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Inhibition of Wnt signalling dose-dependently impairs the acquisition and expression of amphetamine-induced conditioned place preference

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HIGHLIGHTS

- Wnt inhibitor, IWP-2, was microinjected into the NAc of rats.
- Intra-NAc IWP-2 blocked the acquisition and expression of amphetamine-induced CPP.
- Acquisition of CPP was blocked at a lower dose of IWP-2 than the expression of CPP.
- Sensitization to amphetamine was blocked when pre-treated with IWP-2.
- Thus, Wnt signalling is involved in the consolidation of reward-related learning.

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ABSTRACT

The mechanisms by which dopaminergic neurotransmission in the nucleus accumbens (NAc) is involved in incentive learning produced by rewarding stimuli remain unclear. Recently, Wnt signalling has been implicated in synaptic plasticity and learning and memory. Functional interactions between Wnt and dopamine (DA) signalling has been demonstrated using in vitro and tissue physiology approaches, however there remains a lack of in vivo research into the involvement of Wnt in DA-mediated learning in behaving animals. The present study assessed the role of Wnt signalling in DA-mediated incentive learning using the conditioned place preference (CPP) paradigm. We hypothesized that inhibition of Wnt with intra-NAc microinjections of Wnt palmitoylation inhibitor IWP-2 will dose-dependently block the acquisition and expression of amphetamine (AMPH)-induced CPP in rats. Intra-NAc IWP-2 (0.001, 0.05, 1.0 but not 0.0001 μg/0.5 μl/side) prior to conditioning with AMPH (20.0 μg/0.5 μl/side) blocked acquisition of CPP. Intra-NAc IWP-2 (0.05, 0.5, 1.0 but not 0.001 μg/0.5 μl/side) during test following conditioning with AMPH blocked expression but at a higher dose than was need to block acquisition. Sensitization of locomotor activity to AMPH was observed during conditioning and this effect was blocked in groups given IWP-2 prior to AMPH. However, intra-NAc IWP-2 during conditioning did not block the locomotor stimulant effects of AMPH. These results implicate Wnt in DA-mediated incentive learning and suggest that Wnt signalling may be more important for the acquisition of CPP then for its expression. However, mechanisms by which Wnt and DA signalling pathways interact to influence DA-mediated reward-related learning remain to be elucidated.

1. Introduction

Rewarding stimuli activate dopamine (DA) neurons [1] and increase the ability of recently encountered environmental stimuli to elicit approach and other responses in the future, a phenomenon referred to as incentive learning [2,3]. An experimental example is conditioned place preference (CPP). CPP occurs when one of two contextually different chambers is repeatedly paired with a rewarding stimulus and the other with its absence; animals show preference for the chamber associated with the reward in a subsequent choice test [4]. This phenomenon is mediated by dopaminergic neurons projecting from the ventral tegmental area of the midbrain to forebrain targets, particularly the nucleus accumbens (NAc) [5,6]. For example, pairing of one chamber with

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systemic or intra-NAc microinjections of a psychostimulant drug, such as amphetamine (AMPH), enhances dopaminergic neurotransmission and produces CPP [4,7,8]. Similar to other types of learning, incentive learning involves activity-dependent changes in synaptic function and connectivity mediated by specific intracellular events [3,9]. DA-mediated changes in synaptic plasticity in the striatum may be the substrate of incentive learning, however the underlying mechanism remains to be fully elucidated [10].

Wnts are a highly conserved family of secreted glycolipoproteins that has been studied extensively outside the nervous system in early developmental processes [11–13], cell proliferation [14], growth and homeostasis [15,16], and various types of cancer [17–22]. Wnt signalling has emerged in recent years as a crucial player in the central nervous system, with much of the research focused on the role of Wnt in neurodevelopmental processes [23,24]. A small but growing body of data has implicated the canonical Wnt pathway in synaptic plasticity and learning and memory in the adult brain [25–28]; this pathway mediates the transcription of Wnt-target genes by regulating the activation of glycogen synthase kinase 3β (GSK3β) and β-catenin [29,30]. Suppression of endogenous Wnt activity leads to impaired long-term potentiation (LTP) and deficits in memory [26,31]. Aberrancies in Wnt signalling have also been associated with a variety of neuropsychiatric disorders including Alzheimer's disease, schizophrenia, autism and mood disorders [26,31].

To our knowledge, there has been no research on the possible role of Wnt signalling in DA-mediated learning. In fact, there is very little known about the activity of Wnt in vivo on regulating learning and memory formation in animals with most existing studies using in vitro and slice physiology approaches. It appears that signalling components of the canonical Wnt pathway are selectively altered by DA D_2 receptors [32] and are rapidly activated by the administration of AMPH into the rat NAc [33]. DA D_2 receptors increase key components of Wnt signalling cascades including GSK3β and β-catenin [34]. These results suggest functional interactions between the dopaminergic and Wnt signalling pathways at the molecular level, however it is not known whether Wnt interacts with signalling by DA receptors to influence DA-mediated learning in behaving animals.

The aim of the present study was to assess the role of Wnt signalling in the acquisition and expression of DA-mediated incentive learning using the unbiased CPP paradigm. We utilized the small-molecule IWP-2, which is an inhibitor of Wnt production that acts by inactivating Porcupine (Porc), a transmembrane acyltransferase required for the post-translational palmitoylation of Wnt for it to become functionally active [35]. We hypothesized that the inhibition of Wnt signalling in the NAc will dose-dependently impair the acquisition and expression of AMPH-induced CPP. Wnt signalling may be more important for the acquisition of CPP, when incentive learning is taking place, than for its expression. Thus, we hypothesized further that Wnt inhibition will have a greater impact on acquisition than on expression of AMPH-induced CPP.

2. Methods

2.1. Subjects

Experimentally naïve male Wistar rats ($N = 146$) (Charles River, St. Constant, Quebec), weighing 225–250 g upon arrival, were housed in pairs or triplets in clear, Plexiglas cages containing sterilized woodchip bedding changed twice weekly. Animals were housed in a temperature-controlled colony room maintained at 21 °C (+/−2 °C) and humidity of 55% (+/−10%) under a 12-h reverse dark-light cycle (lights on at 19:00–07:00 h). Food (LabDiet 5001, PMI Nutritional International, Brentwood, MO) and water were available ad libitum. Rats were handled daily for at least five days prior to surgery. All experiments were performed around the same time during the dark cycle.

All procedures in this study were carried out in accordance with guidelines of the Animals for Research Act, Canadian Council on Animal Care, and were approved by the Queen's University Animal Care Committee.

2.2. Surgery

Prior to surgery, rats were given subcutaneous injections of analgesics: bupivacaine (2.0 mg/kg) locally on the incision site followed by tramadol (20.0 mg/kg). Rats anaesthetized under an oxygen flow containing 4.5% isoflurane (Fresenius Kabi Canada Ltd, Richmond Hill, Ontario) were placed prone on the stereotaxic unit and steady respiratory rate was maintained during the surgery at 1.5% isoflurane. An incision was made along the midline to expose the skull and the area around bregma was cleaned and dried. Bilateral stainless steel guide cannulae (22 gauge, 7.7 mm long) were implanted into the NAc by drilling holes into the skull with coordinates 1.6 mm anterior to bregma, 1.4 mm lateral to the midline, and 7.7 mm ventral to the skull surface [36]. The cannulae were anchored to the skull with stainless steel screws and dental acrylic. Stainless steel pins (7.7 mm long) were inserted into the guide cannulae to prevent occlusions. Rats were individually housed immediately following surgery and were given post-operative care, including subcutaneous injections of a non-steroidal anti-inflammatory drug, meloxicam (1.0 mg/kg), and an analgesic, tramadol (20.0 mg/kg), and were allowed to recover for 1 week prior to experiments.

2.3. Drugs

D-amphetamine sulphate (AMPH) (Sigma, St. Louis, Missouri) was dissolved in 0.9% sterile saline at a dose of 1.5 mg/kg for intraperitoneal (IP) injections and at a dose of 20 μg/0.5 μl/side for intra-cranial microinjections to the NAc, and prepared on each AMPH conditioning day. IWP-2 (Tocris Bioscience, Bristol, UK) was dissolved in DMSO (dimethyl sulfoxide ≥99.5% (GC), Sigma, St. Louise, Missouri) at doses of either 0.0001, 0.001, 0.05, or 1.0 μg/0.5 μl/side on AMPH conditioning days for groups assessing acquisition of AMPH-induced CPP and at doses of either 0.001, 0.05, 0.5, or 1.0 μg/0.5 μl/side on test day for groups assessing expression of AMPH-induced CPP (see below). Control microinjections consisted of either 0.9% sterile saline or DMSO at 0.5 μl/hemisphere.

2.4. Drug microinjection

Intra-cranial microinjections to the NAc were made with a pair of 10.0 μl microsyringes (Hamilton Co., Reno, NV) mounted on an infusion pump (KD Scientific, Holliston, MA). Stainless steel injection cannulae that extended 1 mm below the guide cannulae to 8.7 mm ventral to the surface of the skull, were attached to the microsyringe via polyethylene tubing. Drug was delivered at a constant rate of 1.0 μl/min over an interval of 30 s and the injection cannulae was kept in place in the guide cannulae for an additional 30 s to promote drug diffusion.

2.5. Apparatus

The four CPP apparatus consisted of a rectangular Plexiglas-covered wooden box with two contextually distinct compartments (38 × 27 × 36 cm) connected by a tunnel (8 × 8 × 8 cm) that could be closed with a removal guillotine-style door. The compartments had distinct combinations of wall patterns of either urethane-sealed wood or black and white vertical stripes (1 cm wide), and had distinct combinations of floor texture of either galvanized steel mesh

or parallel stainless steel rods (1 cm apart). Each box was equipped with six infrared emitters and detectors: two trisecting the long axis of each compartment at a height of 5 cm and two trisecting the tunnel at a height of 3 cm. The locomotion of the rat and the time spent in each compartment was recorded by a 6809 micro-controller. For further details of the apparatus, refer to [37].

2.6. Conditioned place preference (CPP) test

The unbiased CPP procedure consisted of three phases: Preconditioning (three 15-min sessions), conditioning (eight 30-min sessions), and testing (one 15-min session). Animals received one session per consecutive days during the dark phase (07:00–19:00 h).

2.6.1. Controls

Three AMPH control groups were included. To establish the AMPH-induced CPP paradigm, a control group denoted "AMPH systemic", was given AMPH (1.5 mg/kg IP) immediately before drug-conditioning sessions and saline (1.0 ml/kg IP) immediately before vehicle-conditioning sessions. The control for the series of acquisition experiments denoted "ACQ (0.0)" was given intra-NAc microinjections of DMSO (0.5 μl/side) 30 mins prior to being given AMPH (20.0 μg/0.5 μl/side) on drug days, and DMSO (0.5 μl/side) 30 mins prior to saline (0.5 μl/side) on vehicle days. The control for the series of expression experiments denoted "EXP (0.0)" received intra-NAc microinjections of AMPH (20.0 μg/0.5 μl/side) during drug days and saline (0.5 μl/side) on vehicle days of conditioning.

The IWP-2 control group, denoted "IWP-2 alone", received intra-NAc microinjections of IWP-2 (1.0 μg/0.5 μl/side) 30 mins prior to receiving saline (0.5 μl/side) on drug days and DMSO (0.5 μl/side) 30 min prior to saline (0.5 μl/side) apart on vehicle days of conditioning. None of the control groups received any injections on test.

2.6.2. Acquisition of AMPH-induced CPP (ACQ AMPH)

During preconditioning, rats were placed into the CPP apparatus with the tunnel open. During conditioning, rats received two microinjections, 30 mins apart, prior to being restricted to one of the chambers of the CPP apparatus. Days 1, 3, 5, and 7 were the drug-paired conditioning sessions during which rats received intra-NAc microinjections of IWP-2 (0.0001, 0.001, 0.05, or 1.0 μg/0.5 μl/side). After 30 mins, rats received intra-NAc AMPH (20.0 μg/0.5 μl/side) and were then immediately placed into their respective drug-paired chamber. Days 2, 4, 6, and 8 were vehicle-paired conditioning sessions during which intra-NAc microinjections of the IWP-2 vehicle, DMSO (0.5 μl/side) were followed 30 min later by intra-NAc microinjections of saline (0.5 μl/side) and placement into their respective vehicle-paired chamber. Number of beam breaks was recorded as a measure of locomotor activity during conditioning. On test day the tunnel was open.

2.6.3. Expression of AMPH-induced CPP

During preconditioning sessions, rats were placed into the CPP apparatus with the tunnel open. On conditioning days 1, 3, 5, and 7, rats were given intra-NAc microinjections of AMPH (20.0 μg/0.5 μl/side) and immediately restricted to the drug-paired side of the CPP apparatus. On conditioning days 2, 4, 6, and 8, rats were given intra-NAc saline (0.5 μl/side) and immediately restricted to the vehicle-paired side of the CPP apparatus. During the test, rats were administered IWP-2 (0.001, 0.05, 0.5 or 1.0 μg/0.5 μl/side) 30 min prior to being placed in the CPP apparatus with the tunnel open.

2.7. Histological analysis

Rats were euthanized by carbon dioxide exposure and decapitated for brain extraction upon completion of the test phase. Extracted brains were placed in 10% formalin/sucrose solution for a minimum of 1 wk. In a temperature-controlled cryostat, brains were frozen and sliced coronally in 40 μm sections. Alternate brain slices were collected and mounted on gelatin-coated glass slides and were stained with cresyl violet for verification of cannulae placement by an observer blind to the behavioural results. Only data from animals with cannulae located within the NAc were included in the statistical analysis.

2.8. Statistical analyses

Planned paired-samples *t*-tests compared time spent in the to-be-vehicle-paired and the to-be-drug-paired compartments during preconditioning to assess for side bias. Animals (n = 4) that spent ≥700 s and/or ≤200 s in one particular compartment, therefore showing an obvious side bias, during preconditioning were excluded from analysis based on criterion established at the beginning of the study. Planned paired-samples *t*-tests comparing tunnel time during preconditioning vs. test for each group were conducted to evaluate the possibility of change in tunnel time. A decrease in tunnel time during test compared to preconditioning could enhance a putative place preference effect.

Place preference was assessed with the use of a two-way mixed-design analysis of variance (ANOVA) with independent groups and repeated measures on phase (time in drug-paired side on preconditioning days averaged vs. on test). Significant interactions were followed up with simple effects analyses. A CPP effect is observed when the animal spends significantly more time in the drug-paired compartment during test compared to preconditioning sessions.

Locomotor activity during the conditioning sessions was assessed using a three-way mixed-design ANOVA with independent groups and repeated measures on treatment (drug vs. vehicle) and day (1, 2, 3, 4). Where appropriate, significant interactions were followed up by analyses of simple interactive effects and simple main effects. Statistical significance was established at $p < 0.05$.

3. Results

3.1. Histology

Histological examination of the location of the cannula tips revealed that of the 134 rats that underwent surgery, 123 had placements in the target region of the NAc (Fig. 1). Ten rats were removed from the analyses because cannula tips were not in the target region. One rat was excluded because the acrylic skullcap came off during the conditioning phase. Final numbers of rats included in each group are shown in Table 1.

Although all of the rats classified as hits had bilateral cannula placements located in the NAc, examination of Fig. 1 revealed that the rostral-caudal distribution varied among groups. The 3 groups that were prepared by one of us (KX), viz., ACQ 0.001, 0.05 and 1.0, generally had cannulae placed more rostrally than the remaining groups prepared by FI. As discussed below, those 3 groups did not show a CPP. To evaluate a possible relationship between rostral-caudal placement and the CPP effect, we combined all of the central injection rats from groups that showed a CPP (AMPH ACQ control, AMPH EXP control, ACQ 0.0001 and EXP 0.001, see below) and then classified them as rostral (≥1.2 mm anterior to bregma) vs. caudal (≤0.96 mm anterior to bregma) placements in the NAc. This yielded a rostral group of 11 rats and a caudal group of 34 rats with difference (±SEM) scores from preconditioning to test of 108.6 s (±37.3)

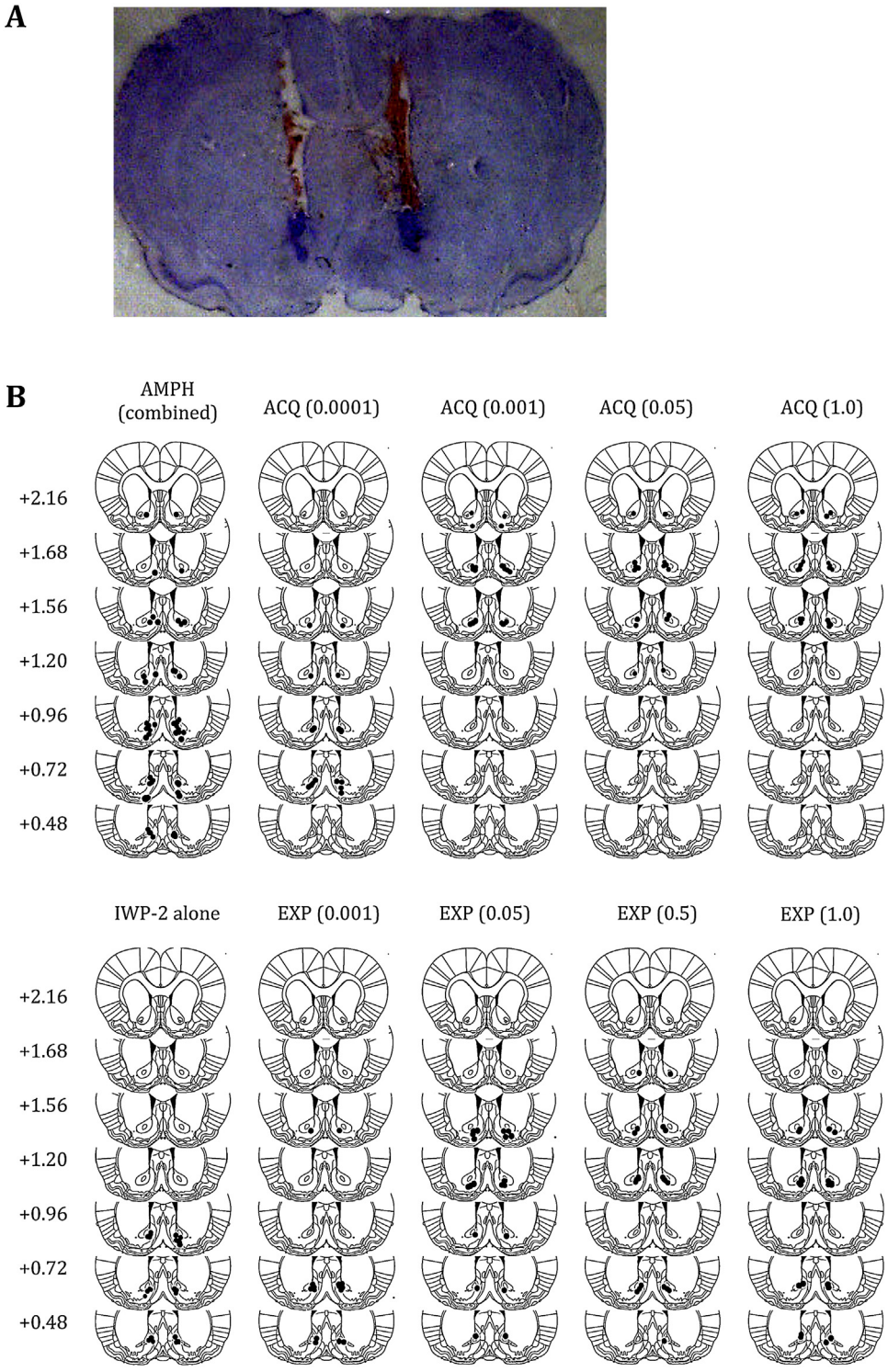

Fig. 1. (A) A representative bilateral NAc injector placement. (B) Reconstructed microinjection sites in the nucleus accumbens (NAc) from all experiments with illustrations adopted from Paxinos [36]. Numbers on the left refer to anterior-posterior distance from bregma in mm. Controls denoted AMPH (combined) includes acquisition (ACQ) and expression (EXP) controls (20.0 μg/0.5 μl/side). Numbers beside ACQ and EXP groups represent IWP-2 dose in μg/0.5 μl/side.

and 97.4 s (±19.3), respectively. Statistical analyses revealed that either rostral or caudal placements in the NAc led to a CPP with AMPH. Thus, two-way mixed design ANOVA on these independent groups with preconditioning and test time on the drug-paired side as the repeated measure revealed a significant main effect of phase ($F(1,43) = 26.49$, $p < 0.001$) but not group or interaction. Dependent *t*-tests conducted separately on each group similarly revealed a significant main effect for phase in the rostral ($t(10) = 2.90$, $p < 0.05$) and caudal groups ($t(33) = 5.04$, $p < 0.001$).

Table 1
Time (s) spent in the to-be-vehicle- and drug-paired sides during preconditioning.

Group (dose)	*n*	Vehicle (S.E.M.)	Drug (S.E.M.)	*t*	*P*
AMPH (combined)	39	425.4 (12.2)	412.3 (12.9)	−0.55	n.s.
AMPH systemic (1.5)	11	445.9 (23.7)	396.3 (28.5)	0.95	n.s.
IWP-2 alone (1.0)	11	434.2 (17.7)	420.8 (17.1)	0.39	n.s.
ACQ (0.0)	10	409.0 (29.3)	403.1 (25.2)	0.13	n.s.
ACQ (0.0001)	8	470.1 (20.2)	376.5 (18.2)	2.44	0.04
ACQ (0.001)	12	434.5 (16.7)	413.6 (14.7)	0.67	n.s.
ACQ (0.05)	9	424.2 (11.4)	429.0 (10.5)	−0.23	n.s.
ACQ (1.0)	12	417.5 (24.6)	426.5 (24.3)	−0.19	n.s.
EXP (0.0)	18	422.0 (15.4)	427.2 (17.3)	−0.16	n.s.
EXP (0.001)	9	410.8 (26.0)	426.8 (20.2)	−0.35	n.s.
EXP (0.05)	10	456.1 (23.4)	392.9 (21.1)	1.42	n.s.
EXP (0.5)	11	445.4 (19.0)	412.5 (14.6)	0.99	n.s.
EXP (1.0)	10	416.7 (50.0)	421.8 (16.4)	−0.17	n.s.

Times shown are averaged over the three pre-conditioning days. Group doses in brackets for systemic is in mg/kg and for intra-nucleus accumbens microinjections are in μg/0.5 μl/side. Numbers beside ACQ and EXP groups represent IWP-2 dose.
ACQ = acquisition, AMPH = amphetamine, EXP = expression, IWP-2 = Inhibitor of Wnt Production-2, n.s. = not significant, and S.E.M. = standard error of the mean.

3.2. Place conditioning

Average time spent in the to-be-drug-paired side compared to the to-be-vehicle-paired side over the three preconditioning sessions was used to evaluate possible side bias. There was no significant difference in all but one group (Table 1). Animals in the ACQ (0.0001) group spent more time in the to-be-vehicle-paired compared to the to-be-drug-paired side during preconditioning ($t(8) = 2.44$, $p < 0.05$). There was no significant difference in time spent in the tunnel from preconditioning to test in all of the treatment groups (Table 2).

CPP was defined by the change in the time spent in the drug-paired side during test compared to the averaged preconditioning sessions. AMPH resulted in an increase in time spent in the drug-paired side during test (Fig. 2A). The three AMPH control groups, i.e., systemic (1.5 mg/kg IP), ACQ control (20.0 μg/0.5 μl/side; Fig. 2B), and EXP control (20.0 μg/0.5 μl/side; Fig. 2C) spent a greater amount of time in the drug-paired side during test [$F(1, 36) = 26.93$, $p < 0.001$ for phase in two-way ANOVA]. As there was no significant effect of group or interaction, the three AMPH control groups were combined into an omnibus control group denoted AMPH (combined) for the rest of the analysis of place conditioning (Fig. 2A).

Acquisition groups (Fig. 2B) given intra-NAc microinjection of IWP-2 prior to AMPH during conditioning revealed a dose-dependent CPP, animals given 0.0 or 0.0001 μg/0.5 μl/side demonstrating the greatest increase. Expression groups (Fig. 2C) conditioned with AMPH and given IWP-2 on test also produced a dose-dependent CPP with the 0.0 and 0.001 μg/0.5 μl/side groups showing the largest increase. The IWP-2 alone group (Fig. 2A) did not show a significant change in time spent in the drug-paired side. These observations were supported by statistical analysis. ANOVA including AMPH (combined), all of the ACQ, EXP and the IWP-2 alone group revealed a significant effect of phase ($F(1, 121) = 22.10$, $P < 0.001$) and interaction ($F(9, 121) = 2.25$, $P < 0.05$). Simple effect analysis of phase using one-way ANOVA for each group revealed a significant increase in time spent in the drug-paired side for the AMPH (combined) group as reported above, ACQ (0.0001) ($F(1, 7) = 7.07$, $p < 0.05$) and EXP (0.001) ($F(1, 8) = 14.23$, $p < 0.01$). Acquisition was impaired at a lower dose of IWP-2, i.e., 0.001 μg/0.5 μl/side, than expression.

3.3. Locomotor activity

Locomotor activity during the conditioning phase was measured by beam breaks on drug and vehicle days for each group (Fig. 3). Groups that received AMPH showed increased locomotor activity on drug days compared to vehicle days. The IWP-2 alone group that did not receive AMPH on drug days did not show a difference in locomotor activity between drug and vehicle days. Sensitization of locomotor activity from drug day 1–4 was observed for the AMPH (combined) and EXP groups (Fig. 3C). Little change in activity was observed on drug day 1 vs. 4 for the ACQ groups given IWP-2 prior to conditioning with AMPH, with the exception of ACQ (0.05) group that appeared to show a decrease (Fig. 3B). Locomotor activity showed little difference between vehicle day 1 vs. 4 in any of the groups.

Statistical analyses support these observations. Locomotor activity was higher [treatment $F(1, 36) = 154.67$, $p < 0.001$] and increased from drug day 1 and 4 compared to vehicle day 1 and 4 of the three AMPH control groups (Table 3) [treatment × day interaction $F(1, 36) = 12.44$, $p \leq 0.001$]. No interactions involving the group variable were observed, so the three AMPH control groups were combined to make an omnibus control group denoted AMPH (combined) that was used in the rest of the analyses of locomotor activity.

The sensitization of locomotor activity from drug day 1–4 observed for the AMPH (combined) and EXP groups but not the IWP-2 alone or ACQ groups yielded a significant day x group interaction ($F(9, 121) = 4.79$, $P < 0.001$) in simple interactive effects ANOVA following observation of a significant treatment × day × group interaction ($F(9, 121) = 3.89$, $P < 0.001$) in a 3-way ANOVA that included the vehicle treatment days. Simple effects analyses of drug day for each group revealed a significant increase for AMPH (combined) ($F(1, 38) = 7.26$, $p < 0.05$), EXP (0.05) ($F(1, 9) = 19.88$, $p < 0.01$), EXP (0.5) ($F(1, 10) = 6.95$, $p < 0.05$) and EXP (1.0) ($F(1, 9) = 19.65$, $p < 0.01$). A significant mean decrease was observed in ACQ (0.05) ($F(1, 8) = 6.36$, $p < 0.05$); the remaining ACQ groups showed no significant change.

4. Discussion

The present study is the first to investigate the role of Wnt signalling in DA-mediated incentive learning. The results show that systemic administration or intra-NAc microinjection of AMPH produces significant CPP in rats. The Wnt palmitoylation inhibitor IWP-2 given during conditioning or on test dose-dependently and respectively impaired acquisition and expression of AMPH-induced CPP. Acquisition was blocked at a lower dose than expression. AMPH produced significantly higher levels of locomotor activity compared to vehicle during conditioning. Groups given only AMPH displayed a sensitization of locomotor activity from drug day 1–4. The acquisition groups that were co-administered IWP-2 on conditioning days with AMPH showed locomotor stimulant effects of AMPH but did not show sensitization of locomotor activity over

Table 2
Time (s) spent in the tunnel during preconditioning vs. test.

Group (dose)	*n*	Preconditioning (S.E.M.)	Tese (S.E.M.)	*t*	*P*
AMPH (combined)	39	55.0 (3.7)	52.4 (5.2)	0.77	n.s.
AMPH Systemic (1.5)	11	57.8 (7.1)	54.5 (7.2)	−0.60	n.s.
IWP-2 alone (1.0)	11	45.1 (4.2)	50.9 (6.4)	−1.108	n.s.
ACQ (0.0)	10	59.2 (10.5)	59.0 (14.5)	0.05	n.s.
ACQ (0.0001)	8	53.9 (4.2)	57.6 (9.0)	−0.45	n.s.
ACQ (0.001)	12	51.9 (5.7)	57.2 (9.0)	−0.97	n.s.
ACQ (0.05)	9	46.8 (7.2)	46.9 (8.1)	−0.01	n.s.
ACQ (1.0)	12	56.0 (5.3)	51.2 (6.0)	0.87	n.s.
EXP (0.0)	18	50.9 (3.8)	43.0 (6.0)	1.36	n.s.
EXP (0.001)	9	62.3 (6.8)	65.1 (10.9)	−0.27	n.s.
EXP (0.05)	10	51.0 (4.0)	59.8 (11.3)	−0.96	n.s.
EXP (0.5)	11	43.2 (5.8)	46.2 (9.8)	−0.35	n.s.
EXP (1.0)	10	61.6 (9.6)	58.9 (9.5)	0.28	n.s.

Times shown for preconditioning are averaged over the three pre-conditioning days. Group doses in brackets for systemic is in mg/kg and for intra-nucleus accumbens microinjections are in μg/0.5 μl/side. Numbers beside ACQ and EXP groups represent IWP-2 dose.
ACQ = acquisition, AMPH = amphetamine, EXP = expression, IWP-2 = Inhibitor of Wnt Production-2, n.s. = not significant, and S.E.M. = standard error of the mean.

Table 3
Locomotor activity measured by number of beam breaks on drug and vehicle days of conditioning for controls.

	AMPH Systemic [1.5 mg/kg] n = 11	ACQ (0.0) [20.0 μg/0.5 μl/side] n = 10	EXP (0.0) [20.0 μg/0.5 μl/side] n = 18
Mean Beam Breaks (S.E.M.)			
AMPH			
Day 1	577.6 (27.5)	625.0 (43.4)	574.9 (43.9)
Day 2	726.2 (55.4)	681.5 (46.6)	709.4 (57.0)
Day 3	660.5 (60.7)	692.3 (38.2)	914.2 (84.8)
Day 4	673.9 (64.8)	703.2 (65.2)	694.2 (73.3)
Mean	636.8 (30.7)	669.9 (28.0)	834.9 (62.5)
Vehicle			
Day 1	289.1 (35.8)	371.7 (25.6)	355.9 (23.1)
Day 2	286.9 (19.2)	298.3 (23.5)	348.1 (27.0)
Day 3	224.5 (29.8)	306.4 (27.6)	385.2 (26.8)
Day 4	188.4 (31.5)	354.9 (31.4)	330.5 (22.1)
Mean	240.1 (25.0)	332.8 (21.7)	371.2 (20.7)

Locomotor activity for amphetamine (AMPH) and Vehicle days 1–4 is expressed in mean beam breaks over 30 min for the three control groups. Doses of AMPH are indicated in square brackets.
ACQ = acquisition, EXP = expression, and S.E.M. = standard error of the mean.

the four drug conditioning days. These data suggest that inhibition of Wnt signalling can dose-dependently disrupt the acquisition and expression of AMPH-induced CPP, with acquisition being more sensitive than expression, and can prevent locomotor sensitization to AMPH.

The CPP paradigm was unbiased, i.e., groups showed no preference for one side of the apparatus over the other during preconditioning with the exception of one group. Rats in the ACQ (0.0001) group preferred the to-be-vehicle-paired side during preconditioning making the least-preferred side the to-be-drug-paired side and unintentionally employing the biased CPP method. The rats in this group spent about the same amount of time in the drug-paired side on test day as the control groups that showed CPP (∼495 s). Total test session time is 900 s but rats spend about 60 s on average in the tunnel; equal time on each side would be 420 s. An average time on the drug-paired side of 495 s indicates that the rats were spending well over half of the test session time on the drug-paired side showing a true side preference. Results indicate that the side bias during preconditioning cannot account for the observed place preference for the ACQ (0.0001) group. Time spent in the tunnel between preconditioning and test was not significantly different; therefore tunnel times did not affect the current findings.

We did not investigate the possible differential role of Wnt signalling in the core and shell subregions of the NAc in CPP. Our cannulae were generally placed at the border of the core and shell sub-regions along the rostral-caudal axis of the NAc and injection may have affected both. A number of studies have shown that the core and shell subregions may differentially contribute to reward-related learning [38], but there has been no consensus on the relative contribution of each. Further research is required to investigate the role of NAc subregions in reward-related learning.

The finding that NAc co-administration of IWP-2 with AMPH during conditioning or prior to the test session dose-dependently impaired acquisition or expression of CPP is consistent with the hypothesis that Wnt inhibition will disrupt incentive learning. This blocking effect of IWP-2 given during acquisition cannot be attributed to additive effects of a possible aversion because intra-NAc IWP-2 alone did not have a significant effect on place preference. Acquisition but not expression of CPP was blocked at the IWP-2 dose of 0.001 μg/0.5 μl/side; expression was disrupted at the higher dose of 0.05 μg/0.5 μl/side. Perhaps Wnt signalling plays a critical role in molecular mechanisms of incentive learning during acquisition but once learning is established it is less reliant on Wnt signalling for its expression.

Microinjections of AMPH into the NAc increased locomotor activity in rats, which is consistent with previous studies [39–42]. IWP-2 did not block this effect revealing dissociation between the effects of Wnt inhibition on CPP and locomotor activity. This suggests that the two behaviours may be mediated by different neural mechanisms. Groups given NAc microinjections of AMPH during conditioning showed increased locomotor activity, i.e., sensitization, over days. Sensitization may reflect the additive effects of incentive learning about cues from the drug-paired side with the unconditioned effect of AMPH [43]. The underlying neural substrates of this phenomenon remain to be understood [44,45].

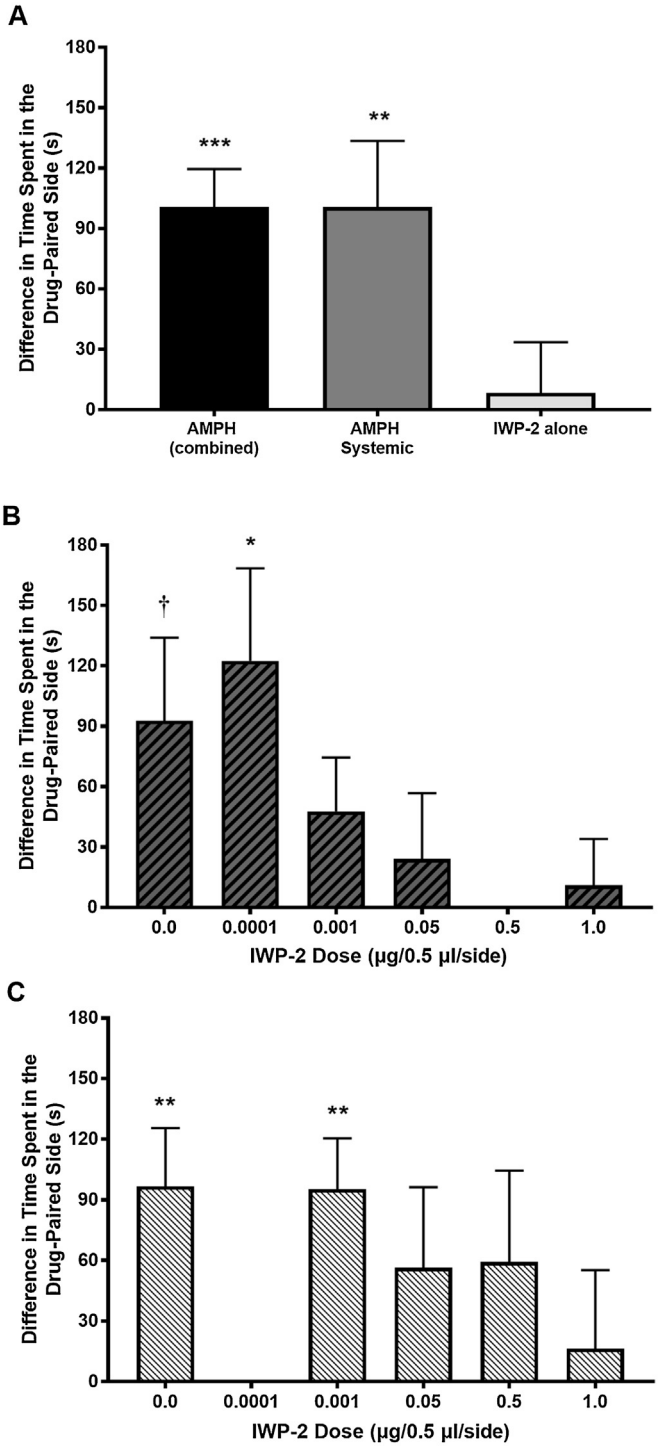

Fig. 2. Mean (±SEM) difference in time (s) spent in drug-paired side during preconditioning (averaged over three sessions) and test session. (A) Amphetamine (AMPH) (combined) is an omnibus group that includes the three AMPH control groups (AMPH Systemic, 0.0 μg/0.5 μl/side IWP-2 groups from B and C): AMPH systemic received IP injections of AMPH (1.5 mg/kg) and the 0.0 μg/0.5 μl/side IWP-2 control groups received microinjections of AMPH (20.0 μg/0.5 μl/side). The inhibitor of Wnt Production-2 (IWP-2) alone group did not receive AMPH. The numbers on the x-axis of (B) and (C) are the doses of IWP-2, in μg/0.5 μl/side. All acquisition (ACQ) groups (B) were conditioned with IWP-2 administered 30 min prior to microinfusion of AMPH on drug days. All expression (EXP) groups (C) were conditioned with AMPH and administered IWP-2 only on test session.
Asterisks above the bar represent a significant difference on test session from preconditioning based on simple effects analyses of each group following observation of a significant group × phase interaction in the analyses of variance of all groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$); † $p = 0.051$.

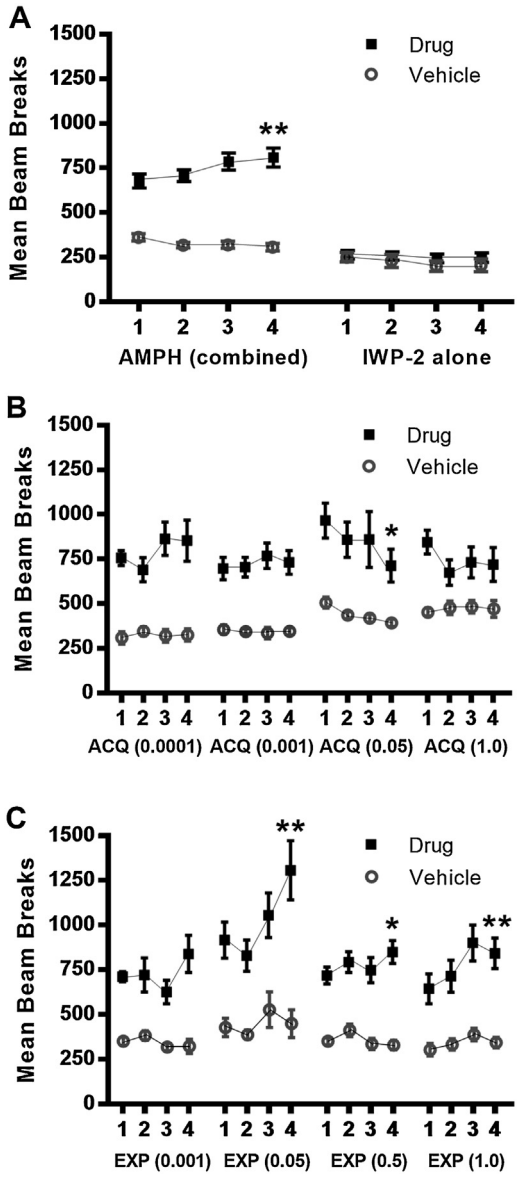

Fig. 3. Mean locomotor activity measured in beam breaks over 30-min conditioning sessions on the four drug and vehicle days for all treatment groups. Control experiments are shown in (A) where amphetamine (AMPH) (combined) is an omnibus group that includes the three AMPH control groups: AMPH systemic that received IP injections of AMPH (1.5 mg/kg) and ACQ (0.0) and EXP (0.0) that received microinjections of AMPH (20.0 μg/0.5 μl/side) on drug days. The Inhibitor of Wnt Production-2 (IWP-2) alone group did not receive AMPH, but received IWP-2 (1.0 μg/0.5 μl/side) on drug days. All acquisition (ACQ) groups shown in (B) were conditioned with IWP-2 administered 30 min prior to AMPH on drug days and were administrated DMSO vehicle followed by saline on vehicle days. The numbers beside ACQ on the x-axis are the doses of IWP-2, in μg/0.5 μl/side. All expression (EXP) groups (C) were conditioned with AMPH on drug days and saline on vehicle days, and on test session, they were microinjected with IWP-2. The numbers beside EXP in the legend are the doses of IWP-2, in μg/0.5 μl/side.
Asterisks above the bar represents a significant difference in locomotor activity between days 1 vs 4 of drug or vehicle based on simple effects analyses of each group following observation of a significant group × phase interaction in the analyses of variance of all groups (*$p < 0.05$, **$p < 0.01$).

Present data show that Wnt signalling is required for the development of locomotor sensitization and compliment the CPP findings implicating Wnt in incentive learning.

Inhibitor of Wnt (IWP) compounds are potent small molecule antagonists that target discrete regulatory steps in the Wnt pathway [46]. IWP-2 targets Porc, a member of the membrane-bound *O*-acyltransferase (MBOAT) family that carries out the

post-translational palmitoylation of Wnt proteins in the endoplasmic reticulum (ER) [46]. Palmitoylation permits Wnt secretion and binding to its receptor, Frizzled (Fzd), leading to intracellular signalling and activation of Wnt target genes [47]. IWP-2 seems to decrease palmitoylation of select Wnts and blocks several Wnt-dependent processes in mouse L-cells, including the phosphorylation of the liporelated-peptide (LRP) 5/6 receptor, phosphorylation of an isoform of dishevelled (Dvl) and the accumulation of β-catenin [46]. To exert its effects, IWP-2 seems to either target the Porc active site or regulates Porc without inducing its destruction or mislocalization to the ER [46]. Although the general consensus is that Porc is required for active Wnt signalling, data from one study show that the knockdown of Porc did not alter Wnt levels nor did IWP-2 treatment inhibit the production of Wnts in a human astrocytic cell line and primary human CD8+ T cells [48]. These opposing findings on the role of Porc and the effect of IWP-2 on Wnt production demonstrate that much work remains to be done in understanding components of the Wnt signalling pathway and identifying reliable pharmacological inhibitors of Wnt.

Wnt signalling is involved in synaptic plasticity and neurogenesis that provide substrates for learning and memory [31,49]. Wnt suppression impairs LTP and Wnt activation facilitates LTP [50–52]. Induction of LTP in hippocampal slices reveals changes in mRNA levels of several Wnt proteins, notably Wnt3a, and the activation of multiple Wnt signalling molecules including β-catenin, Fzd-4, and Dvl-3, and Wnt target genes [51]. Wnt is involved in activity-dependent synaptic remodelling and dendritic arborisation [53]. Wnt and its signalling molecules are present in brain areas of adult neurogenesis such as the subgranular zone of hippocampal dentate gyrus and the subventricular zone [26,49], where Wnt signalling may play a role in the proliferation of neural stem cells and their differentiation into neurons [54,55].

In behaving animals, Wnt signalling was implicated in amygdala-dependent long-term fear memory [25], and in spatial long-term memory of a water maze task involving granule cells of the dentate gyrus [27,56]. Based on these and other results, Wnt signalling is implicated in learning and memory that relies on the amygdala or hippocampus; our findings add the NAc, a ventral striatal region to this list.

Dopaminergic projections to the striatum mediate reward-related learning via D1 receptors of the direct pathway that appear to play a role in the enhancement of corticostriatal synaptic connections and D2 receptors of the indirect pathway [57]. In the developing brain, certain members of the Wnt family regulate distinct aspects of the neurogenesis and development of dopaminergic neurons of the ventral midbrain [58]. Wnt-1, Wnt-3a and Wnt-5a appear to be differentially involved in promoting the proliferation and differentiation of DA progenitors in the ventral midbrain during embryonic development [59–61]. In cell cultures, D2 receptors inhibit Wnt signalling via a direct influence on β-catenin [32]. In in vivo studies, pharmacologic inhibition of D2 receptors increases the Wnt signalling component β-catenin by phosphorylating and thereby inactivating GSK3β [34], and interestingly, in vivo pharmacologic *enhancement* of dopaminergic neurotransmission using AMPH also increases protein levels of β-catenin and phosphorylated GSK3β [33]. These results suggest functional interactions between Wnt signalling and dopamine signalling but further work is needed. In a recent study we showed that selective inhibition of GSK3β dose-dependently blocks the acquisition and expression of AMPH-induced CPP [62] and we now show that inhibition of Wnt produces similar effects. Perturbations in Wnt and its signalling components have been linked to several DA-related neuropsychiatric disorders, such as Parkinson's disease and schizophrenia [63].

In conclusion, we may have uncovered a role for Wnt signalling in DA-mediated reward-related learning. The acquisition and expression of AMPH-induced CPP, a laboratory model of incentive learning, is dose-dependently impaired following intra-NAc microinjections of the Wnt inhibitor, IWP-2. It appears that there is dissociation in the neural mechanisms underlying acquisition and expression of reward-related learning because inhibiting Wnt impairs acquisition at lower doses than expression. Wnt inhibition also blocks the development of AMPH sensitization. These results support existing data that suggest functional interactions between Wnt signalling and the dopamine system. However, there is still much to be specified about this interaction. More detailed studies are needed in order to elucidate the signalling pathways through which Wnt signalling influences DA-mediated reward-related learning.

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