by IVM did not fully agree with the result from genetic deletion of P2X4Rs in the dorsal striatum. This difference in finding may be linked to neurodevelopmental changes in P2X4R KO mice. This hypothesis is supported by several lines of evidence: first, IVM did not increase DARPP-32 phosphorylation in P2X4R KO mice; second, P2X4Rs have been reported to be expressed from postnatal day 1 (Cheung *et al.* 2005); third, the P2X4R KO mice exhibit communication deficits during their pre-adult period (Wyatt *et al.* 2013). Since, phospho-Thr34-DARPP-32 can indirectly increase ERK 1/2 phosphorylation in the striatum (Girault 2012), it was not surprising to see a significant effect of IVM treatment on ERK 1/2 phosphorylation. However, increase in ERK 1/2 phosphorylation was seen in both the genotypes, indicating that IVM's ability to modulate ERK 1/2 phosphorylation is independent of P2X4R function. Interestingly, there was a significant interaction between IVM treatment and genotype for total ERK 1/2 levels, indicating that effect of IVM on total ERK 1/2 expression was dependent upon P2X4R

function. Hence, IVM might be increasing ERK 1/2 phosphorylation in the WT mice by regulating total expression of the protein upon P2X4R potentiation. Although previous investigations have used IVM as a pharmacological tool for studying P2X4R function *in vitro* and *in vivo* (Bortolato *et al.* 2013, Sim *et al.* 2006, Popova *et al.* 2013, Asatryan *et al.* 2010), IVM does have other protein targets in the CNS including GABA_A receptors (Dawson *et al.* 2000), nicotinic acetylcholine receptors (Krause *et al.* 1998) and glycine receptors (Shan *et al.* 2001). Hence, the mechanism by which IVM increases ERK 1/2 phosphorylation in P2X4R KO mice needs further investigation. Similar to the dorsal striatum, IVM modulated DARPP-32 phosphorylation via P2X4R potentiation in the ventral striatum. Moreover, IVM had a tendency to differentially modulate CREB phosphorylation via P2X4R activity in the same brain region. Taken together, using genetic and pharmacological approaches, the data suggests that there is a link between P2X4R activity and DARPP-32 phosphorylation in the striatum and P2X4Rs may have a role in regulating DA neurotransmission in GABAergic MSNs of the striatum via modulating DARPP-32 activity.

The significant increases in DA receptor protein density in the ventral striatum reported herein may underlie the PPI deficits in P2X4R KO mice. PPI measures reduction in startle reflex that occurs when the eliciting acoustic burst is immediately preceded by a weak stimulus and is highly reliable index for measuring sensorimotor gating (Ison & Hoffman 1983). Multiple findings have reported a critical role for DA receptors in regulation of PPI function, of which D2Rs have received considerable attention on basis of findings from pharmacological studies in rats and patient population (Swerdlow *et al.* 1991, Abduljawad *et al.* 1998, Volter *et al.* 2012, Kumari *et al.* 1998). However, gene knockout and pharmacological studies in mice have implicated both D1Rs and D2Rs (Ralph-Williams *et al.* 2003, Ralph-Williams *et al.* 2002, Doherty *et al.* 2008). In the context of findings from the literature, we used both D1R (SCH-23390) and D2R (raclopride) antagonists to identify potential contribution of DA receptors to PPI functioning in P2X4R KO mice. We found that the PPI deficits in P2X4R KO mice were significantly ameliorated by SCH-23390 and raclopride, indicating D1Rs and D2Rs as important modulators of PPI function in mice. The increased density of DA receptors in the ventral striatum, integral to corticolimbic-striato-pallidal circuitry of PPI (Swerdlow *et al.* 2008), of P2X4R KO mice may contribute to PPI dysfunction and that blocking these receptors can reverse the deficit. The pharmacological studies provide insights into the functional consequences of altered DA receptor protein densities on behaviors such as sensorimotor gating in the P2X4R KO mice. Moreover, we reported IVM mediated PPI disruption in WT C57BL/6J mice and its attenuated response in P2X4R KO mice, which further supports a role for P2X4Rs in sensorimotor gating (Bortolato *et al.* 2013). Overall, these studies identify potential interactions between P2X4R function and DA neurotransmission in regulating sensorimotor gating.

The neurochemical and behavioral alterations in P2X4R KO mice could be relevant to multiple psychiatric disorders such as schizophrenia, attentional deficit hyperactivity disorder, Obsessive-Compulsive disorder and bipolar depression. For example, post mortem studies have reported an increase in D2R and DAT expression in psychotic and non-psychotic disorders (Krause *et al.* 2000, Brunswick *et al.* 2003, Pearlson *et al.* 1995, Perez *et al.* 2003). In addition, increased TH expression and presynaptic DA synthesis has been

reported in neuroleptic naïve psychotic patients (Hietala *et al.* 1995, Hietala *et al.* 1999). We observed increased density in D2Rs in the ventral striatum, DAT in both striatal regions and TH in the dorsal striatum in P2X4R KO mice. Perhaps, one of the most notable findings of our study was the identification of increased D2Rs in the striatum, since D2Rs are considered as an important genetic marker for susceptibility to neuropsychiatric diseases (Seeman 2013a, Seeman 2013b, Zhan *et al.* 2011). Additionally, P2X4R KO mice exhibited PPI deficits, an important behavioral biomarker of neuropsychiatric diseases (Seeman *et al.* 2006, Feifel *et al.* 2009, Perry *et al.* 2001, Braff 1993). Interestingly, the upregulation of D2Rs, altered sensitivity to DA receptor acting drugs and PPI deficits in P2X4R KO mice correlate with findings from mouse models linked to psychiatric disorders (Wolinsky *et al.* 2007, Lipina *et al.* 2010, Ralph *et al.* 2001, Kinkead *et al.* 2005). Taken together, the altered DA homeostasis and resultant behavioral deficits induced upon *p2rx4* deficiency suggests a role for this receptor in disorders characterized by DA dysfunction such as schizophrenia, bipolar disorder and attention deficit hyperactivity.

Our findings also suggest that P2X4Rs are involved in other DA dependent functions of the basal ganglia including motor behavior. To test this, we used the 6-OHDA animal model in combination with IVM and P2X4R KO mice, which is a well established model for elucidating DA interactions with other neurotransmitter systems in motor activity (Fox & Brotchie 2000, Xiao *et al.* 2011). Also, this model is used for understanding the pathogenesis of movement disorders including Parkinson's disease and screening of novel therapeutics (Deumens *et al.* 2002, Schwarting & Huston 1996). We found that the L-DOPA induced motor behavior was significantly decreased in 6-OHDA lesioned P2X4R KO mice, indicating that disruption of P2X4R function significantly affected L-DOPA induced behavioral response. This attenuated response in P2X4R KO mice may be due to alterations in DA system in striatonigral circuitry of the basal ganglia. Alternatively, L-DOPA attenuated response could be linked to its faster clearance or metabolism in P2X4R KO mice. This hypothesis will be explored in future studies. Conversely, we demonstrated that pharmacological modulation of P2X4R activity by IVM significantly enhanced L-DOPA induced rotational behavior. A plausible mechanism underlying L-DOPA + IVM response is a synergy between P2X4Rs and D1Rs on MSNs in disinhibition of neurons projecting from the substantia nigra pars reticulata (SNR) to the thalamus, superior colliculus and pedunculopontine nucleus and thereby, producing contralateral rotations. Notably, increased expression of P2X4Rs has been reported in the MSNs of SNR of 6-OHDA treated rats (Amadio *et al.* 2007) and so, these compensatory changes may partially explain the augmented L-DOPA dependent motor response in presence of IVM. IVM did not influence motor behavior independently, suggesting that activation of P2X4Rs alone is not sufficient enough to cause disinhibition of SNR to induce such a response. Unlike D1Rs that are present exclusively on postsynaptic MSNs, P2X4Rs are present both on presynaptic DA neurons and postsynaptic MSNs. The simultaneous activation of P2X4Rs at the presynapses and postsynapses would counteract each other, thus preventing the mice from turning to either side. The lack of effect with IVM alone supports the notion that IVM has a modulatory effect on L-DOPA's motor behavior. In addition to increased behavioral response in WT mice, IVM also enhanced L-DOPA's motor behavior in P2X4R KO mice. However, while comparing the absolute increase in L-DOPA's motor behavior in presence of IVM, we

saw a significant interaction between time and genotype, suggesting an altered effect of IVM between WT and P2X4R KO mice. This finding supports our previous finding that IVM mediates its behavioral effects partially via action on P2X4Rs (Wyatt et al. 2014, Bortolato et al. 2013). Nevertheless, the increase in L-DOPA response in P2X4R KO mice suggests that IVM may be modulating L-DOPA response through a complex network of receptor systems. Overall, the findings suggest that P2X4Rs have a synergistic role in DA modulation of motor control and can alter behavioral responses to dopaminergic drugs. As such, P2X4R allosteric modulators may represent potential adjuvant pharmacotherapies for Parkinson's disease.

In conclusion, the present investigation supports the hypothesis that there are signaling interactions between P2X4Rs and DA neurotransmission in regulation of multiple CNS functions in the basal ganglia. Finally, though at nascent stage, these findings implicate P2X4Rs in neurobiological mechanisms of multiple neurological disorders. There is growing interest in P2XRs as novel drug targets for therapeutic development in psychiatry disorders (Ortiz *et al.* 2015). In support of this hypothesis, others report that the gene for P2X4Rs is located in chromosome 12q24 (Gu *et al.* 2010) which contains several loci that can alter susceptibility to schizophrenia, bipolar disorder and attention deficit hyperactivity (Dawson *et al.* 1995, Jones *et al.* 2002, Bailer *et al.* 2000). Non-synonymous single nucleotide polymorphisms in *p2rx4* gene have been linked to high pulse pressure and age related macular degeneration (Caseley *et al.* 2014) but further investigation is needed before definite conclusions can be drawn regarding P2X4Rs and psychiatric diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

P2X4Rs	P2X4 receptors
P2X4 KO	P2X4 knockout
DA	dopamine
DAT	dopamine transporter
D1Rs	dopamine D1 receptors
D2Rs	dopamine D2 receptors
DARPP-32	dopamine and cyclic AMP regulated phosphoprotein of 32kDa

GPCRs	G-coupled protein receptors
TH	tyrosine hydroxylase
ERK 1/2	extracellular regulated kinase1/2
CREB	cyclic-AMP response element binding protein
PP-1	protein phosphatase-1
PKA	protein kinase A
6-OHDA	6-Hydroxydopamine
SNR	substantia nigra pars reticulata
MSNs	medium spiny neurons
PPI	prepulse inhibition
WT	wildtype
L-DOPA	levodopa
IVM	ivermectin

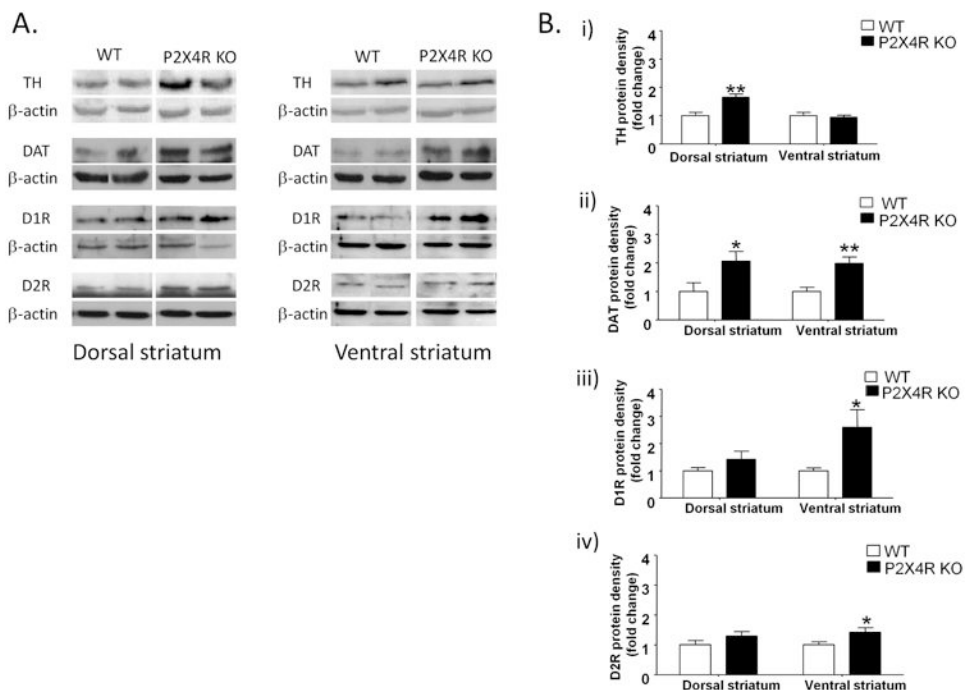


Figure 1. P2X4R KO mice exhibited significant increases in TH protein density in the dorsal striatum, but no changes in the ventral striatum [A & B (i)]; increased DAT protein density in both parts of the striatum [A & B (ii)]; increased D1R [A & B (iii)] and D2R [A&B (iv)] protein densities in the ventral, but no change in the dorsal striatum. The protein levels of DA markers were normalized to β-actin and expressed as arbitrary units (AU). The average of densitometry value of WT samples was arbitrarily normalized to 1. P2X4R KO samples were normalized by dividing each value by the average of WT samples and presented as fold change of P2X4R KO versus WT in that membrane. Values represent mean ± SEM from 5-8 WT, 7-8 P2X4R KO mice for TH, DAT analyses and 11-12 WT, 12-13 P2X4R KO for D1Rs and D2Rs analyses. 2 representative bands from each genotype from the same membrane are shown. * $P < 0.05$, ** $P < 0.01$ versus WT controls. Unpaired Student's t-test

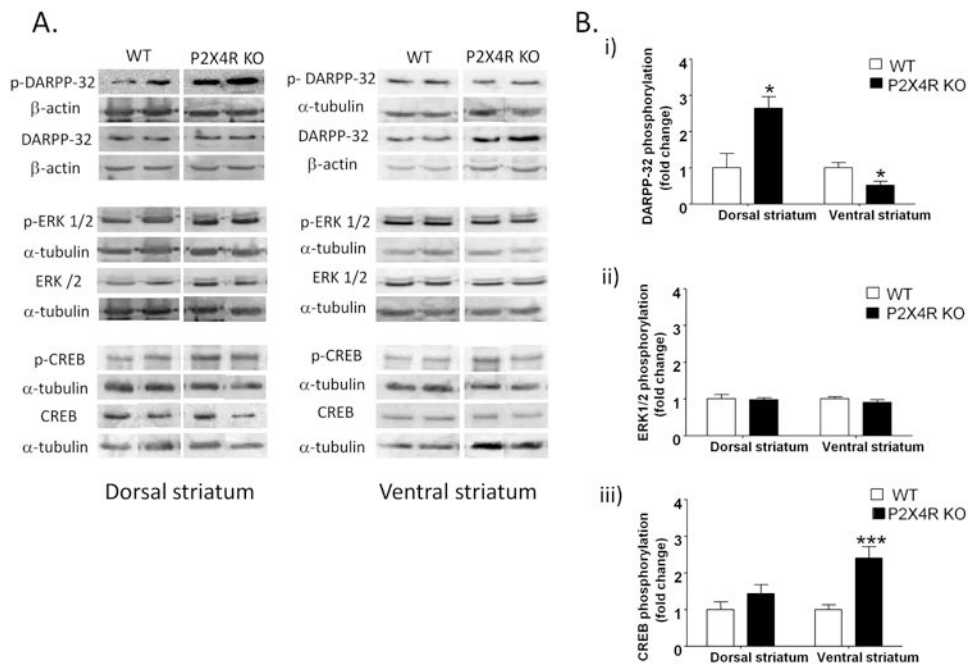


Figure 2. P2X4R KO mice exhibited increased DARPP-32 phosphorylation in the dorsal striatum, but a decrease in the ventral striatum [A & B (i)]; no changes in ERK 1/2 phosphorylation in dorsal or ventral striatum of P2X4R KO mice [A & B(ii)]; increased phosphorylation of CREB in the ventral, but not in the dorsal striatum [A & B(iii)]. Details of normalization and analyses are presented in Figure 1. Values represent mean ± SEM from 3-6 WT and 4-8 P2X4R KO mice for DARPP-32 analysis; 6-8 WT & P2X4R KO for ERK 1/2 and CREB analyses. 2 representative bands from each genotype from the same membrane are shown. * $P < 0.05$, *** $P < 0.001$ versus WT counterparts. Unpaired Student's t-test.

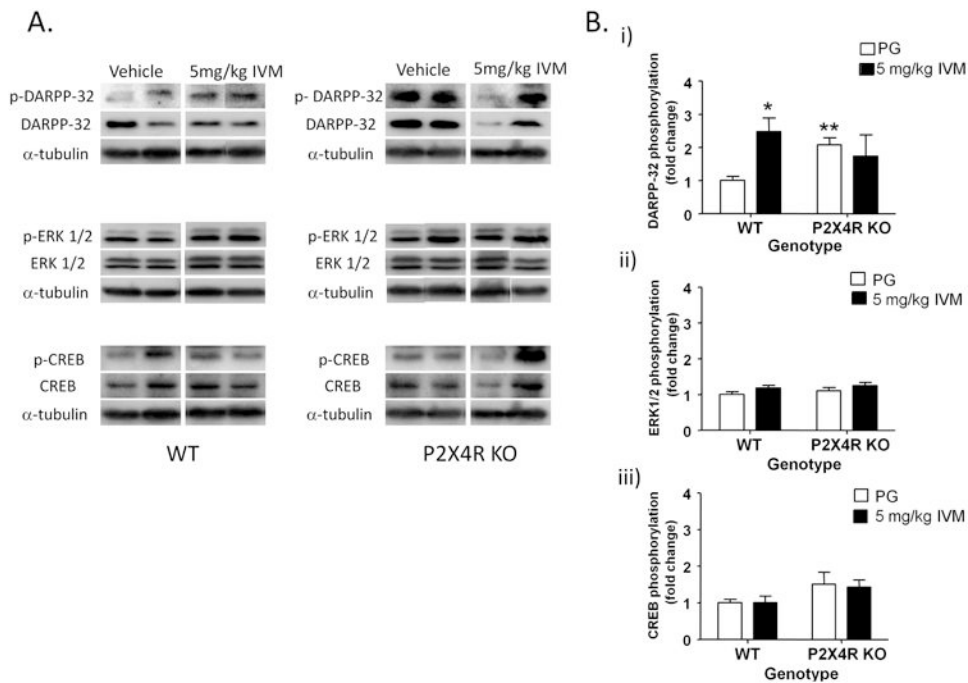


Figure 3.

IVM (5 mg/kg) significantly upregulated DARPP-32 phosphorylation [A & B (i)] via P2X4R potentiation in the dorsal striatum. IVM increased ERK 1/2 phosphorylation independent of P2X4R function [A& B (ii)]. No effect of IVM on CREB phosphorylation [A& B (iii)]. The average of densitometry value of vehicle treated WT samples was arbitrarily normalized to 1. The IVM treated WT mice, vehicle and IVM treated P2X4R KO were normalized by dividing each value by the average of the vehicle treated WT samples. The data is presented as fold change of IVM treated WT, P2X4R KO and vehicle treated P2X4R KO samples versus vehicle treated WT samples in that membrane. Values represent mean \pm SEM from 4-6 WT and P2X4R KO mice per treatment group. 2 representative bands from each treatment group are shown. * $P < 0.05$ versus vehicle treated WT group, Bonferroni post hoc test.

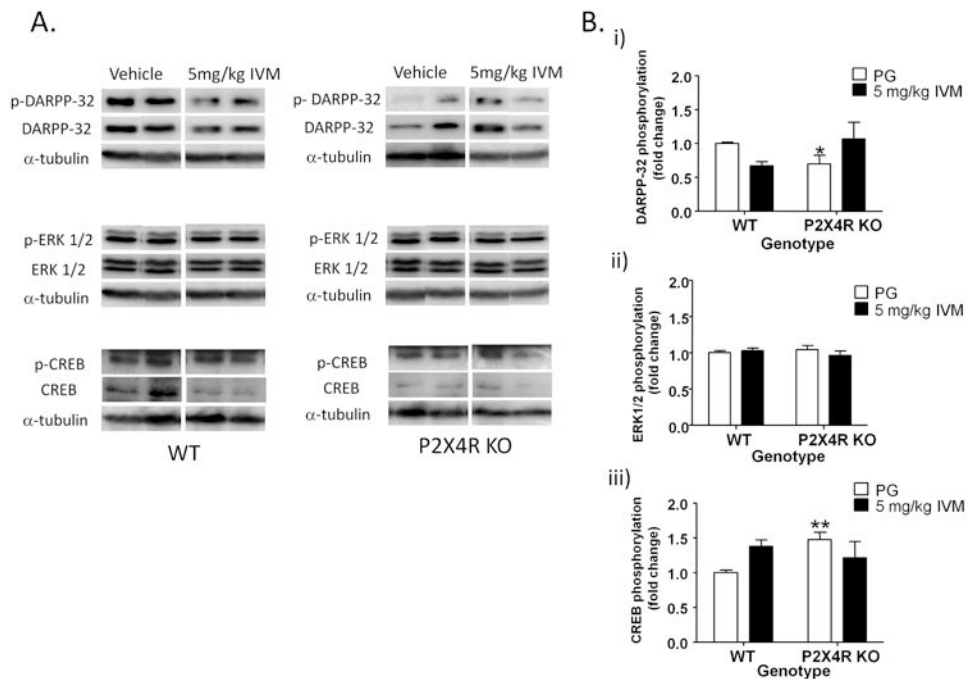


Figure 4.

IVM (5 mg/kg) significantly affected DARPP-32 [A & B (i)] but not, ERK 1/2 [A & B (ii)] or CREB phosphorylation [A& B (iii)], via P2X4R potentiation in the ventral striatum. Details of normalization and analyses are presented in Figure 3. Values represent mean \pm SEM from 4-6 WT and P2X4R KO mice per treatment group. 2 representative bands from each treatment group are shown. * $P < 0.05$ versus vehicle treated WT group, Bonferroni post hoc test.

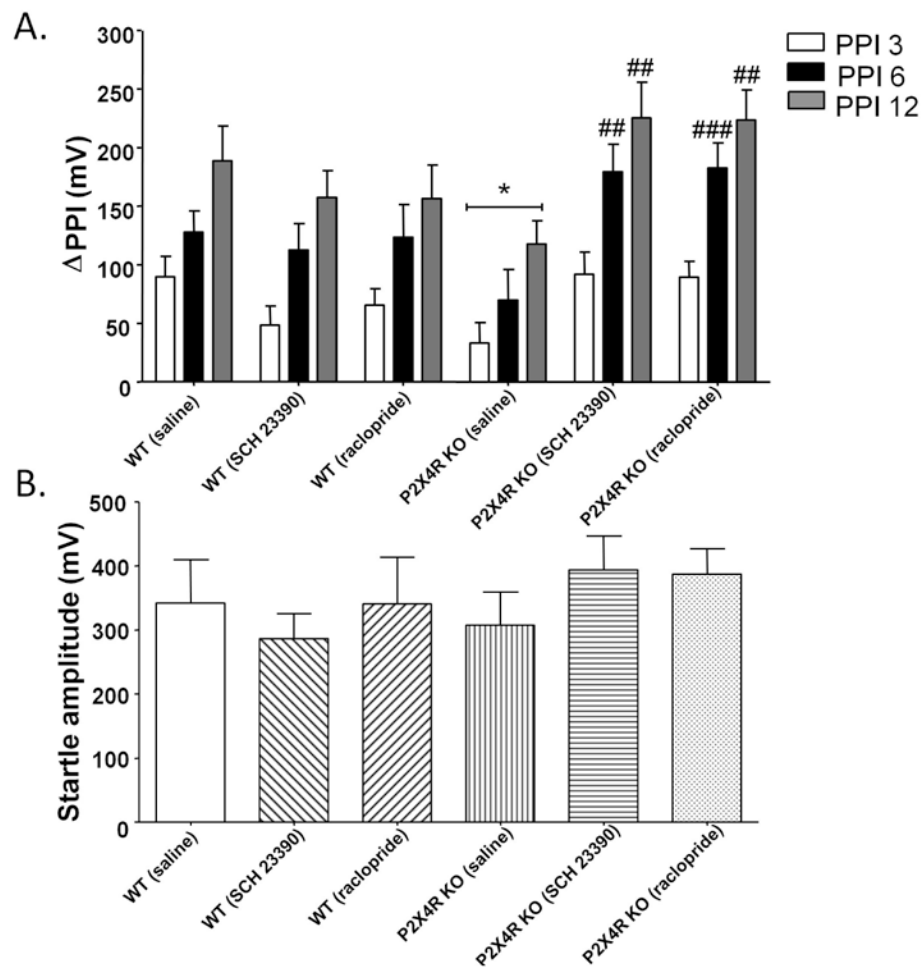


Figure 5. SCH-23390 (1 mg/kg) and raclopride (3 mg/kg) significantly increased prepulse inhibition of acoustic startle reflex in P2X4R KO mice (A) without any changes in startle amplitude (B). There were no changes in PPI in WT mice upon treatment with both antagonists (A). Values represent the mean of PPI and mean startle amplitude \pm SEM from 14 WT (saline), 15 WT (SCH 23390 and raclopride), 14 P2X4R KO (saline) and 16 P2X4R KO (SCH-23390 and raclopride). * $P < 0.05$ versus saline treated WT mice, ## $P < 0.01$, ### $P < 0.001$ versus saline treated P2X4R KO mice, Bonferroni post hoc test.

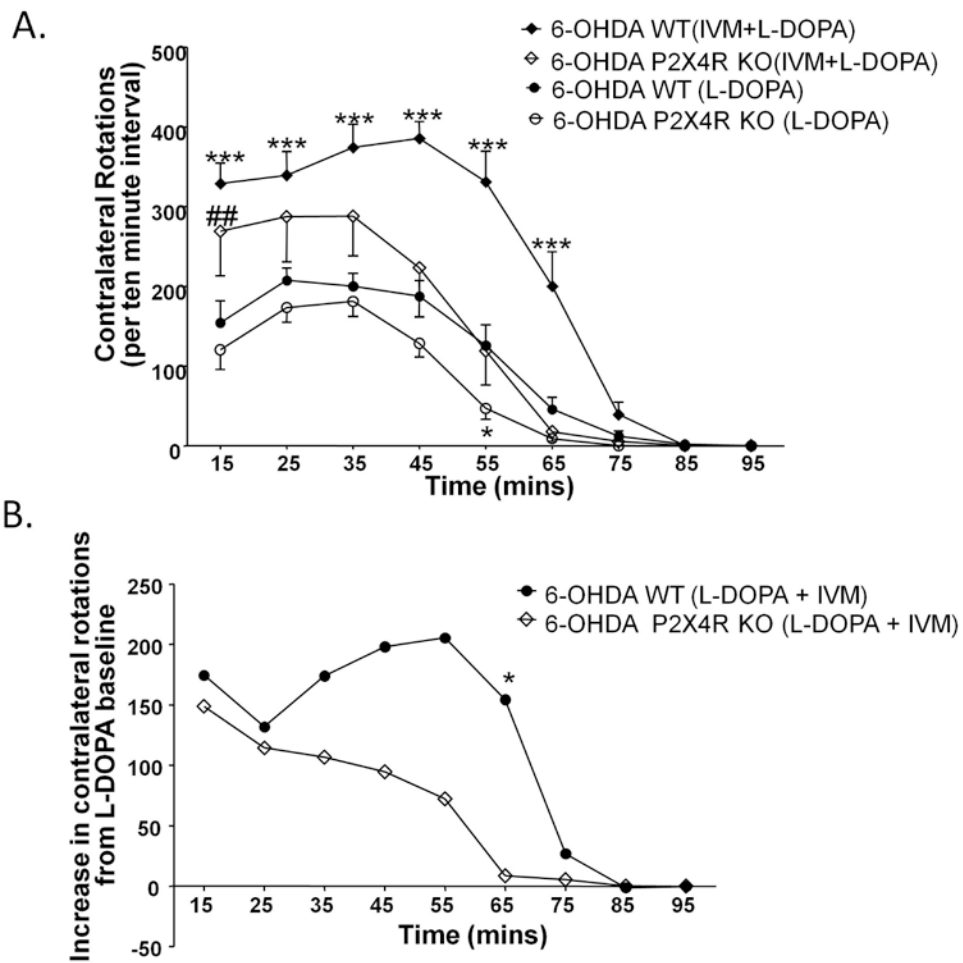


Figure 6. L-DOPA (5 mg/kg) induced rotational behavior is significantly attenuated in P2X4R KO mice. IVM (5 mg/kg) significantly potentiated L-DOPA's effect on the number of contralateral turns in WT and P2X4R KO mice (A). IVM's ability to enhance L-DOPA induced motor behavior was significantly altered in P2X4R KO mice (B). Values on the y-axis represent the mean of number of contralateral turns per 10 minute interval \pm SEM from 14 WT, 8 P2X4R KO. * P <0.05, *** P <0.001 versus L-DOPA treated WT mice, ## P <0.01 versus L-DOPA treated P2X4R KO mice for (A), * P <0.05 versus WT mice for (B), Bonferroni post hoc test.