Addiction and Drug Sensitization (ADS)

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Research Article

Wnt/β-Catenin Pathway on Cocaine-Induced Neuroadaptations: A Novel Target for Therapeutic Opportunities?

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Drug addiction is a chronic and enduring phenomenon that has been extensively investigated in the last decades. 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 [1]. Animal models such as behavioral sensitization, conditioned place preference and intravenous self- administration have been extensively used. The primary difference in these models is the way in which the drug is administered; while the importance of the impact of non-contingent vs contingent drug administration for the molecular basis of addiction is a subject of continuous debate. However, recently it has been described that animal models of addiction (behavioral sensitization vs self- administration) remarkably overlap in terms of neurocircuitry as well as in the molecular changes underlying their respective behavioral responses [2].

Behavioral sensitization is a progressive and enduring enhancement of the motor stimulant effects elicited by repeated administration of psychostimulants [3]. 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 in the prefrontal cortex (PFC) [4-7], while *expression* is linked to changes in the nucleus accumbens (NAcc) [8,9]. A great deal of evidence shows that changes in synaptic plasticity underlie both initiation and expression of behavioral sensitization [2,10,11]. The initiation of sensitization has been shown to be disrupted by Ibotenic acid lesions in both prelimbic and infralimbic regions of the PFC [7]. Moreover, several studies have suggested that cocaine induces a functional decrease of the D_2R in the PFC that would serve to enhance excitatory transmission to subcortical regions [12-15]. Regarding the long-term changes, cocaine induces a decrease in basal glutamate levels in the NAcc that increase after a cocaine challenge [8,16]. These higher levels of glutamate will act on the AMPA receptors (AMPAR) promoting higher behavioral responses [8,17]. Moreover, withdrawal from drug exposure induces changes in the 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 AMPAR relative to NMDAR-mediated currents given by an increase in the surface expression of AMPAR [18-20], as well as changes in dendritic spine density [21-24]. While the mechanisms underlying these structural modifications are not completely clear, the regulation of the actin cytoskeleton, [25,26] together with the activity of small GTPase and the induction of different genes and their targets (eg ΔFosB, NFkB, Cdk5-MEF2, etc) [23] would be involved. Altogether, this evidence indicates that cocaine-induced sensitization is the result of an interaction between dopaminergic and glutamatergic neurotransmission (Figure 1A).

We used the behavioral sensitization paradigm to model addiction-like behavioral responses in order to investigate the role of Wnt (Wingless-related integration site) 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 [27]. The Wnt growth factors belong to a large family of secreted proteins that can signal through different receptors including Frizzled (Fz) [28] and the atypical tyrosine kinase receptors Ror2 and Ryk [29,30]. 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 [31]. 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 [28,32]. While in the absence of Wnt, GSK3β phosphorylates β-catenin marking it for degradation by the proteasome [33]. 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 [34].

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 [35,36]. For instance, Alimohamad et al [37] showed that amphetamine increases GSK3β activity and decreases β-catenin levels in the PFC and in the striatum, while D_2R antagonists produce the opposite effect. Despite the relevance to cocaine effects of dopamine and its

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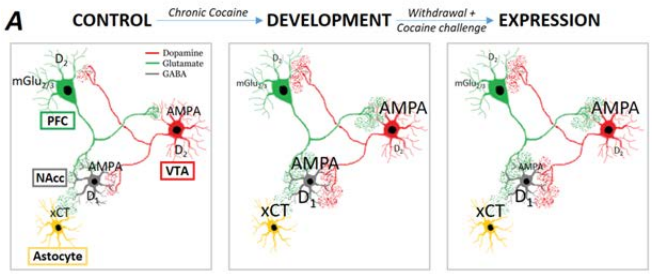

Figure 1A: Summary of cocaine-induced neuroadaptations in the different receptors of the mesocorticolimbic circuitry that underlies behavioral sensitization. xCT: Cystine/ Glutamate exchanger, $mGlu_{2/3}$: metabotropic glutamate receptors, D_2: Dopamine D2-like receptor, D_1: Dopamine D1-like receptor, AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor.

receptors, little was known about the role of Wnt signaling pathways in drug addiction. Furthermore, numerous studies have suggested that the regulation of GSK3β activity might be associated with cocaine-induced neuroadaptations. Not only does cocaine produce changes in GSK3β activity in the striatum, but also inhibitors of GSK3β, both targeted (e.g., SB 216763) and non-selective (e.g., valproate or LiCl), prevent cocaine-induced sensitization [38-40]. Nevertheless, none of these works proposed a link between the changes in GSK3β activity and the Wnt/β -catenin pathway. Therefore, our main goal was to evaluate whether the Wnt canonical pathway was involved in cocaine-induced neuroadaptations related with both, the induction and the expression of behavioral sensitization. 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 [41,42].

As a behavioral model, we worked with a well characterized experimental sensitization scheme developed by the Kalivas Laboratory in the 90's [8] (Pierce, Bell et al. 1996). This schema consists of an *initiation phase*, involving 7 injections of cocaine (2x15mg / kg and 5x30mg / kg, i.p.) administered one per day followed by an *expression phase* evaluated after a cocaine injection (15 mg / kg, i.p.) administered after 3 weeks of abstinence, on day 28. After the injections on days 1, 7 and 28 of the treatment the locomotor activity was recorded for 2hs. The remaining injections (30mg / kg i.p.), between days 2 to 6, were administered in the home cage. We considered that an animal was sensitized when it showed at least a 20% increase in the cocaine-induced locomotor response when comparing the first and last day of treatment (7 for development, 28 for expression) [8] (Pierce, Bell et al. 1996). Different groups of researchers have demonstrated that not all the animals show an increase in their locomotor activity after the sensitization protocol is applied, actually only sixty percent does [8,9,18]. Likewise, we found that about 60% of the chronically treated animals did show the increase in locomotor activity. Based on this criterion we divided the chronic cocaine treated animals into sensitized and non- sensitized according to their locomotor response. Then we investigated whether molecular changes in β-catenin, the final effector of the canonical Wnt pathway, were linked to cocaine-induced development and expression of behavioral sensitization. Therefore, we measured β-catenin levels as a readout for canonical Wnt signaling [32] in brain areas relevant to addiction such as the PFC, the NAcc, the Caudate Putamen (CPu) and the Amygdala (Amyg) in animals sacrificed 24hs after the last injection on day 7 or 28.

Regarding the development of sensitization, our main findings revealed that the animals that received chronic cocaine and developed the behavioral sensitization showed a decrease of β-catenin levels in the PFC, CPu and Amyg when compared to saline treated animals, while no changes were found in the NAcc. Then, and in line with β-catenin observations, GSK3β activity levels were increased in the PFC, CPu and Amyg of sensitized animals. Furthermore, we found that in the PFC the nuclear levels of β-catenin, the mRNA expression of Axin2 (a target gen of the pathway) and the expression of the mRNA of Wnt7b were decreased in sensitized animals. Taken together, these results reveal that the inhibition of the Wnt/β-catenin pathway is an important neuroadaptation for the induction of behavioral sensitization, proposing a new role for this pathway.

When we focused on the expression of behavioral sensitization, we found that chronic cocaine induced an increase on β-catenin levels in the NAcc (both in total homogenates and in the nuclear fraction), a decrease in the CPu and no changes in the PFC when compared to saline treated animals. Once again, all these changes were only present in sensitized animals and entailed a cocaine-induced increase in the activity of the Wnt/ β-catenin pathway in the NAcc.

In order to demonstrate that changes in the activity of the Wnt pathway in the PFC were necessary for developing sensitization we used two pharmacological approaches. The first one involved a systemic treatment with a well-characterized pathway activator as is lithium chloride [43]; while the second one consisted of intracerebral infusions of a pathway inhibitor called Sulindac [44]. Each treatment was administered before each cocaine injection. As expected, we found that LiCl, by preventing β-catenin reduction in the PFC as well as CPu and Amyg, blocked cocaine-induced sensitization. In contrast, by intensifying the pathway inhibition with Sulindac in the PFC, the behavioral sensitization was exacerbated, whereas there was no impact on the behavioral response when infusing Sulindac in the CPu. These findings highlight the relevance of the inhibition of the PFC's Wnt canonical pathway in the initiation of cocaine-induced sensitization.

Finally, we studied the importance of the changes of β-catenin levels induced by cocaine during development in the long-term neuroadaptations that cause the expression of sensitization. In other words, we evaluated the long-term consequences that the pharmacological prevention of β-catenin reduction has during the cocaine treatment. We found that not only the expression of sensitization was blocked 3 weeks after LiCl treatment, but also that the protein levels were modified. Specifically, we found that the LiCl pretreatment by itself induced an increase in β-catenin levels in the NAcc, regardless of the presence of cocaine. Therefore, there was no change on the activity of the pathway after the cocaine injection. These results suggest that it is not the actual level of β-catenin but the *variation* of the pathway activity what impact in the behavioral response, highlighting an important role of the pathway in the cocaine-induced long term neuroadaptations (i.e. expression of behavioral sensitization).

Taken together, these results proposed for the first time that changes in the Wnt/ β-catenin pathway effectors are involved in short and long-term neuroadaptations required for cocaine-induced behavioral sensitization. In the past decade, a relationship between dopamine neurotransmission and intracellular effectors of the Wnt/ β-catenin pathway has been shown [37,45-47]. Taking into account the mounting evidence about changes in dopamine and glutamate neurotransmission that underlies cocaine-induced short and long-term neuroadaptations [2,23,48], it is possible that cocaine-induced changes in Wnt/ β- catenin pathway activity are linked to them. For instance, it has been shown that cocaine induces a functional decrease of D_2R in the PFC that would serve to enhance excitatory transmission to subcortical regions [12-15]. Therefore, it is possible that cocaine-induced inhibition in the Wnt/ β- catenin pathway is related to a functional decrease in dopamine neurotransmission. In fact, Galli et al [49] have recently demonstrated that inducible expression of Dkk-1, a physiological inhibitor of the Wnt/ β-catenin pathway [34], 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 [50]. Moreover, 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 hypothesized that the inhibition in the Wnt/ β-catenin pathway observed in the PFC of sensitized animals may have been associated with a functional decrease of Dvl leading to a disconnection of D_2R. However, when we evaluated β-catenin levels in the PFC after a cocaine challenge on day 28, we found similar levels compared to controls regardless of the behavioral measurement. Interestingly, when we measured levels of β-catenin before the challenge and after a period of abstinence, we found an increase when compared to the control group. In other words, after the abstinence, a cocaine challenge reduced β-catenin to the control level, despite the behavioral outcome (sensitized or non-sensitized). It is possible that D_2R are involved in this mechanism as well as during development, but more work needs to be done to establish the relevance of these changes in cocaine-induced long-term neuroadaptations.

As regards the long-term changes induced by cocaine in the Wnt/ β-catenin pathway, it seems that there are two main modifications in the neurotransmission that could be associated with them: on the one side, the dopaminergic changes on receptor sensitivity and dopamine release in the NAcc [51-54], and on the other, the glutamatergic changes in this same area. In the case of the dopaminergic transmission, it is likely that the increased activation of the D_1R in the NAcc induced by cocaine causes the accumulation of β-catenin through inhibition of GSK3β [55]. This change could, in turn, influence the glutamate transmission. After 3 weeks of withdrawal from repeated cocaine, the surface expression of AMPAR is increased in the NAcc, while a cocaine challenge leads to a decrease in behavioral sensitized animals after the challenge [9,11,18]. Recently, it has been shown that 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 [47]. Then, it is possible that the cocaine-induced increase in β-catenin levels mediated by dopamine in the NAcc activates the pathway as well as facilitates the removal of AMPAR from the surface after the cocaine challenge, giving rise to the expression of behavioral sensitization. On a side note, we emphasized on the long-term effect in this area because our results did not show an immediate influence on the pathway in NAcc. However, it is possible that the fact that we did not sample the NAcc in core and shell could hide small changes.

Interestingly in the CPu, we found that both development and expression of sensitization are associated with a decrease of β-catenin levels in those animals that showed behavioral sensitization after a challenge, while it was significantly increased after 3 weeks of abstinence, similar to what happened in the PFC. Taken together, these results suggest that behavioral sensitization requires a reduction in β-catenin, below basal levels, in order to manifest. However, in the case of development of sensitization in the CPu we also found that nuclear levels of β-catenin were similar to the control ones and forcing the decrease (by infusing an inhibitor) was not enough to induce the behavioral sensitization. In other words, it seems that this decrease is necessary but not sufficient for the development of sensitization. In the case of the expression of sensitization, changes in β-catenin, and probably in the activity of the Wnt canonical pathway, might be more important and could be mediated by the dopaminergic transmission through D_2R. A reduction in striatal D_2R levels have shown after repeated drug exposure in non-human primates [56]. Then, and considering that the antagonism of D_2R is linked to β-catenin accumulation [37] while the activation is associated with β-catenin degradation [57], it is possible that repeated cocaine exposure could facilitate the accumulation of β-catenin found in the CPu of abstinent animals. Although the fact that β-catenin levels in CPu increase during abstinence while they decrease after a challenge may suggest that changes in the activity of the Wnt canonical pathway could be characteristic of the long-term neuroadaptations, further work must be done to fully clarify the role of this pathway in the CPu.

We also demonstrated that the activation of the pathway mediated by LiCl administration before each cocaine injection not only prevented the development of sensitization by restoring β-catenin levels in the PFC, CPu and Amyg, but also prevented the expression of behavioral sensitization by keeping the levels of β-catenin increased in the NAcc. Previously, we proposed that the increase in β-catenin levels in the NAcc, together with it decrease in the CPu, are correlated to 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 [58], then the LiCl results strengthen our previous assumptions that it is the change in β-catenin and the consequent activation of the canonical pathway what matters for the expression of sensitization. To our knowledge, this is the first-time data reported that LiCl has a long-term effect on cocaine-induced behavioral as well as molecular neuroadaptations. The mechanism associated to LiCl long-term effect on cocaine-induced behavioral neuroplasticity might involve distinct effects in the different areas of the motivational circuitry. As mentioned before, 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. These restorations could interfere with the subsequent long-term effects of cocaine. Furthermore, 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 [47] as well as the response to the drug during the expression of cocaine sensitization. Moreover, according to recent evidence, it is also possible that LiCl decreased DA release in the NAcc in response to cocaine [59].

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-NAcc connections as biological substrates of cocaine-induced sensitization (Figure 1B). By using different strategies, we demonstrated the relevance of the inhibition of the Wnt/ β-catenin pathway in the PFC both for short and long-term neuroadaptations induced by cocaine. Specifically, we showed that if we activate the pathway by administering LiCl, then cocaine is not able to induce development or expression of sensitization. Furthermore, only the inhibition of the pathway in the PFC (by Sulindac infusion) exacerbates the development of sensitization, while no effect was found with Sulindac infusions in the CPu. Keeping all these results in mind, we postulate that the initial inhibition of the canonical Wnt pathway induced by chronic cocaine in the PFC results in dramatic changes in the expression of different target proteins of the pathway, altering the communication between the PFC and the NAcc. This modification in the circuitry would be related, after a period of abstinence, with the changes in the activity of the pathway in the NAcc and also with the behavioral response induced by a cocaine challenge.

Since locomotor sensitization in rodents seems to share plastic mechanisms with drug addiction in humans, and corresponds to aspects of drug abuse such as initiation and compulsive drug-seeking behavior [2], our findings suggest that the Wnt canonical pathway may be involved in the early stages as well as in relapse of substance abuse. Although one must always be wary of extrapolating clinical relevance

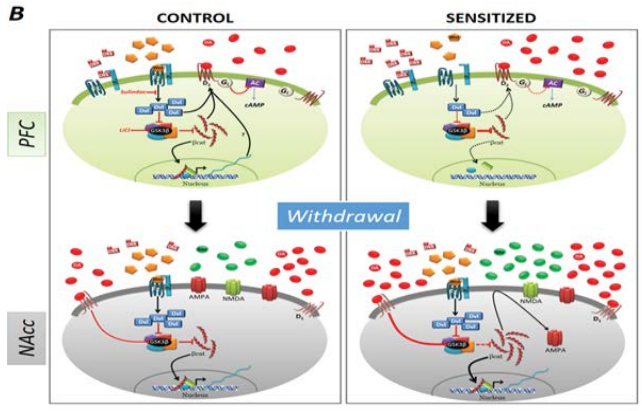

Figure 1B: Schematic representation of the neuroadaptations on Wnt canonical pathway in the PFC and in the NAcc of control and sensitized rats before and after withdrawal of chronic cocaine administration. Under control conditions, in the absence of Wnt, GSK3β phosphorylates β-catenin marking it for 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. Dkk: Dickkopf-1, Dvl: Dishevelled, Fz: Frizzled receptor, D_2: Dopamine D2-like receptor, D_1: Dopamine D1-like receptor, Gi: Inhibitory G protein, AC: Adenylyl Cyclase, βcat: β-catenin, GSK3β: Glycogen synthase kinase 3β, DA: Dopamine, Glu: Glutamate, NMDA: N-methyl-D-aspartate receptor, AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor. Black arrows: activation (filled: normal activity, dotted: reduced activity); red blunted arrows: inhibition (filled: normal activity, thickened: increased activity, dotted: reduced activity).

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

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