INHIBITION OF WNT SIGNALLING DOSE-DEPENDENTLY IMPAIRS THE
ACQUISITION AND EXPRESSION OF AMPHETAMINE-INDUCED
CONDITIONED PLACE PREFERENCE IN RATS

By

Farhana Islam

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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. I hypothesized that inhibition of Wnt with intra-NAc microinjections of the 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 than for its expression. Mechanisms by which Wnt and DA signalling pathways interact to influence DA-mediated reward-related learning remain to be elucidated.
Co-Authorship

Kathleen Xu conducted the surgeries, conditioned place preference behavioural testing, and histology for experimental groups: ACQ (0.001), ACQ (0.05), and ACQ (1.0).

Richard Beninger is the grant holder and supervisor of the research project.
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<tbody>
<tr>
<td>AC</td>
<td>Adenylyl Cyclase</td>
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<td>ACQ</td>
<td>Acquisition</td>
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<td>AMPH</td>
<td>Amphetamine</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
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<td>CaMK</td>
<td>Calmodulin-Dependent Protein Kinase</td>
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<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
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<td>CPP</td>
<td>Conditioned Place Preference</td>
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<td>CREB</td>
<td>cAMP Response Element-Binding Protein</td>
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<td>DA</td>
<td>Dopamine</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>Dvl</td>
<td>Dishevelled</td>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>EXP</td>
<td>Expression</td>
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<tr>
<td>Fzd</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Amino Butyric Acid</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein Coupled Receptors</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen Synthase Kinase-3β</td>
</tr>
<tr>
<td>IWP-2</td>
<td>Inhibitor of Wnt Production-2</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphoid Enhancer-Binding Factor 1</td>
</tr>
<tr>
<td>LRP</td>
<td>Low Density Lipoprotein Receptor-Related Protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>LTD</td>
<td>Long-Term Depression</td>
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<tr>
<td>LTP</td>
<td>Long-Term Potentiation</td>
</tr>
<tr>
<td>MBOAT</td>
<td>Membrane Bound O-Acyl Transferase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MSN</td>
<td>Medium Spiny Neuron</td>
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<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>Porc</td>
<td>Porcupine</td>
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<tr>
<td>TCF</td>
<td>T Cell-Specific Transcription Factor</td>
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<td>VTA</td>
<td>Ventral Tegmental Area</td>
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Chapter 1

General Introduction

Dopaminergic neurotransmission in the mesolimbic pathway of the brain plays a critical role in reward-related learning. However, the neural mechanisms involved in the consolidation of this type of learning remain to be fully understood. Wnt signalling has emerged recently as a crucial player in synaptic plasticity and learning and memory processes of the brain. Recent studies have indicated functional interactions between dopamine (DA) and Wnt signalling at the cellular level, but the possible role of Wnt in DA-mediated learning has not yet been explored. The present thesis assesses the role of Wnt signalling in the consolidation and expression of reward-related learning. The thesis will begin with an overview of reward-related learning and its study within the laboratory using the conditioned place preference (CPP) paradigm. Next, the role of dopamine and its receptor subtypes in reward-related learning will be introduced. Subsequently, Wnt signalling will be discussed, with particular emphasis on its involvement in synaptic plasticity processes and its ties to dopamine signalling. Finally, the rationale for the investigation of Wnt signalling in incentive learning will be established, including the hypothesis for this research.

1.1 Reward-Related Incentive Learning

Reward-related incentive learning is a natural phenomenon that involves the acquisition by previously neutral stimuli of the ability to elicit approach responses as a result of repeated pairing with a rewarding stimulus [1]. The rewarding stimulus may be a
primary reinforcer, such as food, drink, or other resources for which the organism has a
biological need [2]. The ability of organisms to learn to approach predictive cues
signalling biologically important resources is essential for the survival and continuation of
the species [2,3]. Therefore, the brain has evolved a reward system that mediates this
learning.

One of the earliest mentions of a brain “reward system” was by Olds and Milner
[4] who discovered that following implantation of electrodes in the septal and surrounding
regions, animals frequently self-stimulated themselves in these places for long periods of
time by pressing a lever in an operant chamber. However, self-stimulation was not
observed when electrodes were implanted in brain areas associated with sensory and
motor function [5]. These observations seem to suggest that reward-related learning
emerges from the stimulation of particular brain regions, and animals show strong
approach to reward-related stimuli as demonstrated by fast and persistent lever-pressing
for self-stimulation to the point of physical exhaustion [6]. Subsequent drug and electrical
self-stimulation studies have shown that dopaminergic transmission in the mesolimbic
dopamine structures, including the ventral tegmental area (VTA), nucleus accumbens
(NAc), and olfactory tubercle, is particularly important in reward [7]. Presently, it is
becoming more apparent that the ability of the mesolimbic dopamine system to mediate
reward-related learning arises from its interaction with other brain structures such as the
medial prefrontal cortex, ventral pallidum, lateral hypothalamic areas, lateral habenula,
periaqueductal gray, laterodorsal tegmental nucleus and other regions [7].

Pharmacological agents can activate the circuits of the brain that are activated by
natural rewards [2,3]. Psychostimulants, such as cocaine and amphetamine (AMPH),
display potent rewarding properties [3]. Organisms rapidly learn to approach cues in the environment that predict the availability of these psychostimulants, and once learned, these cues motivate drug seeking in human and animal models [3]. Drugs that activate brain reward systems can become addictive. These drugs can “hijack” the natural reward system and become overvalued at the cost of other more biologically beneficial rewards. Animal models using “addictive drugs” are useful for learning the neural mechanism underlying incentive learning and identifying pharmacological targets that could help treat addiction.

1.2 Conditioned Place Preference and Incentive Learning

CPP is often used as a laboratory model to study incentive learning [8]. The CPP apparatus involves two contextually distinct chambers connected by a tunnel. The paradigm involves the pairing of the motivational properties of a drug, which serves as the unconditioned stimulus, with the initially neutral environmental stimuli of one chamber of the apparatus, which becomes the conditioned stimulus [8]. When the animal is exposed to the chamber paired with the drug after conditioning, these stimuli elicits approach responses [8]. A “CPP effect” is observed when the animal prefers to stay longer on the drug-paired chamber after conditioning compared to prior to conditioning. If the drug has no incentive motivational properties, the animal will not demonstrate a preference for the drug-paired compartment of the apparatus. CPP models addictive behaviour in humans in that cues and contexts that predict the availability of addictive drugs are learned, and these cues can then motivate drug seeking [3].
This paradigm can be pharmacologically manipulated in many ways to study various aspects of incentive learning and infer about its neural mechanisms. An experiment that combines a drug with known CPP effects and a drug with unknown CPP effects can reveal the properties of the second drug, and if its mechanism of action is known, the role of specific molecular targets and processes involved in incentive learning can be inferred. In one CPP study, SB 216763, an inhibitor of glycogen synthase kinase-3β (GSK3β), was administered alongside AMPH [9]. When AMPH is administered alone, it produces a CPP, however AMPH in combination with SB 216763 failed to produce a CPP in a dose-dependent manner [9]. These results demonstrate that inhibiting the activation of GSK3β impaired the acquisition and expression of CPP induced by AMPH thereby implicating GSK3β and its associated pathways as important molecular substrates in mediating CPP and incentive learning. In this way, pharmacological manipulations in CPP studies can reveal much about intracellular signalling involved in reward-related learning.

1.3 Nucleus Accumbens Dopamine in Reward-Related Learning

Dopamine is a catecholamine neurotransmitter that is produced in various regions of the brain, including the substantia nigra [10] and the VTA [11]. Dopaminergic projections from the substantia nigra, located at the base of the mesencephalon, to the dorsal striatum forms the nigrostriatal pathway [12]. Dopaminergic projections from the VTA, situated in the ventral portion of the midbrain, to the NAc in the ventral striatum form the mesolimbic dopamine pathway, which will be the focus in this thesis (Figure 1). This pathway is particularly important in reward assessment and has been studied extensively [12]. Investigations using a variety of different methods have converged on
the conclusion that DA release on the NAc by axonal projections from the VTA drives the strengthening of corticostriatal connections that mediate incentive learning produced by natural rewards and addictive drugs [13]. Mesolimbic dopaminergic projections from the VTA also innervates the amygdala, hippocampus, bed nucleus of stria terminalis, lateral hypothalamus, and the prefrontal cortex, which may also be important for reward-related learning and memory [12].

Dopaminergic neurotransmission has been shown to mediate reward-related incentive learning. Pharmacological agents that increase the actions of DA at its receptors, such as AMPH [14] and cocaine [15], or act as DA receptor agonists, such as SKF 82958 [16] and quinpirole [17], have been shown to produce CPP. In contrast, pharmacological agents that are DA receptor antagonists, such as SCH 23390 [18,19] and haloperidol [20], inhibit or attenuate AMPH or cocaine-induced CPP. Dopamine transmission within the NAc is a crucial component in reward-related learning (reviewed in [21]). Biologically important rewards, such as food, increase DA in the NAc and so do addictive drugs [22,23]. Central microinjections of psychostimulants, such as AMPH or cocaine, into the VTA or the NAc produces CPP, whereas central microinjections of the same psychostimulants into other areas such as the prefrontal cortex, caudate, or amygdala, fail to produce CPP [24]. Rats that developed a cocaine-induced CPP following conditioning showed increased NAc DA in response to the cocaine-paired environment after vehicle injection as opposed to the vehicle-paired compartment [25,26]. Taken together, these findings strongly implicate NAc DA in CPP.
1.4 Dopamine-Mediated Intracellular Signalling in Reward-Related Learning

Five DA receptor subtypes have been identified and classified into two separate families: the D$_1$-like receptor family consisting of D$_1$ and D$_5$ receptors and the D$_2$-like receptor family consisting of D$_2$, D$_3$, and D$_4$ receptors [27]. D$_1$-like receptors are found primarily post-synaptically in the neocortex and the hippocampus, however there are also D$_1$ receptors in the caudate, NAc and olfactory tubercule [28,29]. Upon binding of DA to D$_1$-like receptors, there is an increase in cyclic adenosine monophosphate (cAMP) by G$_{\alpha_{s/olf}}$-coupled activation of adenylyl cyclase (AC) and an increase in the activity of protein kinase A (PKA) [30]. D$_2$-like receptors, on the other hand, can be found both pre- and post-synaptically in the caudate, putamen and NAc with high levels of D$_2$ receptors in the substantia nigra and VTA [28]. The D$_2$-like receptors decrease cAMP by G$_{\alpha_i/o}$-coupled inhibition of AC, decrease the activity of PKA and inhibit calcium channels and open potassium channels [27]. Thus, D$_1$-like receptors seem to activate cAMP and PKA, whereas D$_2$-like receptors seem to negatively regulate their concentrations in the cell.

In the circuitry of the basal ganglia, the GABA-containing medium spiny neurons (MSNs) of the striatum can be differentiated into two subpopulations, striatonigral neurons of the direct pathway and striatopallidal neurons of the indirect pathway [31,32]. The striatonigral neurons of the direct pathway predominately express D$_1$ receptors and the substance P neuropeptide in contrast to striatopallidal neurons of the indirect pathway that selectively express D$_2$ receptors and the enkephalin neuropeptide [33]. The NAc neurons of the direct pathway innervate the substantia nigra (from the NAc core), the VTA (from the NAc shell) and also the ventral pallidum, whereas the NAc neurons of the indirect pathway project only to the ventral pallidum [33]. These pathways seem to play
distinct roles in reward-related learning with several CPP studies showing that the inhibition of the direct pathway in the NAc, but not that of the indirect pathway, significantly reduced the ability to elicit CPP [33]. However, the specific roles of the two populations of striatal MSNs in reward learning are not well understood.

The current prevailing hypothesis is that rewarding stimuli elicit bursts of phasic firing in MSNs increasing striatal DA concentrations, which activates D_{1} receptors in the direct pathway neurons and the D_{2} receptors in the indirect pathway neurons [33]. Activation of direct pathway MSNs or the indirect pathway MSNs yields opposing effects on movement and reward-related learning [34]. Persistent stimulation induces long-term potentiation (LTP) and long-term depression (LTD) of glutamatergic transmission at the direct pathway and the indirect pathway MSNs, respectively [33]. The direct and indirect pathways work in concert with one another to grant learning flexibility to acquire reward under changing environmental conditions [33].

1.5 Wnt Signalling

Wnt signalling has been characterized and studied extensively in the fields of developmental and oncology research. Recently, it has emerged as a crucial player in learning and memory and neuroplasticity processes. Wnts are secreted glycolipoproteins that bind the cysteine-rich domain of its seven transmembrane receptor Frizzled (Fzd) complexed with the low density lipoprotein receptor-related protein 5/6 (LRP5/6) at the cell surface [35,36]. Wnt proteins can be classified into two functional groups: Class I Wnts act through the canonical Wnt pathway that activates target genes in the nucleus via the activation of its transcriptional regulator β-catenin and Class II Wnts act through less
characterized noncanonical pathways independent of β-catenin [35,36]. This thesis will focus on the canonical Wnt pathway, which is the more predominant and well-studied pathway.

Upon activation of its receptors by Wnt, a signal is transduced to several intracellular proteins including Dishevelled (Dvl), GSK3β, Axin, Adenomatous Polyposis Coli (APC), and β-catenin (Figure 2). A GSK3β/APC/Axin containing complex in the cytoplasm is regulated by Wnt binding to its receptors, which modulates levels of β-catenin and thus activation of Wnt target genes. A number of Wnt target genes have been identified including developmental regulatory genes, regulators of cell proliferation, growth and homeostasis, and regulators of cell-cell communication. However, there is much about the functions, specificity and biochemical aspects of Wnt signalling that still remain unclear.

1.6 Wnt and Learning and Memory Processes

Synaptic plasticity, which involves changes in synaptic strengthening in response to neural activity, is suggested to mediate learning and memory formation [37]. One of several processes that are involved in synaptic plasticity is LTP, which is described as long-lasting, activity-dependent strengthening of neuronal synaptic connection [37]. The induction of LTP in hippocampal slices has been shown to lead to an increase in messenger RNA (mRNA) of multiple Wnt signalling components, such as Wnt3a, Fzd4, β-catenin and Dvl3 and in the activation of several Wnt target genes [38]. Furthermore, the activation of Wnt signalling can facilitate LTP and the inhibition of Wnt signalling
can suppress LTP, suggesting a critical role for Wnt in activity-dependent synaptic plasticity in the hippocampus [38].

The role of Wnt signalling in synaptic plasticity processes is not completely understood. It has been suggested that Wnt is involved in neuronal synapse formation and dendritic arborisation and remodelling. Dendrite development requires Ca\(^{2+}\) signalling through calmodulin-dependent protein kinases (CaMKs) activated by neuronal activity [39]. The CaMKI cascade includes the activation of transcription factor cAMP response element-binding protein (CREB), which is involved in the activation of select Wnt genes, particularly Wnt-2 [39]. Wnt-2 appears to be the final effector of the activity-dependent CaMK signalling cascade playing an active role in dendritic growth and arborisation [39].

In contrast, blocking Wnt interaction with Fzd using a Wnt inhibitor suppresses activity-dependent dendritic arborisation in hippocampal slices [40]. Wnt signalling also seems to be closely involved in adult neurogenesis in the subgranular zone of the hippocampal dentate gyrus and the subventricular zone of the lateral ventricles [41]. Expression of a dominant negative Wnt in the dentate gyrus reduced neurogenesis in the hippocampus, whereas retrovirus-mediated expression of a stabilized β-catenin enhanced the proliferation of progenitor cells and increased the number of new neurons in the subventricular zone [41].

Very few in vivo studies exist on the role of Wnt signalling in various learning and memory systems. One in vivo study showed that the microinjection of Wnt antagonist, Dickkopf-1, into the amygdala prevented long-term fear memory consolidation in adult mice without altering baseline locomotion or anxiety-like behaviours [42]. In the dentate gyrus, specific inhibition of Wnt signalling using a lentivirus expressing dominant-
negative Wnt produced impairments in the long-term retention of spatial memory in the water maze as well as impairments in a hippocampus-dependent object recognition task [43]. In contrast, certain isoforms of Wnt seem to be elevated in the granule cells of the dentate gyrus following spatial learning in a water maze [44]. The role of Wnt signalling in striatal dependent learning and memory has not been explored.

1.7 Wnt and Dopamine

Data on the possible interaction of Wnt and DA signalling are presently lacking. During embryonic development, certain members of the Wnt family, such as β-catenin, appear to regulate the development of DA neurons by stimulating the proliferation of DA precursors and the differentiation of DA precursors into DA neurons in the ventral mesencephalon [45–47]. β-catenin is also able to bind and activate Nurr1, which is a transcription factor that plays a critical role in development and maintenance of DA neurons [48]. In vivo studies have demonstrated an increase in the cellular levels of canonical Wnt signalling components, Dvl, GSK3β and β-catenin in response to DA D2 receptor inhibition by certain antipsychotics [49,50]. Furthermore, we have previously demonstrated that in vivo pharmacologic enhancement of dopaminergic neurotransmission using AMPH appears to also increase protein levels of β-catenin and phosphorylated GSK3β [51]. These studies taken together seem to suggest functional interactions between Wnt and DA signalling at the cellular and molecular level. However, the role of Wnt in DA-mediated learning and memory has not yet been explored.
1.8 Pharmacological Profiles of IWP-2 and AMPH

In their seminal work, Chen et al. identified nine potent small molecule antagonists of Wnt that target discrete regulatory steps in the Wnt pathway, referred to as inhibitors of Wnt production (IWP) compounds, after screening ~200,000 compounds [52]. IWP compounds share the same core chemical structure and were shown to prevent Wnt production and thus disrupt Wnt pathway responses [52]. IWP-2 antagonizes the Wnt pathway by selectively targeting Porcupine (Porc), a membrane-bound O-acyl transferase (MBOAT) superfamily that has been shown to be required for the palmitoylation and secretion of Wnts [53]. Porc adds a monounsaturated fatty acid, palmitoleic acid, to Wnt proteins at serine at position 209 [53]. This residue is highly conserved among Wnts with studies showing that serine-209 mutation to an alanine causes the retention of the protein in the endoplasmic reticulum (ER) preventing its secretion [54] or produces less active Wnt proteins [55]. An inhibition of Porc function by IWP-2 causes a decrease in lipidated versions of certain canonical and noncanonical Wnts and also causes an inhibition of several Wnt-dependent processes including, phosphorylation of LRP5/6, phosphorylation of Dvl2, and accumulation of β-catenin in mouse-L cells [52]. Furthermore, the block of endogenous Wnt signalling with this drug in culture can be reversed by providing exogenous Wnt proteins [56] or over-expressing Porc [52]. The mechanism of action of IWP-2 is unclear but it does not seem to alter the localization of Porc to the ER or activate its destruction, which suggests that IWP-2 may prevent Wnt production by either inhibiting the Porc active site or regulating its activity [52].

Amphetamine (1-methyl-2-phenethylamine, AMPH) is a psychostimulant that elevates extracellular DA levels by three major mechanisms [57]. First, because AMPH is
highly lipophilic, it is able to enter the CNS readily, and it can also diffuse into nerve terminals through the plasma membrane [58]. Once inside the cell, it facilitates the movement of DA out of vesicles and into the cytoplasm [58]. Second, AMPH is structurally similar to DA, so it is able to bind to the DA transporter (DAT) and competitively inhibit DA uptake [57]. Finally, AMPH can facilitate reverse-transport of DA through DAT into the synaptic cleft independent of action-potential-mediated vesicular release [57]. This AMPH-induced elevation of extracellular DA can eventually result in the saturation of DA receptors and depletion of intracellular DA stores [57]. The D-isomer of AMPH is much more potent than the L-isomer with the half-lives in the whole brain being 1.2±0.1 and 1.3±0.1 h, respectively [59].

1.9 Hypothesis

Recent research implicates Wnt signalling in learning and memory processes and also seems to indicate a possible functional interaction between Wnt and DA signalling. Extensive literature suggests that DA mediates the expression and acquisition of reward-related incentive learning. Thus, the purpose of the present thesis was to investigate whether the Wnt signalling pathway is involved in incentive learning. This was accomplished by studying effects of the inhibition of Wnt signalling by bilateral intra-NAc microinjection of Wnt inhibitor, IWP-2, on AMPH-induced CPP in rats. The CPP paradigm was chosen because it is a reliable measure of incentive learning in the laboratory. Microinjection of AMPH into the NAc has been shown to produce reliable CPP [8,24]. It was hypothesized that the inhibition of Wnt signalling by IWP-2 in the NAc will dose-dependently block the acquisition and expression of AMPH-induced CPP,
and further, Wnt inhibition will block sensitization to AMPH. The acquisition and expression of CPP appear to be mediated by different mechanisms because synaptic changes during the acquisition of learning are much more vulnerable to disruptions than after learning is established [60]. This led to the further hypothesis that the inhibition of Wnt signalling will have differential effects on the acquisition versus the expression of CPP, with acquisition predicted to be blocked at a lower dose of IWP-2 than expression.
Figure 1. Schematic diagram of the major glutamatergic, GABAergic, and dopaminergic pathways to and from the ventral tegmental area (VTA) and the nucleus accumbens (NAc) in the rat brain. The mesolimbic dopamine reward circuit includes dopaminergic projections from the VTA to the NAc. Dopamine is released onto the NAc in response to rewarding stimuli. The NAc is primarily populated by medium spiny neurons and receives glutamatergic afferents from the medial prefrontal cortex (mPFC), hippocampus (Hipp) and amygdala (Amy), as well as other regions. GABAergic efferents of the NAc project onto the VTA. The VTA also receives afferents from the lateral dorsal tegmentum (LDTg), lateral habenula (LHb) and lateral hypothalamus (LH). These other projections onto the VTA and NAc control aspects of reward-related perception and memory [61–63]. Image by Russo & Nessler [62]. Copyright © 2013 Nature Publishing Group. Written permission obtained from publisher to reproduce image in thesis.
Figure 2. Schematic diagram of the canonical Wnt signalling pathway. Under conditions of absent or low Wnt, a GSK3β/APC/Axin containing complex keeps β-catenin levels in the cytoplasm low through continuous proteasomal degradation (left panel) [35]. With the binding of Wnt, the degradation complex is dissociated, and consequently β-catenin accumulates in the cytoplasm and is able to translocate to the nucleus where it interacts with transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) to affect transcription of Wnt target genes (right panel) [35].

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Chapter 2

Manuscript

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

Farhana Islam\textsuperscript{a}, Kathleen Xu\textsuperscript{b}, Richard J. Beninger\textsuperscript{a,b}

\textsuperscript{a}Centre for Neuroscience Studies, Queen’s University, Kingston ON, Canada

\textsuperscript{b}Department of Psychology, Queen’s University, Kingston ON, Canada

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2.1 Introduction

Rewarding stimuli activate dopamine (DA) neurons [64] 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 [1,65]. 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 [8]. This phenomenon is mediated by dopaminergic neurons projecting from the ventral tegmental area of the midbrain to forebrain targets, particularly the nucleus accumbens (NAc) [66,67]. For example, pairing of one chamber with systemic or intra-NAc microinjections of a psychostimulant drug, such as amphetamine (AMPH), enhances dopaminergic neurotransmission and produces CPP [8,14,68]. Similar to other types of learning, incentive learning involves activity-dependent changes in synaptic function and connectivity mediated by specific intracellular events [65,69]. 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 [70].

Wnts are a highly conserved family of secreted glycolipoproteins that has been studied extensively outside the nervous system in early developmental processes [71–73], cell proliferation [74], growth and homeostasis[35,75], and various types of cancer [76–81]. 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 [82,83]. A small but growing body of data has implicated the canonical Wnt
pathway in synaptic plasticity and learning and memory in the adult brain [41,42,44,84]; this pathway mediates the transcription of Wnt-target genes by regulating the activation of glycogen synthase kinase 3β (GSK3β) and β-catenin [85,86]. Suppression of endogenous Wnt activity leads to impaired long-term potentiation (LTP) and deficits in memory [41,87]. Aberrancies in Wnt signalling have also been associated with a variety of neuropsychiatric disorders including Alzheimer’s disease, schizophrenia, autism and mood disorders [41,87].

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 D₂ receptors [50] and are rapidly activated by the administration of AMPH into the rat NAc [51]. DA D₂ receptors increase key components of Wnt signalling cascades including GSK3β and β-catenin [49]. These results suggest functional interactions between the dopaminergic and Wnt signalling pathways at the cellular 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 [88]. 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.

2.2 Methods

2.2.1 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-hour 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.
2.2.2 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 anaesthetised 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 [89]. 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.

2.2.3 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/0.5µl/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/0.5µl/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/0.5µl/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 0.5µl/hemisphere.

2.2.4 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.

2.2.5 Apparatus

The four CPP apparatus consisted of a rectangular Plexiglas-covered wooden box with two contextually distinct compartments (38 x 27 x 36 cm) connected by a tunnel (8 x 8 x 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 [90].

2.2.6 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).

2.2.6a 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 mins prior to saline (0.5 µl/side) apart
on vehicle days of conditioning. None of the control groups received any injections on test.

2.2.6b 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.

2.2.6c 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.

2.2.7 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.

2.2.8 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 \( \geq 700 \) s and/or \( \leq 200 \) 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 $p < 0.05$.

2.3 Results

2.3.1 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. 3). 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. 3 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 (ACQ (0.0), EXP (0.0), 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) and 97.4 s (±19.3), respectively. 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. A dependent t-test 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). Results reveal that either rostral or caudal placements in the NAc lead to a CPP with AMPH.

2.3.2 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 = 0.04). 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. 4A). A two-way mixed-design ANOVA comparing the three AMPH control groups: systemic (1.5 mg/kg IP), ACQ (0.0) (20.0 µg/0.5 µl/side), and EXP (0.0) (20.0 µg/0.5 µl/side) yielded a significant main effect of phase (F(1, 36) = 26.93, p < 0.001) indicating a greater amount of time spent in the drug-paired side during test; there was no significant effect of group (F(2, 36) = 0.46, n.s.) or interaction of phase x group (F(2, 36) = 0.10, n.s.). 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. 4A).

Acquisition groups (Fig. 4B) given intra-NAc microinjection of IWP-2 prior to AMPH during conditioning revealed a dose-dependent CPP, animals given 0.0001µg/0.5 µl/side demonstrating the greatest increase. Expression groups (Fig. 4C) conditioned with AMPH and given IWP-2 on test also produced a dose-dependent CPP with the 0.001µg/0.5 µl/side group showing the largest increase. The IWP-2 alone group (Fig. 4A) did not show a significant change in time spent in the drug-paired side. These observations were supported by statistical analysis. The two-way mixed-design ANOVA including AMPH (combined), all of the ACQ, EXP and the IWP-2 alone group revealed a main effect of phase (F(1, 121) = 22.10, P < 0.001) and a significant phase x group 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., 0.0001 µg/0.5 µl/side, than expression.

2.3.3 Locomotor activity

Locomotor activity during the conditioning phase was measured by beam breaks on drug and vehicle days for each group (Fig. 5). Groups that received AMPH (combined) 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 to 4 was observed for the AMPH (combined) and EXP groups. 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. Locomotor activity showed little difference between vehicle day 1 vs. 4 in any of the groups.

Statistical analyses support these observations. Locomotor activity on drug day 1 and 4 vs. vehicle day 1 and 4 of the three AMPH control groups: systemic AMPH, ACQ (0.0), and EXP (0.0) was compared using a three-way (group x treatment x day) mixed-design ANOVA, that revealed a significant main effect for treatment (F(1, 36) = 154.67, p < 0.001), group (F(2, 36) = 5.54, p < 0.01), and a significant treatment x day interaction (F(1, 36) = 12.44, p ≤ 0.001) (Table 3). 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.

A three-way mixed-design ANOVA comparing locomotor activity on drug day 1 and 4 vs. vehicle day 1 and 4 for AMPH (combined), the ACQ, EXP and IWP-2 alone groups revealed a main effect of treatment \( (F(1, 121) = 411.93, P < 0.001) \), group \( (F(9, 121) = 8.16, P < 0.001) \), and significant interactions of treatment x group \( (F(9, 121) = 6.59, P < 0.001) \), day x group \( (F(9, 121) = 4.34, P < 0.001) \), treatment x day \( (F(1, 121) = 11.75, P \leq 0.001) \), and treatment x day x group \( (F(9, 121) = 3.89, P < 0.001) \). The source of the day x group interaction was isolated for each treatment by tests of simple interactive effects using two-way mixed design ANOVA.

For treatment with drug, a main effect of day \( (F(1, 121) = 8.24, P < 0.01) \), group \( (F(9, 121) = 8.10, P < 0.001) \) and a significant day x group interaction \( (F(9, 121) = 4.79, P < 0.001) \) was observed. Simple effects analyses of 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.

For treatment with vehicle, only a significant group \( (F(9, 121) = 5.41, P < 0.001) \) effect was observed. Pairwise comparisons using Tukey’s post-hoc test revealed that the ACQ (0.05) and ACQ (1.0) groups showed significantly higher activity compared to the other groups.
2.4. 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 to 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 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. 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 [91], 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 [92–94]. 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 [95]. The underlying neural substrates of this phenomenon remain to be understood [96,97]. 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 [52]. 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) [52]. Palmitoylation permits Wnt secretion and binding to its receptor, Frizzled (Fzd), leading to intracellular signalling and activation of Wnt target genes [98]. 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 [52]. 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. 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 [99]. 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 [87,100]. Wnt suppression impairs LTP and Wnt activation facilitates LTP [38,101,102]. 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 [38]. Wnt is involved in activity-dependent synaptic remodelling and dendritic arborisation [39]. 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 [41,100], where Wnt signalling may play a role in the proliferation of neural stem cells and their differentiation into neurons [103,104].

In behaving animals, Wnt signalling was implicated in amygdala-dependent long-term fear memory [42], and in spatial long-term memory of a water maze task involving granule cells of the dentate gyrus [43,44]. 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 [105]. 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 [106]. 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 [45,107,108]. In cell cultures, D₂ receptors inhibit Wnt signalling via a direct influence on β-catenin [50]. In *in vivo* studies, pharmacologic inhibition of D₂ receptors increases the Wnt signalling component β-catenin by phosphorylating and thereby inactivating GSK3β [49], and interestingly, *in vivo* pharmacologic *enhancement* of dopaminergic neurotransmission using AMPH also increases protein levels of β-catenin and phosphorylated GSK3β [51]. 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 [9] 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 [109].

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.
Fig. 3. (A) A representative bilateral NAc injector placement. (B) Reconstructed microinjection sites in the nucleus accumbens (NAc) from all experiments with illustrations adopted from Paxinos [89]. 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.
Table 1
Time (s) spent in the to-be-vehicle- and drug-paired sides during preconditioning

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

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

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/0.5µl/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.
Fig. 4. Mean (±SEM) difference in time (s) spent in drug-paired side during pre-conditioning (averaged over three sessions) and test session. 

(A) Amphetamine (AMPH) (combined) is an omnibus group that includes the three AMPH control groups (gray bars): AMPH systemic received IP injections of AMPH (1.5 mg/kg) and AMPH acquisition (ACQ) control and AMPH expression (EXP) control received microinjections of AMPH (20.0 µg/0.5 µl/side). 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 ACQ groups (B) were conditioned with IWP-2 administered 30 min prior to microinfusion of AMPH on drug days. All EXP groups (C) were conditioned with AMPH and administered IWP-2 only on test session.

Asterisks above the bar represents a significant difference on test session from pre-conditioning based on simple effects analyses of each group following observation of a significant group x phase interaction in the analyses of variance of all groups (*p<0.05, **p<0.01, ***p<0.001).
Fig. 5. Mean locomotor activity measured in beam breaks over 30-minute 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 AMPH acquisition (ACQ) control and AMPH expression (EXP) control 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 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 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 x phase interaction in the analyses of variance of all groups (*p<0.05, **p<0.01).
<table>
<thead>
<tr>
<th>Group (dose)</th>
<th>AMPH Systemic</th>
<th>ACQ (0.0)</th>
<th>EXP (0.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[1.5 mg/kg]</td>
<td>[20.0 µg/0.5 µl/side]</td>
<td>[20.0 µg/0.5 µl/side]</td>
</tr>
<tr>
<td></td>
<td>n=11</td>
<td>n=10</td>
<td>n=18</td>
</tr>
<tr>
<td>AMPH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>577.6 (27.5)</td>
<td>625.0 (43.4)</td>
<td>574.9 (43.9)</td>
</tr>
<tr>
<td>Day 2</td>
<td>726.2 (55.4)</td>
<td>681.5 (46.6)</td>
<td>709.4 (57.0)</td>
</tr>
<tr>
<td>Day 3</td>
<td>660.5 (60.7)</td>
<td>692.3 (38.2)</td>
<td>914.2 (84.8)</td>
</tr>
<tr>
<td>Day 4</td>
<td>673.9 (64.8)</td>
<td>703.2 (65.2)</td>
<td>694.2 (73.3)</td>
</tr>
<tr>
<td>Mean</td>
<td>636.8 (30.7)</td>
<td>669.9 (28.0)</td>
<td>834.9 (62.5)</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>289.1 (35.8)</td>
<td>371.7 (25.6)</td>
<td>355.9 (23.1)</td>
</tr>
<tr>
<td>Day 2</td>
<td>286.9 (19.2)</td>
<td>298.3 (23.5)</td>
<td>348.1 (27.0)</td>
</tr>
<tr>
<td>Day 3</td>
<td>224.5 (29.8)</td>
<td>306.4 (27.6)</td>
<td>385.2 (26.8)</td>
</tr>
<tr>
<td>Day 4</td>
<td>188.4 (31.5)</td>
<td>354.9 (31.4)</td>
<td>330.5 (22.1)</td>
</tr>
<tr>
<td>Mean</td>
<td>240.1 (25.0)</td>
<td>332.8 (21.7)</td>
<td>371.2 (20.7)</td>
</tr>
</tbody>
</table>

Locomotor activity for amphetamine (AMPH) and Vehicle days 1 to 4 is expressed in mean beam breaks over 30 mins 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.
Chapter 3

General Discussion

The results can be summarized as follows: (1) IP and intra-NAc microinjection of AMPH produced a CPP; (2) intra-NAc IWP-2 alone did not produce a CPP; (3) intra-NAc co-administration of AMPH with IWP-2 during conditioning blocked CPP, except at the lowest dose of 0.0001 µg/0.5 µl/side, (4) intra-NAc IWP-2 on test day after conditioning with AMPH blocked CPP, except at the lowest dose of 0.001 µg/0.5 µl/side; (5) all groups showed increased locomotor activity on drug days compared to vehicle days; and (6) AMPH sensitization was observed for AMPH (combined) and all EXP groups, but not for ACQ groups that were pre-treated with IWP-2 during conditioning.

CPP is a reliable behavioural technique with a number of advantages. One major advantage of CPP is that place preference after conditioning is measured in a drug-free state, which eliminates any other effects of the drug besides its rewarding properties that may directly influence the time the animal spends in the previously drug-paired compartment [110]. Another advantage of CPP is that relatively low doses of drug are required thus demonstrating a much greater sensitivity than other behavioural techniques measuring reward-related learning [110]. CPP generally yields a monophasic dose-response curve, which is advantageous because definitive conclusions can be drawn about the direction of change (either increase or decrease) in approach behaviour [111]. Additionally, drugs that produce CPP also show rewarding properties in other behavioural paradigms, thus the CPP task makes consistent predictions on the rewarding properties of drugs [110]. Finally, this behavioural technique allows for the measurement of locomotor
activity as an additional dependent variable to study possible drug-induced locomotor sensitization [111].

The CPP paradigm also has some limitations. One concern with CPP is the extent to which novelty-seeking behaviour influences time spent in the drug-paired chamber on test day. It is possible that the drug administered during conditioning may impair familiarisation to the drug-paired context, thus rendering it more novel than the vehicle-paired context, so on test session, the animal prefers the novel context (the drug-paired chamber) to the familiar context (the vehicle-paired context) [8]. To circumvent this potential issue, the animals can be tested in a CPP apparatus with three distinct compartments, with one compartment paired with drug, one compartment paired with saline and one compartment that the animal has not been previously exposed to [8]. Using this experimental design, if the animal shows a preference for the drug-paired compartment over the novel compartment, it would eliminate novelty-seeking as a confounding factor [8]. Although this set up was not employed, the issue of novelty-seeking behaviour does not seem to be a possible confounding factor in the present study because place preference is not observed for the vehicle-paired side (Appendix Fig. A1). At lower doses of IWP-2, the time spent in the vehicle-paired side decreases significantly on test compared to preconditioning and no significant preference for either the drug-paired side nor the vehicle-paired side following conditioning is observed at higher doses of IWP-2, which posits that IWP-2 is influencing the learning of the pairing of the unconditioned stimulus (rewarding properties of the drug) with the conditioned stimulus (previously neutral environment) and not on novelty-seeking.
Another factor that might influence the outcome of CPP experiments is state-dependent learning, which refers to the fact that the learning of the drug-paired context, which occurs under the influence of the drug, can only be retrieved when the animal is in the same “drugged” state but not a different state [8]. Since animals are conditioned in the drug state and tested in a drug-free state, state-dependency can be a potential confounding factor for the CPP paradigm. To address the issue of state-dependent effects on CPP outcome, animals can be tested under the influence of the drug they were conditioned with, and if they prefer the drug-paired context regardless of “drugged” or drug-free test state, the possibility of state-dependency effects on place preference can be discounted. Although in this study, state-dependent tests were not conducted, the results cannot be attributed to state-dependency effects. This is because AMPH-induced CPP was observed when the animal was in a non-drugged state although conditioning was done under a drugged state for AMPH (combined) and ACQ (0.0001) groups (Figure 4a & 4b). Furthermore, the EXP group given 0.001 µg/0.5 µl/side of IWP-2 on test showed a place preference for the drug-paired context even though IWP-2 was not given during conditioning, yet the same dose blocked CPP when given in ACQ, which posits that the block observed in ACQ is not due to state dependent effects (Figure 4b & 4c).

Finally, the issue of preconditioning side bias can also pose a limitation to the CPP paradigm. A strong preference for one context during preconditioning is concerning because if the drug is paired with the preferred side, a ceiling effect may mitigate CPP, and if the drug is paired with the non-preferred side, it is unclear whether an observed CPP is due to reduced aversion to that context as a result of repeated exposure to it during conditioning or a true place preference. To reduce the effect of baseline preference on the
magnitude of CPP, the apparatus used in conducting the CPP experiments was
constructed to minimize bias in preference for either compartment. An exclusion criterion
was established at the start of the experiment to exclude rats that spent greater than 700 s
or less than 200 s in any one of the two compartments during preconditioning, and
therefore showed a strong side bias. The unbiased design was employed by randomly
assigning compartments of the apparatus as the drug- and vehicle-paired side for each
animal at the beginning of the experiment. Lastly, data was analyzed comparing time
spent in the drug-paired side during preconditioning in comparison to on test day, rather
than comparing time spent on the drug-paired side vs. the vehicle-paired side during test.
This is because the former approach eliminates possible biases due to contextual
differences between the two compartments by comparing the same context. It also directly
measures the effect of the drug on place preference by comparing time spent in the same
context pre- and post-conditioning with the drug. When the data were analyzed using time
spent in drug- vs. vehicle-paired side on test session (Appendix Fig. A2), CPP was
observed for the AMPH (combined) and EXP (0.001) but not ACQ (0.0001) group,
although ACQ (0.0001) showed a significant difference in time spent in the drug-paired
side during preconditioning vs. test. CPP data analyzed in this manner show a much
greater variability between subjects which may mitigate observed CPP and may not have
the same sensitivity as when place preference is measured using difference in time spent
in the drug-paired side preconditioning vs. test (see Results section of the manuscript).

The finding that higher doses of IWP-2 are required to block expression than
acquisition suggest that different neurobiological mechanisms mediate the establishment
and expression of CPP, which is further supported by existing studies [18,112]. The
details of the mechanisms mediating acquisition and expression of incentive learning, however, are not fully understood. It has been suggested that N-methyl-D-aspartate receptor (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors play differential roles in CPP acquisition and expression based on the observation that NMDA receptor blockade abolished acquisition of CPP and AMPA receptor blockade abolished the expression but not the acquisition of CPP \[18,113\]. D_1 and D_2 receptors also display differential roles in CPP acquisition and expression with both D_1 and D_2 receptor antagonists blocking the acquisition of AMPH-induced CPP in a dose-dependent manner, but the effects of the same D_1 and D_2 antagonists administered prior to testing differed with D_1 antagonists blocking expression in the same dose range as they block acquisition, but D_2 antagonists did not block expression in the same dose range which blocked acquisition \[112\]. It is unclear why acquisition of incentive learning displays a greater sensitivity to Wnt inhibition than expression of CPP. It is possible that Wnt is more critical during establishment of learning than during recall, however the finer details of the mechanisms underlying incentive learning and the specific role of Wnt in this framework need to be further elucidated.

Increased locomotor activity in response to AMPH was observed in all groups regardless of whether the group showed or failed to show CPP (Appendix Fig. A3), which suggests that the inhibition of CPP observed in some groups was not due to impaired motor function. These findings further indicate that there exists a dissociation between the effects of IWP-2 on CPP and locomotor activity suggesting that the two behaviours are mediated by different neural mechanisms with IWP-2 mainly exerting its effects on the mechanisms underlying the reward-related learning effects of AMPH. It has
previously been demonstrated that drugs that enhance transmission at DA synapses of the NAc enhance locomotor activity, while drugs that inhibit DA receptors at the NAc reduce locomotor activity [1,114]. Although it is unclear how DA agonists like AMPH are able to produce locomotor stimulant effects, it appears that both D₁ and D₂ receptors seem to be involved with the concomitant stimulation of both receptors in the NAc producing the greatest increase in locomotion [114].

For groups given NAc microinjections of AMPH on drug days of conditioning, increased locomotor activity was observed after the fourth AMPH microinjection in comparison to when AMPH was given for the first time, indicating locomotor sensitization to AMPH. This sensitization phenomenon is another example of incentive learning that occurs when animals repeatedly administered a DA-enhancing stimulant drug in a constant and contextually distinct environment exhibit an increasing behavioural reactivity to the drug when given in the drug-paired environment. Animals that are pre-exposed to the drug but tested in an environment not associated with the drug do not show this increasing behavioural reactivity [95]. Locomotor sensitization to AMPH seems to be associated with enhanced reactivity of DA terminals of the NAc in response to AMPH in the drug-paired environment, however the underlying neural substrates of this phenomenon remain to be understood [96,97]. Present data shows that Wnt signalling is required for the induction of locomotor sensitization because Wnt inhibition during conditioning with AMPH blocked locomotor sensitization in the acquisition groups. These findings provide further evidence for the role of Wnt in incentive learning.

Interestingly, the IWP-2 alone group also showed significantly increased locomotion on drug days in comparison to vehicle days of conditioning although the stimulant effects of
IWP-2 is not as strong as that observed in groups given AMPH (Appendix Fig. A3). Furthermore, ACQ (0.05) and ACQ (1.0) groups showed greater locomotor activity on vehicle days of conditioning compared to all other groups, with ACQ (1.0) showing the greatest overall locomotor activity on vehicle days (Figure 5). Previous research has shown that the inhibition of GSK3β [27] and the overexpression of β-catenin [115] reduced AMPH-induced locomotor activity. Normally, in the presence of Wnt, GSK3β interacts with phosphorylated LRP6 motifs to suppress GSK3β inhibition of β-catenin phosphorylation and activation [116]. Thus, it is possible that the increased locomotor activity observed in the IWP-2 alone group was due to the suppression of Wnt-induced inhibition of GSK3β activity towards β-catenin. Although no IWP-2 microinjections were given on vehicle days of conditioning for the mentioned ACQ groups, it is possible that there were carry-over effects of Wnt inhibition from drug day to the next vehicle day of conditioning. The IWP-2 alone group, however, did not show CPP and locomotor sensitization, which indicates that IWP-2 does not have rewarding effects of its own.

Both D₁ and D₂ receptor agonists are able to produce place preference suggesting that dopaminergic transmission in the direct pathway expressing D₁ receptors and the indirect pathway expressing D₂ receptors are involved in incentive learning [65]. D₁ receptor activation by DA or dopaminergic agents results in the activation of LTP at glutamatergic synapses formed on striatal MSNs, whereas D₂ receptor activation by DA or dopaminergic agents reduces glutamate release resulting in LTD at MSN glutamatergic synapses [117]. DA receptor signalling via G-protein and the Wnt signalling pathway share several intermediate signalling components suggesting possible interactions between these two pathways [50]. D₁ receptor activation by DA results in the stimulation of the cAMP/PKA
pathway [118], which is also activated by the noncanonical Wnt signalling pathway (Appendix Fig. A4) [119]. In contrast, D2 receptor activation by DA results in the suppression of the cAMP/PKA pathway [118], and D2 receptors also appear to be able to selectively inhibit Wnt signalling via direct interaction with β-catenin thereby preventing its nuclear translocation [50]. It is possible that Wnt is differentially modulated in D1 receptor-expressing and D2 receptor-expressing MSNs, with Wnt signalling being stimulated and suppressed by D1 receptor and D2 receptor activation, respectively. Thus, inhibition of Wnt signalling during incentive learning may diminish responsively to DA, especially in D1 receptor-expressing MSNs involving the stimulation of Wnt and associated signalling components, and as a result, the induction of LTP is prevented at glutamatergic synapses of the D1 receptor–expressing MSNs. The details of the functional interaction between Wnt and DA signalling as it relates to incentive learning need to be further explored in future studies.

In conclusion, the present study demonstrates that Wnt signalling is necessary for the acquisition and the expression of intra-NAc AMPH-induced CPP and contributes to AMPH-induced locomotor sensitization, which implicates Wnt signalling in incentive learning. These results support existing research that shows functional interactions between Wnt and DA signalling. Disruptions in Wnt signalling have also been linked to the pathogenesis of neuropsychiatric disorders characterized by dysregulations of the dopaminergic system, including Parkinson’s disease, schizophrenia, and drug addiction [87,120]. The present research has implications for the development of treatment for these disorders. Modulators of Wnt signalling that modify the pathological processes of these disorders might be considered a promising approach for the development of new therapeutic compounds in the future.
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Appendix

A

Difference in Time Spent in the Vehicle-Paired Side (s)

-200 -160 -120 -80 -40 0 40

AMPH (combined)  AMPH Systemic  IWP-2 alone

B

Difference in Time Spent in the Vehicle-Paired Side (s)

-200 -180 -160 -140 -120 -100 -80 -60 -40 -20 0 20

IWP-2 Dose (µg/0.5 µl/side)

0.0 0.0001 0.001 0.05 0.5 1.0

C

Difference in Time Spent in the Vehicle-Paired Side (s)

-200 -160 -120 -80 -40 0 40

IWP-2 Dose (µg/0.5 µl/side)

0.0 0.0001 0.001 0.05 0.5 1.0
Fig. A1. Mean (±SEM) difference in time (s) spent in vehicle-paired side during pre-conditioning (averaged over three sessions) and test session. (A) Amphetamine (AMPH) (combined) is an omnibus group that includes the three AMPH control groups (gray bars): AMPH systemic received IP injections of AMPH (1.5 mg/kg) and ACQ (0.0) and EXP (0.0) control received microinjections of AMPH (20.0 µg/0.5 µl/side). 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 ACQ groups (B) were conditioned with IWP-2 administered 30 min prior to microinfusion of AMPH on drug days. All EXP groups (C) were conditioned with AMPH and administered IWP-2 only on test session.

Asterisks above the bar represents a significant difference on test session from pre-conditioning based on simple effects analyses of each group following observation of group x phase interaction approaching significance (F(9, 121) = 1.95, p = 0.05) in the analyses of variance of all groups (*p<0.05, **p<0.01, ***p<0.001).
Fig. A2. Mean (±SEM) difference in time (s) spent in drug-paired vs. the vehicle-paired side during test. (A) Amphetamine (AMPH) (combined) is an omnibus group that includes the three AMPH control groups (gray bars): AMPH systemic received IP injections of AMPH (1.5 mg/kg) and ACQ (0.0) and EXP (0.0) received microinjections of AMPH (20.0 µg/0.5 µl/side). 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 ACQ groups (B) were conditioned with IWP-2 administered 30 min prior to microinfusion of AMPH on drug days. All EXP groups (C) were conditioned with AMPH and administered IWP-2 only on test session.

Asterisks above the bar represents a significant difference in time between drug-paired vs. vehicle-paired side on test session based on simple effects analyses of each group following observation of a significant group x phase interaction in the analyses of variance of all groups (*p<0.05, **p<0.01, ***p<0.001).
Fig. A3. Mean (±SEM) locomotor activity measured in beam breaks over 30-minute conditioning sessions for all treatment groups. AMPH (combined) is an omnibus group that includes the three AMPH control groups: AMPH systemic, ACQ (0.0), and EXP (0.0).

Asterisk above the bar represents a significant difference in locomotor activity between drugs days and vehicle days based on simple effect analyses of each group following observation of a significant group x phase interaction in analyses of variance of all groups (*p<0.05, ***p<0.001).

AMPH = amphetamine, ACQ = acquisition, EXP = expression, and IWP-2 = Inhibitor of Wnt Production-2 alone. Numbers beside ACQ and EXP groups represent IWP-2 dose in µg/0.5 µl/side.
Fig. A4. Schematic diagram of the noncanonical Wnt/PKA signalling pathway. Wnt activates G-protein and adenylyl cyclase (AC) to increase cyclic adenosine monophosphate (cAMP) levels, which activates protein kinase A (PKA) and transcription factor cAMP-response element-binding protein (CREB) and Wnt-target gene expression.

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