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ORIGINAL ARTICLE

Role of Wnt/β-catenin pathway in the nucleus accumbens in long-term cocaine-induced neuroplasticity: a possible novel target for addiction treatment

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Abstract

Cocaine addiction is a chronic relapsing disorder characterized by the loss of control over drug-seeking and taking, and continued drug use regardless of adverse consequences. Despite years of research, effective treatments for psychostimulant addiction have not been identified. Persistent vulnerability to relapse arises from a number of long-lasting adaptations in the reward circuitry that mediate the enduring response to the drug. Recently, we reported that the activity of the canonical or Wnt/β-catenin pathway in the prefrontal cortex (PFC) is very important in the early stages of cocaine-induced neuroadaptations. In the present work, our main goal was to elucidate the relevance of this pathway in cocaine-induced long-term neuroadaptations that may underlie relapse. We found that a cocaine challenge, after a period of abstinence, induced an increase in the activity of the pathway which is revealed as an increase in the total and nuclear levels of β-catenin (final effector of the pathway) in the nucleus accumbens (NAcc), together with a decrease in the activity of glycogen synthase kinase 3β (GSK3β). Moreover, we found that the pharmacological modulation of the activity of the pathway has long-term effects on the cocaine-induced neuroplasticity at behavioral and molecular levels. All the results imply that changes in the Wnt/β-catenin pathway effectors are long-term neuroadaptations necessary for the behavioral response to cocaine. Even though more research is needed, the present results introduce the Wnt canonical pathway as a possible target to manage cocaine long-term neuroadaptations.

Keywords: cocaine, lithium chloride, long-term neuroadaptations, nucleus accumbens, Wnt canonical pathway.

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Cocaine addiction is a chronic relapsing disorder characterized by the loss of control over drug-seeking and taking, and continued drug use regardless of adverse consequences (APA 2000). Over the years, different animal models have been designed to contribute to the elucidation of the neurobiological processes involved in relapse behavior and to evaluate potential pharmacotherapies that may prevent or reduce the risk of relapse (LaCrosse *et al.* 2016). However, despite almost 50 years of experimental research, effective treatments for psychostimulant addiction have not been

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Abbreviations used: AMPA, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate; CPP, conditioned placed preference; CPu, caudate putamen; Dvl, Disheveled; GSK3β, glycogen synthase kinase 3β; IGF-1R, insulin-like growth factor receptor; LiCl, lithium chloride; LTP, long-term potentiation; NAcc, nucleus accumbens; PFC, prefrontal cortex; PTK7, Tyrosine-protein kinase-like receptor; Ror, receptor tyrosine kinase-like orphan receptor 1; R, receptors; Ryk, receptor-like tyrosine kinase; VTA, ventral tegmental area; Wnt, Wingless-related integration site.

identified. The persistent vulnerability to relapse arises from a number of temporary plastic changes that eventually lead to long-lasting adaptations that mediate the hypersensitivity and enduring response to the drug (Vanderschuren and Kalivas 2000). Abundant evidence suggests that the nucleus accumbens (NAcc) is the locus in the reward circuit where drugs induced the long-term changes that underlie cocaine addiction. The NAcc core receives innervations from the prefrontal cortex (PFC) and the ventral tegmental area. Both glutamatergic and dopaminergic afferents regulate various motivated behaviors thought to model different aspects of drug addiction, including locomotor sensitization, conditioned place-preference, intravenous self-administration, and reinstated drug seeking (Stewart *et al.* 1984; Robinson and Berridge 2000; Feltenstein and See 2008; Sesack and Grace 2010; Stuber *et al.* 2012). In fact, the pharmacological or the optogenetic inhibition of the NAcc core (McFarland and Kalivas 2001; Stefanik *et al.* 2013), as well as the inhibition of its afferents from the dorsal medial PFC, attenuate the behavioral manifestation of cocaine neuroadaptations [i.e. sensitization (Pierce *et al.* 1998; Anderson *et al.* 2003), and reinstatement (McFarland *et al.* 2003)]. Among other changes, cocaine induces a decrease in basal glutamate levels in the NAcc that increase after a cocaine challenge (Pierce *et al.* 1996; Baker *et al.* 2003), which in turn by acting on the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors (AMPAR) promotes higher behavioral responses (Pierce *et al.* 1996; Cornish and Kalivas 2000). Also, withdrawal from drug exposure induces changes in NAcc neurons which can be temporarily reversed by re-exposure to the drug. For instance, during cocaine withdrawal, there is an increase in synaptic strength of AMPA relative to NMDA-mediated current given by an increase in the surface expression of AMPAR (Boudreau *et al.* 2007; Kourrich *et al.* 2007; Wolf and Ferrario 2010), and changes in dendritic spine density (Robinson and Kolb 2004; Shen *et al.* 2009, 2014; Russo *et al.* 2010). While the mechanisms that regulate these structural changes are not clear, the regulation of the actin cytoskeleton appears to be involved (Toda *et al.* 2006, 2010) as well as the activity of small GTPase and the induction of different genes and their targets (e.g. ΔFosB, NFkB, Cdk5-MEF2, etc.) (Russo *et al.* 2010).

Recently, we reported that the activity of the Wnt canonical pathway in the PFC is implicated in facilitating the induction of behavioral sensitization (Cuesta *et al.* 2016). Together with our results, in the past decade, mounting evidence has suggested a link between dysfunction of Wnt signaling and neurological disorders such as Alzheimer's disease, bipolar disorder, and schizophrenia (De Ferrari and Inestrosa 2000; Kozlovsky *et al.* 2002). The Wnt growth factors belong to a large family of secreted proteins, that can signal through different receptors including frizzled (Fz), the atypical tyrosine kinase receptors Ror, Ryk, and tyrosine-protein kinase-like receptor (PTK7) (Clevers and Nusse 2012; Oliva *et al.* 2013; Anastas 2015) as well as insulin-like growth factor receptor (IGF-1R) (Bernis *et al.* 2013). The interaction between Wnt and Fz leads to the phosphorylation of dishevelled (Dvl, first intracellular effector). Downstream of Dvl, the Wnt pathways diverge into three branches: the canonical or Wnt/β-catenin, the planar cell polarity, and the Wnt/calcium pathways (Ciani and Salinas 2005; Oliva *et al.* 2013). The activation of the canonical pathway results in the phosphorylation of GSK3β (Glycogen synthase kinase 3β) leading to β-catenin stabilization and subsequent entrance to the nucleus where it promotes gene expression (Metcalfe and Bienz 2011; Clevers and Nusse 2012). While in the absence of Wnt, GSK3β phosphorylates β-catenin marking it for degradation by the proteasome (Maguschak and Ressler 2012). We, and others, have suggested that regulation of GSK3β activity might be associated with cocaine-induced neuroadaptations. Not only does cocaine produce changes in GSK3β activity, but also inhibitors of GSK3β, both targeted (e.g. SB 216763) and non-selective (e.g., valproate or lithium chloride), prevent the development (Cuesta *et al.* 2016) as well as the expression of behavioral sensitization (Xu *et al.* 2009). However, the relationship between the long-term effects of cocaine, GSK3β, and the Wnt/β-catenin pathway has not been explored. Therefore, our main goal was to evaluate whether the Wnt canonical pathway is involved in long-term cocaine-induced neuroadaptations. In this study, we combined molecular, pharmacological, and behavioral studies in order to evaluate the relevance of the Wnt/β-catenin pathway for cocaine-induced neuroadaptations that may underlie the persistence of addiction**,** looking also into the possibility of considering this pathway as a pharmacological target for addiction.

Methods

Experimental subjects

Male Wistar rats (250–330 g) were purchased from the Vivarium of the Facultad de Ciencias Bioquímicas y Farmaceúticas (Universidad Nacional de Rosario, UNR, Argentina). Rats were group housed in the colony room for at least 7 days before experimental tests started, with food and water *ad libitum*. All experiments were conducted during the light period of a 12 h light/dark cycle and were completed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Facultad de Ciencias Bioquímicas y Farmaceúticas – UNR.

Drugs

Cocaine hydrochloride was purchased from Droguería Saporiti (Buenos Aires, Argentina), while lithium chloride (LiCl) was obtained from Sigma (St. Louis, MO, USA). Cocaine was dissolved in saline while LiCl was in ultrapure water.

Behavioral tests

Motor activity

The testing apparatus consisted of eight acrylic boxes (43 × 43 × 30 cm) equipped with eight infrared photocell beams located 3 cm above the floor. Interruption of any beam resulted in a photocell count. Locomotor activity was recorded during 1 h habituation and the 2 h immediately after the injection. The apparatus and its software were developed by Laboratorio de Investigación Aplicada y Desarrollo, Facultad de Ciencias Exactas, Físicas y Naturales (Universidad Nacional de Córdoba, Argentina).

Tissue preparation

Rats were killed 3 or 24 h after the last injection of cocaine or saline, and their brains were removed. The NAcc, PFC, and caudate putamen (CPu) were dissected according to Heffner *et al.* (1980) using a rat brain matrix. Tissue was kept at −80°C until analysis.

Total homogenates

Tissues were homogenized on ice with radioimmunoprecipitation assay (RIPA) buffer supplemented with phosphatase and protease inhibitors (2 μg/mL aprotinin; 2 μg/mL leupeptin; 1 μg/mL pepstatin; 100 μg/mL phenylmethylsulfonyl fluoride; 1 mM Na_3VO_4; 50 mM NaF), and centrifuged for 5 min at 13 000 g. Protein concentration was measured using the Lowry assay.

Subcellular fractionation

In a different set of animals, brain tissue was dissected and subcellular fractionation was performed as previously described by Cuesta *et al.* (2016).

Western blotting

Protein extracts coming from total homogenates or nuclear fractions were heated to 80°C for 5 min with Laemmli buffer as a reducing treatment. Samples (total homogenate: 10 μg/lane; nuclear fraction: 5 μg/lane) were run in 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membrane. A secondary horseradish peroxidase-conjugated antibody (Sigma) followed overnight incubation with primary antibody (β-catenin (MW 92KDa) 1 : 10 000, phospho-GSK3β-Y216 1 : 8000, total GSK3β (MW 46KDa) 1 : 10 000; BD BioScience, San Jose, CA, USA). Reactivity was detected using enhanced chemiluminescence and quantified using Gel-Pro Plus software package. Total homogenates blots were also incubated with anti-tubulin [(MW 50 KDa) 1 : 14 000; Sigma] or total GSK3β to correct for differences in protein loading.

Experimental procedures

Acute cocaine, chronic cocaine, and saline treatments

Subjects were assigned to one of three conditions: saline, acute cocaine, or chronic cocaine (Fig. 1a). All animals received one injection per day for 7 days, 3 weeks of abstinence, and a final challenge on day 28. Saline group: animals received saline (1 mL/kg i.p). Acute cocaine: rats received saline (1 mL/kg i.p) on days 1–7, and cocaine (15 mg/kg i.p) on day 28. Chronic cocaine: animals received 15 mg/kg cocaine (i.p) on days 1, 7, and 28; and 30 mg/kg cocaine (i.p) on days 2–6. Locomotor activity was recorded after injections on days 1, 7, and 28. From days 2 to 6, animals were injected in their home cages without locomotor activity recording. For the purposes of our study, we separated animals receiving chronic cocaine into sensitized and non-sensitized groups, according to their locomotor responses (Fig. 1b), where cocaine-induced

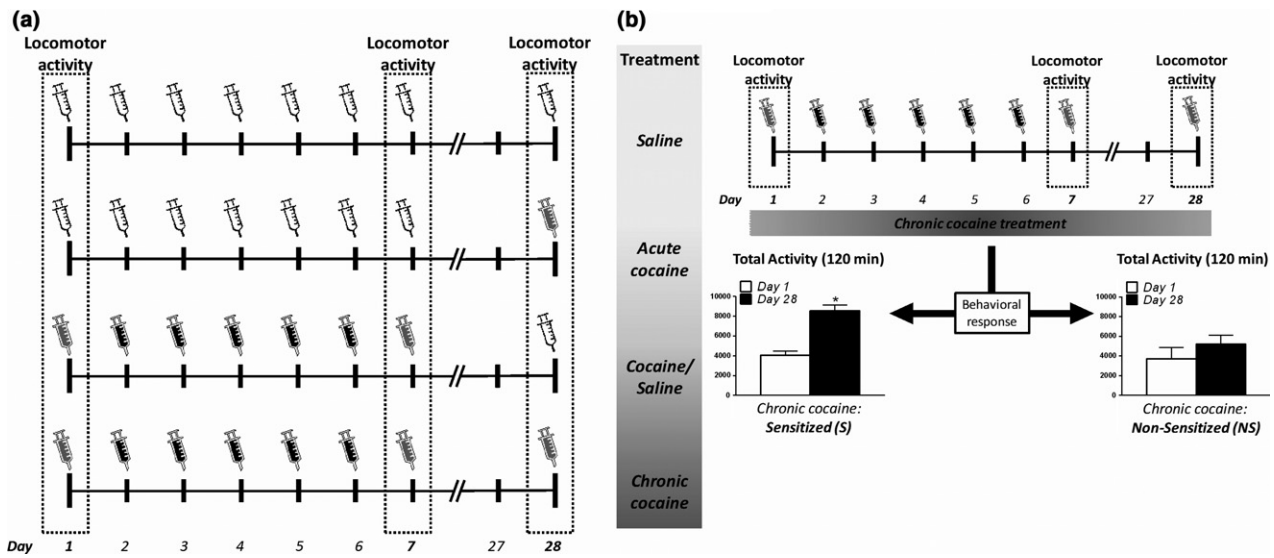

Fig. 1 Schematic descriptions of the treatments. (a) Adult rats received one injection per day for 7 days, 3 weeks of abstinence and a final challenge on day 28. Saline group: all saline injections. Acute cocaine: received saline. Chronic cocaine: received all cocaine injections. Cocaine/Saline: received cocaine on days 1–7, and saline on day 28. On days 1, 7, and 28, all rats were behaviorally tested immediately after the injection. The *white* syringe represents the saline injection (1 mL/kg), the *gray* one represents cocaine in a 15 mg/kg dose, and the *black* one represents cocaine in a 30 mg/kg dose. (b) In the chronic cocaine group, their locomotor responses on each day were compared and animals were divided into Sensitized (S) and Non-sensitized (NS).

sensitization was defined as a minimum of 20% increase in total activity counts on day 28 compared to day 1 (Pierce *et al.* 1996).

Animals were killed 24 h after the last cocaine/saline challenge. Their brains were removed and dissected.

LiCl pretreatments

Animals received LiCl (30 mg/kg i.p.) or Saline (1 mL/kg i.p.) injections 30 min before each saline or cocaine injection (from day 1 to 7), leading to four groups: Saline/Saline, LiCl/Saline, Saline/Cocaine, and LiCl/Cocaine. After 3 weeks of abstinence, on day 28, each group was divided into two subgroups to receive a challenge with cocaine (15 mg/kg i.p.) or saline (1 mL/kg i.p.) (Fig. 4a). The dose of LiCl was chosen based on our previous work (Cuesta *et al.* 2016). Locomotor activity was recorded on days 1, 7, and 28. Animals were killed 24 h after the last cocaine/saline challenge. Their brains were removed and dissected.

Data analysis

Locomotor activity was analyzed using two-way or three-way analysis of variance (ANOVA) with pretreatment, treatment, and time as main factors. Western blots were analyzed using either a *t test* or a one-way analysis of variance (one-way ANOVA). In all cases, the ANOVA was followed by a *post hoc* test with significance set at $p < 0.05$, depending on the number of groups under consideration.

Results

Long-term cocaine-induced neuroadaptations lead to changes in β-catenin expression in NAcc and CPu

In order to investigate the potential role of Wnt/β-catenin pathway on the long-lasting effects of chronic cocaine, we treated the rats with a cocaine regime that produces robust psychomotor sensitization (Pierce *et al.* 1996; Boudreau *et al.* 2007; Cuesta *et al.* 2016). Since it has been previously shown that a percentage of the cocaine treated animals may not manifest psychomotor sensitization (Pierce *et al.* 1996; Boudreau *et al.* 2007; Cuesta *et al.* 2016), we challenged the animals with a cocaine injection 3 weeks after the end of the chronic treatment. The brain areas of the control groups (saline and acute cocaine) as well as the ones of the animals that showed behavioral sensitization were collected after 3 or 24 h of the last injection; the animals of the chronic group that did not show behavioral sensitization were killed after 24 h. Thus, we started by comparing β-catenin levels, the final effector of the canonical Wnt pathway, as a readout of the activity of the pathway in rats that behaviorally showed the psychomotor sensitization after a chronic cocaine treatment against control animals (acute cocaine and saline groups). A one-way ANOVA analysis of data collected 24 h after the last injection on day 28 revealed a significant effect of treatment in the NAcc [$F(2, 19) = 20.53$; $p < 0.001$] (Fig. 2a) and CPu [$F(2, 17) = 6.54$; $p < 0.001$] (Table 1A). The *post hoc* analysis revealed that β-catenin levels were significantly increased in the NAcc, while decreased in the CPu of animals that showed behavioral sensitization compared to control animals. In contrast with our previous results (Cuesta *et al.* 2016), no changes were found in the PFC after the challenge on day 28 (Table 1A). The evaluation of β-catenin expression in animals that were killed 3 h after the last cocaine injection, turned out to be similar to 24 h (Table 1B). Regarding the acute group, it is important to

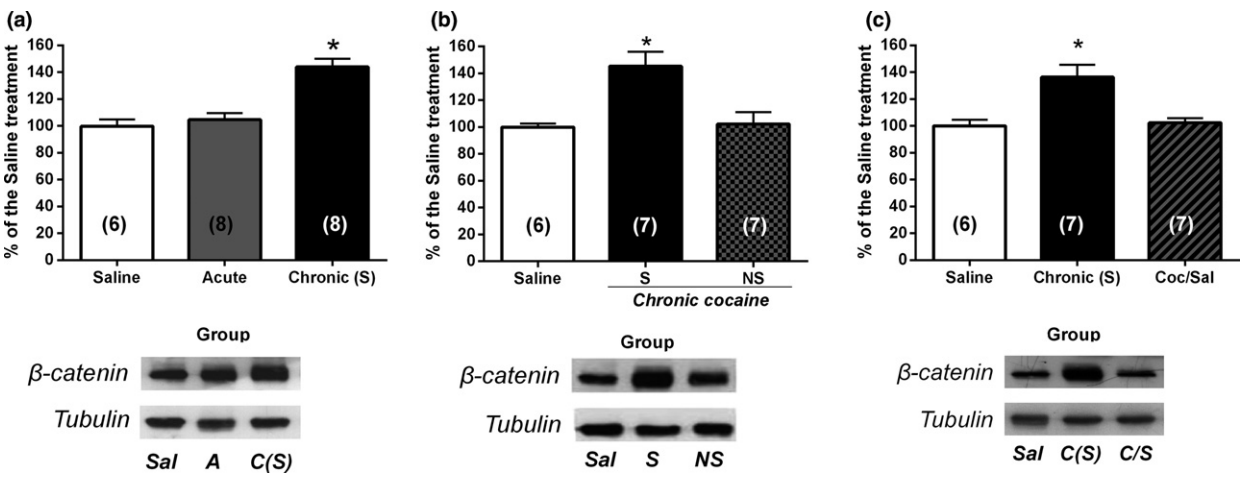

Fig. 2 Long-term cocaine-induced neuroadaptations lead to changes in β-catenin expression in NAcc. (a) First, the levels of β-catenin in the control groups (saline and acute cocaine) were compared against the chronic cocaine group that sensitized [chronic cocaine (S)]. (b) Secondly, in order to study if the changes in β-catenin were related to the cocaine treatment only or to the behavioral display of sensitization, levels of β-catenin in rats from the chronic cocaine group that showed the expression of sensitization were compared with those rats that did not sensitize (NS). (c) Finally, we evaluated if the changes in β-catenin levels needed the cocaine challenge to appear. Then, the effect of cocaine [chronic cocaine (S)] on β-catenin levels was compared to a saline (Coc/Sal) challenge on day 28 in a group of animals chronically treated with cocaine. Bars represent Mean ± SEM from β-catenin levels in the NAcc. Number of animals (*n*) is represented inside each bar. *Significantly different from all the other groups, $p < 0.05$. Bonferroni *post hoc* test.

Table 1 Levels of β-catenin in total homogenates of brain areas collected after the last injection on day 28

(A)		Group		
		Saline	Acute	Chronic (S)
Brain area	CPu	100.0 ± 2.1	89.4 ± 10.3	60.1 ± 7.9[b]
	PFC	100.0 ± 11.1	112.7 ± 6.3	102.8 ± 6.7

(B)		Group		
		Saline	Acute	Chronic (S)
Brain area	NAcc	100.6 ± 4.4	107.1 ± 5.7	146.6 ± 6.5[a]
	CPu	100.0 ± 4.6	86.1 ± 5.7	66.68 ± 6.4[b]
	PFC	100.7 ± 2.1	104.8 ± 5.1	101.9 ± 8.6

(C)		Group		
		Saline	Chronic (S)	Chronic (NS)
Brain area	CPu	100.0 ± 2.1	60.1 ± 7.9[a]	113.1 ± 12.2
	PFC	100.0 ± 11.1	102.8 ± 6.7	105.3 ± 9.2

(D)		Group		
		Saline	Chronic (S)	Coc/Sal
Brain area	CPu	100.0 ± 2.1	60.1 ± 7.9[a]	219.4 ± 12.9[a]
	PFC	100.0 ± 11.1	102.8 ± 6.7	153.6 ± 17.0[b]

(A) and (B) The levels of β-catenin in the control groups (saline and acute cocaine) were compared against the chronic cocaine group that sensitized [chronic cocaine (S)]: (A) CPu and PFC were collected 24 h after the last injection; while in (B) NAcc, CPu, and PFC were collected 3 h after the last injection. (C) β-catenin levels in rats that sensitized (S) were compared with those rats that did not sensitize (NS). (D) Finally, β-catenin levels in chronically treated rats (chronic cocaine (S)) that received a cocaine challenge on day 28 were compared to chronically treated rats that received a saline challenge (Coc/Sal) on the same day. (C) and (D) CPu and PFC samples were collected 24 h after the last injection. Data represent Mean ± SEM from β-catenin levels. Number of animals per group: 6–8

[a]Significantly different from the rest of the groups, $p < 0.05$.

[b]Significantly different from saline, $p < 0.05$. Bonferroni *post hoc* test.

highlight that, as we expected, β-catenin was not different from saline neither at 24 h nor at 3 h after the last injection. So far, these results suggested that a cocaine challenge on day 28 leads to changes in β-catenin levels specifically in the NAcc and CPu of animals that showed long-term adaptations related to an increased behavioral response to cocaine. To study whether those changes in β-catenin were a neuroadaptation necessary for the behavioral manifestation to occur, we compared the levels of β-catenin in brain areas obtained from sensitized animals (S) with non-sensitized animals (NS) and the control group (saline animals), after receiving the corresponding challenge on day 28. A one-way ANOVA analysis of the data revealed a significant effect of the behavioral response on β-catenin levels in the NAcc [$F(2, 17) = 10.17$; $p < 0.01$] (Fig. 2b), and the CPu [$F(2, 17) = 7.73$; $p < 0.005$] (Table 1C), while no differences were found between the groups in the PFC (Table 1C). These results showed that in the NAcc and the CPu, changes in β-catenin levels are a characteristic neuroadaptation for the behavioral expression of sensitization. Then, we investigated if those changes were present before the cocaine challenge on day 28 or were a result of it. To do that, we compared β-catenin levels in saline control animals regarding animals that received a cocaine treatment during 7 days, sensitized and were challenged with saline (Coc/Sal) or cocaine (Chronic) on day 28. A one-way ANOVA analysis of data revealed a significant effect of the challenge on day 28 on β-catenin levels in the NAcc [$F(2, 16) = 16.11$; $p < 0.0001$] (Fig. 2c), CPu [$F(2, 15) = 61.23$; $p < 0.0001$], and PFC [$F(2, 20) = 5.712$; $p < 0.05$] (Table 1D). These data showed that β-catenin levels in the animals that sensitized were different depending on the presence or absence of cocaine: in the NAcc while the levels of β-catenin remain unmodified after the saline challenge regarding the saline control group, the injection of cocaine induced an increase; at the same time, the levels of β-catenin in the CPu and the PFC of animals' challenge with saline were higher than controls, and cocaine induced a decrease. It is important to note here that the Coc/Sal group has been re-exposed to the context where cocaine was administered, which may produce the expectation to the cocaine injection usually reflected as hyperlocomotion. Even though, there is previous evidence that the same cocaine treatment induced conditioned locomotion after 2 weeks of withdrawal (Boudreau *et al.* 2007),we did not find significant differences when comparing the response to saline in animals previously treated with cocaine or saline after 3 weeks of withdrawal (Fig. 4b). In summary, we found that β-catenin levels in the NAcc increase while decrease in the CPu in response to a cocaine challenge only in the animals that expressed cocaine-induced neuroadaptations at a behavioral level. Moreover, the fact that β-catenin expression is not modified in the NAcc and it is increased in the PFC, and the CPu before the cocaine challenge strengthens the idea that the chronic cocaine treatment induces long-term neuroadaptations in the effectors of the Wnt/β-catenin pathway that allow the behavioral display of sensitization.

Cocaine-induced behavioral neuroadaptations are associated with a functional increase of β-catenin in the NAcc

Considering previous evidence that supports an important role of the NAcc in the long-lasting cocaine neuroadaptations that underlie sensitization and addiction, we deepened our study evaluating if the changes in β-catenin levels found in total homogenates of sensitized animals were the result of a

functional increase of the Wnt canonical pathway. Consequently, we first compared the GSK3β activity levels in the NAcc of chronic cocaine sensitized animals and saline controls. Figure 3a showed GSK3β activity levels in the NAcc evaluated as the phosphorylation level of tyrosine 216 (activator site). No changes were found in total GSK3β protein levels (*t* test, $p = 0.14$). However, the ratio GSK3β (Y216):GSK3β total was significantly decreased in chronic cocaine animals compared to saline (*t* test, $p < 0.05$), proposing a reduction in GSK3β activity which is in line with the increase in β-catenin levels found in the NAcc after the expression of cocaine-induced sensitization. Then, fresh brain tissue obtained from another set of animals was subjected to subcellular fractionation and β-catenin was measured in the nuclear fraction (Fig. 3b). Our data showed that β-catenin levels were increased in the nuclear fraction of the NAcc of sensitized animals (*t* test, $p < 0.05$). These results suggest that the behavioral manifestation of cocaine-induced neuroadaptations can be linked to a functional increase of Wnt/β-catenin pathway in the NAcc.

A repeated treatment with systemic Lithium Chloride along with cocaine blocks the long-lasting cocaine-induced neuroadaptations at the behavioral level by changing β-catenin expression in the NAcc

In our previous work, we demonstrated that a repeated treatment with LiCl (a non-specific inhibitor of GSK3β activity) previous to a cocaine injection blocked sensitization preventing changes in β-catenin levels when measured at the end of the chronic cocaine treatment (Cuesta *et al.* 2016). Taking into account the results presented here, we set to determine if LiCl would have a long-lasting effect on the behavioral and molecular response to cocaine. In order to evaluate this, we treated animals with LiCl (30 mg/kg i.p.) or Saline before each cocaine injection during day 1–7 (Fig. 4a). Interestingly, we found that LiCl blocked the increased behavioral response associated with the expression of sensitization conducted 3 weeks after the pretreatment (Fig. 4b) and induced different long-term neuroadaptations in β-catenin levels in NAcc, CPu, and PFC total homogenates (Fig. 5). It is important to mention that animals pretreated with LiCl did not show the development of cocaine-induced sensitization (total activity Day 1 = 6058 ± 360.27; Day 7 = 5940 ± 749.7), as it was previously demonstrated (Cuesta *et al.* 2016). A three-way ANOVA analysis applied to the behavioral response on day 28 showed in Fig. 4b revealed a significant main effect of challenge [$F(1, 49) = 118.69$, $p < 0.0001$], treatment [$F(1, 49) = 13.79$, $p < 0.01$], and a significant interaction between pretreatment × treatment × challenge [$F(1, 49) = 4.28$, $p < 0.05$]. The *post hoc* analysis revealed that, regardless of the pretreatment, the animals showed an increase in their locomotor response compared to the control animals (Sal/Sal/Sal) when they had received cocaine. However, only the animals pretreated with saline showed a significant increase in their locomotor response when comparing the chronic treatment to the acute one. In fact, the response to the cocaine challenge in the chronic cocaine group pretreated with saline was significantly higher than the same response to cocaine in the group pretreated with LiCl. Moreover, when we pretreated this last group with LiCl they showed, on day 28, similar behavioral responses when comparing acute to chronic cocaine. These results indicate that a treatment with LiCl before cocaine injections has long-term effects on the locomotor response to a cocaine challenge after 3 weeks of abstinence, since these animals were unable to express behavioral sensitization.

After 24 h from the last injection on day 28, all the animals were killed and β-catenin was measured in total homogenates

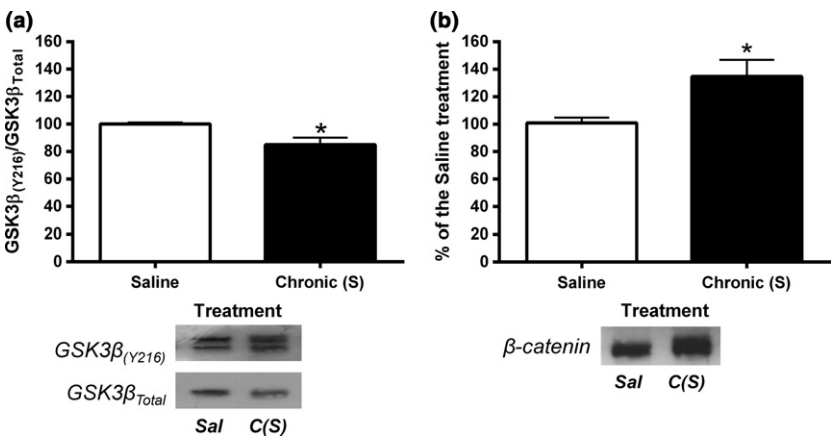

Fig. 3 Cocaine-induced behavioral neuroadaptations are associated with a functional increase of β-catenin in the NAcc. (a) GSK3β activity levels were measured by comparing GSK3β phosphorylated in Y216 and total GSK3β in the NAcc of control and sensitized animals. (b) β-catenin levels in the nuclear fraction of the NAcc obtained by subcellular fractionation of control and sensitized animals. Bars represent Mean ± SEM. Number of animals per group: 6. *Significantly different from Saline treatment, $p < 0.05$; *t* test.

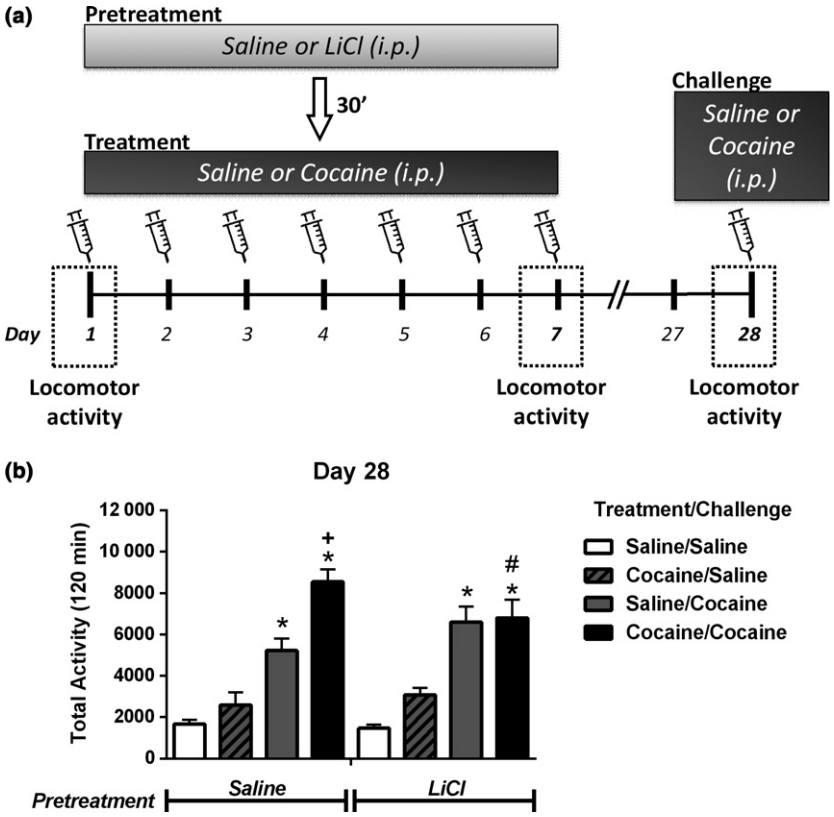

Fig. 4 A repeated treatment with systemic lithium chloride along with cocaine blocks the expression of cocaine-induced behavioral neuroadaptations. (a) Schematic description of the LiCl pretreatment: animals were pretreated with LiCl (30 mg/kg i.p.) or vehicle (saline, 1 mL/kg i.p.) 30 min before each injection of cocaine or saline, and their locomotor activity was tested after injection on days 1, 7, and 28. (b) Total locomotor activity measured on day 28 showed that cocaine induced the behavioral expression of sensitization in animals pretreated with saline while LiCl pretreatment blocked it. Bars represent Mean ± SEM. Number of animals per group: 6–8. *Significantly different Sal/Sal/Sal, $p < 0.01$; $^{+}$significantly different from Sal/Sal/Coc, $p < 0.01$; $^{\#}$significantly different from Sal/Coc/Coc, $p < 0.05$. Holm's Sidak *post hoc* test.

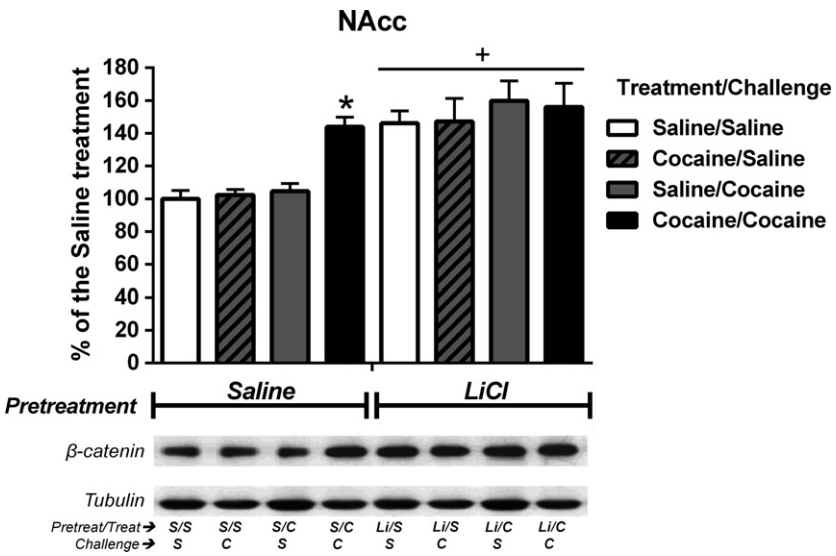

Fig. 5 The activation of Wnt/β-catenin pathway during cocaine treatment with LiCl induced long-term neuroadaptations in β-catenin levels in NAcc. The day after behavioral testing, rats were killed and their brains were dissected to measure β-catenin in total homogenates. Levels of β-catenin in NAcc total homogenates from animals pretreated with saline was significantly increased in the cocaine challenged group, while LiCl pretreated animals showed increase levels of β-catenin in all tested groups. Bars represent Mean ± SEM. Number of animals per group: 6–8. *Significantly different from all other saline pretreated groups, $p < 0.05$; $^{+}$Significantly different from Sal/Sal/Sal group, $p < 0.05$. Holm's Sidak *post hoc* test.

from the NAcc, CPu and PFC (Fig. 5). A three-way ANOVA analysis of data in Fig. 5 revealed the following results for each brain area. In the NAcc (Fig. 5), the analysis displayed a main effect of pretreatment [$F(1, 44) = 33.5, p < 0.001$], and challenge [$F(1,44) = 6.301, p < 0.05$]. The *post hoc* analysis showed a significant increase in β-catenin levels in the NAcc of animals that expressed behavioral sensitization as well as in all the groups that received a LiCl pretreatment compared to the control group (Sal/Sal/Sal). Meanwhile, in the CPu (Table 2), β-catenin levels showed a significant main effect of interaction between pretreatment × treatment × challenge [$F(1, 44)= 34.56, p < 0.0001$]. The *post hoc* analysis revealed significant changes on the β-catenin levels before and after the cocaine challenge, with an increase (Sal/Coc/Sal) before the challenge and a decrease (Sal/Coc/Coc) after, compared to saline animals. Importantly, the pretreatment with LiCl blunted all this modification in β-catenin levels. Finally, in PFC (Table 2), β-catenin levels showed a significant main effect of interaction pretreatment × treatment [$F(1, 41) = 4.85, p < 0.05$]. The *post hoc* analysis only showed a significant increase in β-catenin levels in the PFC of abstinent animals (Sal/Coc/Sal) compared to controls with no changes in β-catenin levels in the PFC of the LiCl pretreated animals.

Discussion

Identifying new targets for therapeutic treatment of addiction has been one of the main foci for drug abuse research during the last decades. In this study, we contribute to this goal by proposing a novel role of the Wnt/β-catenin pathway in cocaine-induced long-term neuroadaptations. Our main findings showed: (i) that chronic cocaine induced an increase on the levels of β-catenin (final effector of the pathway) in the NAcc, a decrease in the CPu and no changes in the PFC, compared to saline-treated animals; (ii) that those changes were present only in animals where chronic cocaine-induced neuronal adaptations were evident at a behavioral level after a cocaine challenge; (iii) that long-term effects of cocaine are related to an increased activity of the Wnt/β-catenin pathway in the NAcc; and, (iv) that a pharmacological modulation of the pathway activity has a long-term effect on cocaine-induced neuroplasticity. Taken together, these results suggest that changes in the Wnt/β-catenin pathway effectors are long-term neuroadaptations required for the behavioral response to cocaine.

Long-term cocaine-induced neuroadaptations lead to changes in Wnt/β-catenin pathway

To the best of our knowledge, this is the first time that the Wnt/β-catenin pathway is associated with cocaine-induced long-term neuroadaptations. While other groups have demonstrated that cocaine treatments induce changes in GSK3β activity, no one has either connected them to the Wnt canonical pathway or evaluated them after 3 weeks of abstinence. For instance, and in line with our results, it has been shown that an acute injection of cocaine induced an

Table 2 Long-term effects of cocaine pretreatment on the levels of β-catenin from total homogenates of CPu and PFC are absent after LiCl pretreatment

(A)

Pretreatment		Saline			
Treatment		Saline		Cocaine	
Challenge Group		Saline Sal/Sal/Sal	Cocaine Sal/Sal/Coc	Saline Sal/Coc/Sal	Cocaine Sal/Coc/Coc
Brain area	CPu	100.0 ± 4.5	89.4 ± 11.8	219.4 ± 15.3[a]	60.1 ± 9.2[b]
	PFC	100.0 ± 3.8	112.7 ± 7.4	153.6 ± 3.3[b]	104.1 ± 4.5

(B)

Pretreatment		LiCl			
Treatment		Saline		Cocaine	
Challenge Group		Saline LiCl/Sal/Sal	Cocaine LiCl/Sal/Coc	Saline LiCl/Coc/Sal	Cocaine LiCl/Coc/Coc
Brain area	CPu	152.5 ± 21.9	119.0 ± 10.3	105.1 ± 6.7	137.7 ± 15.3
	PFC	142.0 ± 4.7	133.5 ± 19.2	118.0 ± 8.6	107.0 ± 7.2

Changes in β-catenin levels showed after saline pretreatment in PFC and CPu total homogenates (A) were absent in LiCl pretreated animals (B). Data in the table represent Mean ± SEM. Number of animals per group: 6–8

[a]Significantly different from all other groups, $p < 0.05$.

[b]Significantly different from Sal/Sal/Sal group, $p < 0.05$. Holm's Sidak *post hoc* test.

increase of GSK3β activity in the CPu of mice (Miller *et al.*, 2009), that can be linked to cocaine-induced conditioned placed preference (Miller *et al.* 2014). Furthermore, in both studies, cocaine behavioral effects were prevented with a specific GSK3β inhibitor (SB216763). On the other hand, two recent works found an increased activity of GSK3β only in the NAcc core after acute cocaine (Miller *et al.* 2014) and 2 weeks after chronic cocaine treatment in drug-free rats (Kim *et al.* 2013). In contrast with our results that showed no changes in β-catenin levels in acute-treated animals compared to control, the data presented in those studies would imply a decrease in β-catenin. This discrepancy could be linked to the way we sampled the area, since we took the whole NAcc instead of discriminating core and shell, or to the sensitivity of the quantification method used by Miller *et al.* (2014). Nevertheless, as we mentioned, none of them proposed a link between these changes in GSK3β activity and the Wnt/β-catenin pathway. Recently, one study found that 24 h after a chronic cocaine treatment, the mRNA expression of Wnt1 and DVL-2 is decreased as well as the protein levels of DVL-2, in the NAcc core (Dias *et al.* 2015). However, and in line with our previous results (Cuesta *et al.* 2016), they could not find any changes in β-catenin levels in the NAcc.

Cocaine-induced changes in Wnt/β-catenin pathway activity could be linked to the dopaminergic and/or the glutamatergic neurotransmission

In the past decade, a relationship between dopamine neurotransmission and intracellular effectors of the Wnt/β-catenin pathway has been shown (Alimohamad *et al.* 2005a, b; Sutton *et al.* 2007; Peng *et al.* 2009). Taking into account the mounting evidence about changes in dopamine and glutamate neurotransmission that underlies cocaine-induced long-term neuroadaptations in the NAcc (Russo *et al.* 2010; Kalivas and Volkow 2011; Steketee and Kalivas 2011), it is possible that cocaine-induced changes in Wnt/β-catenin pathway activity could be linked to them. For instance, it has been shown that D_1 receptor agonist (SKF83959) causes an inhibition of GSK3β activity in neuronal cell culture (Yu *et al.* 2008). Considering that after a period of abstinence from repeated cocaine D_1 receptors exhibited supersensitivity in the NAcc (Henry and White 1991; White *et al.* 1995) and that the drug challenge leads to an exacerbated DA release in this area (Kalivas and Duffy 1993; Williams *et al.* 1996), it is likely that the activation of D_1R causes the accumulation of β-catenin through inhibition of GSK3β. On the other hand, the increase in the release of glutamate induced by a cocaine challenge after withdrawal (Pierce *et al.* 1996; Baker *et al.* 2003) could also be involved, since it has been found that rats chronically treated with mGluR2/3 agonists showed an increase of Wnt canonical pathway effectors (e.g. β-catenin, Dvl-2 and 3, and GSK3β) in PFC and striatum (Sutton & Rushlow 2011). In addition, recently Peng *et al.* (2009) showed that the over-expression of β-catenin in hippocampal cell cultures mimics the effect of increased neuronal activity increasing the total dendritic length and decreasing the density of surface synaptic AMPAR clusters, as well as scaling down mEPSC amplitudes. Intriguingly, after 3 weeks of withdrawal, the surface expression of AMPAR is increased in the NAcc, while a cocaine challenge lead to a decrease, only in those animals that expressed behavioral sensitization after the challenge (Boudreau *et al.* 2007; Boudreau and Wolf, 2005; Thomas *et al.*, 2001). Then, according to Peng *et al.* (2009), cocaine-induced increase in β-catenin could be linked to the surface decrease of AMPA R found in animals that expressed behavioral sensitization. As mentioned before, our results indicate that increased levels of β-catenin in the NAcc are linked to both the expression of sensitization as well as to the cocaine challenge. Therefore, it is possible that cocaine-induced increase in β-catenin levels is mediated by a dopaminergic-glutamatergic interaction in the NAcc, and that this accumulation of β-catenin in the cytoplasm could activate the pathway as well as facilitates the removal of AMPAR from the surface after the cocaine challenge.

Regarding the CPu, we found that a cocaine challenge is linked to a decrease of β-catenin levels in those animals that showed behavioral sensitization compared to controls, and stayed similar to controls when they did not express sensitization in response to cocaine. Moreover, after 3 weeks of abstinence, β-catenin was significantly increased compared to controls. Taken together, these results suggest that behavioral sensitization requires a reduction in β-catenin, below basal levels, in order to manifest. These changes in β-catenin, and probably in the activity of the Wnt canonical pathway, might be mediated by the dopaminergic transmission through D_2R. Preclinical studies in non-human primates have shown that repeated drug exposure is associated with reductions in striatal D_2R levels (Nader *et al.* 2006). Considering that the antagonism of D_2R is linked to β-catenin accumulation (Alimohamad *et al.* 2005b) while a D_2R activation is associated with β-catenin degradation (Min *et al.* 2011), it is possible that the reduction in D_2R levels because of repeated cocaine exposure could facilitate the accumulation of β-catenin found in the CPu of abstinent animals. Interestingly, we previously showed that a similar reduction on β-catenin was present after 7 days of a chronic cocaine treatment and was not related with changes in the activity of Wnt canonical pathway (Cuesta *et al.* 2016). However, the fact that β-catenin levels in CPu increase during abstinence while decrease after a challenge may suggest that changes in the activity of the Wnt canonical pathway could be characteristic of the long-term neuroadaptations. Since our present evidence is not enough to either support or reject this hypothesis, further work must be done to fully clarify the role of this pathway in the CPu.

In the case of PFC, our previous results have shown that cocaine induced changes in Wnt canonical pathway immediately after the chronic treatment were essential for the behavioral sensitization (Cuesta *et al.* 2016). Here, we showed that the day after a cocaine challenge on day 28, β-catenin levels are similar to controls regardless of the behavioral measurement. Interestingly, as in the CPu, after the period of abstinence β-catenin levels were increased compared to the control group. However, in the PFC, the cocaine challenge fails to reduce β-catenin below control level, despite the behavioral outcome (sensitized or non-sensitized). It is possible that D_2R are involved in this mechanism as well; however, more work needs to be done to establish the relevance of these changes in cocaine-induced long-term neuroadaptations.

Long-term effects of LiCl on cocaine-induced neuroplasticity

We have previously demonstrated that LiCl administered before each cocaine injection prevented the development of sensitization by restoring β-catenin levels in PFC, CPu, and amygdala (Cuesta *et al.* 2016). In the present work, we wanted to evaluate the impact of this treatment on cocaine-induced long-term neuroadaptations. We found that LiCl alone not only caused a long-term increase in β-catenin levels in NAcc, but also blocked the changes observed in CPu and PFC. While administered before cocaine, LiCl prevented the expression of behavioral sensitization keeping the levels of β-catenin increased in the NAcc after the cocaine challenge. Moreover, the long-term changes induced by LiCl in the Wnt canonical pathway that prevent cocaine enduring neuroadaptations did not avoid the acute effect of the drug. As we argued above, the increase in the levels of β-catenin in the NAcc together with a decrease in the levels in the CPu compared to control animals were correlated with the behavioral changes. In this scenario, the LiCl results seemed contradictory at first glance. However, if we take into account that it is the fold-change of β-catenin that dictates Wnt pathway activity and not the absolute level (Goentoro and Kirschner 2009), then the LiCl results strengthen our previous assumptions that it is the change in β-catenin and the consequent activation of the canonical pathway that matters for the expression of sensitization. To the best of our knowledge, this is the first evidence of LiCl long-term molecular effects on GSK3β inhibition, keeping β-catenin levels increased. In addition, this is the first time that data reported that LiCl has a long-term effect on cocaine-induced behavioral as well as on molecular neuroadaptations. The mechanism associated with LiCl long-term effect on cocaine-induced behavioral neuroplasticity might involve distinct effects in the different areas of the motivational circuitry. We have shown that the activation of the canonical Wnt pathway blocks the development of behavioral sensitization by restoring the levels of β-catenin in the PFC and the CPu (Cuesta *et al.* 2016). These restorations could interfere with the subsequent long-term effects of cocaine. Furthermore, in this study, we showed that LiCl induced an increase in NAcc's β-catenin levels visible up to 3 weeks after the end of the treatment. So, it is possible that the long- lasting higher levels of β-catenin may reduce the NAcc AMPAR surface expression (Peng *et al.* 2009) as well as the response to the drug during the expression of cocaine sensitization. According to recent evidence, it is also possible that LiCl decreased DA release in the NAcc in response to cocaine (Can *et al.* 2016).

Conclusions

Understanding the long-term neuroadaptations induced by drugs of abuse is fundamental for the development of more successful therapies. In this work, we propose that the long-term activation of the Wnt/β-catenin pathway may be helpful to prevent some cocaine-induced neuroadaptations. Even though it is necessary to study this possibility in animal models of reinstatement/relapse, the introduction of the Wnt canonical pathway as a possible option opens a new door in terms of addiction treatment medication.

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All experiments were conducted in compliance with the ARRIVE guidelines.

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