Addiction Biology

SSA SOCIETY FOR THE STUDY OF ADDICTION

ORIGINAL ARTICLE

doi:10.1111/adb.12377

Wnt/β-catenin pathway in the prefrontal cortex is required for cocaine-induced neuroadaptations

Santiago Cuesta[1,2,3], Maria J. Severin[3], Jorgelina Batuecas[3], Silvana B. Rosso[1,3] & Alejandra M. Pacchioni[1,3]

Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina[1], Douglas Mental Health University Institute, Canada[2] and Área Toxicología, Departamento de Ciencias de los Alimentos y del Medioambiente, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (U.N.R), Argentina[3]

ABSTRACT

Behavioral sensitization is a progressive and enduring enhancement of the motor stimulant effects elicited by repeated administration of drugs of abuse. It can be divided into two distinct temporal and anatomical domains, termed initiation and expression, which are characterized by specific molecular and neurochemical changes. This study examines the role of the Wnt canonical pathway mediating the induction of cocaine sensitization. We found that β-catenin levels in the prefrontal cortex (PFC), amygdala (Amyg) and dorsal striatum (CPu) are decreased in animals that show sensitization. Accordingly, GSK3β activity levels are increased in the same areas. Moreover, β-catenin levels in nuclear fraction, mRNA expression of Axin2 and Wnt7b are decreased in the PFC of sensitized animals. Then, in order to demonstrate that changes in the PFC are crucial for initiation of sensitization, we either rescue β-catenin levels with a systemic treatment of a GSK3β inhibitor (Lithium Chloride) or inhibit Wnt/β-catenin pathway with an intracerebral infusion of Sulindac before each cocaine injection. As expected, rescuing β-catenin levels in the PFC as well as CPu and Amyg blocks cocaine-induced sensitization, while decreasing β-catenin levels exclusively in the PFC exacerbates it. Therefore, our results demonstrate a new role for the Wnt/β-catenin pathway as a required neuroadaptation in inducing behavioral sensitization.

Keywords cocaine neuroadaptations, prefrontal cortex, sensitization, Wnt canonical pathway.

Correspondence to: Alejandra M. Pacchioni, Área Toxicología, Departamento de Ciencias de los Alimentos y del Medioambiente, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario. Suipacha 531, (2000) Rosario, Santa Fe, Argentina. Email: pacchioni.alejandra@conicet.gov.ar

INTRODUCTION

Drug addiction is a chronic and enduring phenomenon that has been extensively investigated in the last decades. Recently, it has been described that animal models of addiction (non-contingent versus contingent drug administration) have remarkable overlap in terms of neurocircuitry as well as in the molecular changes underlying their respective behavioral responses (Steketee & Kalivas 2011).

Behavioral sensitization is a progressive and enduring enhancement of the motor stimulant effects elicited by repeated administration of psychostimulants (Stewart & Badiani 1993). The development of sensitization can be examined as two distinct temporal and anatomical domains termed initiation or induction, and expression. Each one is characterized by specific molecular and neurochemical changes. It has been shown that initiation is associated with changes in the ventral tegmental area (VTA) and prefrontal cortex (PFC) (Schenk & Snow 1994; Tzschentke & Schmidt 1998; Beyer & Steketee 1999; Li *et al.* 1999), while expression is linked to changes in the nucleus accumbens (NAcc) (Pierce *et al.* 1996; Boudreau & Wolf 2005). A great deal of evidence shows that changes in synaptic plasticity underlie both initiation and expression (Vanderschuren & Kalivas 2000; Thomas, Kalivas, & Shaham 2008; Steketee & Kalivas 2011). The initiation of sensitization has been shown to be disrupted by Ibotenic acid lesions in both prelimbic and infralimbic regions of the PFC (Li *et al.* 1999). Moreover, several studies have suggested that cocaine induces a functional decrease of D_2R in the

PFC that would serve to enhance excitatory transmission to subcortical regions (Williams & Steketee 2005; Nogueira, Kalivas, & Lavin 2006; Kroener & Lavin 2010; Liu & Steketee 2011). Together, this evidence has revealed an interaction between dopaminergic and glutamatergic neurotransmission underlying cocaine-induced sensitization.

In the present study, we used a noncontingent drug-administration paradigm, behavioral sensitization, to model addiction-like behavioral responses in order to investigate the role of Wnt factors pathways. Wnt factors signal in axon pathfinding, dendritic development and synapse assembly in both the central and peripheral nervous systems. Wnts also modulate the basal synaptic transmission, and the structural and functional plasticity of synapses in the central nervous system (Salinas 2012). 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 & Inestrosa 2000; Kozlovsky, Belmaker, & Agam 2002). For instance, Alimohamad *et al.* (2005a) showed that amphetamine increases GSK3β activity and decreases β-catenin levels in the PFC and striatum, while D_2R antagonists produce the opposite effect. Despite the relevance to cocaine effects of dopamine and its receptors, little is known about the role of Wnt signaling pathways in drug addiction.

The Wnt growth factors belong to a large family of secreted proteins, which can signal through different receptors including Frizzled (Fz) (Logan & Nusse 2004) and the atypical tyrosine kinase receptors Ror2 and Ryk (Lu *et al.* 2004; Hayashi *et al.* 2009). 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 & Salinas 2005). 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 (Logan & Nusse 2004; Metcalfe & Bienz 2011). While in the absence of Wnt, GSK3β phosphorylates β-catenin marking it for degradation by the proteasome (Maguschak & Ressler 2012) (Fig. 5a). Wnt signaling is also regulated by the presence of a physiological antagonist: Dickkopf-1 (Dkk-1), a secreted protein that specifically blocks the canonical Wnt pathway by binding to LRP6 (Bafico *et al.* 2001). Recently, increased Dkk-1 levels have been linked to deficits in dopaminergic transmission (Galli *et al.* 2014) as well as to neurodegenerative disease (Salinas 2012).

Numerous studies have suggested that regulation of GSK3β activity might be associated with cocaine-induced neuroadaptations. Not only does cocaine produce changes in GSK3β activity in the striatum, but inhibitors of GSK3β, both targeted (e.g. SB 216763) and non-selective (e.g. valproate or LiCl), prevent cocaine-induced sensitization (Perrine, Miller, & Unterwald 2008; Miller, Tallarida, & Unterwald 2009; Miller *et al.* 2014). However, none of these works have established a relationship between cocaine, GSK3β and the Wnt canonical pathway. Therefore, our main goal was to evaluate whether the Wnt canonical pathway is involved in cocaine-induced neuroadaptations such as the induction of behavioral sensitization. In the present study, we combined molecular and behavioral studies with pharmacological strategies in order to evaluate the relevance of the Wnt/β-catenin pathway for cocaine-induced behavioral sensitization.

MATERIALS AND 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, 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) and Sulindac were obtained from Sigma (St. Louis, MO). Cocaine and LiCl were dissolved in saline, while Sulindac was in (2-hydroxypropyl)-β-cyclodextrin (5% w/v).

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 hr habituation and the 2 hrs 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).

Cocaine behavioral sensitization paradigm

The sensitization paradigm consisted of seven daily injections (2 × 15 mg/kg i.p., 5 × 30 mg/kg i.p.) and had been previously used to describe a variety of cocaine-induced neuroadaptations which were later confirmed by cocaine self-administration (Kalivas & Duffy 1993; Pierce *et al.* 1996; Boudreau & Wolf 2005; Boudreau *et al.* 2007; Pacchioni *et al.* 2009). The motor activity was recorded after the first and last injections.

Surgical procedures

One week before the drug or saline treatment, animals were anesthetized with a ketamine (85 mg/kg i.p.)/xylazine (2.5 mg/kg i.p.) mixture, and were placed in a stereotaxic frame (Stoelting, USA). Bilateral cannulae (8 mm, 23 gauge) targeted the PFC or Caudate Putamen (CPu) according to the following coordinates (in mm): AP + 2.9, L ± 0.5, DV-2.0 or AP-0.2, L ± 3.0, DV-3.4, respectively (Paxinos & Watson 1997). Cannulae were secured to the skull using jeweler's screws and dental acrylic. All animals received daily injections of Ketorolac (2 mg/kg i.p.) before anesthesia and for 3 days afterwards, and were allowed to recover for one week.

Microinjection of Sulindac

An injection needle (30 gauge) was introduced into each guide cannula and extended 1.5 mm below the tip. Bilateral infusions of 5 μg/μl/side were made over 180 s, and the injectors were removed 60 s later.

Tissue preparation

Rats were euthanized 3 or 24 hrs after the last injection of cocaine or saline, and their brains were removed. The PFC, NAcc, Amygdala (Amyg) and CPu were dissected according to Heffner, Hartman, & Seiden (1980) using a rat brain matrix. Tissue was kept at −80°C until analysis. Protein concentration was measured using the Lowry assay.

Total homogenates

Tissues were homogenized on ice with RIPA buffer supplemented with phosphatase and protease inhibitors (2 μg/ml aprotinin; 2 μg/ml leupeptin; 1 μg/ml pepstatin; 100 μg/ml PMSF; 1 mM Na_3VO_4; 50 mM NaF), and centrifuged for 5 min at 13 000 *g*.

Subcellular fractionation

In a different set of animals, brain tissue was dissected, and subcellular fractionation was performed as previously described by Pacchioni *et al.* (2009) with slight modifications. More information about the experimental procedure can be found in the Supporting Information (Fig. S1).

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 % SDS-polyacrylamide gel and transferred to nitrocellulose membrane. A secondary horseradish peroxidase-conjugated antibody (Sigma, St. Louis, MO) followed overnight incubation with primary antibody (β-catenin 1:10 000, phospho-GSK3β-Y216 1:8000, total GSK3β 1:10 000; BD BioScience, San Jose, California). Reactivity was detected using enhanced chemiluminescence (ECL) and quantified using Gel-Pro Plus software package. Total homogenates blots were also incubated with antitubulin (1:14 000; Sigma, St. Louis, MO) or total GSK3β to correct for differences in protein loading.

Extraction of mRNA and RT-PCR

Fresh tissue from PFC was homogenized in TRIzol (Invitrogen, Waltham, Massachusetts) and processed according to the manufacturer's instructions. Details on cDNAs synthesis and PCR procedure can be found in the Supporting Information. Primers were selected using Primer3 free software (Rozen & Skaletsky 2000) (Table S1). PCR products were separated on 1 % agarose gel stained with ethidium bromide and then observed under UV light. Optical densities (OD) of PCR products were measured using the Gel-Pro Plus software package and normalized to OD values from 18S. Unless specifically stated, all RT-PCR reagents were from Promega (Madison, WI).

Experimental procedures

Acute cocaine, chronic cocaine and saline treatments

Subjects were assigned to one of the three conditions: saline, acute cocaine or chronic cocaine (Fig. 1a). All animals received one injection per day for 7 days. Saline group: animals received saline (1 ml/kg i.p). Acute cocaine: rats received saline (1 ml/kg i.p) on days 1 to 6 and cocaine (15 mg/kg i.p) on day 7. Chronic cocaine: animals received 15 mg/kg cocaine (i.p) on days 1 and 7, and 30 mg/kg cocaine i.p on days 2 to 6. Locomotor activity was recorded after injection on days 1 and 7. 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 sensitization was defined as a

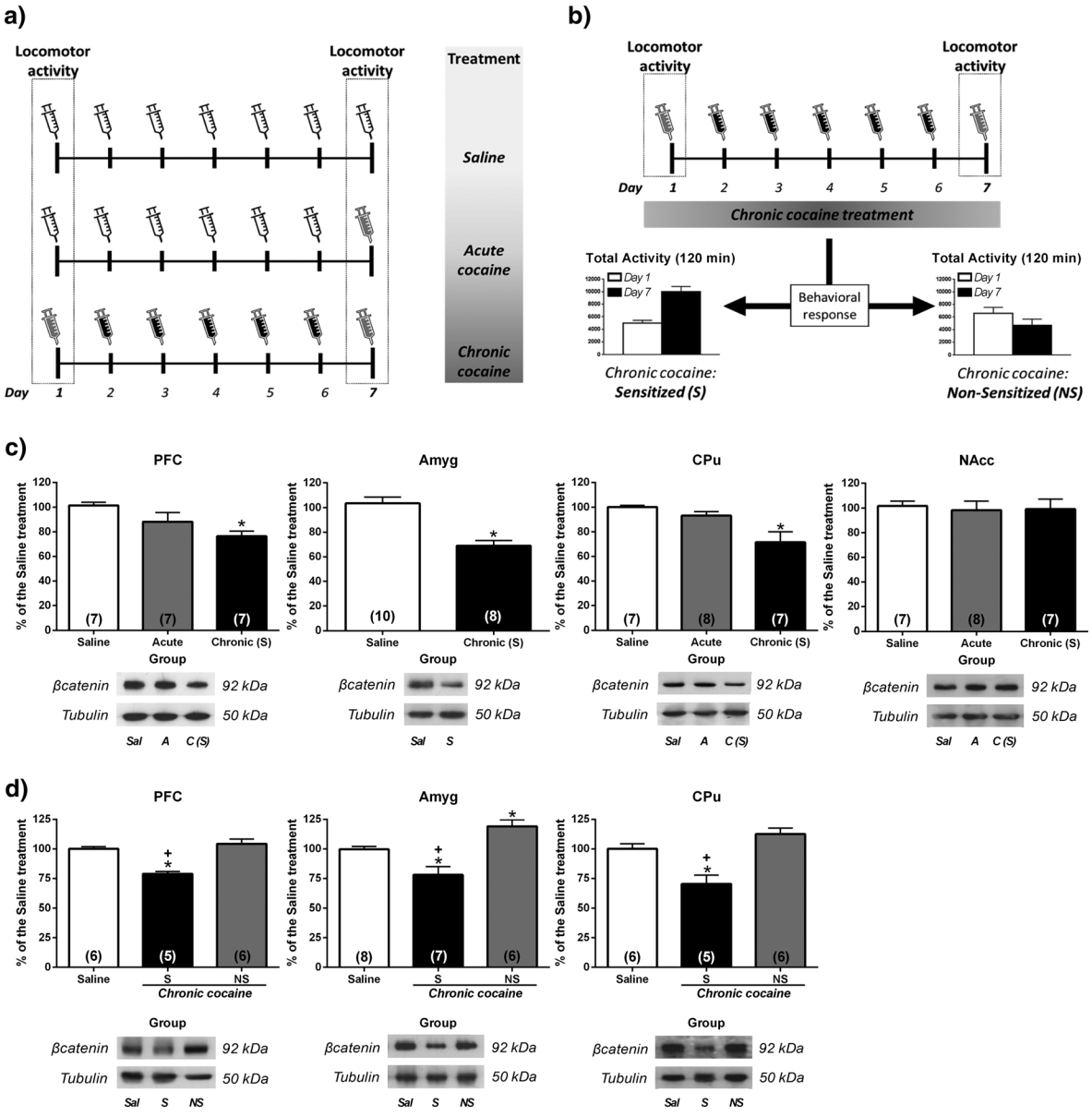

Figure 1 Cocaine-induced sensitization leads to decreased β-catenin expression in PFC, Amyg and CPu. a) Adult rats received: seven saline injections (saline), saline from day 1 to 6 and cocaine on day 7 (acute cocaine), or seven daily injections of cocaine (chronic cocaine). On days 1 and 7 all rats were behaviorally tested immediately after the injection. The white syringe represents the saline injection (1 ml/kg), the grey 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). Levels of β-catenin were measured in brain areas obtained from all treated animals sacrificed on the day after the last injection. c) First, we compared the levels of β-catenin in the control groups (saline and acute cocaine) against the chronic cocaine group that sensitized (chronic cocaine (S)). d) Second, in order to study if the changes in β-catenin were related to the cocaine treatment only or to the behavioral display of sensitization, we compared the β-catenin levels in rats from the chronic cocaine group that developed the sensitization with those rats that did not develop the sensitization (NS). Bars represent mean ± SEM. Number of animals (n) are represented inside each bar. *Significantly different from Saline treatment, $p < 0.05$; + significantly different from Non-sensitized, $p < 0.05$. Bonferroni post hoc test

minimum of 20 % increase in total activity counts on day 7 compared to day 1 (Pierce *et al.* 1996).

LiCl and Sulindac pretreatments

LiCl pretreatment: animals received LiCl (30 mg/kg i.p.) or Saline (1 ml/kg i.p.) injections 30 min before each saline or cocaine injection, leading to four groups: Saline/Saline, LiCl/Saline, Saline/Cocaine and LiCl/Cocaine.

Sulindac pretreatment: animals received Sulindac (5 μg/μl/side) or Vehicle (1 μl/side) infusions 60 min before each saline or cocaine injection, leading to four groups: Vehicle/Saline, Sulindac/Saline, Vehicle/Cocaine and Sulindac/Cocaine.

Locomotor activity was recorded on days 1 and 7. Animals were not separated based on their behavioral response to cocaine.

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 one-way ANOVA followed by Bonferroni's *post hoc* test or a Student's *t* test with significance set at $p < 0.05$, depending on the number of groups under consideration.

RESULTS

Cocaine-induced sensitization leads to decreased β-catenin expression in PFC, Amyg and CPu

Behavioral sensitization to cocaine depends on a number of factors conferring individual vulnerability (Deroche-Gamonet & Piazza 2014). It has been demonstrated that many (60 %), but not all, animals develop sensitization after cocaine exposure (Pierce *et al.* 1996; Boudreau & Wolf 2005; Boudreau *et al.* 2007). Because our behavioral results, described in Table S2, showed a similar pattern, we investigated whether molecular changes in β-catenin, the final effector of the canonical Wnt pathway, were linked to cocaine-induced behavioral sensitization. Therefore, we measured β-catenin levels as a readout for canonical Wnt signaling in brain areas relevant to addiction such as the PFC, NAcc and CPu (Metcalfe & Bienz 2011).

Repeated drug treatment induces neuronal adaptations that result in the development of behavioral sensitization. Thus, we compared the levels of β-catenin in rats that showed sensitization after a chronic cocaine treatment against control animals (acute cocaine and saline groups). Data collected 24 hrs after the last injection, shown in Fig. 1c, revealed that β-catenin expression was significantly decreased in the PFC and CPu of sensitized animals compared to saline animals. Moreover, no significant changes were found in β-catenin levels after acute injection in any of the studied areas compared to saline. A one-way ANOVA analysis of Fig. 1c revealed a significant effect of treatment in the PFC [$F(2, 18) = 5.797$; $p < 0.05$], and CPu [$F(2, 19) = 8.175$; $p < 0.01$]. Based on these results, Amyg tissue was collected from a different set of animals that received chronic, but not acute cocaine treatment, and developed sensitization. A simple comparison revealed a statistically significant decrease in β-catenin levels in the Amyg of sensitized animals compare to saline controls (*t* test, $p < 0.001$). No changes in β-catenin levels were found in the NAcc [(%) Saline: 101.70 ± 4.08, Acute cocaine: 98.38 ± 7.27, Chronic cocaine (Sensitized): 99.29 ± 8.08]. Similar to 24 hrs, when animals were sacrificed 3 hr after the last cocaine injection, β-catenin protein levels were decreased in PFC and CPu, and no changes were found in NAcc (Fig S2). This shows that cocaine-induced sensitization leads to changes in β-catenin expression specifically in the PFC, CPu and Amyg. Next, we conducted a second experiment where we included rats that did not develop sensitization (Fig. 1d). A one-way ANOVA analysis revealed a significant effect of behavioral response on β-catenin levels in the PFC [$F(2, 14) = 20.69$; $p < 0.0001$], Amyg [$F(2, 18) = 14.97$; $p < 0.0001$], and CPu [$F(2, 14) = 13.94$; $p < 0.0005$]. The animals that did not develop behavioral sensitization (Non-sensitized group) did not show changes in the levels of β-catenin compared to saline group, suggesting no changes in the activity of the canonical Wnt pathway. In summary, β-catenin levels are associated with the development of sensitization and the particular brain area being studied. Moreover, the fact that an acute cocaine injection did not modify β-catenin expression strengthens the idea that these changes are a neuroadaptation to repeated cocaine associated with behavioral sensitization.

Cocaine-induced sensitization is associated with a functional decrease of β-catenin in the PFC

To evaluate if the changes in β-catenin levels found in total homogenates of sensitized animals were the result of a functional decrease of the Wnt canonical pathway, we compared the GSK3β activity levels in tissues of cocaine-sensitized animals and controls. Figure 2a shows GSK3β activity levels in the PFC, Amyg and CPu evaluated as the phosphorylation level of Tyrosine 216 (activator site). The simple comparison of the data in Fig. 2a revealed that GSK3β activity was significantly increased in cocaine-sensitized animals compared to saline in all tested areas (*t* test, $p < 0.05$). This indicates that GSK3β is significantly activated in all brain areas that had previously shown a decrease in β-catenin levels after cocaine-induced sensitization. Then, fresh brain tissue obtained from the PFC and CPu of another set of animals was submitted to subcellular fractionation and β-catenin was measured in the nuclear fraction (Fig. 2b). Our data showed that β-catenin levels were only decreased in the nuclear fraction of the PFC (*t* test, $p < 0.05$) but not of the CPu (*t* test, $p = 0.5435$). Considering that β-catenin not only is the final effector of the Wnt canonical pathway, but also has a role as a membrane protein that participates in dendritic remodeling (Clevers & Nusse 2012), we carried out an in-depth analysis by examining the expression of β-catenin in the PFC membrane fraction that showed no changes during cocaine-induced sensitization

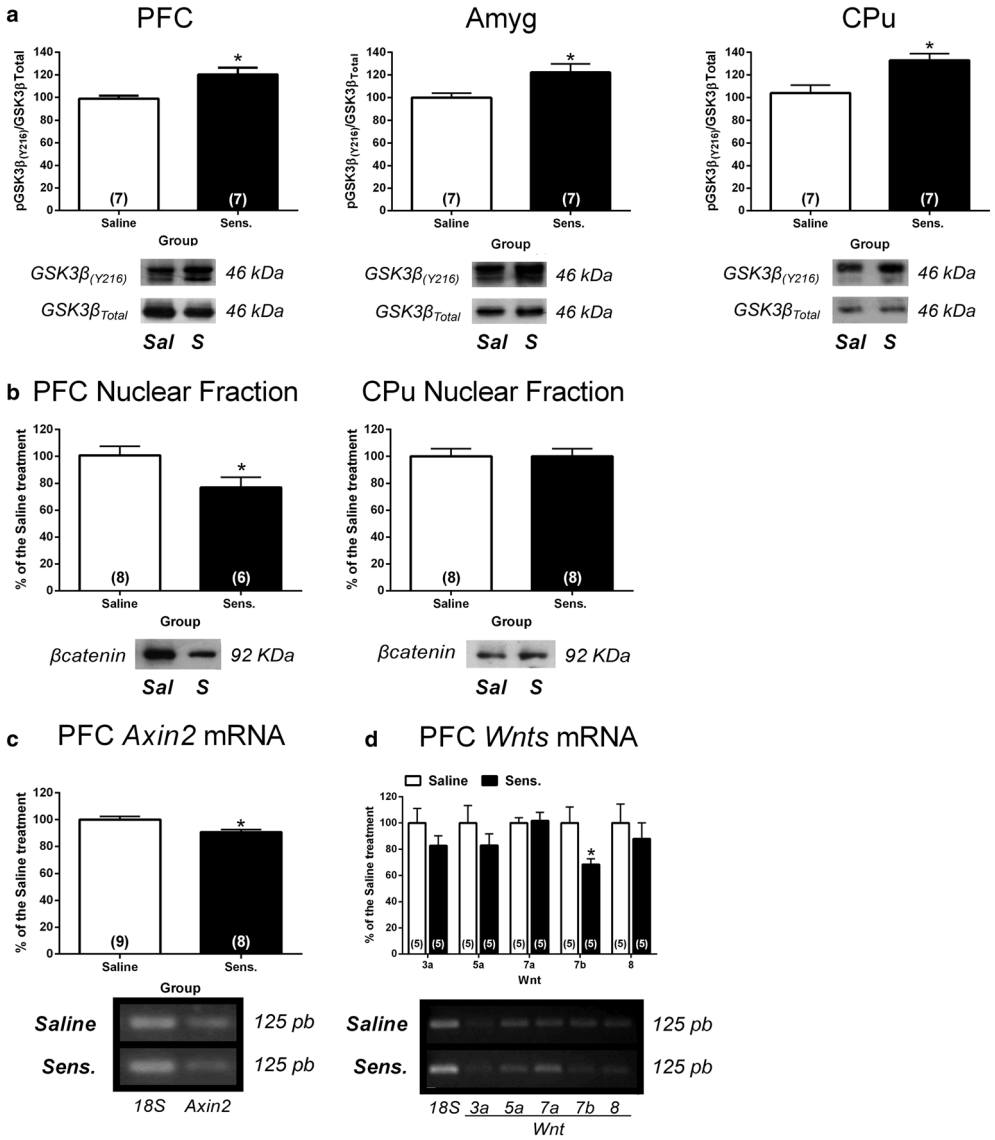

Figure 2 *Cocaine-induced sensitization is associated with a functional decrease of β-catenin in PFC.* a) GSK3β activity levels were measured by comparing GSK3β phosphorylated in Y216 and Total GSK3β through Western blot in the PFC, Amyg and CPu of sensitized animals. *Significantly different from Saline treatment, PFC, $p < 0.01$; Amyg, $p < 0.03$ and CPu, $p < 0.01$, *t* test. b) β-catenin levels in the nuclear fraction of the PFC and the CPu obtained by subcellular fractionation of control and sensitized animal brains areas. *Significantly different from Saline treatment, $p < 0.05$; *t* test. c) mRNA expression of Axin2 and d) Wnt factors were also measured in PFC. *Significantly different from Saline treatment, $p < 0.05$, *t* test. All animals were sacrificed 24 hrs after the last injection. Bars represent mean ± SEM. Number of animals (*n*) are represented inside each bar

(Fig S3). In line with these results, we also found, through RT-PCR, that Axin2 mRNA levels (a β-catenin-targeted gene and a general indicator of Wnt canonical pathway activity (Clevers & Nusse 2012)) was reduced in cocaine-sensitized animals compared to the control group (*t* test $p < 0.01$) (Fig. 2c). Thus far, the results presented here suggest that cocaine-induced sensitization is associated with a functional decrease of β-catenin in the PFC but not the CPu. More studies need to be performed in the Amyg in order to establish whether cocaine-induced sensitization is associated with a functional decrease of β-catenin in this area.

Finally, we investigated if the functional changes in the canonical Wnt pathway found in the PFC, which point to an inhibition of the pathway, were a consequence of decreased expression of Wnt factors. We therefore examined the mRNA expression of different Wnt factors using RT-PCR (Fig. 2d). We analyzed the expression of a variety of Wnt factors, such as 3a, 5a, 7a, 7b and 8 in the PFC tissue, and we found that Wnt7b mRNA

expression was significantly decreased in cocaine-sensitized animals compared to the saline(t test $p < 0.03$).

Preventing β-catenin reduction with systemic lithium chloride blocks cocaine-induced behavioral sensitization

To test the hypothesis that changes in β-catenin levels were responsible for cocaine-induced sensitization, we treated animals with LiCl (30 mg/kg i.p.) or Saline before each cocaine injection. LiCl is a nonspecific inhibitor of GSK3β activity, which means that given before cocaine, it would prevent cocaine-induced decrease of β-catenin levels, and therefore it would block cocaine-induced behavioral sensitization. It is important to note that the dose we administered was lower than the one previously used in the literature (e.g. LiCl 100 mg/kg i.p. (Xu *et al.* 2009)), and that the chosen cocaine-induced sensitization regimen was one that allowed us to study the effect of LiCl. Interestingly, we found that 30 mg/kg i.p. blocked sensitization (Fig. 3a) while preventing the decrease in β-catenin levels in PFC total homogenates (Fig. 3b) as well as nuclear fraction (Fig. 3c). A two-way repeated measures ANOVA of behavioral data of Fig. 3a showed a main effect of treatment [$F(1,40) = 119.53$, $p < 0.0001$], and a significant interaction between pretreatment × treatment × time [$F(1,40) = 13.29$, $p < 0.001$]. Meanwhile, a two-way ANOVA applied on β-catenin levels in PFC total homogenates revealed a significant interaction between treatment and pretreatment [$F(1,32) = 26.77$, $p < 0.0001$]. A similar analysis in nuclear fractions showed a significant effect of pretreatment [$F(1,30) = 17.50$, $p < 0.0005$]. Moreover, we found that LiCl prevented cocaine-induced β-catenin changes in the Amyg and CPu (Fig S4) whereas in the NAcc, LiCl increased β-catenin levels regardless of drug treatment. This was an expected finding suggested by previous studies showing an increase of phosphorylated GSK3β in the NAcc after LiCl (Xu *et al.* 2009; Xu *et al.* 2011) (Fig S4).

Inhibiting PFC's Wnt canonical pathway with Sulindac develops cocaine-induced behavioral sensitization

To test whether or not β-catenin changes were necessary for cocaine-induced behavioral sensitization, we submitted animals to Sulindac infusions (Wnt canonical pathway inhibitor (Lee *et al.* 2009)) in the PFC or CPu before each cocaine injection. After a week of recovery, animals underwent seven daily cocaine injections. Because our previous results showed that cocaine-induced sensitization might involve an inhibition of the Wnt canonical pathway, we used a lower dose of cocaine (7 × 15 mg/kg/day) to avoid a possible behavioral ceiling effect that might have occurred if the previous cocaine regime had been used. As previously done, locomotor activity was tested for 2 h on days 1 and 7. Between days 2 and 6 of the treatment animals also received a bilateral infusion of Sulindac (5 μg/μl/side) or Vehicle in the PFC or CPu, and stayed in their home cages until the cocaine injection. As we anticipated, we found that Sulindac infusions facilitate the development of cocaine-induced sensitization when infused in PFC (Fig. 4a), but not in CPu (Fig S5a). Despite the fact that Sulindac decreased β-catenin levels in both areas, PFC (Fig. 4b) and CPu

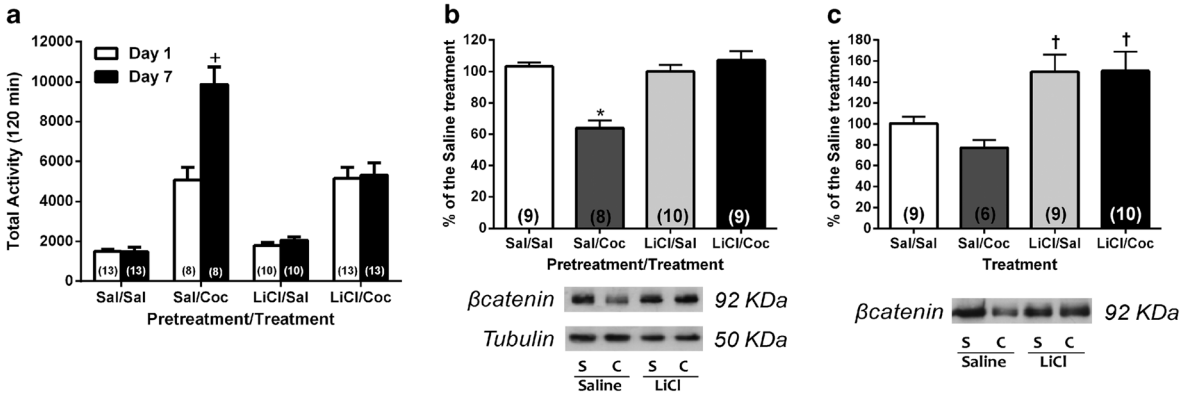

Figure 3 Preventing β-catenin reduction with systemic Lithium Chloride blocks cocaine-induced behavioral sensitization. Animal were pretreated with LiCl (30 mg/kg i.p.) or saline (1 ml/kg i.p.) 30 min before each injection of cocaine or saline; and their locomotor activity was tested after first and last injection. Rats were sacrificed 24 hrs after last injection, their brains were dissected and β-catenin was measured in total homogenates. a) Total locomotor activity measured on day 1 and 7 showed that cocaine induced behavioral sensitization in Sal/Coc, while LiCl pretreatment blocked the initiation of cocaine sensitization. b) β-catenin levels in PFC total homogenates were significantly decreased in Sal/Coc group while LiCl pretreatment restored protein levels. c) Pretreatment with LiCl restored β-catenin levels in PFC nuclear fractions. Bars represent mean ± SEM. Number of animals (n) are represented inside each bar. + Significantly different from day 1, $p < 0.05$; *significantly different from all other groups, $p < 0.05$; † significantly different from Sal/Coc group, $p < 0.05$. Bonferroni post hoc test

(Fig S5b), only changes in the PFC were able to induce sensitization. It is important to point out that Sulindac only modified β-catenin levels in the area where it was administered. No changes were found in other areas such the Amyg (Fig. 4c) or CPu (Fig. 4d) during PFC infusions; nor in the PFC (Fig S5c) or Amyg (Fig S5d) during CPu infusions. A two-way repeated measures ANOVA of behavioral data from Fig. 4a showed significant main effect of treatment [$F(1,30) = 63.56$, $p < 0.001$], and interaction between pretreatment × treatment × time [$F(1,30) = 8.134$, $p < 0.01$]. A two-way ANOVA of β-catenin levels in PFC showed significant effects of treatment [$F(1,21) = 6.552$, $p < 0.05$] and pretreatment [$F(1,21) = 20.40$, $p < 0.0005$] (Fig. 4b).

DISCUSSION

The current study proposes a new role for the Wnt/β-catenin pathway in cocaine-induced neuroadaptations underlying behavioral sensitization. Our main findings were: (1) chronic cocaine induced a decrease of β-catenin levels in the PFC, CPu and Amyg compared to saline treated animals, while no changes were found in the NAcc; (2) those changes were only present when animals showed behavioral sensitization; (3) in line with β-catenin observations, GSK3β activity levels were increased in the PFC, CPu and Amyg of sensitized animals; and, (4) β-catenin levels in nuclear fraction, and mRNA expression of Axin2 as well as Wnt7b were decreased in

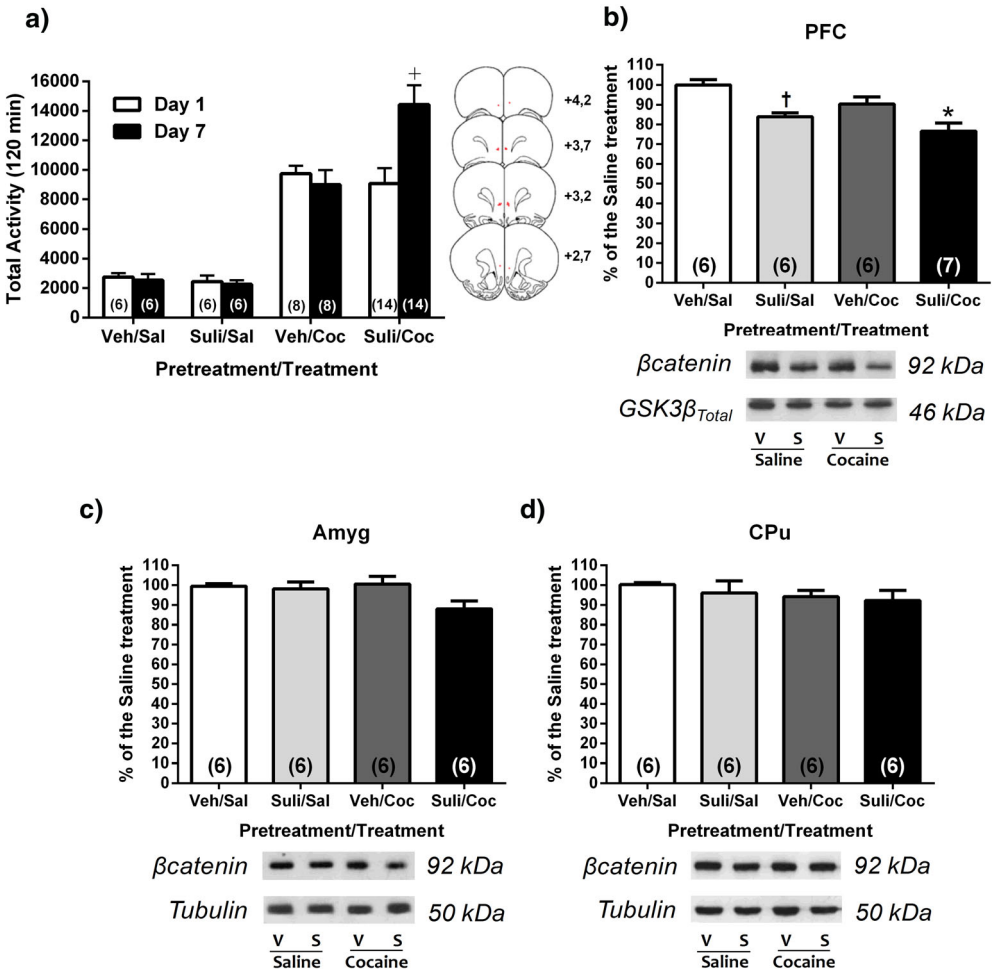

Figure 4 Inhibiting PFC's Wnt canonical pathway with Sulindac develops cocaine-induced behavioral sensitization. Rats were pretreated with Sulindac (5 μg/μl/side) or Vehicle [(2-hydroxypropyl)-β-cyclodextrin] an hour before each injection on days 2 to 5 of the treatment. Locomotor activity was measured for 2 hr after cocaine or saline injections on days 1 and 7. Twenty four hours after the last injection animals were sacrificed and brains were dissected in order to measure β-catenin levels in total homogenates. a) Total locomotor activity measured on day 1 and 7 showed that seven injections of 15 mg/kg i.p. of cocaine in Sal/Coc group did not induce sensitization while it did induce sensitization when a Sulindac pretreatment was given. b) β-catenin levels were measured in total homogenates of PFC, Amyg and CPu from animals infused with Sulindac in PFC and sacrificed 24 hrs after finishing the treatment. Bars represent mean ± SEM. Number of animals (n) are represented inside each bar. + Significantly different from day 1 of same group, $p < 0.001$; *significantly different from Veh/Sal and Veh/Coc group, $p < 0.05$; † significantly different from Veh/Sal group, $p < 0.05$. Bonferroni post hoc test

the PFC of sensitized animals. Our results imply that changes in the Wnt/β-catenin pathway effectors are required neuroadaptations to induce behavioral sensitization, proposing a new role for this pathway (Fig. 5). In order to demonstrate that these changes in PFC are necessary for developing sensitization, we either rescued β-catenin levels with a systemic treatment of a GSK3β inhibitor (LiCl) or inhibited the Wnt/β-catenin pathway with an intracerebral infusion of Sulindac, before each cocaine injection. As expected, rescuing β-catenin levels in the PFC as well as CPu and Amyg blocked cocaine-induced sensitization, while decreasing β-catenin levels exclusively in the PFC exacerbated it. This highlights the relevance of the PFC's Wnt canonical pathway for the initiation of cocaine-induced sensitization.

To our knowledge, this is the first time that the Wnt/β-catenin pathway is associated with cocaine-induced neuroadaptations underlying behavioral sensitization. While other groups have demonstrated that cocaine treatments induce changes in GSK3β activity, no one has connected these changes to the Wnt pathway. For instance, and in line with our results, an acute injection of cocaine induced an increase of GSK3β activity in the CPu of mice (Miller *et al.* 2009). These results suggest a decrease in β-catenin levels, but this was not measured by the authors. Moreover, in the same study GSK3β inhibitors administered prior to a cocaine injection reduced locomotor sensitization (Miller *et al.* 2009) as well as conditioned place preference (Miller *et al.* 2014). While we did not find any β-catenin changes in the NAcc, others found that higher levels of GSK3β activity in the rat NAcc core contributed not only to cocaine-induced hyperactivity (Kim *et al.* 2013) but also to the development of cocaine-induced locomotor sensitization (Xu *et al.* 2009). This discrepancy might be because we sampled the entire area instead of discriminating core and shell.

Repeated cocaine administration progressively enhances locomotor responses, leading to behavioral sensitization (Pierce & Kalivas 1997; Robinson & Berridge 2001). Although the role of sensitization in addiction may be debatable, data collected from sensitization studies has proven to be predictive of the neurochemical circuitry of relapse/reinstatement behaviors (Steketee & Kalivas 2011). The neurobiological basis of cocaine-induced sensitization has been extensively studied. It has been shown that sensitization is the result of a complex series of changes in dopaminergic as well as glutamatergic connections in the mesocorticolimbic pathway: the VTA and PFC seem to be important during initiation, while the NAcc seems to play a role in expression of sensitization (Pierce & Kalivas 1997; Vanderschuren & Kalivas 2000; Steketee & Kalivas 2011). Indeed, it has been shown that Ibotenic acid lesions of the PFC, which encompass both the prelimbic and infralimbic regions, disrupt the induction of sensitization to cocaine (Li *et al.* 1999). We focused our study on the possible role of the Wnt/β-catenin pathway in the initiation of cocaine-induced sensitization. In

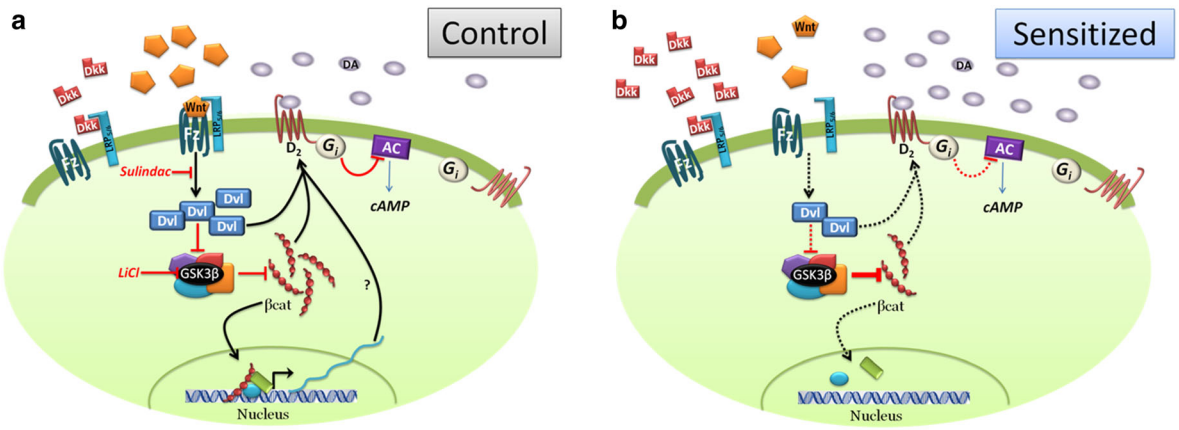

Figure 5 Schematic summary of cocaine-induced neuroadaptations on Wnt canonical pathway in the PFC that underlies behavioral sensitization. a) In the absence of Wnt, GSK3β phosphorylates β-catenin marking it for degradation by the proteasome. Upon activation of the Wnt canonical pathway, GSK3β is inhibited and leads to the stabilization of β-catenin and its subsequent translocation to the nucleus where it regulates the expression of Wnt target genes such as Axin2. It has been shown that both β-catenin and Dvl—and perhaps some of the Wnt target proteins—interact with the D_2R (Sutton *et al.* 2007; Min *et al.* 2011). b) Cocaine-induced behavioral sensitization leads to a functional decrease in β-catenin levels. This inhibition could be produced by the decrease in the expression of the Wnt7b mRNA, or may involve an increase in Dkk-1 release. Any of these two mechanisms would consequently lead to a decrease in Dvl and β-catenin levels. Because D_2R interacts with Dvl and β-catenin, cocaine-induced inhibition in the Wnt/β-catenin pathway could result in the functional decrease in dopamine neurotransmission associated with behavioral sensitization. Dkk: Dickkopf-1, Dvl: Dishevelled, Fz: Frizzled receptor, D2: Dopamine D2-like receptor, Gi: Inhibitory G protein, AC: Adenylyl Cyclase, βcat: β-catenin, GSK3β: Glycogen synthase kinase 3β, DA: Dopamine. Black arrows: activation (filled: normal activity, dotted: reduced activity); red blunted arrows: inhibition (filled: normal activity, thickened: increased activity, dotted: reduced activity)

the PFC, we found changes in Wnt/β-catenin's intracellular effectors and in the expression of genes regulated by β-catenin, both of which correlate with behavioral sensitization. Altogether, these changes suggested that behavioral sensitization is associated with an inhibition of Wnt/β-catenin pathway in the PFC. Furthermore, we also showed that manipulations of this pathway either by a systemic treatment (i.e. LiCl) or an intra-PFC infusion (Sulindac) changed the behavioral response as we expected. That is to say, LiCl reverses β-catenin levels and blocks sensitization while Sulindac decreases β-catenin levels and exacerbates sensitization. It is important to highlight the fact that we blocked the behavioral response with a lower dose of LiCl than the one previously used in the literature (Miller *et al.* 2009; Xu *et al.* 2009) and that we could associate the behavioral response with changes in β-catenin levels. Moreover, the intracerebral infusion of Sulindac only affected the behavior when administered in the PFC but not in CPu, while β-catenin levels were decreased after infusions in both areas. In summary, changes in Wnt/β-catenin pathway in the PFC are crucial for cocaine-induced neuroadaptations that underlie behavior, while changes in the CPu are necessary but not sufficient. Further work must be done to elucidate whether the changes found in the Amyg are necessary for sensitization.

Interestingly, a relationship between dopamine neurotransmission and intracellular effectors of the Wnt/β-catenin pathway has been shown in the past decade. For instance, amphetamine induced a decrease in β-catenin in the VTA while the opposite effect happened after a treatment with a D_2R antagonist not only in the VTA but also in PFC and striatum (Alimohamad *et al.* 2005b,a). As a possible mechanism, Min *et al.* (2011) recently proposed that D_2R inhibits TCF/LEF-1 dependent transcriptional activities by directly interacting with β-catenin, leading to a reduction of its distribution to the nucleus. Our own results do not support this, as we did not see an increase in β-catenin in the membrane fraction. Moreover, other groups found that antipsychotics specifically increased Dvl-3 protein levels (Alimohamad *et al.* 2005b; Sutton *et al.* 2007) and demonstrated an interaction between Dvl-3 and D_2R that may explain the effect of antipsychotics on the Wnt canonical pathway (Sutton *et al.* 2007). Indeed, it has been shown that over-expression of Dvl is sufficient to stabilize β-catenin and increase TCF/LEF-1 mediated gene transcription (Smalley *et al.* 1999; Uematsu *et al.* 2003b,a). In line with this, we showed that repeated intra-PFC infusions of Sulindac, which inhibits Wnt/β-catenin pathway by blocking Dvl's PDZ domain (Lee *et al.* 2009), enhanced cocaine-induced sensitization (Fig. 5b).

Several studies have suggested a role of the PFC in initiation of cocaine sensitization: cocaine induces a functional decrease of D_2R in the PFC that would serve to enhance excitatory transmission to subcortical regions (Williams & Steketee 2005; Nogueira *et al.* 2006; Kroener & Lavin 2010; Liu & Steketee 2011). Therefore, it is possible that cocaine-induced inhibition in the Wnt/β-catenin pathway starts at, or upstream of, Dvl and is related to a functional decrease in dopamine neurotransmission. In fact, Galli *et al.* (2014) have recently demonstrated that inducible expression of Dkk-1, a physiological inhibitor of the Wnt/β-catenin pathway (Bafico *et al.* 2001) in adult mice striatum decreases D_1R and D_2R clusters, leading to deficits in dopaminergic transmission. Hence, another possibility that needs to be tested is whether chronic cocaine decreases Wnt synthesis or increases Dkk-1 levels. However, the fact that we found significantly lower levels of Wnt7b mRNA in the PFC points out to a decrease in Wnt synthesis. Interestingly, Wnt7-Dvl signaling has been associated to presynaptic assembly and neurotransmitter release (Ahmad-Annuar *et al.* 2006) (Fig. 5b).

As far as we know, this is the first time that the Wnt canonical pathway is involved in cocaine-induced neuroadaptations that underlie behavioral changes. Here we described how inhibition of the Wnt/β-catenin pathway is associated with initiation of cocaine-induced behavioral sensitization. Specifically, we found decreased β-catenin levels and increased activity of GSK3β in areas such as the PFC, CPu and Amyg of sensitized animals. In addition, functional inhibition of Wnt/β-catenin pathway was demonstrated in the PFC but not in the CPu. These results suggest that only the changes found in the PFC are associated with the Wnt canonical pathway, while the changes in the CPu might be a result of the structural role of β-catenin. Accordingly, we showed that inhibition of the Wnt canonical pathway at the level of Dvl in the PFC exacerbates initiation of cocaine-induced sensitization. We therefore hypothesize that the inhibition in the Wnt/β-catenin pathway observed in the PFC of sensitized animals may be associated with a functional decrease of Dvl leading to a disconnection of D_2R. Altogether, our results indicate a new role for the Wnt/β-catenin pathway in cocaine-induced neuroadaptations and highlight, once again, the importance of the PFC as a biological substrate of cocaine-induced sensitization. Because locomotor sensitization in rodents seems to share plastic mechanisms with drug addiction in humans, and correspond to aspects of drug abuse such as initiation and compulsive drug-seeking behavior (for review see Steketee & Kalivas (2011)), our findings suggest that the Wnt canonical pathway may be involved in the early stages of substance abuse. Although one must always be wary of extrapolating

clinical relevance from animal data, the considerations discussed above suggest that Wnt pathways constitute a promising target for the development of a preventive treatment for addiction. Consequently, our findings may open a door to new therapeutic strategies in the treatment of cocaine addiction.

Acknowledgements

This work was funded by ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica-Argentina) grant PICT 227-2008 (SBR and AMP). The authors thank Matthew Pokinko for his comments on the manuscript. We thank Florencia Cerchiara and Patricia G. Rivera Podesta for their English technical assistance.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

SC, SBR and AMP were responsible for the study concept and design. SC also supervised and contributed to the acquisition of data, analyzed the data and drafted the manuscript. AMP also provided critical revisions of the manuscript. MJS and JB contributed to the acquisition of data. All authors critically reviewed the content and approved the final version for publication.

Reference

Ahmad-Annuar A, Ciani L, Simeonidis I, Herreros J, Fredj NB, Rosso SB, Hall A, Brickley S, Salinas PC (2006) Signaling across the synapse: a role for Wnt and Dishevelled in presynaptic assembly and neurotransmitter release. J Cell Biol 174:127–139.

Alimohamad H, Rajakumar N, Seah YH, Rushlow W (2005a) Antipsychotics alter the protein expression levels of beta-catenin and GSK-3 in the rat medial prefrontal cortex and striatum. Biol Psychiatry 57:533–542.

Alimohamad H, Sutton L, Mouyal J, Rajakumar N, Rushlow WJ (2005b) The effects of antipsychotics on beta-catenin, glycogen synthase kinase-3 and dishevelled in the ventral midbrain of rats. J Neurochem 95:513–525.

Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA (2001) Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. Nat Cell Biol 3:683–686.

Beyer CE, Steketee JD (1999) Dopamine depletion in the medial prefrontal cortex induces sensitized-like behavioral and neurochemical responses to cocaine. Brain Res 833:133–141.

Boudreau AC, Wolf ME (2005) Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. J Neurosci 25:9144–9151.

Boudreau AC, Reimers JM, Milovanovic M, Wolf ME (2007) Cell surface AMPA receptors in the rat nucleus accumbens increase during cocaine withdrawal but internalize after cocaine challenge in association with altered activation of mitogen-activated protein kinases. J Neurosci 27:10621–10635.

Ciani L, Salinas PC (2005) WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. Nat Rev Neurosci 6:351–362.

Clevers H, Nusse R (2012) Wnt/beta-catenin signaling and disease. Cell 149:1192–1205.

De Ferrari GV, Inestrosa NC (2000) Wnt signaling function in Alzheimer's disease. Brain Res Brain Res Rev 33:1–12.

Deroche-Gamonet V, Piazza PV (2014) Psychobiology of cocaine addiction: contribution of a multi-symptomatic animal model of loss of control. Neuropharmacology 76 Pt B:437–449.

Galli S, Lopes DM, Ammari R, Kopra J, Millar SE, Gibb A, Salinas PC (2014) Deficient Wnt signalling triggers striatal synaptic degeneration and impaired motor behaviour in adult mice. Nat Commun 5:4992.

Hayashi Y, Hirotsu T, Iwata R, Kage-Nakadai E, Kunitomo H, Ishihara T, Iino Y, Kubo T (2009) A trophic role for Wnt-Ror kinase signaling during developmental pruning in *Caenorhabditis elegans*. Nat Neurosci 12:981–987.

Heffner TG, Hartman JA, Seiden LS (1980) A rapid method for the regional dissection of the rat brain. Pharmacol Biochem Behav 13:453–456.

Kalivas PW, Duffy P (1993) Time course of extracellular dopamine and behavioral sensitization to cocaine. I. Dopamine axon terminals. J Neurosci 13:266–275.

Kim WY, Jang JK, Lee JW, Jang H, Kim JH (2013) Decrease of GSK3beta phosphorylation in the rat nucleus accumbens core enhances cocaine-induced hyper-locomotor activity. J Neurochem 125:642–648.

Kozlovsky N, Belmaker RH, Agam G (2002) GSK-3 and the neurodevelopmental hypothesis of schizophrenia. Eur Neuropsychopharmacol 12:13–25.

Kroener S, Lavin A (2010) Altered dopamine modulation of inhibition in the prefrontal cortex of cocaine-sensitized rats. Neuropsychopharmacology 35:2292–2304.

Lee HJ, Wang NX, Shi DL, Zheng JJ (2009) Sulindac inhibits canonical Wnt signaling by blocking the PDZ domain of the protein Dishevelled. Angew Chem Int Ed Engl 48:6448–6452.

Li Y, Hu XT, Berney TG, Vartanian AJ, Stine CD, Wolf ME, White FJ (1999) Both glutamate receptor antagonists and prefrontal cortex lesions prevent induction of cocaine sensitization and associated neuroadaptations. Synapse 34:169–180.

Liu K, Steketee JD (2011) Repeated exposure to cocaine alters medial prefrontal cortex dopamine D-like receptor modulation of glutamate and dopamine neurotransmission within the mesocorticolimbic system. J Neurochem 119:332–341.

Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20:781–810.

Lu W, Yamamoto V, Ortega B, Baltimore D (2004) Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth. Cell 119:97–108.

Maguschak KA, Ressler KJ (2012) The dynamic role of beta-catenin in synaptic plasticity. Neuropharmacology 62:78–88.

Metcalfe C, Bienz M (2011) Inhibition of GSK3 by Wnt signalling —two contrasting models. J Cell Sci 124:3537–3544.

Miller JS, Tallarida RJ, Unterwald EM (2009) Cocaine-induced hyperactivity and sensitization are dependent on GSK3. Neuropharmacology 56:1116–1123.

Miller JS, Barr JL, Harper LJ, Poole RL, Gould TJ, Unterwald EM (2014) The GSK3 signaling pathway is activated by cocaine and is critical for cocaine conditioned reward in mice. PLoS One 9:e88026.

Min C, Cho DI, Kwon KJ, Kim KS, Shin CY, Kim KM (2011) Novel regulatory mechanism of canonical Wnt signaling by

dopamine D2 receptor through direct interaction with beta-catenin. Mol Pharmacol 80:68–78.

Nogueira L, Kalivas PW, Lavin A (2006) Long-term neuroadaptations produced by withdrawal from repeated cocaine treatment: role of dopaminergic receptors in modulating cortical excitability. J Neurosci 26:12308–12313.

Pacchioni AM, Vallone J, Worley PF, Kalivas PW (2009) Neuronal pentraxins modulate cocaine-induced neuroadaptations. J Pharmacol Exp Ther 328:183–192.

Paxinos G, Watson C (1997) *The rat Brain in Stereotaxic Coordinates*, 3rd edn. Academic Press: New York.

Perrine SA, Miller JS, Unterwald EM (2008) Cocaine regulates protein kinase B and glycogen synthase kinase-3 activity in selective regions of rat brain. J Neurochem 107:570–577.

Pierce RC, Kalivas PW (1997) A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. Brain Res Brain Res Rev 25:192–216.

Pierce RC, Bell K, Duffy P, Kalivas PW (1996) Repeated cocaine augments excitatory amino acid transmission in the nucleus accumbens only in rats having developed behavioral sensitization. J Neurosci 16:1550–1560.

Robinson TE, Berridge KC (2001) Incentive-sensitization and addiction. Addiction 96:103–114.

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365–386.

Salinas PC (2012) Wnt signaling in the vertebrate central nervous system: from axon guidance to synaptic function. Cold Spring Harb Perspect Biol 4:1–12.

Schenk S, Snow S (1994) Sensitization to cocaine's motor activating properties produced by electrical kindling of the medial prefrontal cortex but not of the hippocampus. Brain Res 659:17–22.

Smalley MJ, Sara E, Paterson H, Naylor S, Cook D, Jayatilake H, Fryer LG, Hutchinson L, Fry MJ, Dale TC (1999) Interaction of axin and Dvl-2 proteins regulates Dvl-2-stimulated TCF-dependent transcription. EMBO J 18:2823–2835.

Steketee JD, Kalivas PW (2011) Drug wanting: behavioral sensitization and relapse to drug-seeking behavior. Pharmacol Rev 63:348–365.

Stewart J, Badiani A (1993) Tolerance and sensitization to the behavioral effects of drugs. Behav Pharmacol 4:289–312.

Sutton LP, Honardoust D, Mouyal J, Rajakumar N, Rushlow WJ (2007) Activation of the canonical Wnt pathway by the antipsychotics haloperidol and clozapine involves dishevelled-3. J Neurochem 102:153–169.

Thomas MJ, Kalivas PW, Shaham Y (2008) Neuroplasticity in the mesolimbic dopamine system and cocaine addiction. Br J Pharmacol 154:327–342.

Tzschentke TM, Schmidt WJ (1998) The development of cocaine-induced behavioral sensitization is affected by discrete quinolinic acid lesions of the prelimbic medial prefrontal cortex. Brain Res 795:71–76.

Uematsu K, He B, You L, Xu Z, McCormick F, Jablons DM (2003a) Activation of the Wnt pathway in non small cell lung cancer: evidence of dishevelled overexpression. Oncogene 22:7218–7221.

Uematsu K, Kanazawa S, You L, He B, Xu Z, Li K, Peterlin BM, McCormick F, Jablons DM (2003b) Wnt pathway activation in mesothelioma: evidence of Dishevelled overexpression and transcriptional activity of beta-catenin. Cancer Res 63:4547–4551.

Vanderschuren LJMJ, Kalivas PW (2000) Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. Psychopharmacology (Berl) 151:99–120.

Williams JM, Steketee JD (2005) Effects of repeated cocaine on the release and clearance of dopamine within the rat medial prefrontal cortex. Synapse 55:98–109.

Xu CM, Wang J, Wu P, Zhu WL, Li QQ, Xue YX, Zhai HF, Shi J, Lu L (2009) Glycogen synthase kinase 3beta in the nucleus accumbens core mediates cocaine-induced behavioral sensitization. J Neurochem 111:1357–1368.

Xu CM, Wang J, Wu P, Xue YX, Zhu WL, Li QQ, Zhai HF, Shi J, Lu L (2011) Glycogen synthase kinase 3beta in the nucleus accumbens core is critical for methamphetamine-induced behavioral sensitization. J Neurochem 118:126–139.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1 Primers were selected using the Primer3 free software (Rozen & Skaletsky), and their sequences are detailed in the table below.

Table S2 Total locomotor activity measured on first (Day 1) and last day (Day 7) of the treatment during 2 hrs after the injection. Acute Cocaine received Saline from day 1 to 6, and cocaine only on Day 7. As it was previously describes by Pierce *et al.* () most of the animals chronically treated with cocaine showed sensitization. In order to separate animals in sensitized and non-sensitized, the behavioral response on Day 7 should showed at least a 20 percent increase from Day 1 to define sensitization (Pierce *et al.*). (*)Significantly different from the percent of increase of the Non-sensitized animals, $p < 0.005$, *t test*.

Figure S1 Schematic diagram that described the subcellular fractionation experimental procedure.

Figure S2 *Cocaine-induced sensitization leads to decreased β-catenin expression in PFC, Amyg and CPu, 3 hrs after the last injection*. Adult rats received either: seven saline injections (saline), saline from day 1 to 6 and cocaine on day 7 (acute cocaine), or seven daily injections of cocaine (chronic cocaine). On day, 1 and 7 all rats were behaviorally tested immediately after the injection. In the chronic cocaine group, their locomotor responses on each day were compared to divide animals into Sensitized (Sens) and Non-sensitized. However, at this time point only sensitized animals were sacrificed. Levels of β-catenin were measured in brain areas obtained from Sensitized animals sacrificed 3 hrs after the last injection. A-one way ANOVA analysis revealed a significant effect of treatment in the PFC [$F(2, 18) = 3.702$; $p < 0.05$], and, CPu [$F(2, 19) = 8.175$; $p < 0.0001$]. Neither chronic (sensitized) nor acute cocaine treatment produces changes in β-catenin levels in the NAcc [(%) Saline: 99.75 ± 6.62, Acute: 102.90 ± 7.34, Chronic cocaine (Sensitized): 102.40

± 4.47]. *Significantly different from Saline treatment, $p < 0.01$; + Significantly different from the rest of the groups, $p < 0.05$. Bonferroni post hoc test

Figure S3 *Cocaine-induced sensitization is not associated with β-catenin changes in the PFC's membrane fraction.* The PFC's membrane fraction was obtained by subcellular fractionation of fresh tissues coming from a different set of sensitized animals where β-catenin was measured by western blot. A simple comparison between sensitized and saline animals showed that cocaine-induced sensitization produced no changes in β-catenin levels in PFC's membrane fraction (*t* test, $p = 0.5629$). All animals were sacrificed 24 h after the last injection. Bars represent Mean ± SEM. Number of animals (*n*) are represented inside each bar.

Figure S4 *A systemic treatment with lithium chloride restored β-catenin levels in the Amyg and CPu.* 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 first and last injection. Rats were sacrificed 24 hrs after last injection, their brains were dissected and β-catenin was measured in total homogenates. β-catenin levels in the Amyg and CPu total homogenates were significantly decreased in Sal/Coc group while LiCl pretreatment restores protein levels. A two-way ANOVA applied on β-catenin levels in the Amyg revealed a significant effect of pretreatment [$F(1,33) = 11.46$, $p < 0.002$], treatment [$F(1,33) = 13.35$, $p < 0.001$], as well as an interaction between treatment × pretreatment [$F(1,33) = 9.245$, $p < 0.005$]; while in the CPu revealed a significant effect of the pretreatment [$F(1,31) = 11.07$, $p < 0.05$], and treatment [$F(1,31) = 13.89$, $p < 0.001$]. Finally, LiCl treatment induced in the NAcc an increased in β-catenin levels regardless cocaine-induced sensitization. A 2 ways ANOVA revealed a significant effect of pretreatment [$F(1,36) = 21.66$, $p < 0.0001$]. Bars represent mean ± SEM. Number of animals (n) are represented inside each bar. *Significantly different from all other groups, $p < 0.05$; + significantly different from Sal/Coc group, $p < 0.05$. Bonferroni post hoc test.

Figure S5 *Inhibiting CPu's Wnt canonical pathway with Sulindac did not induce cocaine behavioral sensitization.* Rats were pretreated with Sulindac (5 µg/µl/side) or Veh an hour before each cocaine injection, on days 2 to 5 of the treatment. Locomotor activity was measured on days 1 and 7 after cocaine or saline injections. The day after the last injection, animals were sacrificed and brains were dissected. a) Total locomotor activity showed that seven injections of 15 mg/kg i.p. of cocaine did not induce sensitization regardless of the given pretreatment (Veh or Sulindac). A two-way ANOVA for repeated measures revealed a significant main effect of treatment [$F(1,21) = 161.63$, $p < 0.001$]. b) β-catenin levels in the CPu were significantly reduced in animals that received Sulindac infusions before cocaine injections. A two-way ANOVA applied on CPu's data showed a significant effect of treatment [$F(1,19) = 7.764$, $p < 0.05$] and pretreatment [$F(1,19) = 12.98$, $p < 0,09$]. c and d) β-catenin levels did not show any changes in the PFC and Amyg, respectively. Bars represent mean ± SEM. Number of animals (*n*) are represented inside each bar. *Significantly different from Veh/Sal and Suli/Sal group, $p < 0.05$; + significantly different from Veh/Sal group, $p < 0.01$. Bonferroni post hoc test.