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Striatum, Dopamine and Automatism in Cocaine Addiction and Relapse: Pharmacological and Behavioral Investigation

Renan Costa Campos

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Le 18 Décembre 2017

PAR

RENAN COSTA CAMPOS

Né le 27 Août 1986 à Rio Grande/RS – BRÉSIL

POUR L'OBTENTION DU GRADE DE

DOCTEUR

SPECIALITÉ : NEUROSCIENCES

**Striatum, Dopamine et Automatisation dans
l'Addiction à la Cocaïne et dans la Rechute:
Investigation Pharmacologique et Comportementale**

Membres du Jury

Dr. Christelle BAUNEZ, DR1 CNRS (Marseille, France)	Rapporteur
Dr. François GEORGES DR2 CNRS (Bordeaux, France)	Président/Examinateur
Dr. Marcello SOLINAS DR2 CNRS (Poitiers, France)	Rapporteur
Dr. Martine CADOR, DR1 CNRS (Bordeaux, France)	Directrice de thèse

THESIS

PRESENTED AT THE

UNIVERSITY OF BORDEAUX

HEALTH AND LIFE SCIENCES DOCTORAL SCHOOL

18th of December, 2017

by

RENAN COSTA CAMPOS

Born on the 27th of August, 1986 in Rio Grande/RS – BRAZIL

FOR THE DEGREE OF

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Addiction and Relapse: Pharmacological and
Behavioral Investigation**

Members of the Jury

Dr. Christelle BAUNEZ, DR2 CNRS (Marseille, France)	Reviewer
Dr. François GEORGES DR2 CNRS (Bordeaux, France)	President/Examiner
Dr. Marcello SOLINAS DR2 CNRS (Poitiers, France)	Reviewer
Dr. Martine CADOR, DR1 CNRS (Bordeaux, France)	Thesis supervisor

Résumé

L'un des aspects les plus problématiques de l'addiction est la vulnérabilité à la rechute qui persiste même longtemps après la disparition des symptômes de sevrage. Les modèles rongeurs d'auto-administration (AA) démontrent que la réexposition à la drogue, les stimuli associés à la drogue ou le stress sont des déclencheurs majeurs de la rechute. Bien que les différentes catégories de drogues varient dans leurs mécanismes pharmacologiques primaires, elles partagent toutes un effet aigu d'augmentation des niveaux de dopamine (DA) dans le striatum. Après une utilisation répétée, les propriétés addictives des psychostimulants tels que la cocaïne (COC) sont sous-tendues par la mise en place de neuroadaptations persistantes dans le système DA (SDA) mésocorticolimbique. Le striatum est une cible majeure du SDA et, dans cette région, la DA agit sur deux familles de récepteurs (D1R et D2R) séparées positionnées sur les neurones épineux moyens (NEM) et donnant naissance à deux voies de sorties striatales différenciées et ayant des rôles différents dans l'addiction.

Des travaux antérieurs dans notre équipe ont montré une implication différentielle de ces deux sous-types de récepteurs DA sur le rétablissement du comportement de recherche de COC. Alors que l'administration systémique d'un agoniste D2R induisait de puissants effets de rétablissement de recherche de cocaïne sur des rats entraînés à s'autoadministrer de la COC, l'administration systémique de l'agoniste D1R n'avait aucun effet (Dias *et al*, 2003).

Les sous-régions ventrale (noyau accumbens) et dorsale du striatum (caudate putamen) sont modulées par l'innervation dopaminergique du mésencéphale ventral (VTA, substance noire) et la plasticité induite par la cocaïne dans ces circuits est supposée sous-tendre plusieurs aspects du comportement de recherche de drogue (Pierce and Vanderschuren, 2010). C'est pourquoi la première section de ce travail comprend une description de trois expériences réalisées dans le but d'étudier la participation des récepteurs DA du striatum au niveau ventral et dorsal à la réinstallation du comportement de recherche de cocaïne.

Le travail exposé dans cette première section a abouti à la préparation d'un manuscrit qui doit être soumis pour publication. Un résumé des expériences effectuées ainsi que le manuscrit lui-même suivent ci-dessous.

Section I – Investigation pharmacologique: le rôle des récepteurs de la dopamine du striatum dans la réinstallation de la recherche de cocaïne

Expérience 1: Le rôle des récepteurs D1R et D2R du Noyau accumbens (NAcc) sur le rétablissement de la recherche de COC.

Résumé des méthodes: Les rats ont subi une chirurgie pour avoir 1) des cathéters implantés à demeure dans la veine jugulaire pour s'autoadministrer de la cocaïne par voie intraveineuse (COC SA, 250 µg/injection), et 2) des canules implantées à demeure bilatéralement dans le NAcc. Après avoir récupéré de la chirurgie, les animaux ont été entraînés à s'autoadministrer de la cocaïne (COC) selon un programme de renforcement continu en situation opérante, puis soumis à une extinction de la réponse instrumentale, dans laquelle les appuis sur le levier renforcé n'avaient plus de conséquences programmées. Une fois que le comportement de recherche de COC a été éteint, les animaux ont été soumis à différents challenges pharmacologiques tous réalisés en situation d'extinction (pas de délivrance de cocaïne) et au cours desquels le nombre d'appuis sur le levier préalablement associé à la cocaïne a été quantifié. Tout d'abord, nous avons réalisé un challenge avec une administration non contingente de cocaïne (15 mg/kg) COC, qui classiquement induit une réinstallation du comportement d'appui sur le levier préalablement renforcé et qui nous a servi de contrôle interne sur le niveau de réinstallation des animaux. Au cours de challenges suivants, les animaux ont été soumis à des injections bilatérales de différentes doses d'agonistes D1R (SKF82958) ou D2R (Quinelorane) dans le NAcc avant d'être placés dans les cages opérantes.

Résumé des résultats: L'expérience a démontré que l'activation pharmacologique de D1R du NAcc induit le rétablissement de la recherche de COC, mais pas l'activation du D2R du NAcc.

Ces résultats étaient plutôt déroutants, car ils étaient en opposition avec ce qui avait été montré avec l'administration périphérique des agonistes D1R et D2R à savoir que l'activation des D1R dans l'ensemble du cerveau lors d'une administration systémique n'induit pas de rétablissement du comportement de recherche de COC alors que l'activation pharmacologique de D1R exclusivement du NAcc l'induit. A l'inverse, alors que l'activation des récepteurs D2R dans l'ensemble du cerveau lors de l'administration systémique déclenche un puissant rétablissement de la recherche de cocaïne, aucun rétablissement n'est observé lorsque les D2R du NAcc sont activés.

Cette dissociation des effets de réinstallation observés entre l'administration systémique et locale (NAcc) des agonistes D1R ou D2R nous a incités à étudier d'autres localisations

cérébrales où le D2R pourrait agir pour induire une réinstallation du comportement de recherche de cocaïne

Expérience 2: Le rôle des récepteurs D1R et D2R du striatum dorsolatéral (DLS) sur le rétablissement de la recherche de COC.

Résumé des méthodes: Les rats ont subis les mêmes chirurgies que dans l'expérience 1 sauf que cette fois les canules guide ont été implantées dans le DLS. Après avoir récupéré de la chirurgie, les animaux ont subi le même apprentissage et extinction d'autoadministration de cocaïne, et le même challenge avec une administration de cocaïne systémique. Au cours des tests suivants, les animaux ont été soumis à des injections bilatérales de différentes doses de SKF82958 ou de quinélorane au niveau du DLS avant d'être placés dans les cages opérantes en situation d'extinction.

Résumé des résultats: *L'expérience a démontré que l'activation pharmacologique du récepteur D2R du DLS induit le rétablissement de la recherche de COC (avec la plus forte dose de quinélorane sans réelle courbe dose-réponse), alors que l'activation du D1R du DLS n'a montré aucun effet.*

Suite à ces résultats, nous avons décidé de tester la possibilité que l'activation du récepteur D2R du DLS puisse être responsable des effets de réinstallation provoqués par l'activation systémique du D2R. Nous avons donc testé cette hypothèse en administrant par voie systémique l'agoniste D2R (le quinélorane) et en injectant simultanément un antagoniste D2R (raclopride) dans le DLS. Si tel était le cas, en bloquant l'effet du D2R du DLS, nous nous attendions à bloquer, au moins partiellement, la réintégration de la recherche de COC déclenchée par l'activation systémique du D2R.

En accord avec cette hypothèse, nous avons observé que le rétablissement de la recherche de COC induite par l'injection périphérique de quinélorane était effectivement partiellement bloqué par l'injection de raclopride dans le DLS.

Il a ensuite semblé raisonnable d'étudier d'autres doses de quinélorane devraient être étudiées afin d'avoir une idée plus claire des effets locaux des D2R du DLS sur la réinstallation du comportement de recherche de drogues et si la réinstallation induite par l'activation systémique de D2R repose en partie, mais pas exclusivement, sur l'activation de D2R de la DLS.

Ces hypothèses nous ont conduits à la troisième expérience, où nous avons évalué [i] les effets de rétablissement de nouvelles doses de quinélorane dans le DLS; et [ii] si les effets de rétablissement induits par l'activation systémique de D2R seraient contrebalancés par l'injection

d'un antagoniste D1R (SCH23390) dans le DLS; ou d'un antagoniste D1R (SCH23390) ou d'un antagoniste D2R (raclopride) dans le NAcc.

Expérience 3: Le rôle de D1R et D2R de la DLS sur le rétablissement de la recherche de COC.

Résumé des méthodes: Les rats ont été opérés de la même manière que dans les expériences 1 et 2 sauf que cette fois les canules guides ont été implantées dans le DLS pour la moitié des rats, et dans le Nacc pour l'autre moitié. Après avoir récupéré de la chirurgie, les animaux ont subi le même apprentissage et extinction d'autoadministration de COC SA, et le même challenge avec une administration périphérique de cocaïne COC. Les jours suivants, les rats ont été soumis à différents tests, selon le lieu d'implantation des canules guides:

- Groupe DLS : les animaux ont été soumis à des injections bilatérales de deux autres doses de quinélorane dans le DLS avant d'être testés pour la réinstallation, comme précédemment.

Dans une deuxième partie, les animaux ont reçu une injection systémique de quinélorane tout en recevant une administration simultanée de SCH23390 dans le DLS;

- Groupe NAcc : les animaux ont reçu une administration systémique de quinélorane tout en recevant une administration simultanée d'antagonistes D1R ou D2R dans le DLS.

Résumé des résultats: Ces expériences nous ont donné un meilleur aperçu de l'implication du D2R du DLS, et ont démontré que la réinstallation induite par l'activation pharmacologique systémique de D2R est bloquée par l'injection d'un antagoniste D1R ou D2R dans le NAcc ou par l'administration d'un antagoniste D1R dans le DLS.

Finalement, nous avons pu observer que:

- 1) L'activation pharmacologique de D1R du NAcc induit le rétablissement du comportement de recherche de COC chez les rats;
- 2) L'activation pharmacologique de D2R du DLS induit le rétablissement du comportement de recherche de COC chez le rat;
- 3) Les effets réinstallant de l'activation pharmacologique systémique de D2R sont bloqués par [i] l'administration d'antagonistes D1R ou D2R dans le NAcc, ou [ii] l'administration d'un antagoniste D1R dans le DLS, tout en étant diminué par la perfusion d'un antagoniste D2R dans le DLS.

Par conséquent, il semble qu'une activation simultanée des deux sous type de récepteurs dans les deux régions du striatum étudiées est responsable de l'induction du rétablissement du comportement de recherche de COC. Ces effets peuvent éventuellement dépendre de connexions en spirale entre le striatum ventral et dorsal, à travers des boucles ascendantes qui atteignent le mésencéphale.

Ces résultats nous ont incités à examiner les mécanismes comportementaux impliqués dans la rechute. Bien qu'initialement dirigé vers un but, la recherche de COC devient habituelle après une AA prolongée. Nous pensons que cette progression provoque au départ des changements fonctionnels au sein du NAcc, gagnant progressivement les circuits du striatum dorsal. Les activations des D1R dans le NAcc et des D2R dans le DLS ont été associées respectivement aux propriétés renforçantes et la réponse habituelle aux récompenses. Nous avons prévu d'élaborer un protocole qui nous permettrait éventuellement de distinguer une implication respective des D1R dans le NAcc et D2R dans le DLS dans les processus dirigés vers un but vs. les réponses automatiques des processus habituels.

Par conséquent, pour donner une suite à nos travaux précédents, nous avons décidé d'étudier les aspects comportementaux de la recherche de drogue et de la rechute. Dans ce but, nous avons travaillé sur le développement d'un modèle qui nous permettrait de démêler les aspects appétitifs et consommatoires de la recherche de drogue et qui pourrait éventuellement nous permettre d'aller plus loin dans l'implication comportementale des dissociations obtenues dans la section précédente de ce travail, concernant le rôle de D1R et D2R du NAcc et du DLS sur la réinstallation du comportement de recherche de COC. À cette fin, nous avons travaillé sur un modèle de transition du contrôle comportemental vers l'automatisation supposée sous-jacente à l'usage de drogues. Ce travail est exposé dans la section II.

Section II – Investigation comportementale: modélisation de la transition vers l'automatisation du contrôle comportemental

Suite à notre étude pharmacologique, plusieurs questions restaient à résoudre. Jusqu'à présent, bien que montrant une opposition apparente concernant les résultats obtenus par l'activation systémique de D1R et D2R, nos résultats semblent concorder avec la littérature qui soutient l'attribution du D1R du NAcc dans le traitement des aspects motivants de la prise de drogue, ainsi que le rôle du D2R du DLS dans l'automatisation comportementale de la réponse aux drogues d'abus.

Ainsi, en concevant un modèle qui reproduit la transition du contrôle comportemental, nous pourrions être en mesure de répondre à plusieurs questions qui se posent à l'issue de

notre première étude. Par exemple, si les déclencheurs comportementaux de la réinstallation induite par les D2R du DLS suivent les probables incitations motivationnelles attribuées à l'activation du D1R du NAcc; ou si la rechute déclenchée par le D1R du NAcc est influencée par une quelconque trace d'automatisme qui pourrait inciter les rats à reprendre la recherche de COC après la stimulation du D2R du DLS.

Ainsi, nous avons entraîné des rats dans un programme de deuxième ordre avec des renforçateurs alimentaires dans un premier temps afin de dissocier spatialement et temporellement les aspects des réponses appétitives et des réponses consommatoires et d'étudier la transition d'un comportement dirigé vers un but vers un comportement automatisé en agissant à la fois sur la représentation de la récompense et la représentation de l'action menant à la récompense. Une fois établi, ce modèle pourrait être appliqué aux études d'auto-administration de COC et nous aider à éclaircir les questions susmentionnées.

Résumé des méthodes: Initialement, les rats étaient soumis à un programme simple de renforcement avec des renforçateurs alimentaires, dans lequel un levier [appelé désormais «levier de consommation» (*taking lever*; TL)] était disponible. Après l'acquisition de l'association instrumentale, un deuxième levier a été introduit [le «levier de recherche» (*seeking lever*; SL)]. Les réponses sur le SL ont été configurées pour fonctionner selon un intervalle variable de 2 secondes en moyenne. De cette façon, le cycle commençait au début de chaque session: le SL était introduit; la première réponse sur le SL après l'écoulement de l'intervalle variable a donné accès au TL; une réponse sur le TL, livrait une récompense alimentaire. La composante d'intervalle variable a été augmentée entre les sessions, pour atteindre VI30 (c'est-à-dire que l'intervalle était en moyenne de 30 s, allant de 15 s à 45 s).

Les rats ont été entraînés selon ce programme pour être ensuite testés après avoir procédé à la dévaluation de la récompense par satiété spécifique: avant le test, la moitié des animaux ont été exposés au même renforçateur primaire qu'ils recevaient pendant le conditionnement instrumental qu'ils ont pu consommer jusqu'à la satiété, tandis que le groupe témoin a été exposé à la nourriture normale qu'ils avaient accès dans leurs cages d'animalerie. Après avoir été exposés à la nourriture, les animaux ont été placés dans les chambres de conditionnement en situation d'extinction (pas de délivrance de récompense alimentaire ou de présentation de TL) et le nombre de réponses sur le SL a été enregistré.

Résumé des résultats : *Dans ce test, les animaux qui ont été exposés à la récompense avant la séance (et qui ont donc mangé à la satiété) ont montré que leurs actions (appuis sur le SL) sont dépendantes de la valeur actuelle de la récompense alimentaire: le nombre d'appuis sur le SL a diminué spécifiquement pour le groupe dont la récompense a été dévaluée par la satiété, contrairement à nos attentes d'observer un comportement habituel chez les rats.*

Ensuite, dans un deuxième test, les animaux ont subi l'extinction du TL. Le but de cette évaluation était d'étudier l'influence d'une altération de la représentation de l'action conduisant à l'obtention de la récompense. Fait important, cette fois la récompense n'a pas été dévaluée; au lieu de cela, nous avons dévalué la représentation du TL, à travers son extinction.

Dans ce second test, les animaux ont montré un comportement orienté vers un but : les animaux qui ont subi l'extinction du TL ont diminué les appuis sur le SL lorsqu'ils ont été testés en extinction. Ces résultats indiquent que, même si la récompense n'était pas dévaluée, la représentation de la valeur du TL diminuait après qu'une procédure d'extinction du TL en ait modifié sa valeur.

Étant donné que l'utilisation de protocoles à intervalles variables favorise l'établissement de réponses habituelles, nous nous attendions à ce que les rats répondent de façon habituelle, sans prendre en compte la valeur des récompenses (ou du TL) dans leurs actions vers le SL. Cependant, les tests que nous avons effectués jusqu'ici ont montré que ce n'était toujours pas le cas. Puisque nous n'avons pas observé de comportement habituel jusqu'ici, nous avons décidé d'effectuer un régime de sensibilisation à la COC, qui selon la littérature est censé accélérer la transition d'un comportement dirigé vers un but (sensible à la dévaluation) vers un comportement habituel (insensible la dévaluation). Suite à la procédure de sensibilisation, les animaux ont ensuite été replacés dans le programme instrumental de deuxième ordre et testés à nouveaux après une dévaluation de la récompense alimentaire par satiété spécifique.

Les résultats indiquent que le traitement sensibilisant à la cocaïne n'a pas généré un comportement de type habituel puisque le groupe d'animaux sensibilisé et pour qui la récompense a été dévaluée montrent toujours une diminution d'appuis sur le SL.

Étant donné que nous n'avons pas de comportement de type habituel, nous avons décidé de les entraîner pendant 26 sessions supplémentaires et augmenter le VI, augmentant ainsi la probabilité de progresser vers un comportement de type habituel. Ensuite les animaux ont subi le même test après la dévaluation de la récompense alimentaire par satiété spécifique.

Les résultats indiquent que les animaux ne montraient toujours pas un comportement habituel car une diminution des appuis sur le SL a été observée.

Afin de vérifier que l'absence de mise en évidence de comportements habituels n'était pas due à la procédure de dévaluation utilisée, nous avons utilisé un autre paradigme de

dévaluation, l'aversion de goût conditionnée. Dans ce paradigme, la récompense alimentaire est associée à plusieurs reprises aux malaises induits par les injections systémiques de chlorure de lithium. La composante aversive de ce protocole produit un effet de dévaluation plus fort que dévaluation par satiété spécifique.

Une fois de plus, nous n'avons pu montrer un comportement de type habituel car le nombre d'appuis sur le SL a diminué pour les groupes ayant eu le conditionnement aversif, indiquant donc que le comportement de ces animaux dépend bien de la représentation de la récompense.

En conclusion, plusieurs explications peuvent être soulevées pour expliquer l'absence de transition d'un comportement de type dirigé vers un but à un comportement de type habituel (automatique). D'une part, la segmentation des différentes actions (appuis SL, appuis TL et récupération de la récompense dans la mangeoire) et des buts à atteindre pour chacune de ces actions (obtention du TL, obtention de la récompense) et de leurs représentations pourrait avoir empêché l'installation de comportements habituels. En outre, le fait qu'avant les tests les animaux avaient été nourris à la satiété avec la récompense qu'ils recevraient pendant les séances d'entraînement aurait pu leur donner la possibilité d'apprendre la valeur relativement faible de la récompense quand elle était dévaluée, dans un processus d'apprentissage incitatif.

En tout cas, cela pourrait soulever la question de savoir si le changement de comportement est réellement réalisable dans les conditions de notre protocole de deuxième ordre. Néanmoins, même dans le cas contraire, une cible intéressante pour de futures études consisterait à rechercher les raisons pour lesquelles les animaux n'acquièrent pas un comportement habituel même après avoir subi un traitement de sensibilisation COC et un entraînement prolongé au protocole de deuxième ordre.

Section III – Pharmacogénétique: étude préliminaire sur de nouvelles approches pour étudier les voies de sortie striatales dans les rechutes induites par la drogue

Dans cette section, nous avons travaillé à l'établissement d'une nouvelle méthodologie destinée à la manipulation des voies de sorties striatales qui permettrait éventuellement l'étude

de ces voies dans des protocoles tels que ceux développés dans les sections précédentes. Cette technique consistant en l'infusion de vecteurs viraux induisant l'expression de DREADDs dans des régions cérébrales spécifiques permet l'activation ou l'inhibition de voies neurales spécifiques par une administration périphérique de son ligand CNO, dans une méthodologie moins invasive et moins stressante pour les animaux traités.

Logiquement, comme le travail effectué dans les sections précédentes traitait de l'importance des récepteurs DA dans le striatum ventral et dorsal, nous avons commencé cette section en travaillant sur les voies striatales qui pourraient être liées à notre travail pharmacologique et pourraient être étudiées dans de futures études. En effet, les récepteurs DA D1R et D2R ne sont pas co-exprimés dans les NEMs. Dans le NAcc la cible des voies de sortie est moins clairement définie avec les NEMs-D1 projetant vers la substance noire (SN) et aire tegmentale ventrale (VTA) et les deux NEM-D1 et NEM-D2 projetant sur le pallidum ventral (VP).

Compte tenu de cette ségrégation incomplète des deux voies concernant les récepteurs DA, une approche chimio-génétique pourrait établir, par exemple si la réinstallation du comportement de recherche de COC observée après stimulation du D1R de la NAcc était due spécifiquement au recrutement des projections ciblant la VTA, ou influençant en quelque sorte l'équilibre des NEM-D1 et NEM-D2R projetant vers le VP, ou les deux. Cela pourrait être éclairci avec l'utilisation de DREADDs, une fois que nous parvenons à cibler avec succès l'une ou l'autre voie indépendamment de l'expression des récepteurs de DA, soit en provoquant sa stimulation ou son inhibition.

Résumé des méthodes: Ainsi, grâce à l'infusion de traceurs neuronaux rétrogrades, nous avons pu définir les régions où les deux virus (AAV DREADD et CAV-Cre) doivent être injectés pour avoir les mêmes cellules infectées dans deux régions différentes: la NAcc où les corps cellulaires sont trouvés, et les cibles de l'une ou l'autre voie, à savoir le VP et le VTA. Une fois cette étape franchie, nous avons commencé des essais comportementaux, avec perfusion virale et expression de DREADD excitateurs (G_q) dans les voies NAcc-VP ou NAcc-VTA. Pour étudier le comportement résultant de l'activation de l'une ou l'autre voie, nous avons choisi d'évaluer la locomotion des animaux, étant donné que la stimulation des NEM striataux des voies directes et indirectes exercerait respectivement un effet inhibiteur ou excitateur sur le comportement.

Résumé des résultats : *En ce qui concerne nos tests comportementaux, nous n'avons pas pu observer de différences dans l'activité des animaux traités avec CNO après avoir reçu des*

DREADD dans la voie NAcc-VP. D'autre part, une augmentation légère mais significative de l'activité locomotrice a été observée suite à l'injection d'animaux avec des DREADD dans la voie NAcc-VTA. L'absence de résultats comportementaux probants pourrait être expliquée par une recombinaison trop faible des DREADD pour que leur activation par le CNO puisse s'exprimer comportementalement.

Enfin, le principal obstacle au développement des travaux de cette section a été la mise en place d'une méthode fiable de quantification de la recombinaison virale afin de définir un seuil précis pour l'analyse des données comportementales issues des expériences DREADDs, ce qui devrait constituer une priorité pour les études futures utilisant cette méthodologie. La technologie DREADD reste néanmoins intéressante à développer car elle est très puissante dans sa spécificité et sa multiplicité de circuits interrogeables, dans le cadre des neurosciences comportementales et plus précisément ici des circuits neuronaux impliqués dans le processus addictif et de la dépendance aux drogues d'abus.

Abstract

For many, drug taking may continue on an occasional basis for a long time; however, some individuals lose control of their drug use and are unable to stop. The transition from casual use to addiction is accompanied by drug-induced changes in the brain, followed by associated changes in behavioral functions. One of the most problematic aspects of addiction is the enduring vulnerability to relapse that persists even long after withdrawal symptoms have abated. Rodent models of drug self-administration (SA) show that re-exposure to the drug itself, drug associated cues or stress are major triggers of relapse. While different classes of drugs vary in their primary pharmacological mechanisms, they all share an acute effect of raising dopamine (DA) levels in the striatum. Following repeated use, the addictive properties of psychostimulants such as cocaine (COC) are believed to take place through the induction of neuroadaptations within the mesocorticolimbic DA system.

The striatum is a major target of the DA system, where DA acts on two families of metabotropic receptors (D1-like or D2-like) that are segregated into two pathways of medium spiny neurons (MSNs) and have different roles in addiction and relapse. For instance, while systemic D2 receptor (D2R) stimulation induces reinstatement of COC seeking in rats, D1 receptor (D1R) stimulation does not. DA signaling in the nucleus accumbens (NAcc) responds to rewarding and aversive stimuli; in turn, the dorsolateral striatum (DLS) plays a key role in the transition to compulsive use, and the habitual aspects of drug-seeking after prolonged drug SA. Despite several works examining their role in relapse, the results remain somewhat unclear. Given their critical but differential involvement in COC seeking, here we investigated the role of D1R and D2R receptors of the NAcc and DLS in relapse, employing pharmacological manipulations, as well as assessing their protein expression using an animal model of COC SA. Our results showed a double dissociation between the actions of both DA receptors (DARs) in the striatum. Pharmacological activation of the D1R, but not D2R of the NAcc induces reinstatement of COC seeking, whereas the same effect is triggered by the activation of D2R, but not D1R of the DLS. Also, the reinstating effects of the systemic D2R stimulation is blocked by D1R or D2R antagonists injected into the NAcc or D1R antagonist into the DLS, while being blunted by the D2R inhibition in the DLS. These results convey an interaction between both receptor subtypes, likely relying on ascending spiraling connections associating the ventral and the dorsal striatum through midbrain-reaching loops. Finally, we found the reinstatement of COC seeking elicited by D1R or D2R agonists in either region is not due to changes in DAR expression.

These results enticed us to examine the behavioral mechanisms underpinning reinstating behavior. Though initially goal-directed, COC seeking is argued to become habitual after extended training. This progression is believed to initially elicit functional changes within the NAcc, and gradually hijack the circuitry of the dorsal striatum. The activation of D1R in the NAcc and D2R in the DLS has been associated with the processing of rewarding properties and habitual responding for drugs, respectively. Therefore, we aimed to assess whether the reinstatement of COC seeking triggered by the D2R stimulation within the DLS would involve incentive motivational or reinforcing processes likely underlying those induced by the D1R stimulation within the NAcc. Also, we aimed to assess whether the D1R stimulation within the NAcc involves the overtaking of the behavioral control by habitual stimulus-response mechanisms which may be involved in the reinstatement of drug-seeking after the D2R stimulation in the DLS. To this end, we worked on a model of transition of behavioral control from a goal-directed system to the eventual automatization believed to underlie drug use by training rats in a chained schedule of reinforcement with food rewards. Also, rats were submitted to a COC sensitizing regimen that is believed to accelerate the shift in behavioral control. Our results showed that even though it is possible to establish a second order schedule with food rewards, extended training under this protocol does not elicit habitual behavior, even after repeated COC exposure. We then began a preliminary chemopharmacological study to assess the role of the pathways comprised by D1R- and D2R-bearing MSNs to tackle their role beyond the DARs in relapse. Finally, the conflation of pharmacological techniques and behavioral models can represent a promising approach in the search for the basis of drug addiction and relapse.

List of Abbreviations

5-HT	5-hydroxytryptamine (Serotonin)
A – O	Action – outcome association
AAV	Adeno-associated virus
AF	Alexa fluor
AL	Active lever
AMPAr	α -amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptor
AMPH	Amphetamine
BDNF	Brain derived neurotrophic factor
BG	Basal ganglia
BLA	Basolateral nucleus of the amygdala
BNST	Bed nucleus of the <i>stria terminalis</i>
cAMP	Cyclic adenosine monophosphate
CAV	Canine adenovirus
CAV-Cre	Canine adenovirus expressing Cre-recombinase enzyme
CeA	Central nucleus of the amygdala
CNO	Clozapine- <i>N</i> -oxide
COC	Cocaine
CPP	Conditioned place preference
CREB	cAMP response element binding protein
CRF	Corticotrophin-releasing factor
CS	Conditioned stimulus
CTB	Cholera toxin subunit B
D1R	D1 dopamine receptor subtype
D2R	D2 dopamine receptor subtype
DA	Dopamine/dopaminergic
DARPP-32	32-kDa DA- and cAMP-regulated phosphoprotein

DAT	Dopamine transporter
DLS	Dorsolateral striatum
DMS	Dorsomedial striatum
DREADD	Designer receptor exclusively activated by designer drug
DS	Dorsal striatum
ERK	Extracellular signal-regulated kinase
FI	Fixed interval
FR	Fixed ratio
GABA	γ -aminobutyric acid
GLU	Glutamate/glutamatergic
GP	Globus pallidus
GPCR	G protein coupled receptor
GPe	Globus pallidus <i>pars externa</i>
GPI	Globus pallidus <i>pars interna</i>
i.p.	Intraperitoneal
i.v.	Intravenous
IL	Inactive lever
LiCl	Lithium chloride
LP	Lever press(es)
LTP	Long term potentiation
mAChR	Muscarinic acetylcholine receptor
mGluR2/3	Group II metabotropic glutamate receptor
mPFC	Medial prefrontal cortex
MSN	Medium spiny neuron
N-AC	<i>N</i> -acetylcysteine
NAcc	Nucleus accumbens
NaCl	Sodium chloride
NE	Norepinephrine

NET	Norepinephrine transporter
NMDAR	<i>N</i> -methyl- <i>D</i> -aspartate receptor
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PKA	Protein kinase A
PP2B	Calmodulin-dependent protein phosphatase 2B
RFP	Red fluorescent protein
RI	Random interval
RR	Random ratio
S – R	Stimulus – response association
SA	Self-administration
SEM	Standard error of the mean
SERT	Serotonin transporter
SL	Seeking lever
SN	Substantia nigra
SNc	Substantia nigra <i>pars compacta</i>
SNr	Substantia nigra <i>pars reticulata</i>
TL	Taking lever
TO	Time out
TrkB	Tropomyosin receptor kinase B
VI	Variable interval
VP	Ventral pallidum
VTA	Ventral tegmental area

CONTENTS

INTRODUCTION	1
1. DRUG ADDICTION	1
2. THEORIES OF DRUG ADDICTION	3
2.1. THE PSYCHOMOTOR STIMULANT HYPOTHESIS	3
2.2. THE ABERRANT LEARNING HYPOTHESIS	3
2.3. THE HEDONIC ALLOSTASIS HYPOTHESIS.....	5
2.4. THE INCENTIVE SENSITIZATION HYPOTHESIS.....	5
3. COCAINE: A PSYCHOSTIMULANT	7
4. DOPAMINE SYSTEM.....	10
4.1. DOPAMINE, REWARD AND MOTIVATION.....	10
4.2. FUNCTIONAL NEUROANATOMY OF THE DOPAMINERGIC SYSTEM	13
4.3. DOPAMINERGIC RECEPTORS	14
5. THE STRIATUM	17
5.1. THE VENTRAL STRIATUM AND THE NUCLEUS ACCUMBENS	20
5.2. THE DORSAL STRIATUM	22
5.3. THE STRIATUM IN ADDICTION AND THE TRANSITION OF BEHAVIORAL CONTROL.....	23
6. ANIMAL MODELS OF DRUG REINFORCEMENT, CONSUMPTION, ADDICTION AND RELAPSE	26
6.1. BEHAVIORAL SENSITIZATION.....	27
6.2. CONDITIONED PLACE PREFERENCE	29
6.3. DRUG SELF-ADMINISTRATION	30
6.3.1. Simple schedules of reinforcement.....	30
6.3.2. Random Ratio/Random Interval schedules	31
6.3.3. Progressive Ratio schedules	31
6.4. MODELING OF DRUG ADDICTION	32
6.4.1. Escalation of COC self-administration	32
6.4.2. Addiction-like behavior.....	32
7. EXTINCTION-BASED REINSTATEMENT MODELS	33

7.1. CUE- AND CONTEXT-INDUCED REINSTATEMENT	36
7.2. STRESS-INDUCED REINSTATEMENT	37
7.3. PRIMING-INDUCED REINSTATEMENT.....	38

PROBLEM STATEMENT42

OBJECTIVES AND AIMS	43
---------------------------	----

MATERIAL AND METHODS.....47

1. SUBJECTS47

2. SURGICAL PROCEDURES47

2.1. INTRAVENOUS CATHETER IMPLANTATION (SECTION I).....	47
---	----

2.2. INTRACEREBRAL SURGERIES (SECTION I)	48
--	----

3. APPARATUS49

3.1. OPERANT CONDITIONING CHAMBERS (SECTIONS I AND II).....	49
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3.1.1. Operant conditioning for cocaine (Section I).....	50
--	----

3.1.2. Operant conditioning for food pellets (Section II).....	51
--	----

3.2. ACTIVITY CAGES (SECTION I; ANNEX)	52
--	----

4. MATERIAL52

4.1. DRUGS	52
------------------	----

4.2. FOOD OUTCOMES.....	53
-------------------------	----

5. STATISTICAL ANALYSES.....53

SECTION I – PHARMACOLOGICAL INVESTIGATION: THE ROLE OF DOPAMINE RECEPTORS OF THE STRIATUM IN THE RELAPSE OF COCAINE SEEKING56

SECTION OVERVIEW:.....	56
------------------------	----

ABSTRACT	61
----------------	----

INTRODUCTION	62
--------------------	----

MATERIAL AND METHODS64

SUBJECTS	64
----------------	----

SURGERY	64
APPARATUS	65
COCAINE SELF-ADMINISTRATION AND REINSTATEMENT TESTS	66
DRUGS	67
EXPERIMENTAL DESIGN	67
1ST SET OF RATS: NUCLEUS ACCUMBENS	67
2ND SET OF RATS: DORSOLATERAL STRIATUM	68
3RD SET OF RATS: DORSOLATERAL STRIATUM AND NUCLEUS ACCUMBENS	68
HISTOLOGY	69
DATA ANALYSES	69
RESULTS	69
EXPERIMENT I: REINSTATEMENT ELICITED BY D1R, BUT NOT D2R, AGONIST INFUSED INTO THE NACC	69
EXPERIMENT II: REINSTATEMENT ELICITED BY D2R, BUT NOT D1R, AGONIST INFUSED INTO THE DLS	72
EXPERIMENT III: REINSTATEMENT ELICITED BY D2R AGONIST INFUSED INTO THE DLS	75
EXPERIMENT IV-A: INFUSION OF D1R ANTAGONIST INTO THE DLS BLOCKS, WHILE D2R ANTAGONIST BLUNTS, COCAINE-SEEKING INDUCED BY PERIPHERAL INFUSION OF D2R AGONIST	77
EXPERIMENT IV-B: INFUSION OF D1R OR D2R ANTAGONISTS INTO THE NACC BLOCKS REINSTATEMENT OF COCAINE-SEEKING INDUCED BY PERIPHERAL INFUSION OF D2R AGONIST	79
DISCUSSION	80
ADMINISTRATION OF D1R, BUT NOT D2R, AGONIST INTO THE NACC INDUCES REINSTATEMENT OF COCAINE- SEEKING	81
INFUSION OF D2R, BUT NOT D1R, AGONIST INTO THE DLS INDUCES REINSTATEMENT OF COC-SEEKING	83
INFUSION OF D1R ANTAGONIST INTO THE NACC OR DLS BLOCKS REINSTATEMENT OF COCAINE-SEEKING INDUCED BY PERIPHERAL INFUSION OF D2R AGONIST	85
INHIBITION OF D2R WITHIN THE NACC BLOCKS, WHILE WITHIN THE DLS BLUNTS, THE REINSTATING EFFECTS OF THE SYSTEMIC ADMINISTRATION OF D2R AGONISTS	86
ACKNOWLEDGEMENTS	87
FUNDING AND DISCLOSURE	87
REFERENCES	88
SUPPLEMENTARY INFORMATION	93

**SECTION II – BEHAVIORAL INVESTIGATION: MODELLING THE TRANSITION TO THE
AUTOMATIZATION OF BEHAVIORAL CONTROL96**

1. INTRODUCTION.....96

1.1. ASSOCIATIVE LEARNING..... 97

 1.1.1. Pavlovian conditioning – prediction of events 97

 1.1.2. Instrumental conditioning – encoding causal relationship between actions and outcomes..... 97

1.2. GOAL DIRECTED VS. HABIT BASED BEHAVIOR 98

1.3. ASSESSMENT OF BEHAVIORAL CONTROL..... 100

1.4. SECOND-ORDER SCHEDULE OF REINFORCEMENT 102

1.5. OBJECTIVES..... 104

2. MATERIALS AND METHODS105

2.1. EXPERIMENTAL DESIGN..... 105

2.2. SECOND ORDER SCHEDULE OF REINFORCEMENT WITH FOOD PELLETS AS REWARDS 105

2.3. OUTCOME DEVALUATION BY SPECIFIC SATIETY 107

2.4. EXTINCTION OF THE PELLET SEEKING/TAKING LINK 107

2.5. COCAINE SENSITIZATION..... 108

2.6. OUTCOME DEVALUATION BY CONDITIONED TASTE AVERSION..... 108

3. RESULTS.....109

3.1. SECOND ORDER SCHEDULE WITH FOOD PELLETS AS REWARD 109

 3.1.1. Second-order schedule acquisition..... 109

 3.1.2. Outcome devaluation by specific satiety..... 109

 3.1.3. Extinction of the pellet seeking/taking link 111

 3.1.4. Behavioral Sensitization 113

 3.1.5. Outcome devaluation by specific satiety: limited training [post-sensitization – VI30]..... 113

 3.1.6. Outcome devaluation by specific satiety: extended training [post sensitization; VI60]..... 115

 3.1.7. Outcome devaluation by conditioned taste aversion..... 116

4. DISCUSSION117

4.1. THE GOAL OF SEEKING ACTIONS AND TAKING RESPONSES 118

4.2. THE CASE FOR INCENTIVE SALIENCE 120

4.3. CHUNKING OF ACTIONS AND HIERARCHICAL DISPOSITION OF GOAL-DIRECTED AND HABITUAL SYSTEMS 121

4.4. MANIPULATION OF ACTIONS DISTAL OR PROXIMAL TO THE GOAL	122
4.5. PROTOCOL ISSUES AND COC SENSITIZATION	123
4.6. FINAL CONSIDERATIONS.....	125

SECTION III – PHARMACOGENETICS: PRELIMINARY STUDY ON NOVEL APPROACHES TO INVESTIGATE STRIATAL OUTPUT PATHWAYS IN DRUG INDUCED RELAPSE129

1. INTRODUCTION.....	129
1.1. ACCUMBAL D1R AND D2R CELL POPULATIONS – ANATOMICAL INVESTIGATION.....	130
1.2. PHARMACOGENETIC MANIPULATION – DREADDs.....	131
2. MATERIAL AND METHODS.....	134
2.1. SUBJECTS.....	135
2.2. STEREOTAXIC SURGERY: TRACER INFUSION.....	135
2.3. VIRUS INFECTION FOR DREADDs EXPRESSION.....	136
2.4. LOCOMOTOR ACTIVITY ASSESSMENT	137
2.5. PERFUSION, BRAIN SLICING AND IMMUNOHISTOCHEMISTRY	138
3. RESULTS.....	138
3.1. NEURONAL TRACING.....	138
3.2. PHARMACOGENETIC MANIPULATION	140
3.2.1. Behavioral assessment.....	140
3.2.2. Confirmation of DREADD recombination.....	142
4. DISCUSSION	143

SYNTHESIS AND CONCLUSIONS.....150

1. REMINDER OF THE AIMS.....	150
2. SUMMARY OF RESULTS	150
2.1. PHARMACOLOGICAL INVESTIGATION: THE ROLE OF DOPAMINE RECEPTORS OF THE STRIATUM IN THE RELAPSE OF COCAINE SEEKING	150
2.2. BEHAVIORAL INVESTIGATION: MODELLING THE TRANSITION TO THE AUTOMATIZATION OF BEHAVIORAL CONTROL.....	151

2.3. PHARMACOGENETICS: PRELIMINARY STUDY ON NOVEL APPROACHES TO INVESTIGATE STRIATAL OUTPUT PATHWAYS IN DRUG INDUCED RELAPSE	153
3. FINAL CONSIDERATIONS	155
3.1. WHY DO RATS RELAPSE?	155
3.2. THE BEHAVIORAL CONTROL UNDER SECOND-ORDER SCHEDULES	157
3.3. SECOND-ORDER SCHEDULE WITH COC REINFORCEMENT	159
3.4. CONCLUSION	159
<u>REFERENCES</u>	<u>162</u>
<u>ANNEX</u>	<u>185</u>
1. QUANTIFICATION OF D1R AND D2R IN THE NACC AFTER COC SA	185
2. MATERIAL AND METHODS	185
2.1. SUBJECTS, SURGERY AND COC SA	185
2.2. PROTEIN QUANTIFICATION: WESTERN BLOT ANALYSES	186
3. RESULTS	186
3.1. ACQUISITION AND EXTINCTION OF COCAINE SELF-ADMINISTRATION.....	186
3.2. COC SA DOES NOT ALTER EXPRESSION OF D1R OR D2R OF THE NACC OR DS	187

INTRODUCTION

Introduction

1. Drug addiction

It is true that for some people drug taking may continue on an occasional basis for a long period of time; however, some individuals lose control of their drug use and are unable to stop. They compulsively search for and take drugs in spite of the obviously harmful personal and social consequences. The transition from casual to compulsive patterns of drug use and to addiction is accompanied by many drug-induced changes in the brain and associated changes in psychological functions (Robinson and Berridge, 2003) that frequently persist beyond detoxification, particularly in individuals severely afflicted by it. The behavioral effects of these brain changes may be evidenced in the repeated relapse episodes and intense drug craving (American Psychiatric Association, 2013; Everitt, 2014).

Thus, defined as a compulsion to take a drug with loss of control over drug taking despite adverse consequences, substance use disorders are now increasingly regarded as disorders of the brain, with specific neurobiological, molecular and behavioral characteristics that have environmental, drug-induced and genetic determinants of vulnerability (Kreek et al, 2002). Perhaps the most problematic aspect of addiction is the high risk of relapse to drug use that persists even in abstinent addicts long after any withdrawal symptoms have abated and, perhaps, for a lifetime (Stewart, 2000). Therefore, the key questions in addiction are why some susceptible individuals undergo a transition from casual drug use to compulsive patterns of drug use; why do addicts find it so difficult to stop using drugs; and what are the causes of relapse (Robinson and Berridge, 2003).

Drug addiction has also been proposed to involve elements of impulsivity, especially on early stages and compulsivity on late stages. As the subject shifts from impulsivity to compulsivity, a shift is also undergone from positive reinforcement to negative reinforcement on the drive of motivated behavior (Koob and Le Moal, 2008a). These elements of impulsivity and compulsivity yield an addiction cycle comprising three stages, conceptualized as interacting with each other, to become progressively intense and leading to the pathological state conceived as addiction (Figure 1):

- A) Binge/intoxication: heavily involving positive reinforcement and the basal ganglia (BG) (described below);

- B) Withdrawal/negative affect: involving key elements of the extended amygdala¹ and negative reinforcement;
- C) Preoccupation/anticipation: involving elements of the prefrontal cortex (PFC), is deemed a key element of relapse, often linked to the construct of craving.

Furthermore, the latest edition Diagnostic and Statistical Manual of Mental Disorders [DSM-5; (American Psychiatric Association, 2013)] combines Abuse and Dependence into a single unified construct and measures severity on a continuous scale from mild (2–3 symptoms endorsed), moderate (4–5 symptoms endorsed) and severe (6 or more symptoms endorsed) out of 11 total symptoms (Koob and Volkow, 2016; Robinson and Adinoff, 2016).



Figure 1. The addiction cycle. Adapted from Koob and Le Moal (2008b).

Over the course of addiction, the changes in brain circuits imposed by long-term exposure (often years) to drugs do not seem to revert to a 'pre-drug' state, even when the body is cleansed of the drug, thus long-term treatment beyond detoxification is usually necessary (O'Brien, 2005). Because of its chronic nature, the high vulnerability to relapse even after long abstinence and well after symptoms of withdrawal have disappeared remains as a major clinical problem in the treatment of drug addiction.

¹ The *extended amygdala* includes not only the central nucleus of the amygdala (CeA), but also the bed nucleus of the stria terminalis (BNST) and a transition zone in the NAcc shell. These regions share certain cytoarchitectural and circuitry similarities (McDonald *et al*, 1999)

2. Theories of Drug Addiction

It has become clear that the consumption of drugs elicits several neuroadaptations throughout the brain, with the related modifications in behavioral functions, which contribute to the enduring nature of the addictive state. Over the last decades several different theoretical approaches have been developed in the intent of unraveling the mechanisms involved in the process of drug-dependence. Although each of these theories has been deemed incomplete and unable to explain all of the data obtained so far, they are not incompatible with each other. Instead, each one of them captures different aspects of the process and so they provide a series of potential causes for addictive behavior.

Thus, in order to understand the pathophysiology of addiction, it is necessary to address questions that require the integration of behavioral, systemic, cellular and molecular points of view. With this purpose, several theories of drug addiction have been developed throughout the last decades, and what follows is a brief description of some of the most relevant so far:

2.1. *The Psychomotor Stimulant hypothesis*

The core of this theory sustains that the reinforcing effects of drugs, and consequently their addiction liability, can be predicted from their ability to induce psychomotor activation. It sustains that all drugs that act as positive reinforcers activate a common biological mechanism associated with locomotion and that this mechanism is comprised by dopaminergic fibers that project from the midbrain to limbic and cortical regions. Importantly, this very same circuitry is implicated in brain reward. Hence, the authors sustain that the locomotor effects and the positive reinforcing effects of these drugs are homologous, suggesting that they derive from the activation of a common mechanism, which is also responsible for the approach and reinforcement that are associated with natural rewards such as food or water for hungry or thirsty animals (Wise and Bozarth, 1987).

2.2. *The Aberrant Learning hypothesis*

This theory sustains that addiction develops, at least in part, because of aberrant learning and memory, arguing that chronic drug exposure leads to long-lasting changes in the circuits underlying normal learning and memory mechanisms and this happens through their direct pharmacological actions on multiple neurotransmitter systems (Torregrossa *et al*, 2011).

The learning component of this hypothesis is based on the general concept of positive reinforcement, which assimilates two main processes identified with conventional reinforcers (Dickinson, 1985). The first is a process based upon knowledge of the relationship between an action and its outcome. When controlled by this process, instrumental behavior takes the form of intentional, goal-directed actions which are performed because the individual 'knows' that these *actions* give access to the *outcome* – being the latter frequently a reinforcer. The second is the *stimulus–response* (S–R) mechanism by which reinforcers strengthen an association between the response and the contextual/discriminative stimuli that are evoked concomitantly. Behavior controlled by this process is composed of habitual responses that are elicited automatically by these discriminative stimuli and, importantly, is elicited independently of the current value of the outcome.

In the transition from initial drug use, a drug is initially taken voluntarily because of its reinforcing effects, characterizing a goal-directed behavior. The loss of control over its consumption happens as it becomes a S–R (rather than *action-outcome*; A–O) association eventually becoming an over-learned/habitual behavior which eventually culminates in a compulsion (Everitt and Robbins, 2013). Physiologically, this transition from goal-directed actions to more habitual modes of responding in drug seeking behavior is proposed to result from a transition from prefrontal cortical to striatal control over responding, and from ventromedial to dorsolateral striatal sub regions (Voorn *et al*, 2004), in a dopamine (DA)-dependent manner [(Everitt and Robbins, 2005) Figure 2].

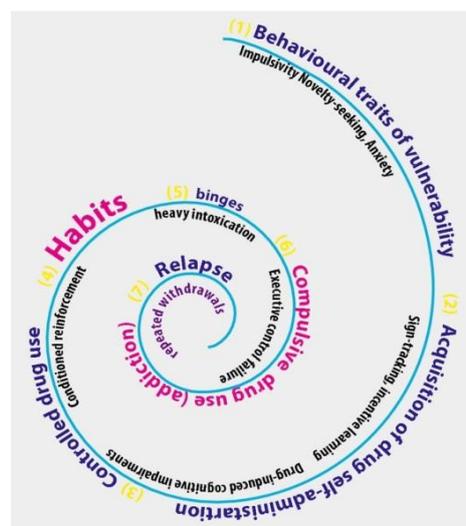


Figure 2. The processes that may underlie the transition from voluntary drug seeking, through loss of control over drug use, and the emergence of compulsive drug seeking habits. Although depicted as a spiral, these processes may also occur in parallel. The notion of vulnerability is also captured in the spiral, determining that some but not all individuals ultimately seek and take drugs compulsively, in part through the loss of inhibitory control over drug seeking habits. Adapted from Everitt (2014).

2.3. *The Hedonic Allostasis hypothesis*

This theory is based on the opponent process theory of motivation, proposed by Solomon and Corbit (1974), who postulated that hedonic, affective, or emotional states, once initiated, are automatically modulated by the central nervous system through mechanisms that reduce the intensity of hedonic feelings. Within this theoretical frame, two motivational processes were defined: the *A-process* and the *B-process*. From a drug-taking perspective, the initial positive hedonic response resultant from drug use is hypothesized to be opposed by a negative hedonic response in a devolution aiming for the homeostatic balance within brain motivational systems (Edwards and Koob, 2010). Thus, for instance, the first few self-administrations of an opiate drug produce a pattern of motivational changes similar to that of euphoria (the *A-process*), which is followed by a decline in intensity. Then, after the drug wears off, an opposing, aversive negative emotional state emerges (the *B-process*) (Koob and Le Moal, 2008a).

In turn, the hedonic allostasis hypothesis sustains that initial drug use is primarily controlled by the drug's rewarding effects, in a process of positive reinforcement based on the activation of mesolimbic DA projections to the nucleus accumbens (NAcc) and amygdala. Eventually the chronic drug use leads to decreases in its rewarding effects and the recruitment of stress-related systems, with the release of corticotrophin-releasing factor (CRF), as well as dynorphins and norepinephrine in several brain areas. This stage is characterized by the manifestation of anxiety/aversive-like states, in which the motivation for drug consumption is guided by negative reinforcement. At this point, drugs serve mainly to alleviate negative emotional symptoms induced by drug withdrawal (Edwards and Koob, 2010).

Therefore the individual undergoes a new emotional state, termed the *hedonic allostatic* state, which represents a chronic change in the normal reward set point. In this new set point, counteradaptive processes (such as the opponent *B-process*), which are part of the normal homeostatic limitation of reward function, fail to return to within the normal homeostatic range, ultimately leading to a state of pathology [(Koob and Le Moal, 2001, 2008b) Figure 3].

2.4. *The Incentive Sensitization hypothesis*

The incentive sensitization theory of addiction, proposed by Robinson and Berridge, (1993), proposes that repeated exposure to potentially addictive drugs can persistently elicit changes in circuits that normally regulate the attribution of incentive salience to stimuli, which is involved in motivated behavior. These neuroadaptations render the brain circuits hypersensitive (*sensitized*) in a way that results in pathological levels of incentive salience being attributed to drugs and drug-associated cues. Persistence of incentive sensitization makes pathological

incentive motivation (*wanting*) for drugs last for years, even after the discontinuation of drug use. Importantly, it is suggested that the sensitization of neural systems that mediate incentive salience (drug *wanting*) occurs *independently* of changes in neural systems that control pleasurable effects of drugs (drug *liking*).

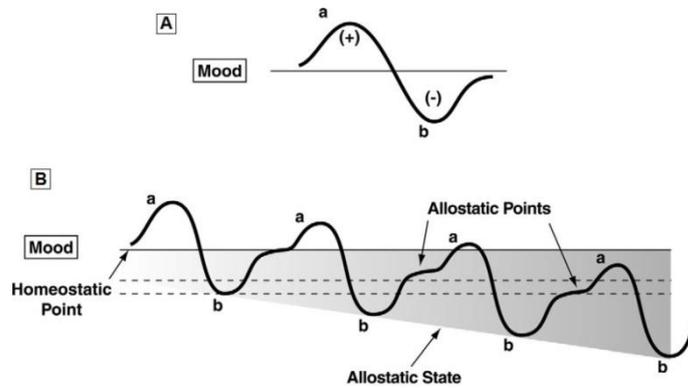


Figure 3. Schematic depiction of Solomon and Corbit's (1974) opponent-process model of motivation that incorporates the conceptual framework of the Hedonic allostasis hypothesis. Both panels represent the affective response to the presentation of a drug. (A) Representation of the initial experience of a drug with no prior drug history, and the a-process represents a positive hedonic or positive mood state and the b-process represents the negative hedonic or negative mood state. The affective stimulus (state) has been argued to be a sum of both an a-process and a b-process. (B) The changes in the affective stimulus (state) in an individual with repeated frequent drug use that may represent a transition to an allostatic state in the brain reward systems (representing a transition to addiction). The apparent b-process never returns to the original homeostatic level before drug-taking begins again, thus creating a greater and greater allostatic state in the brain reward system. The authors sustain that even after a period of protracted abstinence the reward system is still bearing allostatic changes. Adapted from Koob and Le Moal (2001).

More specifically, the theory specifies that encounters with new incentives and rewards normally activate three distinct psychological processes mediated by dissociable neural substrates: *hedonic activation (liking)*, by the unconditioned stimulus, *associative learning* of the correlation between conditioned stimulus and unconditioned stimulus, and *attribution of incentive salience (wanting)* to the conditioned stimulus or its representation on subsequent encounters [(Berridge and Robinson, 1998) Figure 4]. Within this framework, DA is proposed to mediate the '*wanting*' component through the attribution of incentive salience to reward-related/predictive stimuli. Thus, these stimuli and their associated rewards become motivationally '*wanted*' through Pavlovian learning mechanisms (Berridge, 2007).

Therefore in addicts the focus on drugs in particular is produced by an interaction between incentive salience mechanisms with associative learning mechanisms that normally direct motivation to specific targets. However, as learning itself is not enough for the pathological motivation to take drugs, the authors argue that the pathological motivation arises from sensitization of brain circuits that mediate Pavlovian conditioned incentive motivational

processes (i.e. *incentive sensitization*) and that the contextual control over the expression of sensitization provides an additional mechanism that accounts for why addicts '*want*' drugs most particularly when they are in drug-associated contexts (Robinson and Berridge, 1993, 2008).

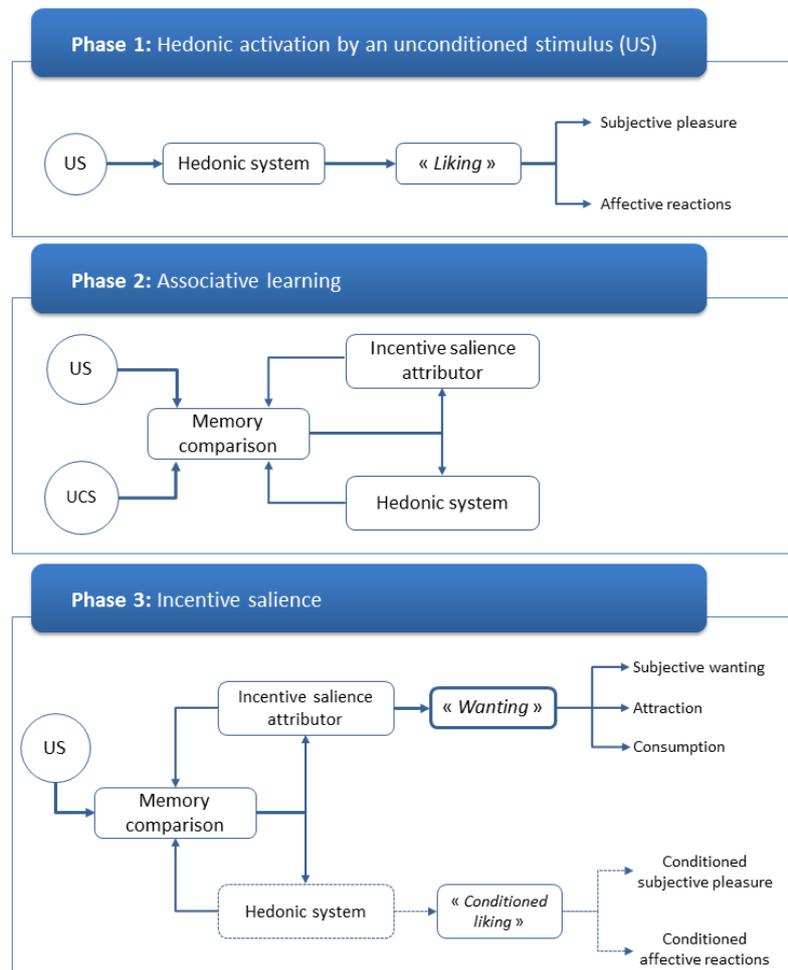


Figure 4. The incentive-sensitization model. Phase 1: the first presentation of a reward or an unconditioned stimulus (US) activates the hedonic system. This activation results in feeling pleasure (*liking*), which is manifested by emotional responses such as subjective pleasure. Phase 2: when a reward (US) and a conditioned stimulus (CS) are presented contiguously and contingently, an association is established between them, and the CS acquires incentive value during this associative learning. Phase 3: after conditioning is established, the presentation of the CS triggers several conditioned responses. The CS has acquired incentive value and thus creates a desire (*wanting*), which is manifested as the attraction to the CS. Individually the CS can also activate the hedonic system and produce conditioned emotional responses. However, only *wanting* is capable to drive and control behavior. Adapted from Berridge and Robinson (1998).

3. Cocaine: a psychostimulant

Most of these theories mentioned above have been developed taking into account data with psychostimulants, such as cocaine (COC), and this work has been developed with COC as a psychostimulant drug model. Psychomotor stimulants or psychostimulants are a broad class

of sympathomimetic drugs that elicit behavioral activation at doses typically employed for therapeutic or recreational purposes (McCreary *et al*, 2015), and their effects can include increased movement, arousal, vigilance, anorexia, vigor, wakefulness, and attention (Wood *et al*, 2014). A number of synthetic stimulants, including amphetamines (AMPH), are useful medications in the treatment of attention deficit hyperactivity disorder, narcolepsy and obesity (Howell and Kimmel, 2008; McCreary *et al*, 2015).

Psychostimulants include drugs with [i] a high potential for abuse and no accepted medical use [e.g. 3,4-Methylenedioxymethamphetamine (MDMA; ecstasy)], [ii] a high potential for abuse which may lead to severe psychological or physical dependence and accepted for medical use with severe restrictions (e.g. AMPH, COC, methamphetamine, methylphenidate) and [iii] a low potential for abuse which may lead to limited physical or psychological dependence with accepted medical use (e.g. armodafinil, dextromethylphenidate, modafinil) (Howell and Kimmel, 2008; McCreary *et al*, 2015). Cocaine itself is still used clinically as a local anesthetic, primarily for eye, ear, nose or throat procedures, whereas most of the stimulant medications used for clinical purposes are closely related to the catecholamine neurotransmitters, norepinephrine (NE) and dopamine (Howell and Kimmel, 2008). It is worth to mention that almost all psychostimulants display abuse potential, while recreational doses are generally much higher than those prescribed for therapeutic applications, consequently carrying greater risks of side effects.

The primary mechanism of action of psychostimulants varies, yet common mechanisms of action are described:

- A) Release of neuronal catecholamines (amphetamines, methylphenidate);
- B) Inhibition of monoamine uptake (amphetamines, cocaine, methylphenidate);
- C) Inhibition of monoamine oxidase (amphetamine);
- D) Binding to extracellular receptors: adrenoceptors (ephedrine, modafinil), serotonin receptors (e.g., 5-HT_{2A/2C} series “hallucinogens”), and adenosine receptors (caffeine).

Thus, monoamines [DA, serotonin (5-hydroxytryptamine; 5-HT) and NE] participate in the behavioral pharmacology and addictive properties of psychostimulants and, in particular, DA plays a primary role in the reinforcing and behavioral-stimulant effects of these drugs in animals and humans (McCreary *et al*, 2015; Tanda *et al*, 2009; Thomsen *et al*, 2009).

Cocaine pertains to a subclass of psychostimulants known as ‘amphetamine-like’, and is consumed by methods such as intravenous (i.v.) injection, snorting or smoking. These drugs share with AMPH a molecular site of action at monoamine transporters (White and Kalivas, 1998; Wood *et al*, 2014).

Being an alkaloid derived from the coca plant *Erythroxylum coca* (Figure 5), the chewing of its leaves originated in Peru at least as early as 3000 BC. Typically extracted in a paste form and converted into a hydrochloride or sulfate salt, COC has been used for centuries, displaying powerful stimulant effects. Once in the body, it produces effects such as euphoria, disinhibition, impulsiveness and high psychomotor activation (Koob *et al*, 2014; Rowbotham, 1988; Wood *et al*, 2014).



Figure 5. The coca plant *Erythroxylum coca*. Coca leaves have been used for thousands of years in Central and South America for their more modest stimulant effects.

The primary physiological mechanism for inactivation of monoamine signaling is transporter-mediated uptake of released monoamine neurotransmitters. Psychostimulants enhance monoamine signaling by interfering with transporter function, but they differ, however, in their relative affinity for DA, 5HT and NE transporters [DAT, SERT, NET; respectively (Grouleff *et al*, 2015; Tanda *et al*, 2009; Vaughan and Foster, 2013)]. Cocaine is an uptake inhibitor with similar affinity for these three transporters, nevertheless its behavioral effects and addictive properties have been linked most closely to enhanced dopaminergic activity. This does not imply that actions at SERT and NET do not contribute shaping the profile of motor or drug-seeking behavior. However, studies with ligands for these transporters have shown that such drugs lack the ability to maintain self-administration behavior in animals or to produce euphoria in humans, properties that are highly associated with actions at DATs and DA receptors (White and Kalivas, 1998; Wood *et al*, 2014).

In this sense, COC is proposed to bind the DAT at a site distinct from DA, thus inhibiting both the binding and transport of DA and sodium into the cytosol. By blocking the reuptake of DA by the DAT, COC acts as an indirect DA agonist, increasing extracellular levels of DA (Carroll *et al*, 1999; Howell and Kimmel, 2008). Cocaine-induced increases in extracellular DA in

the mesocorticolimbic DA system are critical in mediating the behavioral effects of psychostimulants (McCreary *et al*, 2015; Tanda *et al*, 2009). Therefore, within this work, the nigrostriatal and mesocorticolimbic DA system are to be discussed in the framework of the adaptations they undergo following the exposure to drugs of abuse.

4. Dopamine System

While different classes of addictive drugs vary in their primary pharmacological mechanisms, they all share an acute effect of increasing DA concentration in the striatum, particularly in the NAcc [(Di Chiara, 1999; Di Chiara and Imperato, 1988) Figure 6]. Dopamine is a metabolite of the amino acid tyrosine, and has been discovered nearly 60 years ago by Carlsson *et al*, (1957). Taking into account its large anatomical distribution, DA is the predominant catecholamine neurotransmitter in the mammalian brain, involved in a number of neurobiological processes, through which it controls a variety of functions, including locomotor activity, cognition, emotion, feeding, reward, and endocrine regulation. Being a neuromodulator, its actions on DA receptors allows it to modulate the excitability of neurons as well as synaptic plasticity, for instance, by regulating release of neurotransmitters. In the periphery, DA plays important physiological roles in the regulation of olfaction, retinal processes, vascular tone, and hormone secretion, among others (Beaulieu and Gainetdinov, 2011; Missale *et al*, 1998; Tritsch and Sabatini, 2012). As DA is involved in a variety of critical functions, it is of no surprise that multiple disorders have been related to dopaminergic function. These include Parkinson's disease, Tourette's syndrome, schizophrenia, Huntington's disease and, more specifically through its role in motivation and reward mechanisms, drug addiction.

4.1. Dopamine, Reward and Motivation

The mesolimbic DA system is deemed the 'brain reward system', but the role of DA in reward is still a source of debate, and several ideas have been put forward in the attempt to decipher the role of DA in reward processes. These ideas include the activation-sensorimotor hypothesis, the hedonia hypothesis of reward pleasure, reward learning hypotheses, and incentive salience hypothesis of reward 'wanting'.

The activation-sensorimotor hypotheses suggest DA mediates general functions of action generation, effort, movement, and general arousal or behavioral activation. Although it is well established that DA systems play roles in movement activation and control, attention, and

arousal, and these are likely important aspects of behavior, this theory is argued not to account for why DA effects are rewarding, predictive or motivating (Berridge, 2007; Salamone and Correa, 2002).

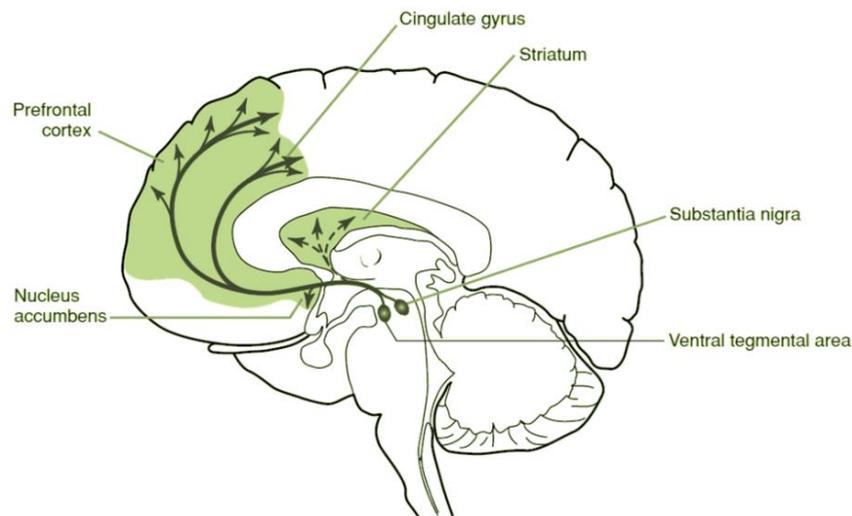


Figure 6. Dopamine projections in the human brain. Projections from the VTA to the NAcc, and PFC, and projections from the SN to the dorsal striatum [caudate and putamen and related structures. Adapted from Hyman *et al* (2006).

Further, the hedonia hypothesis sustains that DA mediates the pleasure produced by natural rewards (e.g. food, sex, or drugs of abuse). In this case, a reduction in the perception of pleasure (i.e. anhedonia) is perceived once DA function is suppressed (Wise, 1980). The anhedonia hypothesis has soon been contested, notably with the use of hedonic facial reactions: though hedonic pleasure is often regarded as exclusively subjective, hedonic stimuli also bring about reactions from brain systems. These reactions, observed in primates and rodent species, share the same rule for generating certain aspects of expression microstructure, and are translated into facial reactions such as tongue protrusions to sweet tastes and negative ‘disliking’ gapes to bitter tastes (Berridge, 2007; Berridge *et al*, 2009; Di Chiara, 2002). This tool allowed for the demonstration that DA-depleted animals still show behavioral hedonic responses (i.e. ‘liking’ reactions) to palatable tastes such as that of sucrose solutions infused orally, thus undermining the idea that DA is exclusively responsible for hedonic processing of rewards (Berridge *et al*, 1989).

Also, the reward learning hypothesis suggests that phasic DA within the striatum associatively reinforces new links between stimulus – response events, acting as a prediction-error or teaching signal that guides learning (Di Chiara, 2002; Schultz, 2007; Schultz *et al*, 1997). One remarkable example is the electrophysiological demonstration, in primates, that in the initial stages of associative stimulus-reward learning DA neurons fire to the presentation of

the reward. This could lead to the previous idea, with the DA transmission coding for the hedonic value of the reward. However, as learning occurs, DA firing is drawn to the presentation of the reward-predicting stimulus rather than to the delivery of the reward itself. It could be thus suggested that [i] the reward itself does not activate the DA system, and [ii] the stimulus associated to the obtaining of a reward can acquire a DA-activating aspect (Schultz *et al*, 1997). Regarding this hypothesis, it has been argued that DA neurons may not be the source of their own learning-related changes in firing patterns. Instead, their associative signals may be a consequence, not a cause, of learning elsewhere in the brain, as signals received by DA neurons are likely to be highly processed previously by forebrain structures before DA cells get learning-relevant information (Berridge, 2007).

Finally, the incentive salience hypothesis asserts that reward is a composite construct, containing a number of components, namely wanting, learning and liking. Within this premise is introduced the concept of 'wanting', for which DA is argued to be responsible. In this case, DA by mediating the attribution of *incentive salience* to reward-associated stimuli renders them and their associated reward motivationally 'wanted'. It is worth noting that 'wanting' by itself would not suffice for reward to occur, and therefore the incentive salience still needs the two other components: the hedonic representation (i.e. 'liking'), and the predictive learning (Berridge and Robinson, 1998, 2003; Smith *et al*, 2011). In the case of human addicts, drugs such as heroin and COC are argued to cause real-life 'wanting' without 'liking', given long-lasting sensitization of the brain mesolimbic systems: addicts may take drugs compulsively even when they do not draw much pleasure from them. Therefore, incentive salience and sensitization concepts are combined in this theory, which does not deny that drug pleasure, withdrawal, or habits are indeed triggers of drug consumption, but suggests that it is rather the sensitized 'wanting' that underpins compulsive long-lasting addiction and relapse (Berridge, 2004). One of the critics attributed to this hypothesis, as argued by Di Chiara (2002), concerns the fact that the attribution of incentive salience is the result of a phasic stimulus-bound activity of DA neurons temporally placed in series between its triggering stimulus and the response. This assumption would contrast with the time-relationship between stimulus-bound burst activity in DA neurons and movement-related activity in BG output neurons.

Thus, the role of DA in normal behavior is still subject to debate, and though its role in addiction has been demonstrated in several ways, still much remains to be discovered. A brief description of the anatomical and pharmacological properties of the DA system, approaching the bases of its role in drug reward and addiction, follows below.

4.2. Functional Neuroanatomy of the Dopaminergic System

Within the mammalian brain, the three following dopaminergic pathways have been most studied and better described; arising from the three groups of DA-containing cells (Figures 6; 7):

- o *Nigrostriatal pathway*: arising from *substantia nigra (SN) pars compacta (SNc)*; group A9) and reaching the dorsal striatum (DS);
- o *Mesolimbic and mesocortical pathways* (collectively termed mesocorticolimbic pathway): arising from ventral tegmental area (VTA) and retrorubral area (RRA; groups A10 and A8, respectively) and reaching mostly limbic structures, such as the ventral portion of the striatum (including the NAcc), amygdala, olfactory tubercle, hippocampal formation and prefrontal cortex;
- o *Tuberoinfundibular pathway*: Arising from neurons of the arcuate nucleus and the tuberal region of the hypothalamus (groups A12 and A14) reaching the median eminence.

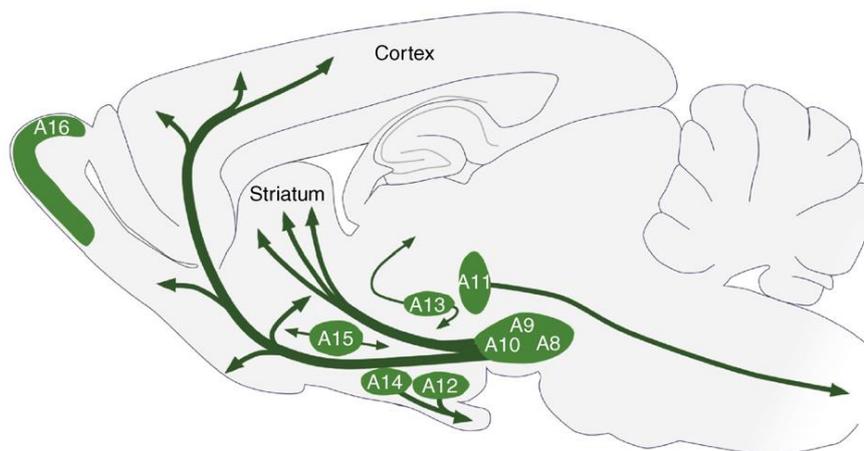


Figure 7. Main dopaminergic pathways within the rat brain. Adapted from Björklund and Dunnett (2007).

The striatum, as an important target on the DA system, is divided into several subregions, each endowed with different neuroanatomical and functional aspects; these are described in detail on §4.

Dopamine neurons are reported to display three main patterns of activity: a slow, irregular, ‘tonic’ mode; and a ‘phasic’ firing pattern (Floresco *et al*, 2003; Grace *et al*, 2007). The ‘tonic’ DA state is characterized by the spontaneous firing state of DA neurons, which provides the stable basal levels of extracellular DA. Whereas this pattern of firing is deemed *pacemaker*, these low basal levels are strongly regulated and maintained within a narrow concentration in the striatum. Conversely, a burst-firing pattern represents the ‘phasic’ DA response, which is

dependent on GLU afferents and is characterized by a high-amplitude transient signal. Phasic DA transmission is proposed to be the signal that mediates rapid behaviorally relevant activation of the DA system by relevant stimuli; and by raising extracellular DA levels, it is believed to be a key component of the reward circuitry. Also, phasic DA transients that result from burst firing are believed to activate the low-affinity striatal D1R, whereas the tonic dopamine levels arising from spontaneous firing is believed to sustain the activation of high affinity D2R (Baik, 2013; Grace *et al*, 2007; Juarez and Han, 2016; Surmeier *et al*, 2011).

4.3. Dopaminergic receptors

Once released from presynaptic terminals, the physiological actions of DA are mediated by membrane receptors belonging to the family of seven transmembrane domain G protein-coupled receptors (GPCRs), whose activation leads to the formation of second messengers, and activation or repression of specific signaling pathways (Figure 8). The DA receptors were initially subdivided into two major groups, based on their ligand selectivity and positive or negative modulation of adenylate cyclase activity and consequent cyclic adenosine monophosphate (cAMP) production: D1 and D2 receptors (D1R; D2R), respectively (Sealfon and Olanow, 2000). Eventually five subtypes of DA receptors have been characterized (D1 through D5) on the basis of their structural, pharmacological, and biochemical properties, of which D1 and D5 are classified as D1-like, whereas D2, D3 and D4 are classified as D2-like receptors (Beaulieu and Gainetdinov, 2011).

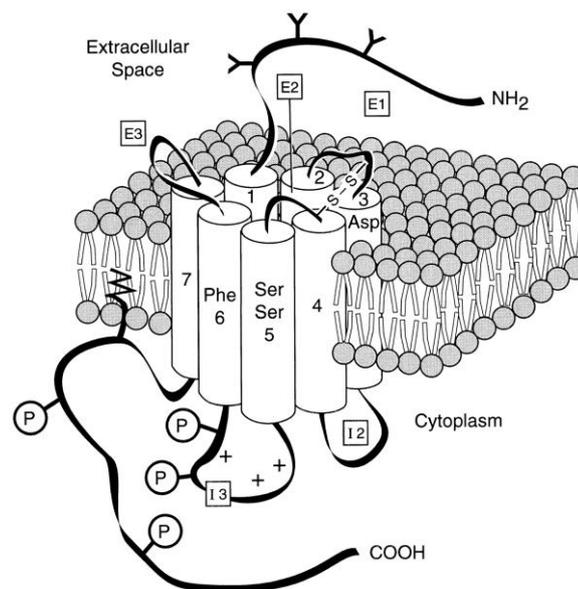


Figure 8. Structure of the dopaminergic receptor. Residues involved in dopamine binding are highlighted in transmembrane domains. Potential phosphorylation sites (P) are represented on intracellular loop (I3) and on COOH terminus. Adapted from Missale *et al* (1998).

It is commonly accepted that D1-like receptors are found exclusively postsynaptically in DA-target cells. The D1R is the most widespread DA receptor subtype, being found in the striatum (both dorsal and ventral portions), hypothalamus and thalamus. Interestingly, in other areas where the D1R is found in high densities (e.g. entopeduncular nucleus and SN *pars reticulata*), no mRNA is found, suggesting that its presence occurs mainly in projections, preferentially from γ -aminobutyric acid (GABA) releasing neurons. The D5R are much less abundant in the central nervous system and are expressed in the cortex, SN, hypothalamus, hippocampus and thalamus; very low level of expression has also been observed in the medium spiny neurons (MSNs) of the striatum. Also, D1R is reported to have the lowest affinity for DA, while D2R have been argued to have a 10- to 100-fold greater affinity for DA than the D1R-like receptor family (Beaulieu and Gainetdinov, 2011; Missale *et al*, 1998).

The D2R receptors, on the other hand, are expressed both pre- and post-synaptically on DA-receptive cells. The highest levels of expression in the brain are found within the DS, NAcc, and olfactory cortex and tubercles, but they are also expressed in the cortex, septum, amygdala, hippocampus, hypothalamus, VTA and SN. The D3R has a more limited pattern of expression, with the highest levels being found in limbic areas, such as the shell of the NAcc, the olfactory tubercle and islands of Calleja, not being expressed in the DS or cortex. The D4R has the lowest levels of expression in the brain, being found in the frontal cortex, amygdala, hippocampus, hypothalamus, GP, SNr and thalamus. Presynaptically, it is thought that D2R act as autoreceptors on DA neurons, mediating critical feedback inhibition of the circuit by limiting DA neuron firing and neurotransmitter release, as well as reducing DA synthesis and packaging. Within the striatum, D2 autoreceptors are found in pre-synaptic terminals of DA projection neurons, where they also elicit potent inhibition of DA release, notably in the presence of COC (Congar *et al*, 2002; Dobbs *et al*, 2017; Holroyd *et al*, 2015). Furthermore, D2R have two isoforms generated by the alternative splicing of the same gene. These isoforms, termed D2_L and D2_S (for 'long' and 'short'), are identical except for an insert of 29 aminoacids in the third intracellular loop of the D2_L, a domain believed to serve as an interaction site for G proteins or to participate in the interaction of the receptor with specific second messengers, although a role in resistance to receptor desensitization and internalization has also been suggested (Beaulieu and Gainetdinov, 2011; Dobbs *et al*, 2017; Missale *et al*, 1998).

As GPCRs, all DA receptors activate heterotrimeric G proteins, but the second messenger pathways and effector proteins activated by both receptor classes vary greatly and often mediate opposite effects. D1R trigger the activation of G $\alpha_{s/oif}$ family of G proteins, which then act stimulating the production of cAMP through the activation of adenylyl cyclase, leading to the activation of protein kinase A (PKA). In contrast, D2R activates the G $\alpha_{i/o}$ family of G proteins leading to the inhibition of adenylyl cyclase and thus limiting PKA activation. In turn, PKA

mediates most of the effects of D1R by phosphorylating and regulating the function of a wide range of cellular substrates, such as voltage-gated K^+ , Na^+ and Ca^{2+} channels, ionotropic GLU and GABA receptors as well as transcription factors. One of the major substrates of PKA is the DA- and cAMP regulated phosphoprotein (DARPP-32). This phosphoprotein is highly expressed in striatal MSNs and, when phosphorylated by PKA, amplifies PKA signaling by inhibiting protein phosphatase 1, which in turn antagonizes the actions of PKA. In addition, dephosphorylation by the calmodulin-dependent protein phosphatase 2B (PP2B) helps convert DARPP-32 into an inhibitor of PKA signaling [(Baik, 2013; Beaulieu and Gainetdinov, 2011; Tritsch and Sabatini, 2012) Figure 9]. Also, the co-localization of DA and GLU receptors increases the complexity of neuronal interactions within the striatum. In this process, DA crucially influences both the induction and reversal of neuroplasticity at corticostriatal synapses, with D1R and D2R appearing to be involved in both, *long-term potentiation* and *long-term depression*² (LTP and LTD, respectively) processes (Wickens, 2009).

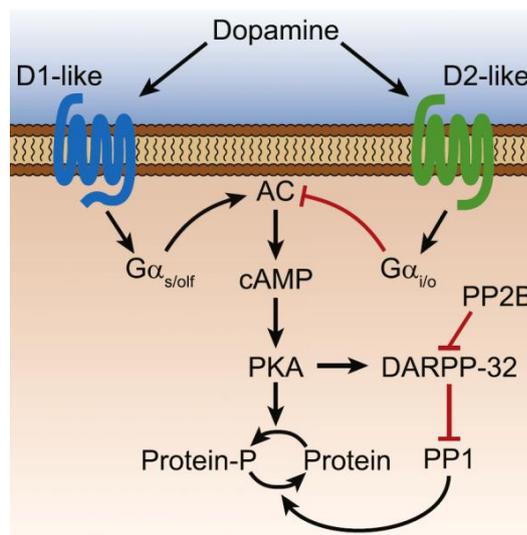


Figure 9. Intracellular DA signaling pathways. Representation of the cAMP/PKA-dependent pathways recruited by DA receptors. Black and red arrows indicate activation and inhibition, respectively. Both D1R and D2R are represented in the same cell for convenience. AC, adenylyl cyclase; PP2B, calmodulin-dependent protein phosphatase 2B. Adapted from Tritsch and Sabatini (2012).

Cocaine alters dendritic spine morphology of striatal MSNs, in a process which is generally associated with changes in synaptic strength. In this process, COC exposure induces

² *Long-term potentiation*, (LTP) and *long-term depression* (LTD) refer to mechanisms of synaptic plasticity characterized by long-lasting increase and decrease, respectively, in synaptic strength, which is a basic property of most excitatory synapses throughout the central nervous system. Both are associated with the trafficking of GLU receptors culminating in changes in dendritic spine morphology. Thus, LTP includes the formation and enlargement of dendritic spines, and insertion of AMPARs at the synapses, whereas retraction or contraction of spines, and decreases and AMPARs are associated with LTD (Kauer and Malenka, 2007).

alterations in PKA signaling which have been related with sensitization and reinstatement, and many of these actions are mediated by differential actions of DA receptors (Calabresi *et al*, 2007; Scofield *et al*, 2016; Wolf *et al*, 2003). For instance, D1R receptor is believed to enhance the NMDAR response through the activation of PKA and DARPP-32, while together D1R and NMDAR have been shown to contribute significantly to neuronal and morphological changes induced by repeated exposure to COC (Ortinski *et al*, 2013; Ren *et al*, 2010; Wang *et al*, 2012). Also on the interaction between D1R and NMDARs, repeated COC treatment strongly activates the extracellular signal-regulated kinase (ERK) in the striatum, in a D1R- and NMDAR-dependent manner. In turn, ERK³ is suggested to be involved in COC-induced behavioral effects and has been identified as a common downstream target for both the DA and GLU receptor pathways (Bertran-Gonzalez *et al*, 2008; Ren *et al*, 2010; Valjent *et al*, 2000, 2006; Wang *et al*, 2012). In contrast, D2R stimulation reduces opening of voltage-dependent Na⁺ channels and promotes the opening of K⁺ channels modulates Ca²⁺ homeostasis and related signaling in MSNs by decreasing Ca²⁺ influx. In addition to inhibiting adenylyl cyclase by coupling to G_q subunits, D2R has also been associated with the regulation of protein kinase C (PKC) activity, which participates in COC seeking (Beaulieu and Gainetdinov, 2011; Ortinski *et al*, 2015; Perez *et al*, 2011; Schmidt *et al*, 2013; Surmeier *et al*, 2007). While pharmacological stimulation of D2R in the NAcc shell has been shown to reinstate COC-seeking, at least in part through the activation of PKC, the D1R agonist-mediated reinstatement is PKC independent (Ortinski *et al*, 2015). Finally, repeated COC exposure increased PKA activity and abolished the D2R modulation of Ca²⁺ homeostasis both after 3 or 21 days of COC abstinence, indicating enduring neuroadaptations of MSNs in COC-sensitized rats (Perez *et al*, 2011).

5. The striatum

The effects of psychostimulants on DA transmission mediate both the motor stimulant and reinforcing effects of these drugs, with a critical participation of the striatum, which represents a major target of the mesolimbic and nigrostriatal DA pathways. It is through the DA transmission within the striatum, along with alterations in the neurochemistry of this structure, that the effects induced by psychostimulant has been proposed to promote self-administration and, eventually, drug seeking (Pierce and Kalivas, 1997; Vezina and Leyton, 2009).

³ ERK is one isoform of the mitogen activated protein kinases, which responds to extracellular stimuli and regulates cell proliferation and differentiation. The phosphorylated ERK is translocated to the nucleus where it controls the expression of several immediate early genes through regulating cAMP response element binding protein [CREB (Wang *et al*, 2012)].

The striatum is the largest nucleus of the basal ganglia (BG), and a major integration site of the cortico-basal ganglia-thalamic circuit, and as such, is generally divided into ventral and dorsal portions. It receives a large variety of inputs, being the DAergic input critical to shaping the activity of its networks. In humans, the DS is subdivided into caudate nucleus and putamen. The caudate nucleus receives inputs mostly from cortical regions (prefrontal and orbitofrontal cortices) while the putamen receives mainly excitatory inputs from sensorimotor areas. The ventral portion of the striatum acts as a motor-limbic interface; it is composed of NAcc and olfactory tubercle, receiving projections from prefrontal areas subserving limbic and autonomic functions (i.e. orbitofrontal cortex and anterior cingulate area) and limbic structures (e.g. hippocampus, amygdala, VTA), being involved in motor, emotional and motivational processing [(Gerfen and Surmeier, 2011; Joel and Weiner, 2000; Soares-Cunha *et al*, 2016b) Figure **10A**].

In rodents, the striatum is also composed by dorsal and ventral subregions. As a whole, the DS receives DAergic inputs mainly from SNc, with sparse projections from the VTA. It is subdivided into dorsomedial striatum (DMS) and dorsolateral striatum (DLS). The DMS mainly receives interoceptive-related inputs from the prelimbic area of the medial PFC (mPFC), whereas the DLS receives predominantly sensorimotor-related information from the motor cortex. The ventral striatum is composed by the NAcc, which is subdivided into core and shell regions, predominantly receiving DAergic inputs from the VTA as well as inputs from prelimbic and infralimbic regions, respectively [(Joel and Weiner, 2000; Soares-Cunha *et al*, 2016b; Voorn *et al*, 2004) Figure **10B**].

The vast majority (~95%) of the neuronal cell population within the striatum is comprised of inhibitory, GABAergic medium spiny projection neurons, which can be divided into two equally sized populations. This division is mainly based on their projection targets, peptide co-transmitters and DA receptor expression: in the DS (Figure **11A; B**), the MSNs of the *direct pathway* project to the nuclei at the interface between the basal ganglia and the rest of the brain, including the GP *pars interna* (GPI) and the SNr. These MSNs express D1R, along with M4 cholinergic receptors and the neuropeptides dynorphin and substance P. On the other hand, MSNs of the *indirect pathway* reach the interface nuclei via an indirect, multisynaptic circuit that includes the GP *pars externa* (GPe), and the subthalamic nucleus (STN). Also, along with D2R, these neurons co-express A_{2A} adenosine receptors and the neuropeptide enkephalin (Gerfen and Surmeier, 2011; Yager *et al*, 2015; Yin *et al*, 2008; Yin and Knowlton, 2006). Classically, these two striatal MSN populations are thought to have opposing effects on basal ganglia output. Activation of the D1R-MSNs causes a net excitation of the thalamus resulting in a positive cortical feedback loop, thereby acting as a 'go' signal to initiate behavior. In contrast, activation of the D2-MSNs causes the inhibition of thalamic activity resulting in a negative

cortical feedback loop, therefore serving as a 'brake' to inhibit behavior (Calabresi *et al*, 2014; Yager *et al*, 2015).

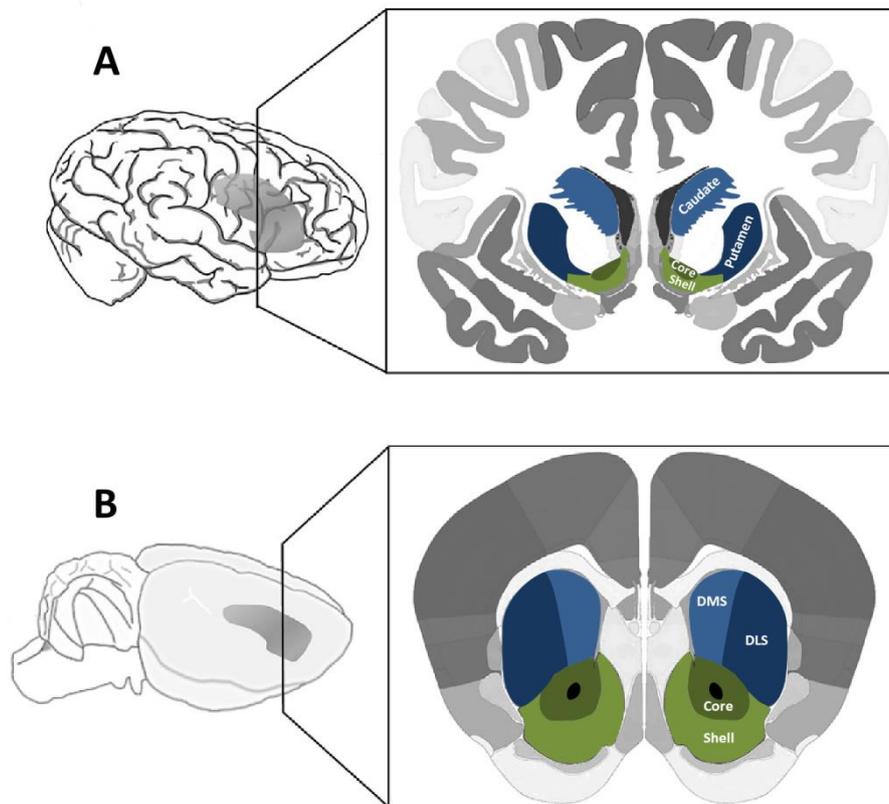


Figure 10. Simplified depiction of human and rodent striatum. The human dorsal striatum includes caudate and putamen; ventral striatum includes the NAcc (subdivided into core and shell) and olfactory tubercle (A). The rodent striatum is divided in dorsal and ventral regions; the dorsal striatum is subdivided in dorsomedial (DMS) and dorsolateral (DLS) portions; the ventral striatum is comprised by the NAcc, with core and shell portions (B). Adapted from Soares-Cunha *et al* (2016b).

Concerning the NAcc (Figure 11C; D), although these two striatal output pathways are also present, the efferent targets of the MSNs are not the same as those in the DS, and the pathway segregation is less established. Both D1R-MSNs and D2R-MSNs in the NAcc project to VP, whereas D1-MSNs alone project to ventral mesencephalon output structures (SNr and VTA), also sending axon collaterals to the VP (Smith *et al*, 2013; Yager *et al*, 2015).

In addition to the two types of MSNs expressing D1R and D2R, there are several subtypes of interneurons representing approximately 5% of the total number of cells in the striatum in rodents. Except for the aspiny cholinergic neurons that project to the MSNs, they are all GABAergic, targeting the soma of MSNs (Gerfen and Surmeier, 2011; Grillner and Robertson, 2016).

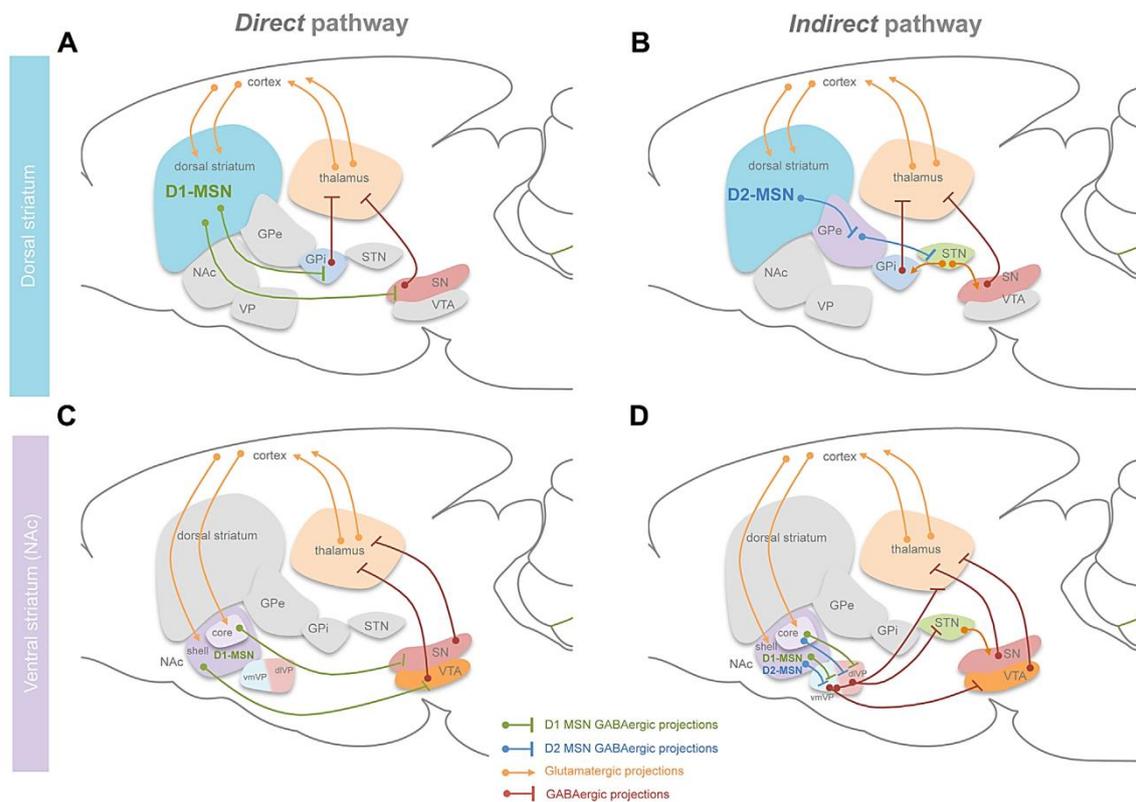


Figure 11. Simplified representation of striatal direct and indirect pathways. (A) In the dorsal striatum, D1R-MSNs from the direct pathway project directly to the basal ganglia output nuclei, [SNr and the GPi]. The inhibitory GABAergic effect leads to disinhibition of the thalamus, which in turn projects to the cortex. (B) In contrast, activation of D2R-MSN indirect pathway, which project indirectly to the SNr via the GPe and the STN, inhibits thalamic output to the cortex. Dopaminergic input from the SNc modulates corticostriatal transmission by exerting a dual effect on MSNs, depending on their receptor expression (D1R or D2R). (C) Within the NAcc there is a similar direct/indirect dichotomy akin to the dorsal striatum. The D1R-MSN direct pathway involves NAcc projections to the SNr/VTA and from there to the mediodorsal thalamus. (D) The indirect circuit projects to the VP and STN before reaching the output nuclei. However, the indirect pathway NAcc-VP projections have major contributions of both D1R and D2R types of MSNs. Modified from Soares-Cunha *et al* (2016b).

5.1. The Ventral striatum and the Nucleus Accumbens

The nucleus accumbens is a structure in the rostromedial forebrain considered a major input nucleus of the ventral striatum. Conceptualized as an essential interface between limbic and motor systems, within the NAcc, DA was originally proposed to serve as a modulator in the translation of motivation into action (Mogenson *et al*, 1980).

Analysis of NAcc connectivity and histochemical profile showed that it is a heterogeneous structure that can be divided into two main subregions, the core and the shell (Ikemoto, 2007; Zahm, 2000; Zahm and Brog, 1992). Both core and shell receive inputs from the amygdala, globus pallidus (GP) and ventral pallidum (VP), but they differ substantially in the density of their cortical inputs: the core receives projections mostly from the prelimbic and anterior cingulate cortices, whereas the shell receives projections predominantly from the ventromedial (infralimbic cortex) and ventrolateral (anterior insular) cortices (Zahm, 2000; Zahm and Brog, 1992). Their

outputs also differ considerably: while the core projects to the dorsolateral VP, GP and SN, the shell projects to subcortical limbic structures, such as the lateral hypothalamus, VTA and ventromedial VP (Ikemoto, 2007; Zahm and Brog, 1992).

As a whole, the NAcc has an essential role modulating the processing of rewarding and aversive stimuli. In a simplistic perspective, one may assume that rewarding stimuli evoke an increase in DA levels within the NAcc, whereas aversive stimuli evoke the opposite response by diminishing DA release in the NAcc (Soares-Cunha *et al*, 2016b). Thus, through its DAergic innervation the NAcc has a key role mediating the reinforcing effects of stimulant drugs, as all major drugs of abuse activate this system increasing extracellular levels of DA (Di Chiara, 1999; Wise, 2004). Early behavioral pharmacology studies revealed that the NAcc is directly involved in the development and expression of several addiction-related phenomena, including incentive and psychomotor sensitization (Robinson and Berridge, 2008), self-administration (Steketee and Kalivas, 2011) and relapse/reinstatement models (Bossert *et al*, 2013). Therefore, because of the involvement of ventral striatal DA in incentive salience attribution and the motivational influence of environmental stimuli on behavior, this augmented release of DA in the NAcc has been hypothesized to contribute to exaggerated drug ‘wanting’, leading to excessive pursuit of drugs (Nordquist *et al*, 2007; Robinson and Berridge, 1993, 2003).

A functional specialization of the NAcc subterritories has been also proposed, in which core and shell mediate actions based on their inputs (Di Chiara, 2002; Saddoris *et al*, 2013). The NAcc core shows similarities to the DS, acting on voluntary motor functions; together with its circuitry NAcc core plays a prominent role in selecting and executing motor responses to stimuli whose motivational significance has been recently updated, thus integrating motivational value of reward-related stimuli and context-dependent associations, regardless of valence (appetitive vs. aversive), and translating these into motor actions (Bassareo *et al*, 2002; Di Chiara, 2002). These functions are evidenced in studies showing that lesions in the NAcc core impair [i] the ability of drug-associated Pavlovian stimuli to support operant responding (Ito *et al*, 2004); [ii] the selection and initiation of actions based on the reward value in instrumental learning for food reinforcers (Corbit *et al*, 2001; Smith-Roe and Kelley, 2000); or [iii] cue-induced reinstatement of food or cocaine seeking behavior (Floresco *et al*, 2008; Fuchs *et al*, 2004).

The shell, on the other hand, shares similarities with the ‘extended amygdala’, participating on motivational mechanisms; together with its circuitry, the shell plays an important role in the control of reward prediction and reward learning (Goto and Grace, 2008; Kelley, 2004). In this sense, DA responsiveness in the NAcc shell is argued to depend on positive valence (appetitive as opposed to aversive stimuli) and novelty. The release of DA in the shell by unpredicted rewards has been argued to associate the discriminative properties of the rewarding stimulus with its biological outcome. That is, the DA response triggered by a palatable

food might serve to associate food taste to its post-ingestive consequences (Bassareo *et al*, 2002; Di Chiara, 2002). These roles are also exemplified by studies showing that initial responding for sucrose (first 20 minutes of a 60 minutes session) increased DA levels in the shell, but not in the core, returning to basal values despite sustained responding for the remainder of the sessions. This suggests that the elevation of DA is dissociated from the operant responding *per se* or the actual intake of sucrose (Bassareo *et al*, 2015, 2017). Also, NAcc shell has been argued to be involved in the inhibition of inappropriate behaviors in goal-directed action (Ambroggi *et al*, 2011), as implied by the enhancement of cued reinstatement of food (Floresco *et al*, 2008) and COC seeking (Di Ciano *et al*, 2008) after its transient inactivation. Also, the shell has been argued to be the primary site where psychostimulants exert their reinforcing actions, as COC and AMPH are able to sustain intra-structure self-administration in rodent models, likely through the mediation of their rewarding effects (Chevrette *et al*, 2002; Rodd-Henricks *et al*, 2002).

5.2. *The dorsal Striatum*

As the DS is a large structure, a functional dissociation has been suggested for its subareas in terms connectivity, mechanisms of synaptic plasticity (Gerdeman *et al*, 2003; Partridge *et al*, 2000) and functionality (Yin and Knowlton, 2006). The DMS correlates to the caudate of primates and is part of the 'associative striatum', whereas the DLS correlates to the putamen of primates and composes part of the 'sensorimotor striatum' (Balleine *et al*, 2007, 2009; Yin *et al*, 2008).

The DMS has been shown to be a critical locus for the acquisition and expression of the action–outcome associations (i.e. flexible/goal-directed behavior) in instrumental conditioning (Corbit and Janak, 2010; Yin *et al*, 2005b). It has been shown that within the DMS the glutamatergic NMDARs, involved in the induction of LTP in cortico-striatal projections, are also critical for the encoding of action–outcome associations (Yin *et al*, 2005a). In contrast, DLS-lesioned rats trained to press a lever for sucrose display increased sensitivity to outcome devaluation, even when rats were trained on interval schedules (Yin *et al*, 2004), known to promote the weakening of the relation between response and outcome delivery, thus favoring the emergence of habitual control over reward seeking [(Dickinson *et al*, 1983) further discussed on Section II]. Therefore, as their performance was reduced following devaluation of the goal, instrumental performance was controlled by goal-expectancy. Furthermore, with the same training DMS-lesioned rats were also insensitive to outcome devaluation, implying that the DMS is not critical for habit learning (Yin *et al*, 2004).

Actions that are initially goal-directed can eventually become insensitive to their consequences. Therefore, it is now well accepted that the control over behavior undergoes a shift from this initial goal-directed to a habitual process, and the latter has been postulated to reflect the increasing establishment of S–R associations (Dickinson, 1985). This transition in behavioral control from goal-directed to habitual S–R processes has been shown to be accompanied by a switch in the engagement of the neural activity from the DMS early in training towards the engagement of the DLS later in training (Yin *et al*, 2009). Importantly, the DAergic input has been shown to be important in both early-training DMS mediated performance, and DLS-mediated habitual behavior (Darvas and Palmiter, 2010; Faure, 2005; Gruber and McDonald, 2012; Kravitz *et al*, 2012; Vanderschuren *et al*, 2005; Vicente *et al*, 2016; Yin *et al*, 2009).

5.3. *The striatum in addiction and the transition of behavioral control*

Prior repeated exposure to psychostimulants has been shown to facilitate habitual responding for food reinforcers, and it is suggested that COC administration accelerates the development of habits. For instance, prior COC treatment has been shown to hinder the ability of rats to guide their behavior by action – outcome (A – O) association in a Pavlovian conditioning paradigm (Schoenbaum and Setlow, 2005). Also, previous exposure to AMPH or COC accelerates stimulus – response (S – R) learning (Nordquist *et al*, 2007), also disrupting the acquisition of goal-directed behavior (Corbit *et al*, 2014a; LeBlanc *et al*, 2013; Nelson and Killcross, 2006).

Along the development of addiction, drug seeking is argued to progress through A – O responding, to an automatic maladaptive stimulus-response structure. Thus, initially consisting in a goal-directed behavior, performance of actions is determined by explicit knowledge of its consequences, as the drug is sought after and consumed for its reinforcing/rewarding properties. Then drug seeking is believed to become triggered and maintained by drug-associated stimuli, eventually becoming inflexible (Everitt *et al*, 2008; Everitt and Robbins, 2005).

The initial goal-directedness over COC-seeking after a moderate amount of training has been demonstrated by Olmstead *et al* (2001). In a set of experiments using a chained schedule of intravenous COC SA, habitual COC seeking was tested by devaluing the final link of a drug seeking/taking chained schedule. In this protocol, Olmstead and colleagues were able to dissociate *taking* from *seeking* components of COC consumption: responding on the drug *seeking lever* provided access to the *taking lever*, rather than to COC itself. In turn, responses

on the drug *taking lever* delivered a COC injection. As devaluation (through extinction) of the *drug taking* component was assessed, decreased responding during the *drug seeking* indicates that behavior is goal-directed, rather than habitual (further discussed on Section II). Olmstead *et al* (2001) were therefore able to show that COC seeking is initially mediated by the knowledge of the contingency between the seeking response and the access to an effective drug-taking response [i.e. an A – O association (Olmstead *et al*, 2001)].

Though initially a goal-directed process, COC seeking is argued to become habitual after extended training (LeBlanc *et al*, 2013; Zapata *et al*, 2010). This progression to habitual control of behavior is also believed to be accompanied by a transition within the circuits responsible for behavioral output. Indeed, a widespread hypothesis for the neurocircuitry underlying addiction proposes that it initially depends on the ventral striatum (including the NAcc), but progressively engages and hijacks the circuitry in the DS, which is normally required for habitual learning (Korpi *et al*, 2015; Takahashi *et al*, 2007; Yin and Knowlton, 2006). More specifically, the functional changes occurring in the NAcc caused by COC exposure are believed to spread progressively to more dorsal and lateral domains of the striatum over the course of prolonged COC use (Pierce and Vanderschuren, 2010; Takahashi *et al*, 2007).

This initial processing of the NAcc and progressive recruitment of the medial portions of the dorsal striatum (i.e. DMS) and finally the DLS has DA as a critical player in this process (Murray *et al*, 2012a; Nelson and Killcross, 2013). Well-established cue-controlled COC seeking is correlated with increased extracellular DA levels within the DLS (Ito *et al*, 2002) and severely impaired by DA blockade in the same region (Belin and Everitt, 2008). On this matter, Zapata *et al* (2010) managed to show that after protracted, but not limited, training COC seeking becomes resistant to reinforcer devaluation but can be restored by transient inactivation of the DLS. Therefore, as previously demonstrated with food rewards, the shift in the control of DA transmission to the DLS has also been shown to happen on the performance of COC-seeking behavior.

Indeed, initial exposure to COC SA was correlated with changes in cerebral metabolic response in the ventral striatum of rhesus monkeys, whereas prolonged exposure resulted in a metabolic response that expanded to encompass the entire DS [(Porrino *et al*, 2007) Figure 12]. This possibility is also supported by anatomical evidence that the neuronal circuits including ventral and dorsal subregions of the striatum are interconnected and process information via parallel as well as integrated feedforward connections (Haber, 2014; Haber *et al*, 2000). In fact, ventral portions of the striatal area regulate the DAergic innervation of more dorsal regions through “spiraling” connections with the midbrain. In this frame, the NAcc shell projects to DA neurons in the VTA that innervate shell and the more dorsally situated core regions of the NAcc. In turn, neurons of the NAcc core innervate DA neurons projecting to both the core itself and its

contiguous dorsal region, and so on, in a serially cascading pattern finally encompassing more lateral parts of the DS, where dopaminergic innervation is increased during habitual drug seeking behavior (Belin and Everitt, 2008; Haber *et al*, 2000; Heimer *et al*, 1997; Zahm, 2000).

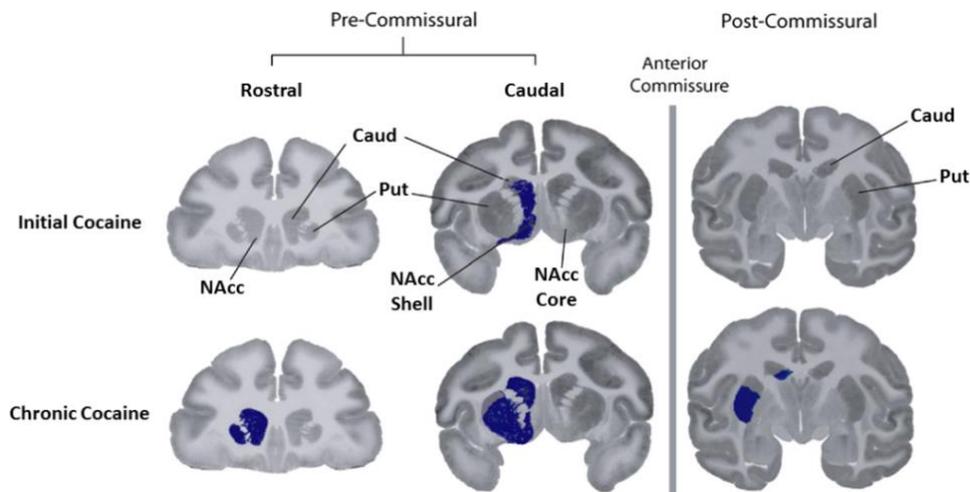


Figure 12. Areas of cerebral metabolic response produced by self-administered cocaine in the striatum of rhesus monkey. Representative autoradiograms of glucose uptake in coronal sections of rostral and caudal striatum of rhesus monkeys. Top panel are effects of initial (5 days) COC SA, bottom panel are effects of chronic (100 days) cocaine self-administration. Blue coloring superimposed upon the grayscale autoradiograms represents the location of significant changes in rates of glucose utilization (effects were bilateral but are depicted on one hemisphere). Caud, caudate; Put, putamen. Adapted from Porrino *et al* (2007).

Finally, the participation of DA in these spiraling connections has also been demonstrated in the context of COC seeking. In another elegant study with unilateral lesion of NAcc core and contralateral DA inhibition within the DLS, in a protocol which aimed for the functional disconnection of the serial interactions between ventral and dorsal striatal domains (Belin and Everitt, 2008; Figure 13). This procedure selectively decreased COC seeking in rats tested after extended training under a second-order schedule of reinforcement. Interestingly, the same disconnection treatment did not impair goal-directed control for sucrose in rats trained under an fixed ratio (FR) 1 schedule of reinforcement⁴ of sucrose reward (Belin and Everitt, 2008; Everitt *et al*, 2008).

⁴ In *fixed ratio* schedules of drug reinforcement, a specific number of responses are necessary to provide one drug infusion. In the case of FR1 schedules, one operant response delivers one reward [in the case mentioned, one drug infusion (Allain *et al*, 2015)].

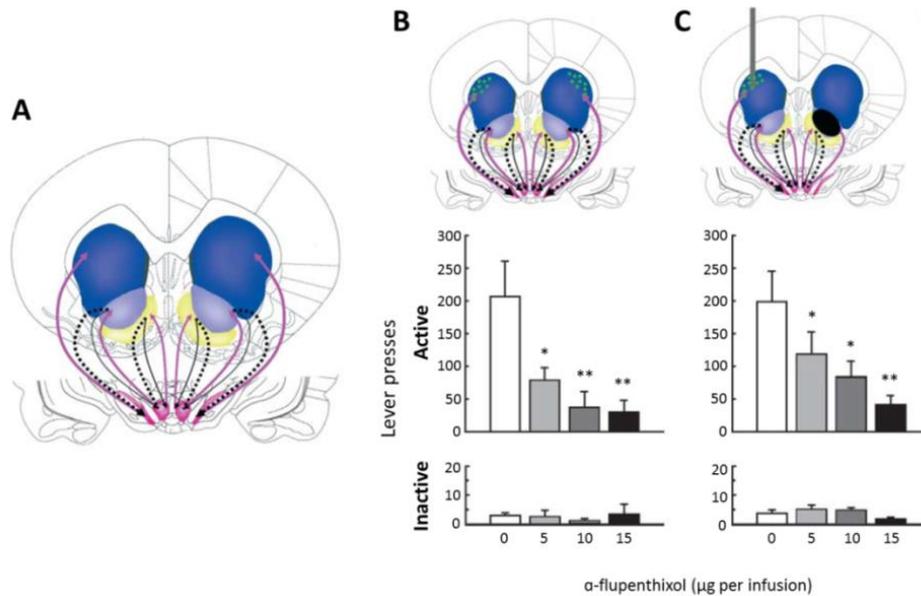


Figure 13. Serial processing within the striatum on the establishment of habitual cocaine-seeking habits. Representation of DA-dependent spiralling circuitry functionally connecting the ventral with the dorsal striatum in the rat. The spiralling loop organization is depicted as the alternation of pink and black arrows from the ventral to the more dorsal parts of the circuit. From the NAcc shell (yellow) to NAcc core (light blue) via the VTA (pink, central) and from the NAcc core, via the SN (pink, lateral) to the DS (dark blue; **A**). Bilateral infusions of the DA antagonist α -flupenthixol (green dots) into the DLS dose dependently decreased COC-seeking under a second-order schedule of COC reinforcement (**B**). Disconnecting the NAcc core (through quinolinic acid-induced lesions) from the DA innervation of the DLS (α -flupenthixol infusion) also impairs habitual COC seeking. When α -flupenthixol is infused in the DLS contralateral to the lesioned NAcc core, it blocks the DAergic innervation from the midbrain, impairing the output structure of the spiralling circuitry on the non-lesioned side of the brain (**C**). Therefore, this asymmetric manipulation disconnects the NAcc core from the DLS bilaterally, and greatly decreases COC seeking. Adapted from Everitt *et al* (2008).

6. Animal Models of Drug Reinforcement, Consumption, Addiction and Relapse

As an exclusively human disease, addiction involves a variety of factors, comprising social and economic to developmental and molecular aspects, and it is thus not possible for an animal model to capture the full complexity of elements involved in it. Indeed, the chronic exposure to stimulants is known to produce neurobiological adaptations in the DA system. Even though the changes elicited by drug exposure may vary according to the paradigm used [some important variables include whether the administration is contingent or non-contingent, with continuous or intermittent exposure, and the dose of the drug administered (Allain *et al*, 2015)], animal models have been proved especially invaluable for the understanding of specific components and neural mechanisms subserving drug seeking and drug taking behaviors. In this framework, much of the research on the effects of drugs of abuse in animals derives from three major

approaches: behavioral sensitization, conditioned place preference (CPP) and COC SA (Ewald and LaLumiere, 2017; Shalev *et al*, 2002).

6.1. Behavioral Sensitization

One notable effect of drugs of abuse is their ability to increase locomotor activity which is associated with hyper-responsiveness of the DA neurotransmission in the mesoaccumbens system, in a process argued to involve DA receptors. The term *sensitization* regards the enhanced response to a stimulus after repeated exposure to that stimulus (Steketee and Kalivas, 2011). In turn, when repeatedly administered, the ability of drugs to produce these effects becomes enhanced so that re-exposure to the drug, even months later, produces greater DAergic and behavioral activation than seen initially, in a phenomenon termed *behavioral/psychomotor sensitization* (Vanderschuren and Pierce, 2010; Wise and Bozarth, 1987). In this framework, sensitization is a useful paradigm for studying addiction processes because it is an easily observable output of the neural circuitry thought to underlie the incentive-motivational aspects of drug-seeking that facilitate the transition to addiction (Robinson and Berridge, 2001, 2008). Thus, assessing behavioral sensitization typically involves repeated administration by the experimenter of a drug, commonly a psychostimulant, in a specific environmental context, and the dependent measure is usually locomotor activity. Animals that received drugs show a much more pronounced increase in locomotor activity to a challenge dose of drug than controls that had received only repeated vehicle injections (Pierce and Kalivas, 1997; Steketee and Kalivas, 2011).

This enhanced behavioral response has been observed after repeated administration to several drugs of abuse, such as COC, AMPH, nicotine, morphine and Δ -9-tetrahydrocannabinol (Bassareo *et al*, 2013; Ginovart *et al*, 2012; Goutier *et al*, 2015; Jung *et al*, 2013; Kalivas and Duffy, 1990; Knackstedt and Kalivas, 2007; Scofield *et al*, 2016; Steketee and Kalivas, 2011), and has been reported to last for at least a year (Paulson *et al*, 1991). In this phenomenon, the mesoaccumbens DA system is believed to mediate motivational influences on behavior, and the repeated drug exposure is also argued to cause brain motivational circuitry to become persistently hyper-responsive, or *sensitized*, to drugs and drug-associated stimuli, which in turn become excessively “wanted” (Berridge, 2007; Scofield *et al*, 2016; Vanderschuren and Pierce, 2010). Besides, as the mesocorticolimbic DA pathway is also argued to invigorate appetitive behaviors, including seeking and consumption of abused drugs, long lasting enhancements within this pathway lead to enduring heightening of appetitive behavioral output. Therefore, behavioral sensitization is argued to be analogous to behaviors that are characteristic of drug addiction (Vezina and Leyton, 2009).

The development of behavioral sensitization includes two distinct phases: initiation and expression. Initiation consists in the immediate neural events that induce behavioral sensitization, during which the increased behavioral response following repeated COC drug exposure is associated with increases in extracellular DA concentration in the NAcc. The expression occurs after cessation of drug administration. It refers to the neuroadaptations that are consequent of the initial neurochemical events and are manifested by the persistent hyper-responsiveness subsequently to drug treatment (Baik, 2013; Steketee and Kalivas, 2011; Vanderschuren and Kalivas, 2000).

Neurochemically, it is hypothesized that the repeated drug exposure decreases the sensitivity of D2R autoreceptors within VTA DA neurons, which elicit transient increases of DA release within the VTA (Kalivas and Duffy, 1993). Released DA acts on D1R to enhance GLU release from mPFC projecting neurons in the VTA, inducing LTP in DA neurons (Bocklisch *et al*, 2013; Huang *et al*, 2007; Sesack and Grace, 2010; Williams and Steketee, 2004). In turn, repeated exposure to the drug reduces basal GLU levels in the NAcc, which is argued to enhance GLU α -amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptor function (Madayag *et al*, 2007). The reduced basal levels of GLU seen in sensitized animals seem to allow for enhanced GLU release in the NAcc in response to drug challenge. Acting on enhanced AMPA receptors, GLU promotes behavioral sensitization, a process shown to be blocked by the restoration of basal NAcc GLU levels with N-acetylcysteine⁵ [N-AC; Further discussed on §7.2.3 (Kupchik *et al*, 2012; Madayag *et al*, 2007; Moussawi *et al*, 2009; Murray *et al*, 2012b)].

The neurocircuitry, as well as the neurochemical and neuropharmacological mechanisms underpinning behavioral sensitization and relapse to drug seeking are similar; nonetheless, studies have not been entirely consistent regarding a role for sensitization in reinstatement. Indeed, DAergic drugs that reinstate drug seeking in animals extinguished from cocaine self-administration (COC SA) have been shown to also evoke sensitized psychomotor response in these same animals, whereas drugs that do not induce reinstatement do not produce sensitization (Dias *et al*, 2003; de Vries *et al*, 1998, 1999, 2002). However, animals trained to self-administer COC under both, a long- (6h/day) and a short-access (1h/day) paradigm develop behavioral sensitization to COC, but only long access animals exhibit reinstatement of drug-seeking behavior in response to drug-priming injections (Ahmed and Cador, 2006). Also, using a similar protocol of long versus short access, the ability of COC priming to reinstate COC-

⁵ *N-acetylcysteine* is a cystine prodrug that increases extracellular GLU by stimulating the glial cystine/GLU exchanger (Corbit *et al*, 2014a).

seeking has been shown not to be dependent on behavioral sensitization or escalation⁶ of drug seeking (Knackstedt and Kalivas, 2007). As for DA receptors, systemic priming with a D2R agonist resulted in a decrease in locomotor activity and an increase in drug-seeking behavior, whereas priming with a D1R agonist increased locomotor activity but failed to reinstate drug-seeking behavior (Dias *et al*, 2003; Koeltzow and Vezina, 2005). Therefore, sensitization is likely elicited by repeated exposure to drugs occurring during SA, but it does not always coincide with enhanced reinstatement of drug seeking behavior (Steketee and Kalivas, 2011).

6.2. Conditioned Place Preference

Conditioned place preference is a classical conditioning procedure in which animals are initially trained to associate one distinctive environment with a drug, by repeatedly pairing injections of the drug with the specific context, whereas a vehicle injection is paired with another environment. After repeated pairings, rodents will 'prefer' the drug-paired context, or otherwise, once given access to the full apparatus, a parameter estimated from the amount of time spent in that area reflecting a conditioned place *preference* or *avoidance*, respectively (Nygard *et al*, 2017; Xu *et al*, 2017).

For instance, in one study groups of rats were given pairings in one chamber with COC and in other chamber with saline, and the acquired preference for the drug-paired environment was extinguished by repeated pairings between saline injections and the previously drug-associated environment. After extinction, priming injections of COC reinstated the extinguished CPP for the drug, suggesting that drugs may induce relapse by renewing the incentive value of drug-associated cues [in this case, the environment paired with COC injections (Mueller and Stewart, 2000)]. Conversely, in a study using morphine-dependent rats, it was shown that naloxone (an opiate antagonist known for its precipitating effects on morphine withdrawal) was able to induce conditioned place aversion in these rats but not in control rats, suggesting that the desire to avoid the naloxone-precipitated withdrawal state contributes to the compulsive nature of opiate abuse (Frenois *et al*, 2002). Therefore, the CPP procedure allows for the assessment of the conditioning of drug reinforcement, also providing information regarding the positive and negative reinforcing effects of drugs.

⁶ *Escalation* consists in a model devised to reproduce the escalating, dysregulated character of consumption and eventual loss of control over drug-taking observed in addicted individuals. In this model, animals are typically allowed to self-administer psychostimulants such as cocaine for extended periods (e.g. 6 hours per day), and thus gradually and increase drug intake across sessions (Ahmed and Cador, 2006; Ahmed and Koob, 1998).

Advantages of the CPP reinstatement model include [i] the relative ‘independence’ of instrumental performance, so that nonspecific motor effects of pharmacological manipulations are less likely to influence behavior; and [ii] the fact that this paradigm does not require the expertise of intravenous surgery and the care needed to maintain catheter patency. On the other hand, one major disadvantage of CPP models is that the drug is administered by the experimenter and, thus, the animal has no choice in whether to receive it (Steketee and Kalivas, 2011).

6.3. Drug Self-Administration

As opposed to CPP, the self-administration variant of the reinstatement model requires animals to engage in an instrumental behavior (e.g. lever press or nose poke) that elicits the delivery of the drug. This enables animals to decide when to engage in drug-seeking behavior, also allowing for the assessment of questions such as the strength of the motivation to obtain the drug, or the nature of the behavior [e.g. goal-directed or habit based (Siciliano and Jones, 2017; Soares-Cunha *et al*, 2016a; Zapata *et al*, 2010)]. Since the procedures developed by the pioneering work of Weeks (1962), i.v. self-administration of drugs is generally accepted as a gold standard for modeling drug abuse. Overall, it is well accepted that self-administration of addictive drugs by animals supports the concept that drugs act as universal reinforcers and that animal models are an accurate reflection of what occurs in the human brain (Köks, 2015). Within this field, many self-administration procedures have been developed to model distinct aspects of the addiction process and to assess the consequences of drugs on neural function (España and Jones, 2013), eventually to define the NAcc and adjacent ventral striatal structures as a key site for the reinforcing effects of stimulants and other drugs of abuse (Everitt, 2014).

In this paradigm, animals initially learn to emit an operant response (typically a lever press) that is reinforced by an i.v. infusion of a drug of abuse (Myers and Carlezon, 2010). Drug availability is typically signaled by an environmental stimulus, so the injection is accompanied by the exhibition of cues (more commonly a visual or an auditory stimulus, such as a light or a tone, respectively). Also, the pattern of responding required for each i.v. infusion is determined by the schedule of reinforcement established, which can be classified as follows:

6.3.1. Simple schedules of reinforcement

In simple, *fixed ratio* (FR) schedules, the number of responses required for drug infusions is set at a fixed number. In a continuous reinforcement schedule, such as FR1, every operant response (being it lever press, nose poke or equivalent) is followed by a reward delivery. In

these cases, the dependent variables are the number of rewards obtained or the rate of responding during a session (Roberts *et al*, 2013). In the case of drug SA under these schedules, rats will develop a highly stable pattern of drug SA in a limited access situation, but the rate of consumption is inversely related to the size of the unit dose. That is, as the unit dose is increased or decreased, rats will decrease or increase their SA rate, respectively, presumably to compensate the changes in the unit dose. If the dose remains consistent, changes in responding may be interpreted as changes in the reinforcing potency of the drug (Roberts *et al*, 2013).

6.3.2. *Random Ratio/Random Interval schedules*

The term 'random ratio' refers to the use of probability (ratios) in the reward schedule in which each response has a specified probability of being rewarded. As it arranges a linear correlation between behavior and reward rates, even with extended training the random ratio schedule is argued not to be suitable for the induction of habitual behavior. On the other hand, the relative disconnection between response rate and reinforcement observed in random interval schedules allows for the development of automatisms, usually when animals are over-trained. In this regard, eventually along training animals no longer experience the behavior-reward correlation, and as a result, their performance no longer controlled by the knowledge of this relation. This happens as, by definition, these schedules consist of random time intervals with a specified average during which responses are not reinforced; the first response after this time interval has elapsed is rewarded. This establishes that a reward will become available with a constant probability in each time unit (e.g. each second), instead of a precise number of lever presses [as it is the case for RR schedules (Dickinson, 1985; Nordquist *et al*, 2007)].

6.3.3. *Progressive Ratio schedules*

The term 'progressive ratio' describes a schedule in which the response requirements are increased after the delivery of each reinforcer, until responding becomes erratic or ceases completely. This schedule yields an assessment termed 'breaking point' which consists in the largest ratio requirement attained, or the number of ratios completed per session. In the case of drug SA experiments, the breaking point represents the maximum effort that an animal will expend in order to receive a particular reinforcer, and this estimation serves an index of the reinforcing strength of a self-administered drug, working as a function of the motivation of the animal to obtain it (Richardson and Roberts, 1996; Stafford *et al*, 1998).

6.4. Modeling of drug addiction

New models of addiction have emerged in the last decades, and these are based on self-administration procedures that have been devised to assess the essence of psychological constructs believed to be involved in addiction. These aspects involve [i] the escalation of drug intake; [ii] maintained drug use despite adverse consequences; [iii] the compulsive aspect of consumption; [iv] establishment of drug seeking habits controlled by drug-associated stimuli in the environment; and [v] the enduring vulnerability to relapse after voluntary abstinence (Belin-Rauscent *et al*, 2015). Two notorious models are described below.

6.4.1. Escalation of COC self-administration

Limited drug access (e.g. 1 h/day) yields a remarkably stable rate of consumption (see §5.3.1.), which persists even after months of testing. However, when rats are provided with extended access (6 h/day) to COC SA, COC intake progressively increases across days, above control levels, in an effect that persists several weeks (Ahmed and Koob, 1998). This excessive level of drug intake is estimated to be developed by about 70% of animals trained with long-access of COC SA, as compared to less than 12% of animals under restricted access. In this sense, the extended access to COC SA is argued to induce the development of a number of behavioral changes that resemble some of the behavioral symptoms of COC addiction. For instance, rats with extended access to COC SA show enhanced motivation for COC, as shown by higher breakpoints (Paterson and Markou, 2003), as well as higher resistance to suppression of COC seeking by exposure to footshock-paired conditioned stimulus (Vanderschuren and Everitt, 2004), when compared with rats that are trained under limited access, suggesting they are willing to expend more efforts and to take more risks to obtain the drug than control animals (Ahmed, 2005; Ahmed and Cador, 2006).

6.4.2. Addiction-like behavior

In the attempt to model drug addiction in animals, Deroche-Gamonet *et al* (2004) devised a method that takes into account addiction-like behavioral criteria in rats that resemble three of the essential diagnostic criteria for addiction, as defined by the DSM-IV. The protocol involved an extended training of COC SA in a time frame of about 3 months, and the criteria considered are [i] inability to refrain from seeking the drug, as assessed during periods when the drug is signaled as unavailable; [ii] increased motivation to take the drug, as measured in a progressive ratio schedule; and [iii] maintained drug use despite harmful consequences, as measured by the persistence in responding for drug SA when drug delivery was associated with punishment. In this model, rats positive for none of the criteria are argued to be resistant to addiction, whereas

rats meeting all three addiction-like criteria are considered addicted; the latter represent 15-20% of the population, a proportion that is similar to that suggested to be observed in human populations. Based on this model, the authors sustain that addiction results from the interaction of two variables: [i] the degree of exposure to drugs, as the addiction-like behavior appear after extended access to COC; and [ii] the interindividual degree of vulnerability, as despite similar drug intake, addiction-like behavior is not observed in all individuals that undergo extended access to COC SA (Belin-Rauscent *et al*, 2015; Deroche-Gamonet *et al*, 2004).

7. Extinction-based reinstatement models

The rigid nature of drug-seeking habits and the associated difficulty to reestablish inhibitory control over them has been argued to be a critical feature of addiction. As drug seeking becomes progressively controlled by drug-associated stimuli present in the environment, subjective states of craving are argued to ensue. This frequently culminates in drug seeking and relapse, even without the pleasurable effects anticipated by earlier association with drug experiences (Garavan *et al*, 2000). In the learning context, *reinstatement* refers to the recovery of a learned response (e.g. lever-pressing behavior) that occurs when a subject is exposed, non-contingently, to the unconditioned stimulus (e.g. food), after extinction. Within the addiction literature, reinstatement typically refers to the resumption of drug seeking following a period of abstinence (Shaham *et al*, 2003; Stewart, 2008).

Using monkeys, initial studies on reinstatement of AMPH or COC self-administration by drug-priming were conducted in the 1970s (Gerber and Stretch, 1975; Shalev *et al*, 2002; Stretch *et al*, 1971), whereas in rodents initial studies were developed during the early 1980s (de Wit and Stewart, 1981, 1983). This model became arguably the most valid animal model of relapse, providing a well characterized preclinical model with relevance towards understanding drug relapse in humans, since conditions that are reported to provoke relapse in humans will also reinstate extinguished drug taking behavior in laboratory animals (Shaham *et al*, 2003).

Studies carried out in humans and laboratory animals have demonstrated that a return to drug use can be precipitated by three distinct types of stimuli:

- o Exposure to an environmental stimulus (i.e. a discrete or contextual cue) that was previously associated with the drug experience (Augur *et al*, 2016; Bossert *et al*, 2009; Cruz *et al*, 2014; Prasad and McNally, 2016);
- o Exposure to stressors (Blacktop *et al*, 2016; Brown *et al*, 2012; Shalev *et al*, 2010);

- o Exposure to a pharmacological stimulus (i.e. the drug itself or a pharmacologically related agent) that induces some component of the drug experience (Vassoler *et al*, 2008; Venniro *et al*, 2016; Yee *et al*, 2011).

In the reinstatement model (see Figure 14), animals initially learn to self-administer the drug of abuse and then are subjected to a period of drug abstinence, in which the drug is no longer available. During this period, animals may simply be left in their home cages (Cameron *et al*, 2016; Caprioli *et al*, 2017; Lu *et al*, 2014) or (as is often the case) be re-exposed to the context where the drug was previously delivered (the operant chambers), for the extinction of seeking behavior (Heinsbroek *et al*, 2017; Pomierny-Chamiolo *et al*, 2017). By definition, extinction is the reduction in drug seeking that occurs when the contingency between drug seeking behavior or drug predictive stimuli and the delivery of the drug reward is broken. Thus, following the initial training phase, lever pressing may be extinguished over the course of one or more sessions in which animals are given access to the levers, but the drug remains unavailable. Learning in this period is reflected by decreasing rates of lever pressing along sessions. The purpose of extinction is to reduce the rate of lever pressing so as to reach a low baseline against which operant responding can be compared when the subjects are re-exposed to relapse-inducing stimuli allowing. This allows therefore the experimenter to detect the

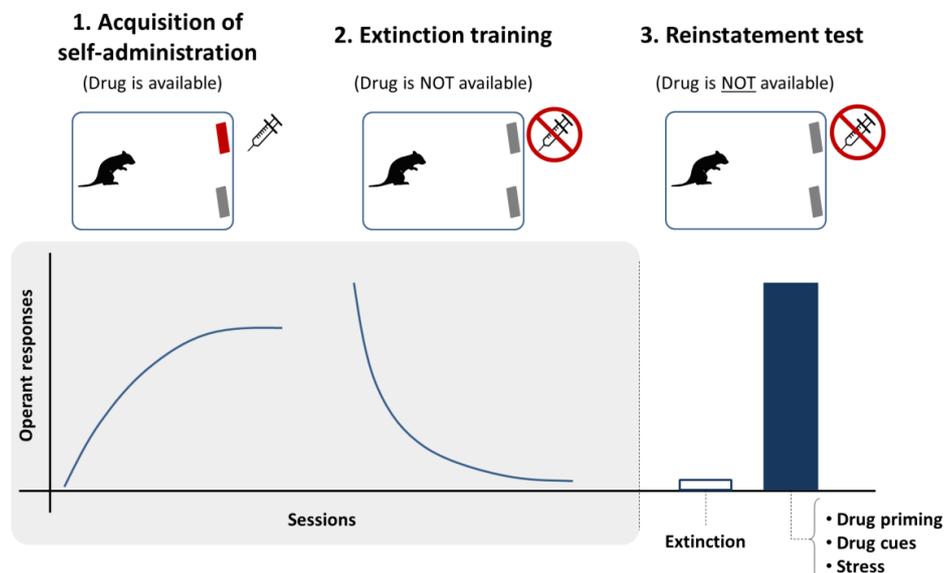


Figure 14. The reinstatement model of relapse. The Y-axis represents operant responses, used as an index of drug-seeking. (1) In the first phase animals are trained to self-administer the drug by performing an operant behavior (i.e. lever pressing), and drug delivery is usually accompanied by a stimulus (typically a cue light or a tone). (2) The second phase consists of extinction training, during which lever pressing no longer delivers the drug and animals learn to inhibit COC-seeking responses. (3) In the reinstatement phase animals are presented to different 'triggers' that induce reinstatement, such as the cue previously associated with drug delivery, a mild stressor (e.g. a foot shock), or to the drug itself. The reinstatement itself provides evidence that extinction involves new learning, rather than erasure of the original COC-seeking memory. Adapted from Keiflin (2008).

modulation of drug-seeking behavior during subsequent test sessions. Finally, tests for reinstatement usually take place once animals reduce their responding to very low levels during extinction training and, during this period lever presses are also not reinforced (Feltenstein and See, 2009; Myers and Carlezon, 2010; Shalev *et al*, 2002).

Also, several variants of the reinstatement model have been used throughout the years. The difference between these variants is the temporal organization of the three phases: acquisition training, extinction and reinstatement (Shalev *et al*, 2002). In the 'between-sessions' scheme, the three phases are developed across several days; in the 'within-sessions' variant all the phases take place subsequently, on the same day, during the same session; finally, in the 'between-within' scheme the training phase lasts several days, while extinction and reinstatement phases are held on the on the same day, during the same session (Figure 15).

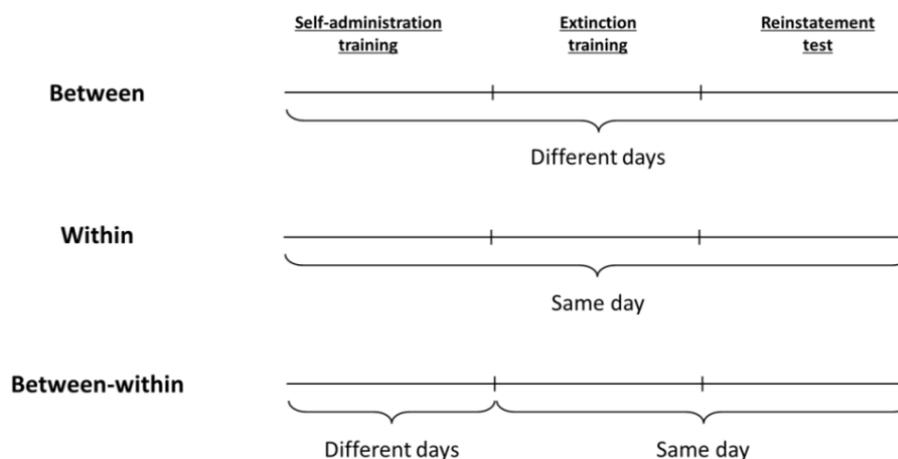


Figure 15. Depiction of the timeline of different reinstatement procedures. Adapted from Shalev *et al* (2002).

Thus, in animal models trained to self-administer drugs and then subjected to a period of extinction training (or simply to the passage of time), the presentation of cues that have been explicitly paired with drug delivery, brief exposure to a stressor or an experimenter-delivered injection of the drug all result in an increase in drug-seeking behavior (Shalev *et al*, 2002; Stewart, 2008). Clearly in a non-laboratory environment, the reestablishment of drug-seeking after abstinence occurs before exposure to the drug itself, and is instigated by environmental cues, thoughts or stressors. However, it is well established that the re-exposure to the drug incites further seeking; thus, it is relevant to study under an experimental setting how the action of the drug itself increases subsequent seeking. Also, it is worth to mention that the mechanisms undergone by the brain whether the period of abstinence is accompanied by extinction training or not are not necessarily the same (Fuchs *et al*, 2006). Extinction training

aims for the reduction of drug seeking. However, rather than erasing drug seeking, it is a form of new learning in which new contingencies are established between the behavioral response and the outcome, in such a way that the response eventually becomes inhibited. Thus, as the animal learns to withhold lever pressing, extinction training also involves new learning-based neuroadaptations in several components of the relapse circuitry, with important participation of mPFC and NAcc (Dhonnchadha *et al*, 2012; Knackstedt *et al*, 2010; Millan *et al*, 2011; Self *et al*, 2004; Sutton *et al*, 2003; Szalay *et al*, 2011).

7.1. Cue- and Context-induced reinstatement

Discrete stimuli such as odors and visual stimuli thus serve as conditioned stimuli (cues), eliciting neural and neurochemical responses, emotional and motivational responses, as well as behavioral responses, such as approach (Stewart, 2008). Contexts or environments where drugs are taken can have similar effects. Although these stimuli are paired with the effects of drugs whenever they are self-administered, their effectiveness can best be studied using classical conditioning procedures where environments or discrete stimuli are explicitly paired with injections of a drug (Crombag and Shaham, 2002; Stewart, 2008). Discrete stimuli paired either with passive infusions (Pavlovian conditioning) or with response-contingent presentation of a drug (instrumental learning) can come to serve as conditioned reinforcers, maintaining behavioral responses such as lever pressing in the absence of drugs. Thus, through a process of associative learning, cues that predict the availability/non-availability of drugs acquire incentive motivational properties that can differentially control drug seeking and drug taking behaviors (Di Ciano *et al*, 2008; Crombag *et al*, 2002, 2008; Shaham *et al*, 2003).

In the conditioned-cued model of reinstatement, stimuli (e.g. light, tone, odor) previously paired with the self-administered drug can be presented in the absence of drug reinforcement following extinction and/or abstinence. The magnitude of operant responding on the previously drug-paired operandum can then serve as an index of conditioned-cued reinstatement of drug-seeking behavior [(Bossert *et al*, 2013; See, 2005) Figure 16]. Several lines of research have implicated the amygdala in the acquisition, consolidation and expression of cocaine-cue associative learning that maintains conditioned-cued reinstatement (See, 2005). Besides the central (CeA) and basolateral nuclei of the amygdala (Kruzich and See, 2001; Stefanik and Kalivas, 2013), studies investigating the circuitry underlying cued-reinstatement also suggest the involvement of dorsal mPFC (and its GLUergic projection to) NAcc core (McGlinchey *et al*, 2016; Stefanik *et al*, 2015), the bed nucleus of the stria terminalis (BNST) (Buffalari and See, 2011), as well as their DA input from VTA (Bossert *et al*, 2013; Feltenstein and See, 2009, 2013; Mahler and Aston-Jones, 2012; Stewart, 2008).

Also, environmental contexts previously associated with drug intake can provoke relapse to drug use in humans. However, until recently the role of contextual cues in preclinical models of relapse to drug use has received scant attention (Crombag *et al*, 2008; Shalev *et al*, 2002). The role of the environmental context in reinstatement of drug seeking can be studied in a procedure in which animals are trained to self-administer a drug in one context (A), and each drug infusion is paired with an explicit discrete drug cue (e.g. light or a compound tone-light cue). Drug-reinforced lever responding in the presence of the cue is extinguished in a different (non-drug) context (B), distinct from the drug-associated one in its tactile, visual, auditory and olfactory characteristics. Next, context-induced reinstatement of drug seeking is assessed through the re-exposure of the animal to the drug associated context [context A (Crombag *et al*, 2002, 2008)]. Given the role of context, the hippocampus also serves an important role in context-induced reinstatement of drug seeking (Luo *et al*, 2011; McGlinchey and Aston-Jones, 2017), as well as the NAcc shell (Cruz *et al*, 2014), the BLA (Hamlin *et al*, 2008), the PFC and their DA inputs from VTA and SN (Khoo *et al*, 2017).

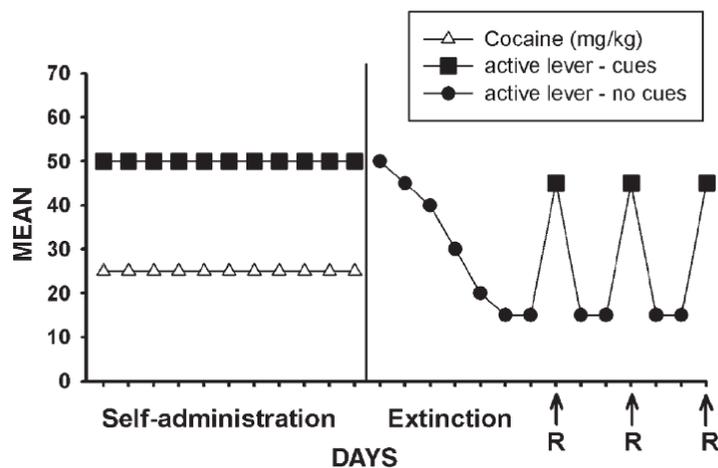


Figure 16. Representation of the cue-induced reinstatement. Experimental paradigm for reinstatement of drug-seeking behavior showing representative phases of acquisition, extinction, and reinstatement tests (R). Subjects are presented with discrete drug-paired cues on each self-administration session. Cues are then presented at the reinstatement test sessions to determine conditioned drug-seeking behavior. Obtained from See (2005).

7.2. Stress-induced reinstatement

Human research indicates that the life stress is not only a risk factor in the development of addiction but also a trigger for relapse to drug use (Ouimette *et al*, 2007; Sinha *et al*, 1999). Stressors are reported to reinstate drug seeking in the operant self-administration reinstatement procedures on animals with a history of COC (Erb *et al*, 1996; Mantsch *et al*, 2016; McReynolds

et al, 2014; Ogbonmwan *et al*, 2015; Shaham *et al*, 2003), alcohol (Lê *et al*, 2017), heroin (Sadeghzadeh *et al*, 2015), nicotine (Grella *et al*, 2014), and methamphetamine (Ferland *et al*, 2016). Also, the methods of these works include both environmental stressors, such as intermittent footshock, food deprivation, and swim stress; and pharmacological stressors such as corticotropin releasing factor (CRF), and yohimbine (Brown *et al*, 2012; Mantsch *et al*, 2016).

Interestingly, the exposure to an intermittent footshock stressor that reinstates drug seeking (Shalev *et al*, 2010) does not reinstate food reward seeking in male rats trained to self-administer food pellets (Ahmed and Koob, 1997), or sucrose pellets (Mantsch and Goeders, 1999). The reasons for the 'selective' effect of intermittent footshock stress on reinstatement of drug vs food reward are unknown, though one possibility is that while certain physiological effects of intermittent footshock promote reward seeking, other effects of footshock induce physiological states that inhibit food intake and seeking (Mantsch *et al*, 2016; Shaham *et al*, 2000). For instance, the release of CRF is associated with exposure to stressors (Bale and Vale, 2004), and may also cause inhibition of food intake (Sekino *et al*, 2004).

Using the reinstatement approach in rodents, the neurocircuitry involved in stress-induced cocaine seeking has begun to be defined. As other reinstating stimuli (e.g. priming injections of COC and cocaine-associated cues), stress-induced reinstatement appears to involve a corticostriatal GLUergic projection from the prelimbic cortex to the NAcc core, which likely regulates striato-pallidal outputs to produce drug seeking (Capriles *et al*, 2003; Mantsch *et al*, 2014; Sinha, 2001). Also, DAergic projections to the mPFC arise from the VTA, which appears to be a key integration site at which inputs that relay stress- and drug context/cue-related information converge. Brain structures upstream from this mesocortical pathway that are necessary for stress-induced relapse appear to include the BNST, CeA, and the NAcc shell (Mantsch *et al*, 2016; Shaham *et al*, 2003), which collectively compose the 'extended amygdala' complex.

7.3. Priming-induced reinstatement

The reinstatement of drug craving or seeking by priming injections of the abused or training drug, drugs of a similar class, or drugs that activate pathways in common with the training drug is a robust phenomenon in humans and laboratory animals (Robinson and Berridge, 1993; Stewart, 2008). In the drug-priming-induced reinstatement procedure, the effect of non-contingent injections of the self-administered drug, or other drugs, on reinstatement of the operant response in the self-administration or CPP procedures is determined after extinction of the drug-reinforced learned behavior (Venniro *et al*, 2016). Within this model, the priming

injection has been argued to renew the significance or salience of the drug-related environmental stimuli through its incentive properties, drawing the animal to approach the manipulandum and to engage in operant behavior. Thus, after extinction, a priming injection of the previously self-administered drug could be said to renew the salience of the lever and surrounding stimuli (Goddard and Leri, 2006; Mueller and Stewart, 2000; Stewart, 2000). An alternative explanation could involve the retrieval of habitual control over behavior, with relapse occurring as a result of automated processes, independent of motivational processes (Tiffany, 1990). In this framework, one might suggest that drug administration evokes related interoceptive emotional states that may serve as cues in the promotion of drug seeking (Belin and Everitt, 2008; Vanderschuren and Everitt, 2004; Zapata *et al*, 2010). In any case, multiple neuronal systems are involved in relapse induced by drug-priming, cues, and stress, implying that multiple psychological processes are likely to be involved (Shalev *et al*, 2002).

As COC exerts its stimulating and rewarding effects by acting as an indirect agonist of DA as it blocks DATs and enhances DA levels in the synapse, it is therefore not surprising that the systemic administration of DA reuptake inhibitors also reinstate COC seeking (Schenk, 2002; Schenk and Partridge, 1999). Also, it is now widely known that DA transmission, as well as D1R and D2R receptors, play a critical role in COC priming-induced reinstatement of drug-seeking. However despite conveying that both receptor subtypes are differentially involved in priming-induced reinstatement, conflicting results have emerged. Specifically, D2R agonists strongly reinstate COC seeking (Dias *et al*, 2003; Self *et al*, 1996; de Vries *et al*, 1999, 2002), and potentiate COC seeking when administered prior to a COC priming, whereas D2R antagonists attenuate priming induced reinstatement (Fuchs *et al*, 2002; Khroyan *et al*, 2000; Self *et al*, 1996; Spealman *et al*, 1999). In contrast, peripheral administration of D1R agonists does not induce reinstatement by itself; in fact, systemic administration of D1R agonists *or* antagonists attenuate COC-seeking induced by priming COC injection (Alleweireldt *et al*, 2002, 2003; Dias *et al*, 2003; Khroyan *et al*, 2003; Norman *et al*, 1999, 2002; Self *et al*, 1996; de Vries *et al*, 1999). Although the mechanisms underlying these conflicting results remain unclear, hypotheses include differing efficacies of the D1R agonists tested, D1R modulation of satiation through the saturation of primary rewarding processes (Baik, 2013; Philibin *et al*, 2011; Schmidt *et al*, 2005).

Studies on the role of DA in reinstatement of drug-seeking behavior have focused primarily on the NAcc. Given its role in mediating drug primary rewarding effects, as well as the attribution of incentive value to reward-conditioned stimuli (Schmidt *et al*, 2005) it has been attributed a critical role in priming-induced reinstatement. Reversible inhibition of the NAcc core, or inhibition of VTA DA neurons, the primary DA afferent of the NAcc, blocks COC priming-induced reinstatement (McFarland and Kalivas, 2001), whereas intra-NAcc DA infusion has also

been shown to reinstate COC seeking behavior (Cornish and Kalivas, 2000). Yet, conflicting results permeate the participation of both D1R and D2R within the striatum in reinstatement (their role in reinstatement is subject of further discussion on Section I).

Furthermore, an important role of GLU projections from the prelimbic mPFC to the NAcc core has been identified in priming-induced reinstatement of drug-seeking. Similarly to what occurs in behavioral sensitization (see §5), the repeated exposure to the drug in COC SA decreases basal GLU levels within the NAcc, and reversing this effect by systemic N-AC injections has been shown to decrease COC priming-induced reinstatement. In this process, N-AC has been shown to normalize extra synaptic GLU transmission, controlled by glial cystine/GLU exchanger, as it restores tonic stimulation of group II metabotropic GLU receptors (mGluR2/3) located on presynaptic neurons. These GLU release-regulating presynaptic mGluR2/3 autoreceptors reduce synaptic release of GLU by PFC afferents into the NAcc core, preventing sensitization as well as COC priming-induced reinstatement (Bossert *et al*, 2013; Kupchik *et al*, 2012; Moussawi *et al*, 2009; Steketee and Kalivas, 2011).

Regarding the COC priming-induced reinstatement circuitry, it has been argued that neuronal circuits centered in the NAcc and DS are interconnected and process information via parallel and integrated connections. The NAcc shell is indirectly connected to the dorsal PFC and the NAcc core through the VP and mediodorsal thalamus. In turn, the NAcc core is connected more dorsal portions of the striatum, through the spiraling connections (see § 6.3). further structures involved in the priming effects of priming induced reinstatement of COC seeking include the mPFC (and its GLU projections to the NAcc) (Capriles *et al*, 2003; Kerstetter *et al*, 2015; Kupchik *et al*, 2012), the ventral subiculum (Sun and Rebec, 2003; Vorel *et al*, 2001), central and basolateral nuclei of the amygdala (Alleweireld *et al*, 2006) and the VP (Mahler *et al*, 2014), as well as their DAergic input from the VTA and SNc [(McFarland and Kalivas, 2001; Schmidt *et al*, 2005) Figure 17].

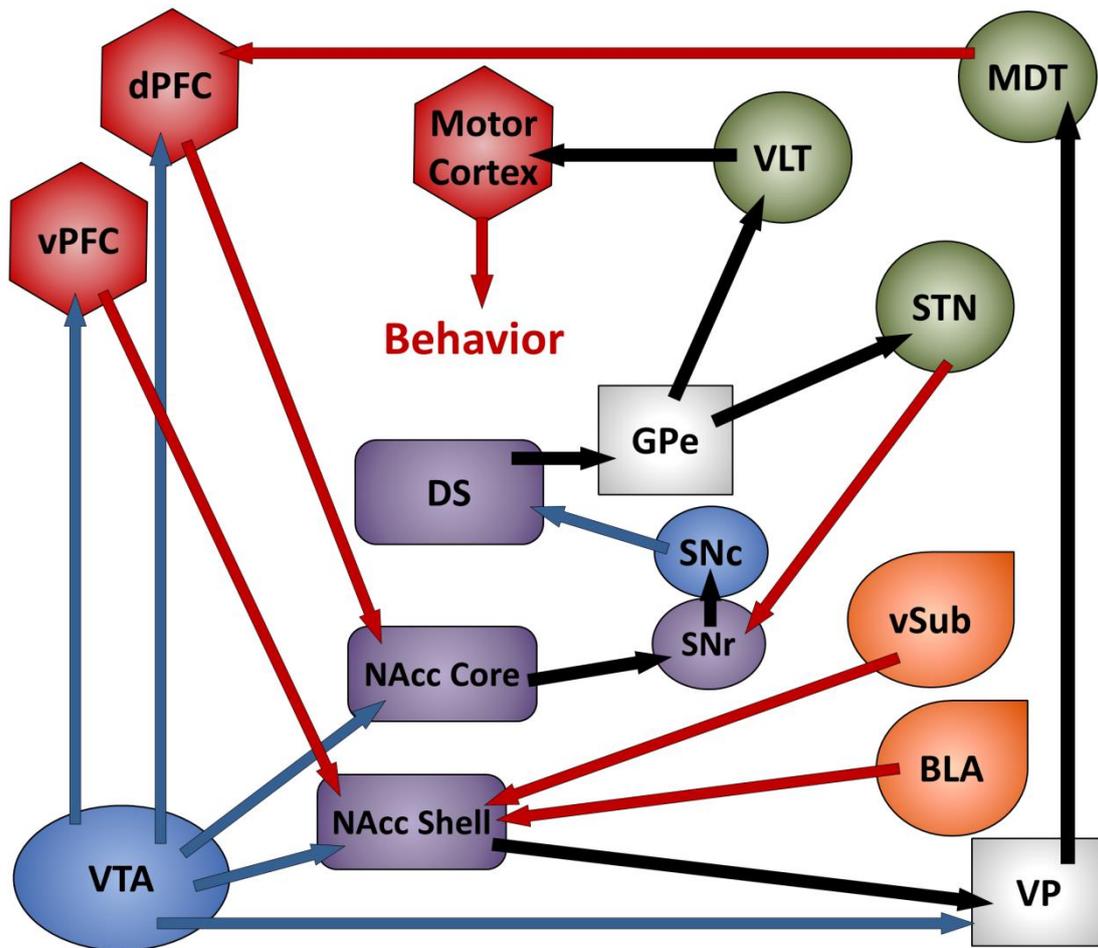


Figure 17. Anatomical representation of the circuitry mediating COC priming-induced reinstatement. The ventral PFC projects to the NAcc shell, which communicates with the dorsal PFC via serial projections to the VP and mediodorsal thalamus. The dorsal PFC projects to the NAcc core, which relays information to the DS via the SNr. The DS influences the motor cortex via the GP and ventrolateral thalamus. Dopaminergic inputs from the VTA to several of these nuclei modulate the flow of information through these circuits (Red arrows: DA projections; blue arrows: DA projections; black arrows: GABA projections). CeA/BLA: central and basolateral nuclei of the amygdala; DS: dorsal striatum; GPe: external globus pallidus; MDT: mediodorsal thalamus; dPFC: dorsal prefrontal cortex; vPFC: ventral prefrontal cortex; SNr: substantia nigra pars reticulata; STN: subthalamic nucleus; VLT: ventrolateral thalamus; VP: ventral pallidum; vSub: ventral subiculum; VTA: ventral tegmental area). Modified from Schmidt *et al* (2005).

Problem statement

In the last decades, significant progress has been achieved regarding the different aspects of addiction and relapse. Indeed, it is believed that the exposure to drugs engenders persistent neuroadaptations which are considered to be responsible for the dependence and the vulnerability to relapse. Nonetheless, several aspects underlying drug-seeking and drug-taking behavior are still not clear, and their discovery and comprehension will allow for the improvement of the treatments for addiction.

Animal models permit the study of both neuroadaptations as well as their behavioral manifestation. Indeed acute re-exposure to the drug has been identified as a major determinant of relapse. In animal models of relapse, it is known that one of the most powerful events for reinstating drug-seeking or drug-taking behavior is a priming injection of the drug itself or drugs of a similar class. In turn, the DA system has been shown to have a critical participation in the development of drug addiction. Notably, its action on the striatum has been investigated on both normal behavior and in addiction, yielding invaluable information. Considerable advances have been achieved in the last decades on the search of mechanisms underpinning the enduring vulnerability to relapse that afflicts addicted subjects.

Though several hypotheses have been put forward, the reasons why repeated exposure to drugs eventually makes subjects vulnerable to relapse remain somehow elusive. Dopamine receptors have been shown to play a differential role in striatal circuits both in normal behavior and addictive processes. Adding to the complexity of their actions, the different subareas of the striatum are also known to play differential roles in behavior and addiction. Yet, the specific mechanisms that involve dopamine receptors of different striatal subregions in relapse are not totally clear.

Hypotheses concerning the development of drug addiction have focused on gradual adaptations in the brain conceiving the concept that control over drug use gradually evolves from voluntary drug use to automatized habitual consumption, which eventually becomes compulsive. In support of hypothesis, the sensitization to cocaine or amphetamine has been shown to accelerate the formation of habits during behavioral training for food rewards, conveying that the neuroadaptive changes induced by these drugs are likely to be involved in habit formation. This progression is believed to be paralleled by the initial influence of functional changes exerted by drugs within the ventral striatum, and the gradual shift of behavioral control towards the automatization guided by the dorsal striatal circuitry.

Previous work from our lab and others has shown that the stimulation of the D2 dopamine receptors elicits a strong reinstating effect on animals subjected to extinction training after cocaine self-administration. Conversely, the stimulation of D1 receptors does not elicit reinstatement by itself. Thus, one might raise the question as to what is the role exerted by dopamine receptors of striatal subregions in the reinstatement of cocaine seeking behavior induced by the systemic stimulation of dopamine receptors. Furthermore, taking into account the differential roles assigned to both ventral and dorsolateral striatal subregions in reward/motivation and habitual performance, respectively, one might ask which behavioral mechanisms drive subjects to the resumption of the search for drugs of abuse.

Objectives and aims

Taking these questions into account, the work presented in this thesis has been devised with the aim of assessing the role of D1 and D2 dopamine receptor subtypes from the ventral and dorsal striatum on the reinstatement of cocaine seeking behavior, as well as to build a model of the transition of behavioral control from a goal-directed system to the eventual automatization believed to underlie drug consumption using a second-order schedule of reinforcement with food reinforcers. We also investigated the contribution of a COC sensitization regimen to the establishment of habitual behavior. This protocol is intended to tease apart the appetitive and consummatory aspects that drive drug consumption and shed some light to the underpinnings of cocaine seeking behavior and relapse. To this end, the following questions are to be approached:

- o **Section I – Pharmacological investigation: the role of Dopamine receptors of the ventral and dorsal striatum in the relapse of cocaine seeking.**

Here the aim was to examine the participation of the D1 and D2 subtypes of dopamine receptors on the reinstatement of cocaine-seeking. The assessment of their relative contribution within the nucleus accumbens and dorsolateral striatum (as a follow up of a work started previously in the lab) is described on Section I;

- o **Section II – Behavioral investigation: Modelling the transition to the automatization of behavioral control.**

In this section the development of a second order schedule of reinforcement with palatable food pellets as reinforcers is described. The aim here is to establish a model that would allow for the investigation of the transition of behavioral control to the establishment of automatisms, regarded as a major feature of addiction. This

model would allow for the dissociation of appetitive and consummatory responses and, once established, could be applied to COC SA, thus shedding light in the behavioral determinants of the relapse observed in animals studied on Section I;

o **Section III – Pharmacogenetics: Preliminary study on novel approaches to investigate addiction and relapse**

Finally, in this section is described preliminary data obtained in the attempt to establish a chemogenetic methodology that would allow for the assessment of D1R and D2R bearing pathways of the nucleus accumbens and, eventually, of the dorsolateral striatum. This methodology would enable us to examine the role of these pathways beyond the actions of D1R and D2R on the reinstatement of cocaine seeking behavior.

MATERIAL AND METHODS

Material and methods

1. Subjects

For the experiments performed, male Wistar rats (HAN) were used as experimental subjects (Charles River Laboratories, France). Upon arrival at the laboratory, the age of these animals was from 5 to 6 weeks old and their weight between 225 and 275g. Rats were housed in collective cages in groups of 2 and were allowed at least one week familiarization with the vivarium before procedures began. Food and water are available *ad libitum*, unless otherwise stated (food conditioning experiments, Section II; animals had access limited to 18g chow in their home cages, to be maintained on 90% of their free feeding weight). All the animals were periodically weighed to control for health issues.

The home cages were located in a temperature (22 °C) and humidity (60%) controlled environment and subjected to a reverse 12 h light/dark cycle (lights on from 20:00 to 08:00 h). All experiments were conducted during the dark phase of the light/dark cycle (activity phase in rats) and performed in accordance with the European directive (86/609/EEC) and with approval of the *Centre National de la Recherche Scientifique* (CNRS; ethical commission number 50; approval number 50120123-A), concerning the use of laboratory animals. Animals were manipulated every day in the intent of reducing their stress in the beginning of the experimental sessions and all efforts were made to minimize the number of animals used as well as their suffering.

2. Surgical procedures

2.1. Intravenous catheter implantation (Section I)

Animals were deeply anesthetized with isoflurane (4–5% induction, 1–2% maintenance) and an indwelling catheter (SILASTIC tubing; 0.64 mm inner diameter, 1.19 mm outer diameter; Dow Corning Co., USA) was surgically implanted into the right jugular vein. The proximal end of the catheter was secured to the vein with surgical silk sutures and passed subcutaneously to the top of the back, where it exited into a connector (modified 22ga cannula). The distal end of

the catheter was connected to stainless steel tubing encased in dental cement anchored with a square of mesh (Small Parts Inc., USA). After surgery, animals were flushed daily with 0.2 ml of an ampicillin solution (0.1 g/ml Totapen; ConvaTec, Paris, France) containing heparin (300 IU/ml) to maintain patency. During drug SA sessions, animals' catheters are connected by spring-covered Silastic tubing through a fluid swivel to a syringe pump located outside the chamber. Rats were given 7 days of recovery before the start of behavioral experiments.

2.2. Intracerebral surgeries (Section I)

Stereotaxic coordinates were determined in accordance with the cerebral region targeted, being variable according to the age and size of the animals. They were predetermined with the aid of an atlas (Paxinos and Watson, 2006), and verified in two surgical trials. Following observations, the coordinates were corrected before the beginning of each experiment.

Bilateral implantation of stainless steel guide cannulae was carried out immediately after the implantation of the indwelling catheter into the right jugular vein for SA experiments. Still under deep anesthesia, the animals were fixated in a stereotaxic apparatus (Kopf, USA) and the connective tissue was retracted exposing the skull. The coordinates were measured from bregma and two holes were drilled for the bilateral implantation of guide cannulae (23ga) following the previously observed coordinates, which were positioned 1.5 mm above the injection site for DLS experiments and 2.5 mm above the injection site for NAcc experiments. For the fixation of the guiding cannulae to the skull, dental cement was used, with the addition of four screws. Stainless steel stylets (0.3 mm in diameter, 10 mm in length) were slid into the guide cannulae to avoid obstruction and removed right before intracerebral injections. Surgery recovery period was around seven days. Cannulae placement aiming for either the DLS or the NAcc were established by the following coordinates (Figure **18A; B**):

Dorsolateral striatum:

- + 0.8 mm anteroposterior (AP), from bregma
- ± 3.4 mm mediolateral (ML), from bregma
- 3.8 mm dorsoventral (DV), from the surface of the skull

Nucleus Accumbens:

- +1.5 mm AP, from bregma
- ± 1.3 mm ML, from bregma
- 4.0 mm DV, from the surface of the skull

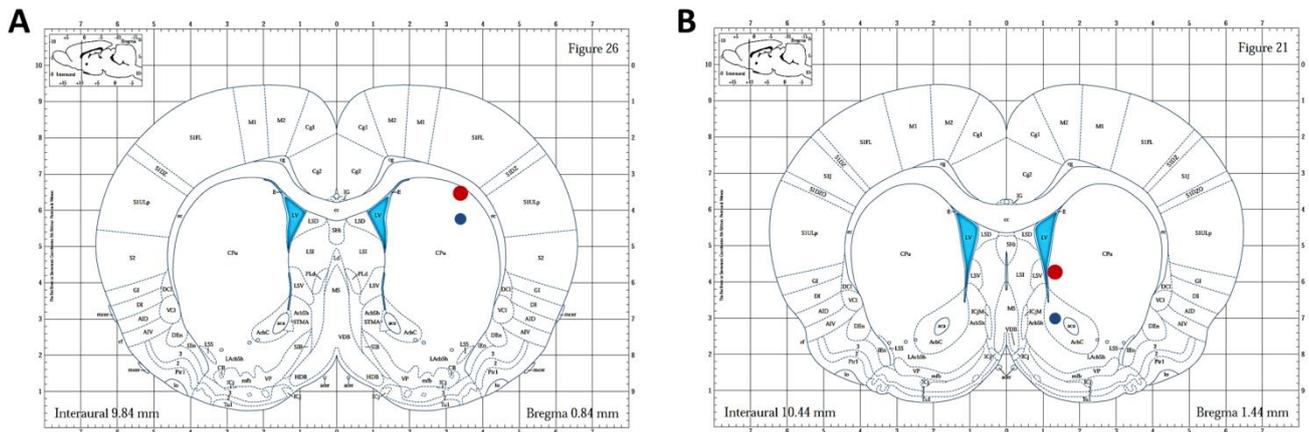


Figure 18. Aims of the injection sites for pharmacological stimulation of DA receptors performed in Section I. Intended cannulae positions (red dots) and injection sites (blue dots) in the DLS (A) and NAcc (B) for infusions of D1R and D2R agonists. Adapted from Paxinos and Watson (2006).

3. Apparatus

3.1. Operant conditioning chambers (Sections I and II)

Operant conditioning for food pellet or COC reinforcement was conducted in twelve standard operant conditioning chambers (30 cm height × 40 cm length × 36 cm depth, Imetronic, Pessac, France) which also allowed for the recording of locomotor activity of the animals (Figure 19). The experimental chambers were located in an experimental room equipped with white noise generators. Each of them was individually housed in wood attenuation boxes fitted with ventilation fans and with two clear Plexiglass walls on the front and back sides and two opaque panels on the left and right sides. The floor consisted of 6 mm diameter steel bars spaced 15 mm apart, center-to-center. Two retractable levers (0.03 N force required for activation) were located on either side of the left panel (7 cm above the floor) counterbalanced as active lever/inactive lever (for cocaine reinforcement experiments; vide infra) or seeking lever/taking lever (for food pellet reinforcement experiments) across left and right (Figure 20). For food pellet reinforcement experiments a food magazine, from which food rewards were delivered, was located at the center of the same wall. Illumination of a soft house-light bulb (2W) signaled the start of the sessions.



Figure 20. Operant conditioning chamber fitted in wood attenuation boxes.

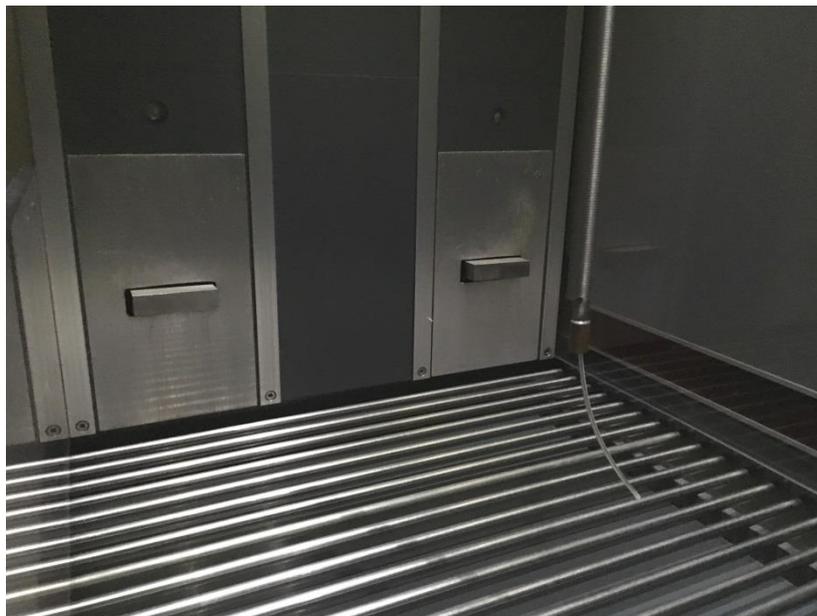


Figure 19. Interior of conditioning chamber, with two retractable levers and the metal spring through which saline or COC solutions were intravenously injected.

3.1.1. Operant conditioning for cocaine (Section I)

During self-administration sessions, animals were connected by their catheter through a Silastic tube protected by a metal spring with a swivel to a 10 ml syringe fitted into an external pump. Responses in the active lever activated the external pump (Figure 21) delivered intravenous saline or cocaine infusions (0.25 mg per infusion) in a volume of 100 μ l over 3s. Each infusion was paired with a cue light located above the lever designated as active lever and

a time out period of 20 s, during which responses had no effect. The second lever was designated as inactive lever and pressings on this lever resulted in no effect.



Figure 21. External pump for i.v. saline or COC infusions.

3.1.2. Operant conditioning for food pellets (Section II)

The cages described for COC SA were similar to those used for food pellet reinforcement. However, each response in the *taking lever* activated a pellet dispenser located outside the cage which delivered one palatable food pellet into the food magazine (Figure 22), located midway between the two levers. Number of lever presses and food pellets distributed, as well as locomotor activity were recorded.



Figure 22. Operant conditioning cages for food pellets with two levers in each side of a food magazine.

3.2. Activity cages (Section I; Annex)

Locomotor activity assessments were conducted in twenty four locomotor activity cages (35 cm height × 25 cm length × 25 cm depth; Imetronic, Pessac, France) located in a dark experimental room equipped with white noise generators. The door, floor, and ceiling of each experimental cage consisted of wire mesh and the side walls were made of 10 mm thick clear Plexiglass. Infrared beams automatically recorded the locomotor activity of the animals (Figure 23).



Figure 23. Locomotor activity cages.

4. Material

4.1. Drugs

- Cocaine hydrochloride (Cooperative Pharmaceutique Française, France)
- SKF 82958 (D1R agonist)
- SCH 23390 (D1R antagonist)
- Quinelorane (D2R agonist)
- Raclopride (D2R antagonist)

Drugs were dissolved either in sterile saline (NaCl 0.9%) or phosphate-buffered saline (PBS). Cocaine was delivered intraperitoneally (i.p.; 15 mg/kg for challenges or 30 mg/kg for sensitization procedures; Sections I and II, respectively) in volumes of 1 ml/kg body weight in all experiments. All control treatments consisted of an equivalent volume of either saline or phosphate buffered saline (PBS). The D1R DA agonist SKF 82958 was infused either into the NAcc or into the DLS, depending on the experiment, in doses of 0; 0.05; 0.1; 0.5 or 1 µg/0.5 µL/side. The D2R DA agonist Quinelorane was delivered either i.p. (0.25 mg/kg) or intra cerebrally, either into the NAcc or DLS, in doses of 0; 0.05; 0.1; 1; 1.5; 2.5 and 5 µg/0.5 µL/side. The D2R antagonist Raclopride was infused into the DLS (5 µg/0.5 µL/side) and the D1R antagonist SCH 23390 was infused into the NAcc (1 µg/0.5 µL/side).

4.2. *Food outcomes*

For second order schedule experiments rats were given food pellets [Pellets formula "P": Test Diet 1811155 (5TUL) AIN-76A, rodent tablet 45 mg] as food outcome.

5. Statistical Analyses

Statistical analyses were performed using STATISTICA (StatSoft; v. 7.1), Statview (SAS Institute; v. 5.0) or Prism (GraphPad Software; v.6.01). Statistical analyses are described in detail in each section; in all cases statistical significance was considered when $p < 0.05$.

SECTION I:

**Pharmacological investigation: the role of
Dopamine receptors of the striatum in the
relapse of cocaine seeking**

Section I – Pharmacological investigation: the role of Dopamine receptors of the striatum in the relapse of cocaine seeking

Section overview:

Previous work in our team has shown a dissociable effect between DA receptor subtypes on reinstatement of COC seeking behavior. Whereas the systemic administration of a D2R agonist induced strong reinstating effects on rats trained to self-administer COC, the systemic administration of D1R agonist did not (Dias *et al*, 2003). Given that both the ventral and dorsal subregions of the striatum are influenced by dopaminergic inputs from the midbrain, and cocaine-induced plasticity in these circuits is hypothesized to underlie several aspects of drug seeking behavior (Pierce and Vanderschuren, 2010), we decided to investigate the role of DA receptors of different regions of the striatum, namely the nucleus accumbens and the dorsolateral striatum on the reinstatement of COC seeking behavior.

With this aim, 3 experiments were performed and resulted in the preparation of a manuscript that is to be submitted for publication. A summary of the experiments performed as well as the manuscript itself follow below.

Experiment 1: The role of D1R and D2R of the NAcc on reinstatement of COC seeking.

Methods summary: Rats were operated to have indwelling catheters implanted in the jugular vein for COC SA, and were also bilaterally implanted with cannulae, aiming for the NAcc. After recovering from surgery, animals trained to self-administer COC under a continuous reinforcement schedule, and then submitted to extinction training, in which lever presses had no programmed consequences. Once COC-seeking behavior was extinguished, animals were submitted to a COC challenge, which served as classical probe for reinstating behavior. On the following tests, animals were submitted to bilateral injections of either D1R (SKF82958) or D2R (Quinelorane) agonists through the cannulae aiming for the NAcc before being placed in the operant chambers to access for potential reinstating effects elicited by either DA receptor

agonist. Importantly, all the tests were carried out in extinction; that is, lever presses were not reinforced.

Results summary: The experiment demonstrated that the pharmacological activation of D1R of the NAcc induces reinstatement of COC seeking, but the same activation of the D2R of the NAcc does not.

These results were rather puzzling, as they were stark opposition to what has been shown with the peripheral administration of D1R and D2R agonists. In summary, thus far we had observed that: [i] the systemic pharmacological activation of D1R does not induce reinstatement of COC seeking behavior, but [ii] the pharmacological activation of D1R exclusively of the NAcc does. Additionally, [iii] whereas the systemic activation of the D2R receptors triggers a potent reinstating response in the rats tested, [iv] the same was not true when the same agonist was administered locally, into the NAcc.

This apparent dissociation of reinstating effects observed between systemic and local (NAcc) administration of D1R or D2R agonists enticed us to further investigate where else could the systemically administered D2R agonist be acting to induce reinstatement, if not within the NAcc.

Experiment 2: The role of D1R and D2R of the DLS on reinstatement of COC seeking.

Methods summary: Rats were operated in the same manner as in Experiment 1 except that this time the guiding cannulae were implanted aiming for the DLS. After recovering from surgery, animals underwent the same training and extinction of COC SA, and COC challenge. On the following tests, animals were submitted to bilateral injections of either SKF82958 or quinolorane through the cannulae, this time aiming for the DLS before being tested, as previously.

Results summary: The experiment demonstrated that the pharmacological activation of D2R of the DLS induces reinstatement of COC seeking (though in this experiment rats reinstated COC seeking only with the highest dose of quinolorane, not displaying an expected dose-response curve), but the same activation of the D1R of the DLS does not.

Following these results, we decided to test the possibility that the activation of D2R of the DLS could be responsible for the reinstating effects elicited by the systemic activation of D2R. So we tested this hypothesis by peripherally administering the D2R agonist (quinolorane, which was known to induce reinstatement) and simultaneously injecting a D2R antagonist (raclopride) into the DLS. If this was the case, by blocking the effect of D2R of the DLS we were expecting to, at least partially, block the reinstatement of COC seeking triggered by the systemic activation of D2R.

As a result, we observed that the reinstatement of COC seeking induced by peripheral injection of quinolorane was only partially blocked by the infusion of raclopride into the DLS.

From that, it seemed reasonable to assume that [i] other doses of quinolorane should be investigated, so we have a clearer idea of the reinstating effects elicited by the activation of the D2R of the DLS; and [ii] the reinstatement induced by the systemic activation of D2R relies partially, but not exclusively, on the activation of D2R of the DLS.

These assumptions moved us to the third experiment, where we assessed [i] the reinstating effects of further doses of quinolorane into the DLS; and [ii] if the reinstating effects induced by systemic activation of D2R would be counteracted by the infusion of a D1R (SCH23390) into the DLS; or by either SCH23390 or raclopride into the NAcc.

Experiment 3: The role of D1R and D2R of the DLS on reinstatement of COC seeking.

Methods summary: Rats were operated in the same manner as in Experiment 1 and 2 except that this time the guiding cannulae were aiming for the DLS in half of the rats, while the other half received cannulae implantation in the NAcc. After recovering from surgery, animals underwent the same training and extinction of COC SA, and COC challenge. On the following days, rats were submitted to different tests, according to their cannulae implantation:

- DLS group: animals were submitted to bilateral injections of two other doses of quinolorane into the DLS before being tested for reinstatement, as previously. On a second part, animals were systemically infused with quinolorane while receiving a simultaneous infusion of a SCH23390 into the DLS;
- NAcc group: animals were systemically infused with quinolorane while receiving a simultaneous infusion of a D1R or D2R antagonists into the DLS.

Results summary: *These experiments gave us a better overview on the activation of the D2R on the DLS, and demonstrated that the reinstatement induced by the systemic pharmacological activation of D2R is blocked by the infusion of either a D1R or a D2R antagonists in the NAcc or by the administration of a D1R antagonist in the DLS.*

Finally, we were able to observe that:

- 1) The pharmacological activation of D1R of the NAcc induces reinstatement of COC seeking behavior in rats;
- 2) The pharmacological activation of D2R of the DLS induces reinstatement of COC seeking behavior in rats;

- 3) The reinstating effects of the systemic pharmacological activation of D2R is blocked by [i] the infusion of either D1R or D2R antagonists into the NAcc, or [ii] the infusion of a D1R antagonist into the DLS, while being blunted by the infusion of a D2R antagonist into the DLS.

Therefore, it appears that a conflating activation of both subtype receptors in both regions of the striatum is responsible for the induction of reinstatement of COC seeking behavior. These effects might possibly rely on spiraling connections between ventral and dorsal striatum, through ascending loops that reach the midbrain. An interesting target for future studies would be to assess whether the reinstatement of COC seeking triggered by the D2R stimulation within the DLS also involves the incentive motivational or reinforcing processes likely underlying those induced by the D1R stimulation within the NAcc. Additionally, it would also be useful to investigate whether the D1R stimulation within the NAcc also involves the overtaking of the behavioral control by habitual stimulus-response mechanisms which may be involved in the reinstatement of drug-seeking after the D2R stimulation in the DLS.

Original article

Title: Double dissociation between the actions of dopamine D1 and D2 receptors of the ventral and dorsolateral striatum on the reinstatement of cocaine seeking behavior

Authors: Renan Costa Campos¹, Carine Dias¹, Florence Darlot^{1,2}, Martine Cador^{1,2}

¹ Université de Bordeaux, F-33076 Bordeaux, France

² CNRS, Institut de Neurosciences Cognitives et Intégratives d'Aquitaine, UMR 5287, F-33076 Bordeaux, France

Corresponding author:

Martine Cador (martine.cador@u-bordeaux.fr)

Institut de Neurosciences Cognitives et Intégratives d'Aquitaine (INCIA)

UMR 5287 CNRS/Univ. Bordeaux

Université de Bordeaux-Site Carreire, BP31

146 rue Léo Saignat

33076 Bordeaux, France

ABSTRACT

Although the factors responsible for the resumption of drug taking in human addicts are not completely understood, acute re-exposure to the drug has been identified as a major determinant of relapse. It is hypothesized that cocaine-induced plasticity in both nucleus accumbens (NAcc) and dorsolateral striatum (DLS) circuits underlies several aspects of drug seeking behavior, under the influence of dopamine (DA) receptors. Several works investigated the role of D1 and D2 DA receptors (D1R; D2R, respectively) of the NAcc and DLS on reinstatement, but results remain somewhat controversial. Given their importance, we assessed the reinstating effects of infusions of D1R (SKF82958) or D2R (quinelorane) agonists within the NAcc or DLS after the extinction of cocaine self-administration (COC SA). To further investigate their role on reinstatement, we infused either a D1R (SCH23390) or a D2R (raclopride) antagonist within the NAcc or DLS with the simultaneous systemic administration of quinelorane, known to induce reinstatement. The infusion of SKF82958 induced reinstatement when infused into the NAcc, but not into the DLS; quinelorane had no effects when injected into the NAcc, but induced relapse when infused into the DLS. Also, while the infusion of SCH23390 into the NAcc or DLS blocks the reinstatement induced by peripheral infusion of D2R agonist, raclopride administration into the NAcc blocks, while within the DLS blunts, the reinstating effects of the systemic administration of D2R agonists. The results point to a double dissociation between DA receptors of the NAcc and DLS in the induction of relapse; and highlight the interaction of D1R and D2R within both structures in the reinstatement of cocaine seeking.

Keywords:

Self-administration; Nucleus Accubens, Dorsolateral striatum; Reinstatement; Cocaine; Addiction

INTRODUCTION

Although the factors responsible for the resumption of drug taking in human addicts are not completely understood, acute re-exposure to the drug has been identified as a major determinant of relapse. In an animal model of relapse, an acute “priming” injection of the drug results in a robust reinstatement of a formerly acquired and then extinguished drug self-administration (SA) behavior (for reviews, see Shaham *et al*, 2003; Stewart, 2000). It is now established that drugs abused by humans increase synaptic dopamine (DA) in the mesolimbic system (Di Chiara, 1999; Torregrossa and Kalivas, 2008), which is responsible for regulating motivation and reward (Wise, 2004). In fact, the repeated exposure to addictive substances elicits changes at both molecular and cellular levels in this pathway on the neurobiological process culminating in addiction (Robinson and Berridge, 1993). One of the major targets of the DAergic pathway is the striatum, where DA acts on five subtypes of metabotropic membrane receptors which have so far been identified. These receptors have been categorized as either D1-like (D1 and D5) or D2-like (D2, D3, and D4) with basis on their structural and pharmacological properties (for review, see Beaulieu and Gainetdinov, 2011).

Being both the ventral and dorsal striatum influenced by dopaminergic inputs from the midbrain, it is hypothesized that cocaine-induced plasticity in these circuits underlies several aspects of drug seeking behavior (Pierce and Vanderschuren, 2010). Dopamine signaling in the nucleus accumbens (NAcc) is believed to respond to rewarding and aversive stimuli. However, although many studies suggest a rewarding/reinforcing role for the D1 receptors (D1R) and an aversive/punishing role for the D2 receptors (D2R) (Hikida *et al*, 2013; Kravitz *et al*, 2012; Lobo *et al*, 2010; Volman *et al*, 2013), some results do not support this dichotomy (Soares-Cunha *et al*, 2016; Steinberg *et al*, 2014; Trifilieff *et al*, 2013). On reinstatement, several works investigated the role of NAcc and its DA receptors, but the results remain somewhat controversial, possibly due to the many differences between the protocols used. Reversible

inactivation of the NAcc has been shown to block cue induced reinstatement, an effect not observed with the inactivation of NAcc shell (Fuchs *et al*, 2004). On the other hand, prior D1R or D2R inhibition within the NAcc shell have been shown to block cocaine priming-induced reinstatement, while the same treatment did not affect the NAcc core (Anderson *et al*, 2003, 2006). Also, whereas the administration of D1R or D2R agonists into the NAcc shell have been shown to be enough to induce the reinstatement of cocaine seeking (Bachtell *et al*, 2005; Schmidt and Pierce, 2006), conflicting results from DA agonists within the core have been found (Bachtell *et al*, 2005; Schmidt *et al*, 2006). Thus, the role of DA receptors of the NAcc and its sub regions on reinstatement remains subject to debate.

The DLS is known to play a key role in the transition to compulsive use (Everitt *et al*, 2008), being responsible for the habitual aspects of drug-seeking after prolonged drug self-administration (Belin and Everitt, 2008; Zapata *et al*, 2010). The role of its DA input on relapse has also been suggested by studies with opioids and psychostimulants. Whereas cue- and priming-induced heroin seeking are blocked by reversible inactivation of the DLS (Rogers *et al*, 2008), the D1R of the DLS have been implicated in the context induced reinstatement of heroin seeking. Furthermore, the reversible inactivation or DA blockade within the DLS has been shown to attenuate context and cue-induced reinstatement of cocaine seeking (Fuchs *et al*, 2006; See *et al*, 2007). Finally, the demonstration that the reinstatement of cocaine seeking was also blocked by the inactivation of substantia nigra, the primary DA input into the DLS, further supports a critical role of DLS DA in relapse behavior (See *et al*, 2007). Yet the specific role of D1R and D2R within the DLS on relapse of cocaine seeking are not clearly established.

The DA receptors themselves add one more aspect to the function of NAcc and DLS on reinstatement, as both D1 and D2 receptors (D1R and D2R) play an essential but differential role in the reinstatement of cocaine seeking behavior. Whereas the systemic administration of D2R antagonists reduces priming-induced reinstating effects of cocaine (Schenk and Gittings, 2003; Spealman *et al*, 1999; Weissenborn *et al*, 1996), D2R agonists are able to strongly reinstate cocaine seeking behavior (Dias *et al*, 2003; Self *et al*, 1996; Weissenborn *et al*, 1996).

On the other hand, D1R agonists do not induce reinstatement by themselves, (Dias *et al*, 2003; Self *et al*, 1996; Spealman *et al*, 1999; de Vries *et al*, 1999, 2002), they rather reduce the reinstating effect of a cocaine priming, which is also demonstrated by the administration of D1R antagonists (Alleweireldt *et al*, 2003; Self *et al*, 1996; Spealman *et al*, 1999).

Together, these data convey that both D1R and D2R play differential roles within both striatal subregions, and show they are crucial for the reinstatement of cocaine seeking and relapse. Yet the mechanisms and the specific regions where these receptors act to induce the reinstatement of cocaine seeking remain elusive. Given their importance, we sought to tease apart the reinstating effects of both DA receptor subtypes within the two striatal regions (i.e. NAcc and DLS) as well as their participation on the systemic D2R agonist-induced reinstatement of cocaine seeking (Dias *et al*, 2003).

MATERIAL AND METHODS

Subjects

Male Wistar rats (Charles River Laboratories, France) weighing 225–275g were used. Rats were housed in cages in groups of 2 in a temperature (22 °C) and humidity (60%) controlled environment, subjected to a reverse 12 h light/dark cycle (lights on from 20:00 to 08:00 h). Food and water were available *ad libitum*. All experiments were conducted during the dark phase of the light/dark cycle (activity phase in rats) and performed in accordance with the European directive (86/609/EEC) and with approval of the *Centre National de la Recherche Scientifique* (CNRS), concerning the use of laboratory animals.

Surgery

Intravenous catheter implantation

Animals were deeply anesthetized with isoflurane (4–5% induction, 1–2% maintenance) and an indwelling catheter (SILASTIC tubing; Dow Corning, Midland, MI) was surgically implanted into the right jugular vein. The proximal end of the catheter was secured to the vein with surgical silk sutures and passed subcutaneously to the top of the back, where it exited into a connector (modified 22 gauge cannula). The distal end of the catheter was connected to stainless steel tubing encased in dental cement anchored with a square of mesh (Small Parts Inc., USA). Animals were flushed daily with 0.2 ml of an ampicillin solution (0.1 g/ml Totapen; ConvaTec, Paris, France) containing heparin (300 IU/ml) to maintain patency. Rats were given 7 days of recovery before the start of behavioral experiments.

Intracerebral surgeries

Bilateral implantation of stainless steel guide cannulae was carried out immediately after the implantation of the indwelling catheter into the right jugular vein for SA experiments. The rats were implanted with bilateral 26-gauge guide cannulas targeting the NAcc (anteroposterior AP) + 1.5 mm; mediolateral (ML) \pm 1.4 mm; dorsoventral (DV) - 4.0 mm ventral), or the DLS (AP + 0.8 mm; ML \pm 3.4 mm; DV -3.8 mm) with coordinates relative to bregma (Paxinos & Watson, 2006). The guide cannulae were aimed at either 1.5 mm (for DLS) or 2.5 mm (for NAcc) above the target region. For the fixation of the guiding cannulae to the skull, dental cement was used, with the addition of four screws. Animals were monitored and weighed daily for 10 days after surgery and were allowed to recover for at least 1 week prior to operant training.

Apparatus

Operant conditioning for cocaine reinforcement was conducted in twelve standard operant conditioning chambers (30 cm height \times 40 cm length \times 36 cm depth, Imetronic, Pessac, France). The experimental chambers were located in an experimental room equipped with white noise generators, and each of them was individually housed in wood attenuation boxes

fitted with ventilation fans. They had two clear Plexiglass walls on the front and back sides and two opaque panels on the left and right sides and the floor consisted of 6 mm diameter steel bars spaced 15 mm apart, center-to-center. Two retractable levers were located on either side of the left panel (7 cm above the floor) counterbalanced as “active” lever (AL) and “inactive” lever (IL). Illumination of a soft house-light bulb (2W) signaled the start of the sessions. During SA sessions, animals were connected by their catheter through a Silastic tube protected by a metal spring with a swivel to a 10 ml syringe fitted into an external pump. Responses in the AL activated the external pump and delivered intravenous saline or cocaine infusions. Each infusion was paired with a cue light located above the AL.

Cocaine self-administration and reinstatement tests

Acquisition and extinction phases

Rats were placed in the operant chambers and trained to lever press on an FR1 schedule for cocaine (250 µg/infusion; COC SA, n=17 for first set of NAcc experiments and n=5 for the second set of NAcc experiments; n=19 for the first set of DLS experiments and n=6 for the second set of DLS experiments) or saline (SAL SA, NAcc first set n=12, and n=4 for second set; DLS first set, n=12; and DLS second set, n=6) SA in daily 2-h sessions. Each session started with a free non-contingent infusion. Depression of the AL resulted in the delivery of 100 µl drug solution over a period of 4 s and illumination of a cue light above the active lever, while the house light was switched off. Each injection was followed by a 20-s time out period during which lever responding was without consequence. Depression of the “inactive” lever was recorded but had no programmed consequences. After 15 training sessions on average, COC SA rats achieved stable responding (less than 10% variation over the last three SA sessions). Subsequently, all animals underwent 15 days on average of extinction sessions during which each active lever press resulted in the presentation of the cue light and the infusion of saline for both groups. At the end of the extinction period, the number of lever presses had stabilized at a low level of responding.

Reinstatement phase

Responding on levers was recorded during the 2-h reinstatement sessions. Each active lever responding elicited the illumination of the cue light and the infusion of saline; the responses on the inactive lever had no consequences. First, on two consecutive sessions, rats were submitted either to a saline or a cocaine (15 mg/kg, i.p.) challenge. Subsequently, the ability of different DA receptor agonists to reinstate cocaine-seeking behavior was assessed in five test sessions conducted every 3 days and interspersed by two extinction sessions. Using a microinfusion syringe pump, four different doses of the D1R agonist, SKF82958 (0.05; 0.1; 0.5 or 1 µg/0.5 µL/side), six doses of the D2/D3R agonist, quinolorane (0.05; 0.1; 1; 1.5; 2.5 or 5 µg/0.5 µL/side), the D2R antagonist raclopride (5 µg/0.5 µL/side), the D1R antagonist SCH23390 (1 µg/0.5 µL/side), or phosphate-buffered saline (PBS) were bilaterally infused over 53 s either into the NAcc or the DLS, following a latin square design. Infusion cannulae were left in place for another 60 s to allow for drug diffusion and rats were placed into the operant chambers immediately after microinfusions).

Drugs

Cocaine hydrochloride (Cooperative Pharmaceutique Française, France), (±)chloro-APB hydrobromide (SKF82958) and R(+)-SCH-23390 hydrochloride (SCH23390) were dissolved in sterile 0.9% saline solution; Quinelorane dihydrochloride and S(-)-Raclopride (+)-tartrate salt were dissolved in PBS (all drugs were purchased from RBI-Sigma, USA).

Experimental design

1st set of rats: Nucleus Accumbens

The first set of rats was submitted to the surgical procedure for intravenous catheter and intra-cerebral cannulae implantation. Once recovered from surgery, the animals underwent the

acquisition on the cocaine self-administration (COC SA) paradigm, followed by extinction training. After attaining stable responding on the extinction phase, these animals participated on Experiment I. Rats underwent cocaine and saline challenges, in a counterbalanced manner. In subsequent days, interspersed by extinction sessions, animals were submitted to intracerebral infusions of different doses of SKF82958 or quinolorane into the NAcc, immediately followed by 2h of reinstatement tests, carried out in extinction (see **Figure 1A**). Subsequent sets of rats followed the same procedures, up to cocaine and saline challenges.

2nd set of rats: Dorsolateral striatum

The second set of rats was used for Experiment II and IVA. For Experiment II, the animals received intra-DLS infusions of SKF82958 or quinolorane (**Figure 2A**). After the conclusion of Experiment II, Experiment IVA consisted in the reversal test, with systemic administration of quinolorane and simultaneous intra-DLS infusion of raclopride (**Figure 4D**).

3rd set of rats: Dorsolateral striatum and Nucleus Accumbens

The third set of rats was initially divided into two groups, differing in regions targeted on cannulae implantation: DLS or NAcc. The DLS group participated on Experiment III, receiving intra-DLS infusions of 2 doses of quinolorane, as well as the control treatment (**Figure 3A**). Then, animals underwent systemic administration of quinolorane and simultaneous intra-DLS infusion of SCH23390, in Experiment IVA (see **Figure 4B**). The NAcc group participated on Experiment IVB consisting in the reversal test, with systemic administration of quinolorane and simultaneous intra-NAcc infusion of either SCH23390 raclopride (**Figure 5A**).

Histology

After completion of behavioral testing, animals received a lethal dose of sodium pentobarbital (Ceva Santé Animale) and were perfused transcardially with a 10% formaldehyde solution. Next, the brains were removed and stored in a 30% sucrose-formalin solution for 72h. Coronal sections (60 μ m) were sliced on a freezing microtome, and after being mounted onto gelatin-coated slides, they were left to dry for 24h to be then stained with thionin. Cannula placements were verified under a light microscope and section reconstructions were drawn in reference to the atlas of (Paxinos and Watson, 2006; **Supplementary Figure S1**).

Data analyses

Responding during the acquisition and extinction sessions was analyzed using a two-way analysis of variance (ANOVA) with days and levers as repeated measures. Responding during the reinstatement phase was analyzed using a three-way ANOVA with group as the between-subjects factor (SAL SA versus COC SA) and challenge or dose (PBS versus Cocaine or PBS versus SKF82958, quinelorane, SCH23390 or raclopride) and lever (active versus inactive lever) as within-subjects factors. Following each overall ANOVA, significant main effects were further analyzed by multiple comparisons Newman-Keuls *post hoc* test.

RESULTS

Experiment 1: Reinstatement elicited by D1R, but not D2R, agonist infused into the NAcc

Rats of the COC SA group acquired stable responding on the AL [comparison of the last three days of acquisition: $F_{(2,32)} = 2.27$; n.s.; averages over the 3 days (\pm SEM): AL 22.24 ± 1.89 ; IL 1.24 ± 0.48], while the SAL SA group maintained a low level of responding on

both levers (AL 3.33 ± 0.82 ; IL 1.72 ± 0.61). During the extinction phase, where cocaine was replaced by saline, the COC SA group decreased substantially AL presses, eventually stabilizing at a low level of responding [comparison of the last three days of acquisition: $F_{(2,32)} = 1.85$; n.s.; averages over the 3 days (\pm SEM): AL 8.41 ± 0.99 ; IL 2.00 ± 0.60 ; SAL SA: AL 2.78 ± 0.73 ; IL 0.92 ± 0.25 ; data not shown. **Figure 1.1A** shows the outline of experiment 1].

The three-way ANOVA comparing saline and cocaine challenges at the end of extinction training shows a significant interaction of group \times challenge \times lever [$F_{(1,27)} = 18.11$; $p < 0.001$]. Further two-way ANOVA within COC SA group showed a challenge \times lever interaction [$F_{(1,16)} = 26.54$; $p < 0.001$] and post hoc Newman-Keuls confirmed the cocaine challenge elicited the reinstatement of responding on the AL within the COC SA group in comparison with the saline challenge ($p < 0.001$) and with its IL ($p < 0.001$). Also, challenge \times group interaction [$F_{(1,27)} = 17.80$; $p < 0.001$] and post hoc analyses showed COC SA had also more AL presses in comparison with the SAL SA group ($p < 0.001$; **Figure 1.1B**). Responding on the IL showed no interactions whatsoever.

Intra-NAcc administration of different doses of SFK 82958 elicited a dose-dependent increase of AL presses within the COC SA group, as indicated by the interaction group \times dose \times lever [$F_{(4,52)} = 4.12$; $p < 0.01$]. Newman-Keuls post hoc analyses indicate the COC SA group increased AL presses in a dose-dependent manner with all doses of the D1R agonist increasing AL presses in comparison with the dose 0 [lever \times dose interaction $F_{(4,32)} = 12.94$; $p < 0.001$; NK: dose 0/0.05: $p < 0.05$; 0/0.1: $p < 0.001$; 0/0.5: $p < 0.001$; 0/1: $p < 0.001$] and also in comparison to the IL (NK: $p < 0.01$ for all doses). No effects were observed within the IL presses of the COC SA group. A dose \times lever interaction close to significance was observed within the SAL SA group due to a marginal increase in AL presses [$F_{(4,20)} = 2.70$; $p = 0.06$] under the doses of 0.1 and 0.5 of the D1R agonist, in comparison with the control treatment (**Figure 1.1C**).

Concerning the D2R agonist, intra-NAcc administration of different doses of Quinelorane elicits main effect of lever [$F_{(1,12)}=17.72$; $p=0.001$] but no group effect nor group x dose x lever interaction [$F_{(4,48)}=0.86$; n.s.; **Figure 1.1D**].

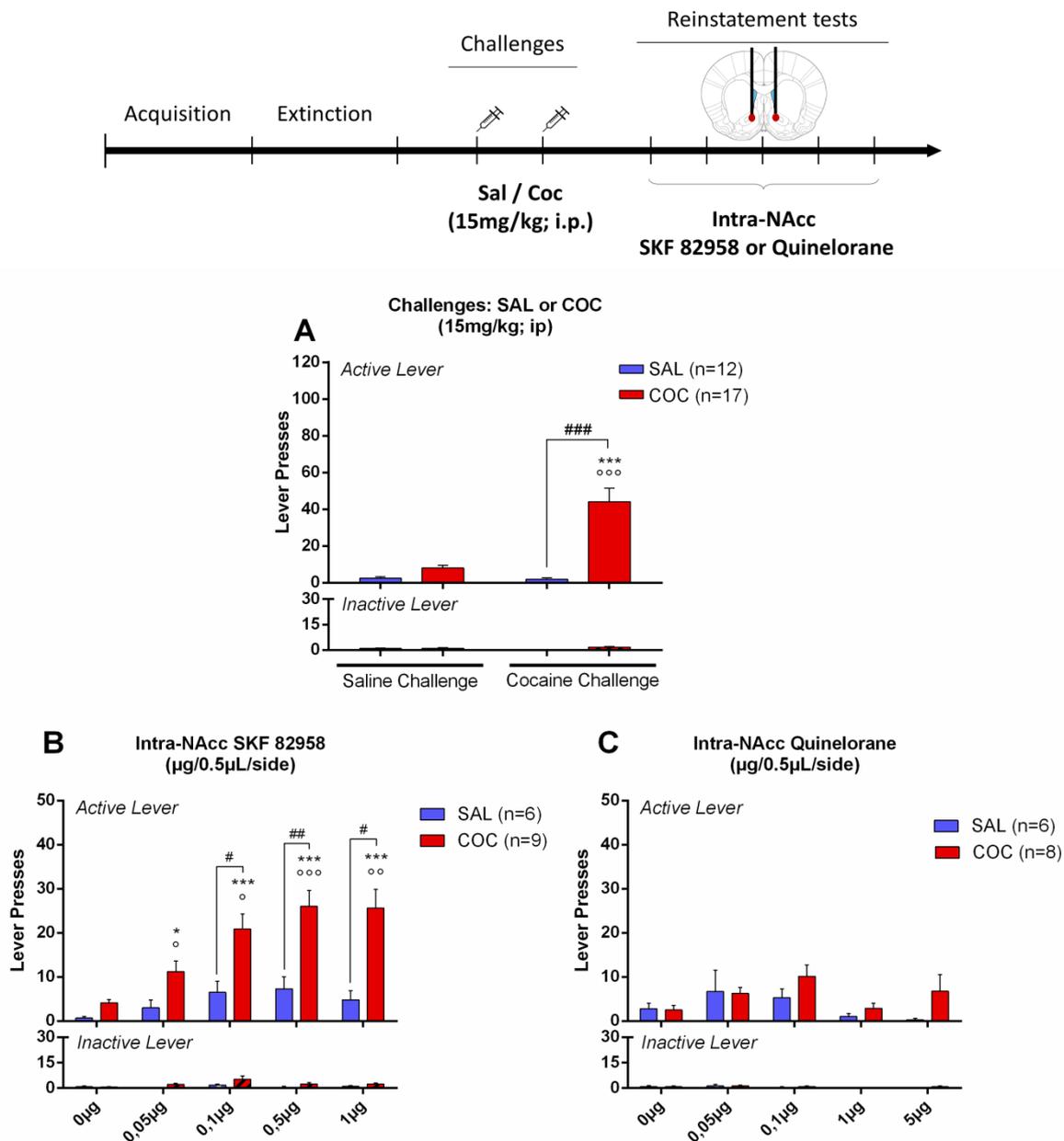


Figure 1.1. Intra-NAcc infusion of the D1R agonist, but not D2R agonist, induces reinstatement of cocaine-seeking (experimental design: **A**). Active lever responses to cocaine or saline challenge for SAL SA (n=12) and COC SA (n=17) animals (**B**). D1R agonist SKF82958 dose-dependently induces the reinstatement of cocaine seeking in the COC SA group (red bars, n=9) but not the SAL SA group (blue bars, n=6) when injected into the NAcc (**C**). Intra NAcc infusion of the D2R agonist quinelorane does not induce reinstatement of cocaine seeking in the COC SA group (n=8; SAL SA: n=6; **D**). Data are expressed as mean + SEM. * $p<0.05$; *** $p<0.001$: dose/treatment effect [versus dose 0 (PBS)]; # $p<0.05$; ## $p<0.01$; ### $p<0.001$: group effect; ° $p<0.05$; °° $p<0.01$; °°° $p<0.001$; lever effect (three-way ANOVAs followed by Newman-Keuls *post hoc* tests).

Experiment II: Reinstatement elicited by D2R, but not D1R, agonist infused into the DLS

Rats of the COC SA group acquired stable responding on the AL [last three days of acquisition: $F_{(2,36)}= 0,24$; n.s.; average over the 3 days (\pm SEM): AL 48.88 ± 5.57 ; IL 0.37 ± 0.19], while the SAL SA group maintained a low level of responding on both levers (AL 5.42 ± 1.51 ; IL 2.86 ± 0.65). During the extinction phase, the COC SA group decreased substantially AL presses, eventually stabilizing at a low level of responding [comparison of the last three days of acquisition: $F_{(2,36)}= 2.50$; n.s.; average over the last 3 days (\pm SEM): AL 9.25 ± 0.74 ; IL 1.49 ± 0.37 ; SAL SA: AL 3.83 ± 0.85 ; IL 1.22 ± 0.35 ; data not shown]. **Figure 1.2A** shows the outline of experiment 1.

The comparison of saline and cocaine challenges at the end of extinction training with a three-way ANOVA shows a significant interaction of group x challenge x lever [$F_{(1,29)}=20.18$; $p<0.001$]. Two-way ANOVA showed a challenge x lever interaction [$F_{(1,18)}=34.55$; $p<0.001$]. Newman-Keuls *post hoc* test showed the cocaine challenge elicited the reinstatement of responding on the AL in comparison with the saline challenge ($p<0.001$) and with its IL ($p<0.001$). Another two-way ANOVA showing challenge x group interaction [$F_{(1,29)}=20.59$; $p<0.001$] followed by post hoc analyses showed COC SA group had more AL presses than the SAL SA group ($p<0.001$) in response to the cocaine challenge. Responding on the IL showed a challenge x group interaction [$F_{(1,29)}=4.31$; $p=0.05$] but Newman-Keuls post hoc test did not identify significant differences within this parameter. Given the low averages of IL presses by both groups, this interaction is not likely to have interfered on the reinstating effects observed [saline challenge: SAL IL $0.50 (\pm 0.34)$; COC IL $0.50 (\pm 0.19)$; cocaine challenge: SAL IL $0.17 (\pm 0.17)$; COC IL $3.88 (\pm 2.29)$; **Figure 1.2B**].

Intra-DLS administration of different doses of the D1R agonist SKF82958 showed a lever x group interaction [$F_{(1,15)}=5.85$; $p<0.05$] and post hoc analyses showed the COC SA group showed a general increase on AL compared to SAL SA and to its IL ($p<0.001$ for all comparisons). However, no group x dose x lever interaction was found [$F_{(4,60)}=0.18$; n.s.]. Also, no interactions have been observed regarding responding on the IL (**Figure 1.2C**).

As for the D2R agonist, Intra-DLS administration of different doses of quinolorane showed an interaction of group x dose x lever [$F_{(4,48)}=5.95$; $p=0.001$]. Newman-Keuls post hoc analyses indicated the COC SA group increased AL presses when injected with the highest dose of quinolorane in comparison with all the doses [$F_{(4,28)}=8.03$; $p<0.001$; NK: $p<0.001$ for all doses] and also in comparison with the IL (NK: $p<0.01$ for all doses). Further two-way ANOVA and post hoc analyses showed that AL presses of COC SA group were also superior in comparison with SAL SA group [dose x group interaction; $F_{(4,48)}=6.14$; $p<0.001$; NK: $p<0.001$ for all doses]. No interactions have been observed regarding responding on the IL (**Figure 1.2D**).

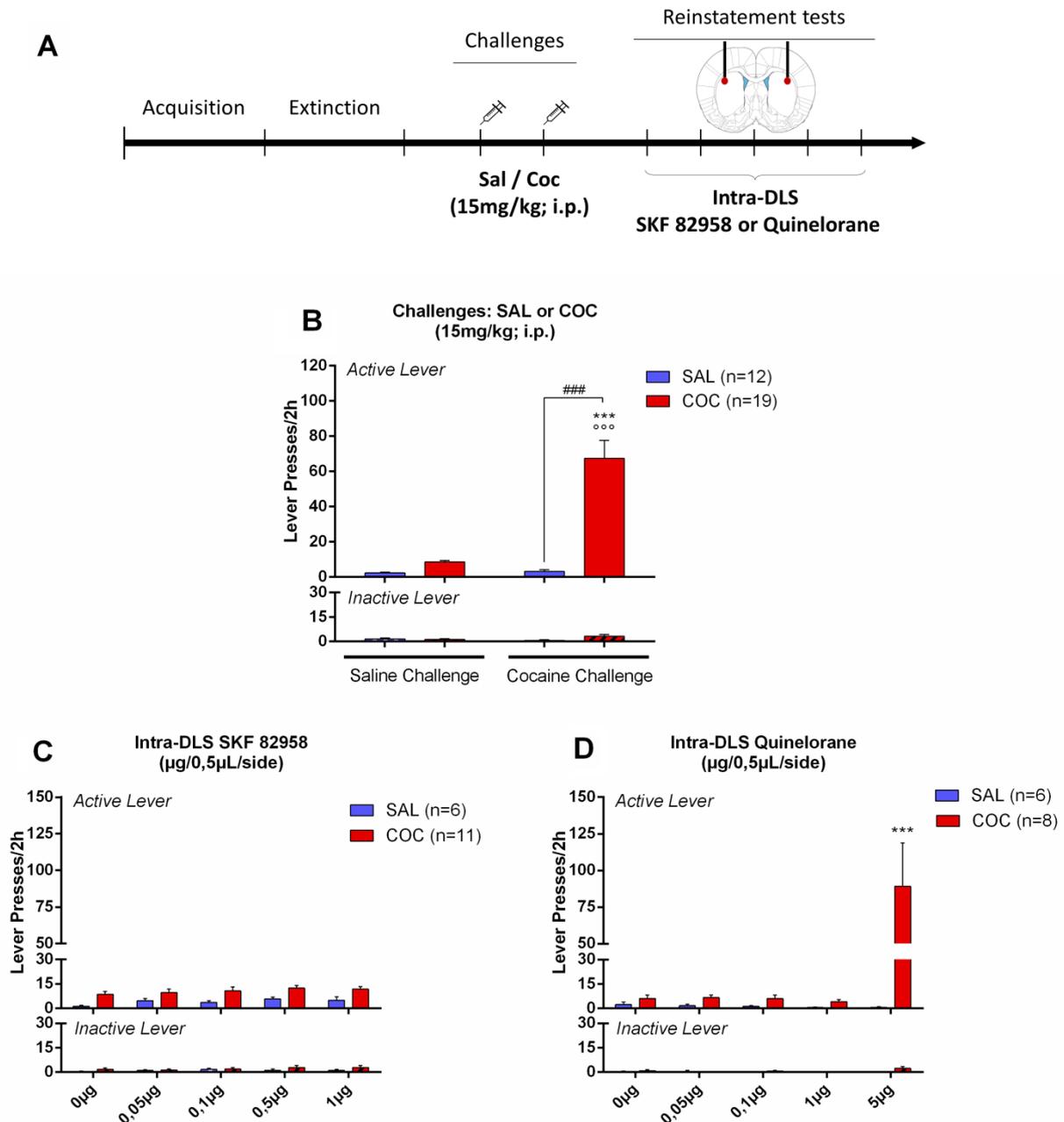


Figure 1.2. Intra-DLS infusion of the D2R agonist, but not D1R agonist, induces reinstatement of cocaine-seeking (experimental design: **A**). Active lever responses to cocaine or saline challenge for SAL SA (n=12) and COC SA (n=19) animals (**B**). D1R agonist SKF82958 did not induce reinstatement of cocaine seeking in the COC SA group (red bars, n=11; SAL SA: blue bars, n=6) when injected into the DLS (**C**). Intra DLS infusion of the D2R agonist quinelorane induces reinstatement of cocaine seeking in the COC SA group (n=8; SAL SA: n=6) in the highest dose tested (**D**). Data are expressed as mean + SEM. *** p<0.001: dose/treatment effect [versus dose 0 (PBS)]; ### p<0.001: group effect; °°° p<0.001; lever effect (three-way ANOVAs followed by Newman-Keuls *post hoc* tests).

Experiment III: Reinstatement elicited by D2R agonist infused into the DLS

Indeed, our results showed that reinstatement of cocaine seeking was induced only by the highest dose of quinolorane administered into the DLS. So we performed another experiment in which we administered intermediate doses of quinolorane aiming to further investigate the reinstating effects caused by the infusion of the D2R agonist into the DLS. Thus, we trained another set of rats in the same protocol of COC SA as in the previous experiments (see experimental design on **Figure 1.3A**).

As previously, rats in the COC SA group acquired stable responding on the AL [last three days of acquisition: $F_{(2,18)}=2.01$; n.s.; average over the 3 days (\pm SEM): AL 34.30 ± 4.17 ; IL 0.30 ± 0.15], while low level of responding on both levers by the SAL SA group was observed throughout the acquisition (AL 3.77 ± 0.59 ; IL 1.93 ± 0.45). Next, the COC SA group extinguished AL presses steadily throughout the next phase, also stabilizing at a low level of responding [comparison of the last three days of acquisition: $F_{(2,20)}=0.13$; n.s.; average over the last 3 days (\pm SEM): AL 10.07 ± 1.94 ; IL 2.27 ± 0.61 ; SAL SA: AL 2.87 ± 0.73 ; IL 0.50 ± 0.18 ; data not shown].

The comparison of saline and cocaine challenges at the end of extinction training with a three-way ANOVA shows a significant interaction of group x challenge x lever [$F_{(1,18)}=19.02$; $p<0.001$]. Two-way ANOVA showed a challenge x lever interaction [$F_{(1,9)}=19.81$; $p<0.001$]. Newman-Keuls post hoc test showed the cocaine challenge elicited the reinstatement of responding on the AL in comparison with the saline challenge ($p<0.001$) and with its IL ($p<0.001$). Another two-way ANOVA showing challenge x group interaction [$F_{(1,18)}=20.21$; $p<0.001$] followed by post hoc analyses showed COC SA group had more AL presses than the SAL SA group ($p<0.001$) in response to the cocaine challenge. Responding on the IL showed no interactions whatsoever (**Figure 1.3B**).

Intra-DLS administration of different doses of the D2R agonist, quinolorane showed an interaction of group x dose x lever [$F_{(2,18)}=6.92$; $p<0.01$]. Newman-Keuls post hoc analyses indicated the COC SA group increased AL presses when injected with the both doses of

quinelorane in comparison with the control treatment [lever x dose interaction; $F_{(2,8)}=5.56$; $p=0.01$; NK: $p<0.01$ for both doses] and also in comparison with the IL (NK: $p<0.05$ for all doses). Further two-way ANOVA and post hoc analyses [dose x group interaction; $F_{(2,18)}=6.78$; $p<0.01$] showed that AL presses of COC SA group were not superior in comparison with SAL SA group in the dose of $1.5\mu\text{g}$ [NK: $p=0.17$], and was marginally significant for the dose of $2.5\mu\text{g}$ [NK: $p=0.08$]. No interactions have been observed regarding responding on the IL (**Figure 1.3C**).

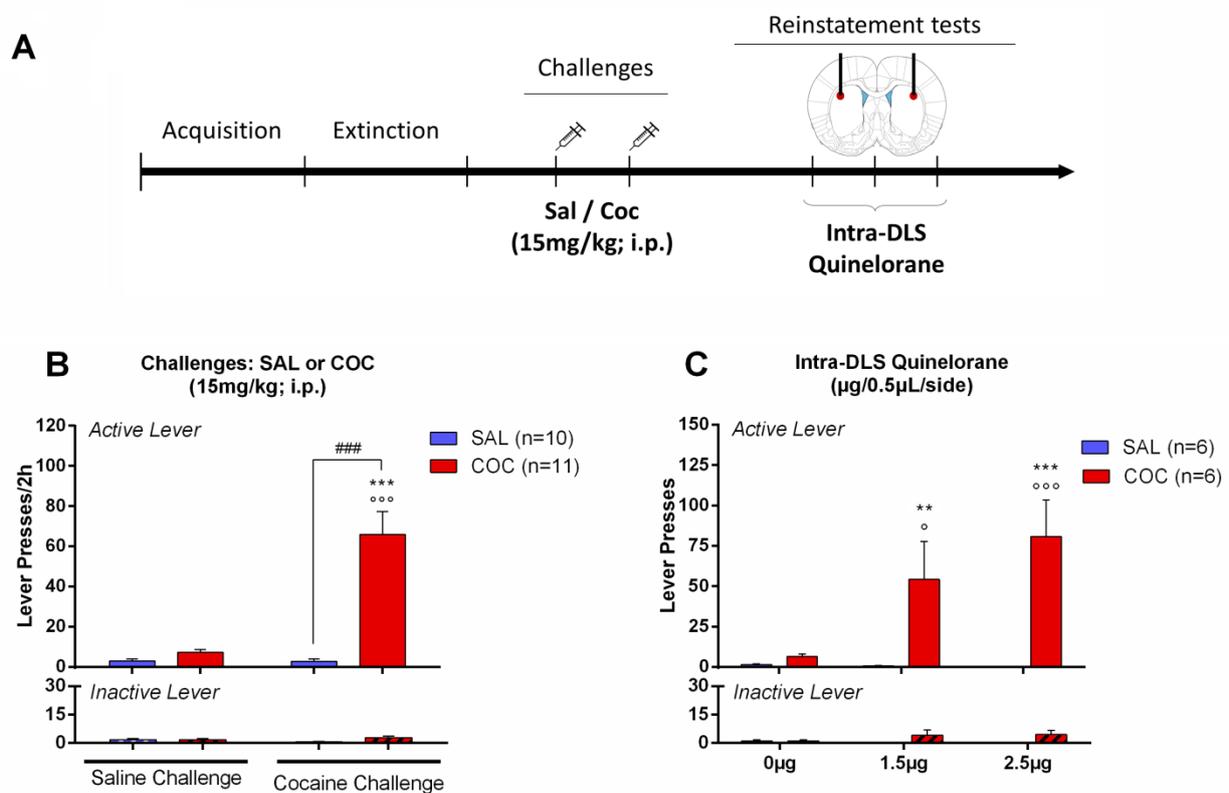


Figure 1.3. Intra-DLS infusion of the D2R agonist induces reinstatement of cocaine-seeking (experimental design: **A**). Active lever responses to cocaine or saline challenge for SAL SA (n=10) and COC SA (n=11) animals (**B**). D2R agonist quinelorane dose-dependently induces the reinstatement of cocaine seeking in the COC SA group (red bars, n=6) but not the SAL SA group (blue bars, n=6; **C**). Data are expressed as mean + SEM. ** $p<0.01$; *** $p<0.001$: dose/treatment effect [versus dose 0 (PBS)]; ### $p<0.001$: group effect; ° $p<0.05$; °°° $p<0.001$; lever effect (three-way ANOVA followed by Newman-Keuls *post hoc* tests).

Experiment IV-A: Infusion of D1R antagonist into the DLS blocks, while D2R antagonist blunts, cocaine-seeking induced by peripheral infusion of D2R agonist

Our lab and others have previously shown that the peripheral administration of the D2R agonist quinolorane induces reinstatement of cocaine seeking (Dias *et al*, 2003; Self *et al*, 1996; Weissenborn *et al*, 1996). Given that our results showed that the injection of quinolorane in the DLS also induced reinstatement of cocaine seeking, we assessed whether the D2R in the DLS were responsible for the reinstatement observed with the peripheral injection of this D2R agonist. Thus, in two sets of rats (from experiments 2 and 3) we performed a systemic administration of the D2R agonist quinolorane with the simultaneous intra-DLS injection of either the D1R antagonist SCH23390 or the D2R antagonist raclopride (see **Figures 1.4A** and **1.4C** for experimental outlines).

Three-way ANOVA showed a group x lever x antagonist interaction [$F_{(1,9)}=9.97$; $p=0.01$], while further two-way ANOVA [group x antagonist interaction: $F_{(1,9)}=10.24$; $p=0.01$] and post hoc analyses showed the peripheral administration of the D2R agonist quinolorane induced an enhancement of AL responding in the COC SA group in comparison with SAL SA group (NK: $p<0.01$). This confirms previous studies showing the strong reinstating effects of the peripheral infusions of D2R agonists on cocaine-seeking (Caine *et al*, 1999; Khroyan *et al*, 2000). Post hoc analyses also showed simultaneous intra-DLS infusion of D1R antagonist SCH23390 blocked the reinstatement of AL pressing induced by the peripheral infusion of quinolorane on the COC SA group (NK: $p<0.01$), being comparable to the SAL SA group (NK: $p=0.37$; **Figure 1.4B**).

For the intra-DLS infusion of raclopride, a three-way ANOVA showed a group x lever x antagonist interaction [$F_{(1,29)}=7.04$; $p=0.01$]. Further two-way ANOVA [group x antagonist interaction: $F_{(1,29)}=7.91$; $p<0.01$] and post hoc analyses showed the peripheral administration of quinolorane induced again an enhancement of AL responding in the COC SA group in comparison with SAL SA group (NK: $p<0.001$). Post hoc analyses also showed simultaneous intra-DLS infusion of D2R antagonist raclopride blunted the reinstatement of AL pressing

induced by the peripheral infusion of quinolorane on the COC SA group (NK: $p < 0.001$), though still being superior in comparison to the SAL SA group (NK: $p < 0.05$). Responding on IL in both experiments showed no interactions whatsoever (Figure 1.4D).

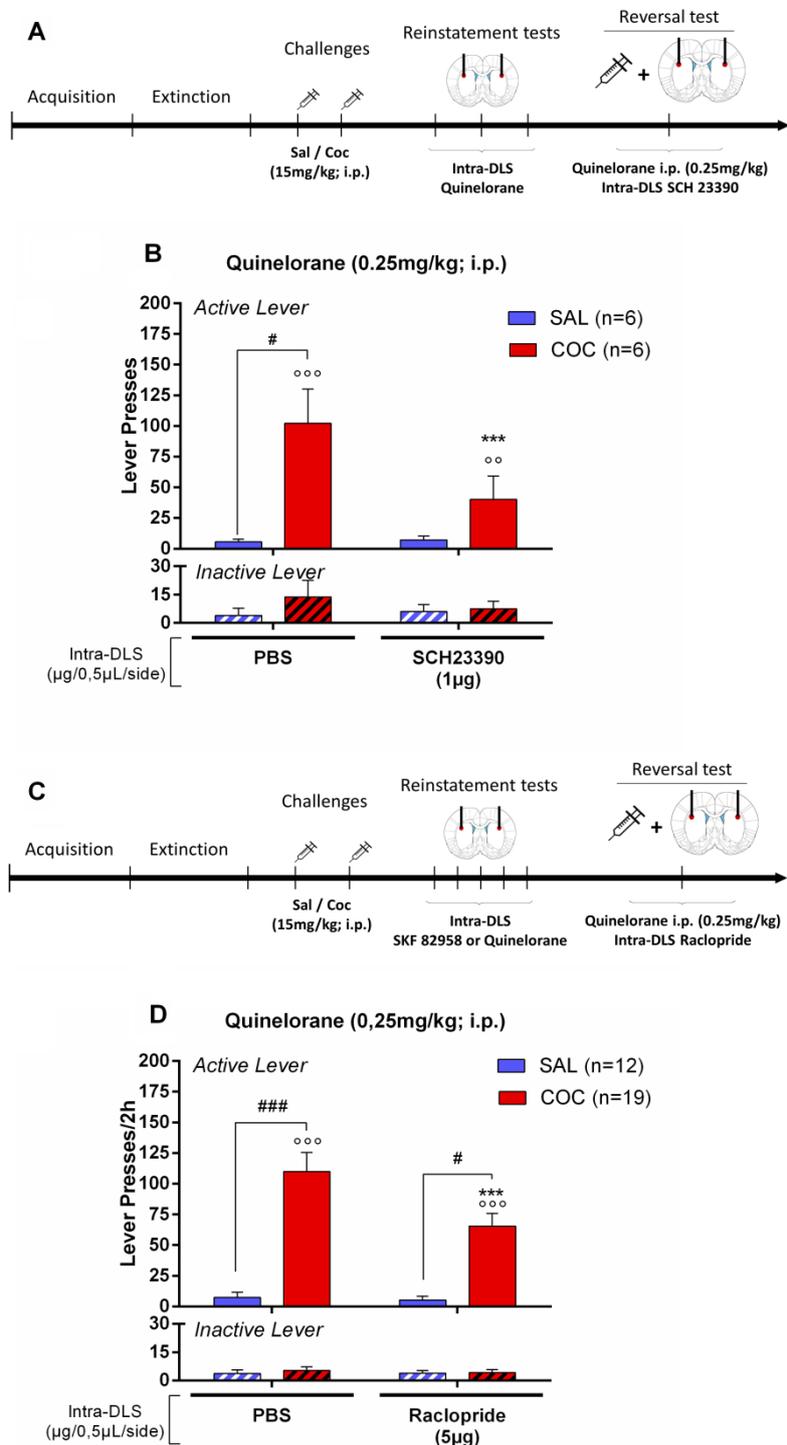


Figure 1.4. Intra-DLS infusion of the D1R antagonist blocks, while D2R antagonist blunts, D2R agonist-induced reinstatement of cocaine-seeking (experimental designs: **A**; **C**). D1R antagonist SCH23390 blocks reinstatement induced by systemic injection of D2R agonist quinolorane on the COC SA group (red bars, $n=6$; blue bars: SAL SA, $n=6$; **B**). D2R antagonist raclopride blunts reinstatement induced by systemic injection of D2R agonist quinolorane on the COC SA group (red bars, $n=19$; blue bars: SAL SA, $n=12$; **D**). Data are expressed as mean + SEM. *** $p < 0.001$: treatment effect (versus PBS); # $p < 0.05$; ### $p < 0.001$: group effect; °° $p < 0.01$; °°° $p < 0.001$: lever effect (three-way ANOVAs followed by Newman-Keuls *post hoc* tests).

Experiment IV-B: Infusion of D1R or D2R antagonists into the NAcc blocks reinstatement of cocaine-seeking induced by peripheral infusion of D2R agonist

The infusion of a D1R antagonist into the NAcc shell blocked the reinstatement of cocaine seeking induced by the infusion of the D2R agonist into the same region (Schmidt and Pierce, 2006). As we have previously observed the stimulation of the D1R within the NAcc induced reinstatement of cocaine seeking, we assessed whether the D1R in the NAcc participated in the process of induction of reinstatement observed with the peripheral injection of this D2R agonist. Thus, with another set of rats we performed the same systemic treatment with quinolorane (0.25 mg/kg, IP), this time with the simultaneous injection of either the D1R antagonist SCH23390 or the D2R antagonist raclopride in the NAcc (see **Figure 1.5A** for experimental design).

Three-way ANOVA showed a lever x antagonist x group interaction [$F_{(2,14)}=10.05$; $p<0.01$], while further two-way ANOVA [antagonist x group interaction: $F_{(2,14)}=9.44$; $p<0.01$] and post hoc analyses showed the peripheral administration of the D2R agonist quinolorane induced an enhancement of AL responding in the COC SA group in comparison with SAL SA group (NK: $p<0.05$). This confirms previous studies and our own showing the strong reinstating effects of the peripheral infusions of D2R agonists on cocaine-seeking (Dias *et al*, 2003; Khroyan *et al*, 2000; Weissenborn *et al*, 1996). Post hoc analyses also showed simultaneous intra-NAcc infusion of either SCH23390 (NK: $p<0.001$) or raclopride (NK: $p<0.01$) completely blocked the reinstatement of AL pressing induced by the peripheral infusion of quinolorane on the COC SA group, with the COC SA group showing similar average of AL presses as the SAL SA group, under the same treatments (SCH: NK $p=0.90$; Quin: NK $p=0.81$). Responding on IL showed no interactions whatsoever (**Figure 1.5B**).

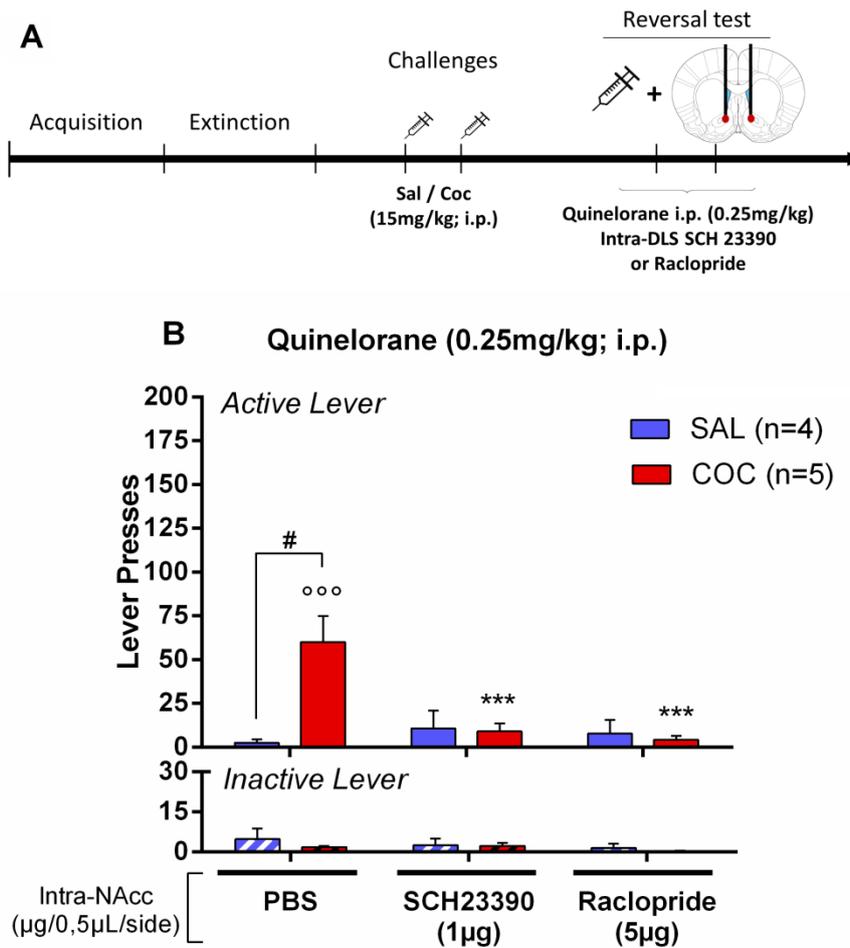


Figure 1.5. Intra-NAcc infusion of the D1R antagonist SCH23390 or the D2R antagonist raclopride (experimental design **A**). D1R or D2R block the reinstatement of cocaine seeking induced by systemic injection of D2R agonist quinlorane in the COC SA group (red bars, n=5; blue bars: SAL SA, n=4; **B**). Data are expressed as mean + SEM. *** $p < 0.001$: treatment effect (versus PBS); # $p < 0.05$: group effect; °° $p < 0.01$; °°° $p < 0.001$: lever effect (three-way ANOVA followed by Newman-Keuls *post hoc* test).

DISCUSSION

The present work shows a double dissociation between the actions of D1R and D2R in the ventral and dorsolateral portions of the striatum, respectively, on the reinstatement of cocaine seeking in rats. Our results show that within the NAcc, administration of a D1R, but not D2R, agonist induces reinstatement; whereas within the DLS, administration of a D2R, but not D1R, agonist induces reinstatement of drug-seeking. Also, the reinstating effects of the systemic administration of D2R agonists is blocked by D1R or D2R antagonists injected

into the NAcc and by the infusion of D1R in the DLS, while it is blunted by the D2R inhibition in the DLS.

Administration of D1R, but not D2R, agonist into the NAcc induces reinstatement of cocaine-seeking

Our results obtained by the infusion of the D1R agonist SKF82958 into the Nacc add to the literature showing the importance of the D1R-MSN pathway of the NAcc on the reinstatement of cocaine-seeking (Bachtell *et al*, 2005; Hobson *et al*, 2013) by showing the reinstating effects of the pharmacological stimulation of D1R within the NAcc. The infusion of D2R agonist, on the other hand, did not induce reinstatement of cocaine-seeking when injected in the same region.

Intra-Nacc DA infusion has been shown to reinstate cocaine seeking (Cornish and Kalivas, 2000), the same effect elicited by the stimulation of D1R within the NAcc in our study. While weakening the transmission at D2R-MSNs, the repeated COC administration has been shown to potentiate transmission at D1R-MSNs (Creed *et al*, 2016), through the strengthening of their glutamatergic (GLUergic) inputs onto NAcc D1R-MSNs (Bock *et al*, 2013) as well as to increase D1R sensitivity within the NAcc (Henry and White, 1991). The latter effect to our knowledge has not been shown to happen within the DLS. Both of the aforementioned processes likely enhance D1R mediated signaling of the D1R-MSN direct pathway arising from the NAcc, which contributes to the induction of cocaine-seeking (Hobson *et al*, 2013).

As for the involvement of the DAergic mesolimbic circuitry on reinstatement, the inactivation of the DA projection from the VTA to the NAcc leads to a reduction in cocaine-seeking behavior (See *et al*, 2007). In fact, as opposed to D2R bearing-MSNs, D1R-MSNs of the NAcc project preferentially to GABAergic neurons within the VTA which, in turn, innervate VTA-DA neurons (Bocklisch *et al*, 2013). This D1R-MSN direct-pathway is potentiated by cocaine treatment, resulting in an enhancement of GABA release from D1R-MSNs onto VTA-

GABA neurons, and thus disinhibiting VTA-DA neurons (Bocklisch *et al*, 2013). Taking into account the importance of the DAergic input into the NAcc, one can assume that the activation of D1R by the intra-NAcc injection of SKF82958 may indirectly disinhibit DA-VTA neurons, thus enhancing DA levels on the ventral striatum, leading to reinstatement of drug-seeking behavior (Bocklisch *et al*, 2013; Phillips *et al*, 2003).

Concerning the D2R action within the NAcc, our results are in disagreement with previous study showing the infusion of a D2R agonist into this region induces reinstatement of cocaine seeking (Bachtell *et al*, 2005). This discrepancy might be due to differences in the methodology as, for instance, the previous study used a within-session reinstatement procedure, while in our work the animals undergo at least 2 weeks of extinction sessions before reinstatement tests are carried out. Also, pharmacological differences between the D2R agonist used in both studies may contribute to the conflicting results, as in our study we used quinolorane while Bachtell *et al* (2005) have used 7-OH-DPAT, which have considerable, or even higher, affinity for the D3R as compared with D2R (Malmberg *et al*, 1994).

For the ineffectiveness of D2R stimulation within the NAcc to induce reinstatement, potential D2R downstream mechanisms for cannot be ruled out, such as the decreased availability of the inhibitory G protein subunits $G_{\alpha i}$ and $G_{\alpha o}$ (but not stimulatory $G_{\alpha s}$) within the NAcc caused by chronic COC treatment (Nestler *et al*, 1990). Also, D2R have been shown to interact with adenosine A2A receptors (A2AR) within the ventral striatum, where both receptors are coexpressed (Svenningsson *et al*, 1997). Within the Nacc, A2AR stimulation has been shown to reduce the D2R relapse-mediating signaling (O'Neill *et al*, 2012). Specifically, a significant reduction of the D2R high affinity state is observed within the NAcc after COC SA, likely as COC treatment increases the antagonistic A2A-D2R interactions within the NAcc (Pintsuk *et al*, 2016b). This leads to the suggestion that this interaction may hinder any reinstating effects that the D2R agonist may have when administered within the

NAcc after COC SA (Bachtell and Self, 2009; Borroto-escuela *et al*, 2016; Pintsuk *et al*, 2016b).

Infusion of D2R, but not D1R, agonist into the DLS induces reinstatement of COC-seeking

The participation of the DAergic input to the DLS on reinstatement is suggested by several works. Exposure to cocaine cues induce increases in extracellular DA in the dorsal striatum of rodents and humans, with this increase being positively correlated with self-reports of craving in cocaine-dependent subjects (Gabriele *et al*, 2012; Garavan *et al*, 2000; Ito *et al*, 2002; Volkow *et al*, 2006). Also, the involvement of the DLS DA in cocaine associations progressively increases with experience (Ito *et al*, 2002). Within this region, DA mediates cue-controlled cocaine seeking (Ito *et al*, 2002), whereas intra-DLS infusion of the DA receptor antagonist α -flupenthixol impairs COC SA under a second-order schedule of reinforcement (Vanderschuren *et al*, 2005). Furthermore, reversible inactivation of the DLS attenuates cocaine seeking after abstinence, as well as after exposure to a discrete cue or context following extinction training (Fuchs *et al*, 2006). Importantly, the DAergic input into the DLS is known to be a critical component of drug seeking characterized as habitual or compulsive (Belin and Everitt, 2008; Volkow *et al*, 2006), and its action has been shown to be critical in habitual cocaine seeking behavior (Zapata *et al*, 2010).

Regarding the striatonigral pathway, the infusion of a D1 antagonist does not increase cocaine self-administration in rats (Caine *et al*, 1995), while previous studies showed that COC SA increases the expression of dynorphin within the DLS (Steiner and Gerfen, 1993). Within the DLS, Dyn is argued to act as a compensatory response to repeated/excessive D1R stimulation, as its expression seems to dampen the effects of subsequent D1R stimulation. Notably, this process seems to predominate within the DLS, in comparison with the NAcc (Steiner and Gerfen, 1996), and thus it may have contributed to the ineffectiveness

of the D1R agonist in inducing reinstatement of COC seeking when injected into the DLS, as opposed to the NAcc.

As for the DLS indirect pathway, in the present work we use a D2R agonist quinolorane, which is known to also exert agonistic effects on D3R (Ireland *et al*, 2005). However, the reinstating effect observed by the intra-DLS infusion of this compound is not likely underlain by its actions through D3R, since this receptor subtype is scarcely, if at all, expressed in the DLS (Stanwood *et al*, 2000). On the other hand, the D2R is shown to be highly expressed within the dorsal striatum and specifically within the DLS, as compared to DMS (Yin *et al*, 2009).

One possibility is that the stimulus-response process believed to take place within the DLS after COC SA (Gabriele and See, 2011; See *et al*, 2007; Zapata *et al*, 2010) may be in play in the reestablishment of the cocaine seeking behavior with a significant contribution of D2R (Corbit *et al*, 2014b; Pintsuk *et al*, 2016a). This is suggested by the demonstration that the activation of D2R within the DLS is crucial for the expression of alcohol seeking (Corbit *et al*, 2014b). These data go along with the postulation that following extended training, performance becomes independent of D1R, whereas the activation of D2R within the DLS remains critical (Yin *et al*, 2009). Even though our protocol does not involve extended training, these data might shed some light into the possible mechanisms underlying the reinstatement induced by the D2R agonist, as opposed by D1R agonist, infusion into the DLS observed in our study.

Furthermore, COC SA increases the proportion of D2R in the high affinity state within the dorsal region of the striatum (Briand *et al*, 2008). Within the ventral striatum, on the other hand, the over-expression of the D2R DA receptor decreases COC SA (Thanos *et al*, 2008), suggesting that any potential increased signaling through D2R in this region is inversely associated with the rewarding properties of cocaine. Additionally, the latter effect might also have contributed to the lack of reinstating effects observed after the infusion of quinolorane into the NAcc in our study.

Infusion of D1R antagonist into the NAcc or DLS blocks reinstatement of cocaine-seeking induced by peripheral infusion of D2R agonist

The systemic stimulation of D2R is known to be a powerful trigger of cocaine-seeking behavior, whereas the stimulation of D1R does not elicit relapse (Dias *et al*, 2003). The results shown in the present study suggest that this effect is not mediated by the D2R of the NAcc alone, since the injection of quinolorane in this region did not induce reinstatement. Instead, while the participation of D2R of the NAcc cannot be ruled out, our results suggest that the reinstating effects of systemic administration of the D2R agonist are most likely dependent on concomitant D1R activation within NAcc and DLS. Our assumption is based on our results showing that the inhibition of the D1R within the dorsolateral or the ventral domains of the striatum blocked this process.

This blockage highlights the importance of the cooperative activity of both D1R and D2R of the striatum on the systemic D2R stimulation-induced reinstatement of cocaine-seeking. Other examples of the necessary role for D1R on the expression of D2R-mediated effects have also been shown. For instance, the facilitation of conditioned reward by bromocriptine, a D2R agonist, has been shown to depend on D1R activity, as the pretreatment with D1R antagonist blocks this effect (Ranaldi and Beninger, 1995). Similar studies evidenced the interaction between both DA receptors on the reinstatement of cocaine-seeking by showing that the reinstatement induced by intra-NAcc shell infusion of D2R agonist was completely blocked by either pre- (Schmidt and Pierce, 2006) or simultaneous treatment with D1R antagonist SCH23390 (Bachtell *et al*, 2005).

We argue that this influence may be due to the interaction between MSN collateral projections (Kang *et al*, 2017), and less likely from D2R autoreceptors on DAergic projections (Kharkwal *et al*, 2016). Within these collateral projections, the activation of D2R-MSNs can produce substantial inhibitory postsynaptic response on neighboring D1R-MSNs within the striatum, thus restraining D1R-MSN output, while conversely, the decreased activity of D2R-MSNs disinhibits D1R-MSNs (Dobbs *et al*, 2016; Kharkwal *et al*, 2016). Thus, the activation

of D2R by peripherally injected quinolorane, among other effects, also induces the disinhibition of D1R-MSN direct pathway, both likely acting in concert to the induction of addictive behaviors (Dobbs *et al*, 2016; Kharkwal *et al*, 2016), and this process is likely counteracted by the administration of SCH23390 in the NAcc or DLS.

Inhibition of D2R within the NAcc blocks, while within the DLS blunts, the reinstating effects of the systemic administration of D2R agonists

Within the DLS raclopride was not able to completely block the systemic quinolorane-induced reinstatement. This suggests that whereas the stimulation of D2R within the DLS can reinstate drug seeking behavior, the reinstatement induced by systemic D2R agonist may rely on additional mechanisms. Similar observation can be drawn by the participation of the D1R within the DLS in our study, not being able to induce reinstatement by itself, but impeding systemic quinolorane reinstatement once pharmacologically blocked.

The additional mechanisms underlying our puzzling results might involve interactions with other neurotransmitter systems as well as other brain regions (McFarland and Kalivas, 2001). For instance, the participation of ENK, which is co-released by D2R-MSNs and binds to μ -opioid receptors within the VP influencing GABA transmission (Kupchik *et al*, 2014) and reinstatement of cocaine seeking (Tang *et al*, 2005). Other examples include the marked influence of D2R on the cortical GABAergic input to the striatum (Bamford *et al*, 2004; Brady and O'Donnell, 2004; Yamamoto and Davy, 1992), shown to be crucial for cocaine reinstatement (Park *et al*, 2002; Sun and Rebec, 2005), as well as the participation of cholinergic system in interneurons of the NAcc (Yee *et al*, 2011) and the spiraling striato-nigro-striatal circuitry, also critical for the expression of cocaine-seeking habits (Belin and Everitt, 2008).

In a way, it seems that the relapse-inducing systemic D2R stimulation eventually conflates within the NAcc finally requiring the activation of both D1R and D2R, to then elicit the behavioral output. Whenever one of the pathways is blocked, the relapse is hindered.

This adds another layer of complexity for the dopaminergic connectivity linking ventral with dorsal striatum, shown to be crucial for the development and performance of cocaine-seeking behavior that represent a hallmark of drug addiction (Belin and Everitt, 2008).

Finally, whereas the activation of D1R in the NAcc have been associated with the rewarding properties of drugs (Kravitz *et al*, 2012; Lobo *et al*, 2010), the D2R in the DLS have been associated with habitual responding for alcohol (Corbit *et al*, 2014b). One possible target for future studies would be to assess whether the reinstatement of cocaine seeking triggered by the D2R stimulation within the DLS also involves the incentive motivational or reinforcing processes likely underlying those induced by the D1R stimulation within the NAcc. Additionally, it would also be useful to investigate whether the D1R stimulation within the NAcc also involves the overtaking of the behavioral control by habitual stimulus-response mechanisms which may be involved in the reinstatement of drug-seeking after the D2R stimulation in the DLS.

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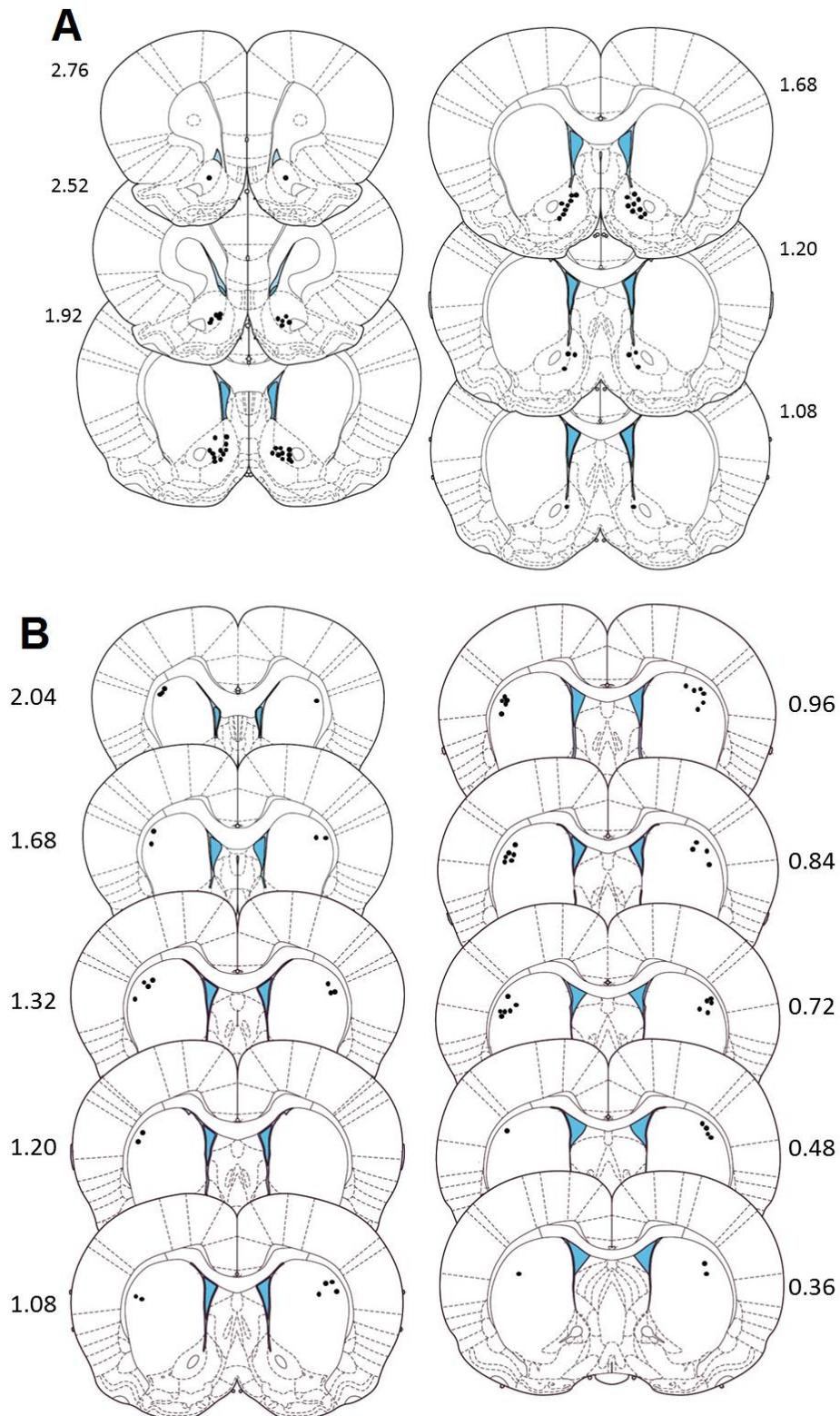
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Supplementary information

S1. Sites of injection in the NAcc (A) and DLS (B).



SECTION II

Behavioral investigation: Modelling the transition to the automatization of behavioral control

Section II – Behavioral investigation: modelling the transition to the automatization of behavioral control

1. Introduction

In the previous section the role of D1R and D2R of the NAcc and DLS in the reinstatement of COC seeking was assessed. Our results suggest a double dissociation between the actions of D1 and D2 DA receptors in the ventral and dorsolateral portions of the striatum, respectively. Within the NAcc, pharmacological activation of D1R, but not D2R, induces reinstatement. Conversely, within the DLS reinstatement is induced by the pharmacological stimulation of D2R, but not D1R. Also, the reinstating effects of the systemic administration of a D2R agonist is blocked by D1R or D2R antagonists injected into the NAcc and by the infusion of D1R in the DLS, while it is blunted by the D2R inhibition in the DLS.

Whereas the activation of D1R in the NAcc has been associated with the rewarding properties of drugs, the D2R in the DLS have been associated with habitual responding for drugs (Lobo *et al*, 2010; Soares-Cunha *et al*, 2016b; Vicente *et al*, 2016). Questions may be raised regarding the behavioral processes underlying the reinstatement of COC seeking triggered by both receptor subtypes, within different regions of the striatum. For instance, it is not known whether the reinstatement triggered by the pharmacological activation of D2R within the DLS involves incentive motivational or reinforcing processes likely underlying those induced by the D1R stimulation within the NAcc. Additionally, it would also be pertinent to investigate whether the D1R stimulation within the NAcc also involves the overtaking of the behavioral control by habitual S – R mechanisms, which may be involved in the reinstatement of drug-seeking after the D2R stimulation in the DLS.

To this end, in this section is described the attempt to devise a model of transition from goal-directed to habitual control of behavior. Once established, this model allows for the examination of the aspects involved in the transition of behavioral control while also teasing apart the different aspects involved in appetitive and consummatory behaviors (i.e. drug seeking and drug taking behaviors). These assessments could permit a detailed investigation of the participation of DA receptors of each striatal domain in the neurobiological mechanisms

subserving the transition from the goal-directed to the habitual control of drug-seeking that is believed to take place in the establishment of addiction. Therefore, below are presented the principles of the learning mechanisms examined, with their neurobiological substrates, and the tools used for the assessment of the behavioral control in our laboratory settings, using rats.

1.1. Associative learning

The associative learning theory characterizes learning as the formation of an association between mental representations of events in the environment. This phenomenon can be dissociated in two basic paradigms of conditioning: Pavlovian (classical) and instrumental (operant). Both Pavlovian and instrumental learning usually occur in parallel during the acquisition and performance of a task, and these two forms of conditioning concern the ways that animals learn to predict and respond to important events in their environments.

1.1.1. Pavlovian conditioning – prediction of events

Pavlovian conditioning mechanisms are responsible for the formation of associations between conditioned and unconditioned stimuli, usually as a result of simple temporal coincidence. Thus, learning to anticipate a reward can occur by associating a discrete stimulus with an outcome. The outcome is provided whatever the animal does, so changes in behavior likely reflect innately determined physiological reactions to predictions of the outcome. Importantly, associations acquired through Pavlovian conditioning are also capable of acting as secondary reinforcers, invigorating responding instrumental to their own presentation while in the absence of the primary reinforcer/reward itself (Balleine *et al*, 2009; Berridge and Robinson, 2003; Di Chiara, 2002; Dickinson and Balleine, 1996).

1.1.2. Instrumental conditioning – encoding causal relationship between actions and outcomes

Whereas Pavlovian conditioning enables an animal to anticipate motivationally relevant events, it is instrumental conditioning that allows control over these events according to needs and desires. In instrumental conditioning the subject associates the execution of an action to the obtainment of a stimulus as reward. Whenever a particular outcome is contingent on a response, the behavior in question is instrumental. This type of associative learning differs from the Pavlovian conditioning, in which the subject learns that the presentation of a stimulus predicts the occurrence of a particular event (stimulus-outcome association). In contrast to Pavlovian, instrumental conditioning depends on the active participation of the subject, being controlled by the contingency between behavior and consequence, in order for learning to take

place (Balleine and O'Doherty, 2010; De Wit and Dickinson, 2009; Yin and Knowlton, 2006). Thus, while Pavlovian conditioning allows for the prediction of an event, the instrumental conditioning allows animals to encode the causal relationship between their actions and outcomes, and actively control their actions according to their anticipation of, and desire for, the outcome (Yin and Knowlton, 2006).

1.2. *Goal directed vs. Habit based behavior*

It is now generally accepted that instrumental conditioning engages two distinct learning processes. In one of them, the choice between actions is determined by the animals' representation of the association between a specific action, its outcome, and the current value of this outcome (i.e. A – O associations). This process is proposed to be critical to the acquisition and employment of *goal-directed actions*. Typically, goal-directed actions are determined by [i] their dependency on the causal relation between acting and the occurrence of some consequence; and [ii] the sensitivity of these actions to changes in the desirability of the goal of an action. Thus, as in this case actions are highly sensitive to changes in the value of their associated outcome, the devaluation of the outcome will reduce the performance rate of an action leading to it (Adams, 1982; Balleine *et al*, 2009; Dickinson and Balleine, 1994).

In contrast, a fundamentally different process occurs when actions are overtrained. In this case the control over behavior undergoes a shift from this initial goal-directed process, to become more rigid or habitual; performance is controlled by a sensorimotor associative process, reflecting the increasing establishment of stimulus-response (S – R) associations. These associations reflect a reduction in the perceived correlation between the performance and the outcome (Balleine *et al*, 2007, 2009; Di Chiara, 2002). Thus, goal-directed and habitual actions differ in two primary ways: [i] they differ in their sensitivity to changes in the value of the consequences previously associated with the action; and [ii] they differ in their sensitivity to changes in the causal relationship between the action and those consequences [Figure 2.1] (Balleine and O'Doherty, 2010).

Habit learning is a natural learning process that allows stereotyped, repetitive motor sequences to be performed with little or no conscious awareness. This type of learning allows cognitive resources to be liberated for the maintenance of more complex actions. It is believed that habit formation involves a switch in the control of behavior from a circuit including the ventral and medial striatum and dorsal PFC to a circuit involving the DLS and ventral medial PFC. Also, despite its automatic character habit learning is not impervious to the eventual adaptive control exerted by its outcome. This means that repeated failure to meet the

requirements for an optimal response in survival terms results in switching back from the habitual to the goal-directed processing of behavior, and then, after stabilization and practice, in the acquisition of a new habit (Di Chiara, 2002; Coutureau and Killcross, 2003; Yin *et al*, 2004, 2005a, 2005b).

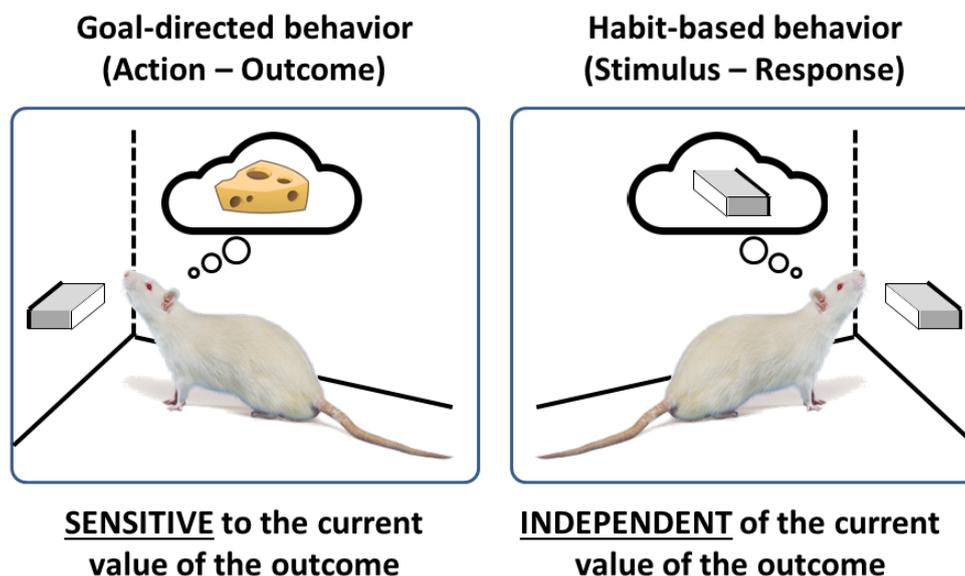


Figure 2.1. Goal-directed vs. habitual behaviors. goal-directed and habitual actions differ in two primary ways: [i] they differ in their sensitivity to changes in the *value of the consequences* previously associated with the action; and [ii] they differ in their sensitivity to changes in the *causal relationship* between the action and those consequences. Adapted from González-Marín (2012).

Thus, though generally advantageous to an organism, habit formation can become pathological when a habit is so intrinsically established that an individual has difficulty refraining from a habitual action even when the outcome of that action has changed. It is hypothesized that addicts form habits related to drug use more rapidly and possibly to a greater degree than non-addicted individuals and in comparison to habits formed for non-drug reinforcers (Everitt and Robbins, 2005; Torregrossa *et al*, 2011).

In fact, goal-directed and habitual actions exist in a dynamic balance that can be biased in one direction or the other by conditions of training or testing that favor acquisition/expression of the A – O versus S – R association. Adams (1982) found that the influence of devaluation on lever pressing was dependent on the amount of training; rats trained to press a lever for a reward initially employ goal-directed behavior, which is controlled by an A – O contingency. After a period of overtraining, the lever pressing by rats appeared to be no longer sensitive to devaluation. This was consistent with the view that A – O learning dominates performance early after acquisition but gives way to an S – R process, as performance becomes habitual. Thus, goal-directed and habit learning processes are simultaneously engaged, but the fact that one or

the other predominates depends on the circumstances during training (Hilário and Costa, 2008; Yin *et al*, 2004, 2005b, 2006).

Furthermore, several experimental conditions, notably exposure to drugs of abuse (Corbit *et al*, 2012, 2014a; Nelson and Killcross, 2006; Nordquist *et al*, 2007; Zapata *et al*, 2010) and the amount of instrumental training (Coutureau and Killcross, 2003; Killcross and Coutureau, 2003; Shan *et al*, 2015) can specifically promote habitual behavior. Also among these factors, the schedule of reinforcement is a convenient way to bias rodent behavior toward goal-directed or habitual responding. Random ratio (RR) schedules of reinforcement have been shown to promote goal-directed behavior whereas random interval (RI) schedules and overtraining promote the development of S – R habits. It has been initially suggested that the differential effect of RR and RI schedules on the control of behavior is related to differences in contingency between the response rate and the reinforcement rate (Dickinson *et al*, 1983), since RI schedules distance the relationship and thus weakens the contingency or the contiguity between response and outcome delivery, eventually rendering performance of actions insensitive to changes in its value (Balleine *et al*, 2009; Dickinson, 1985; Dickinson *et al*, 1983; Everitt *et al*, 2008; Vandaele and Janak, 2017).

1.3. *Assessment of behavioral control*

Two classes of assay have become common in the assessment of instrumental learning. In the first, the A – O contingency (i.e. the degree to which the outcome depends on the action) is manipulated. This is usually done through the contingency degradation paradigm, in which free rewards are introduced independently of any action. Instrumental contingency can be regarded as the probability of reward given a particular action relative to the probability of reward given no action. If these probabilities are the same, the contingency is deemed degraded. If degrading the contingency has no effect on behavior, it can be concluded that behavior is habitual, rather than goal-directed (Halbout *et al*, 2016; Ostlund and Balleine, 2008; Phillips and Vugler, 2011).

In the second class of assays, the value of the outcome is increased or decreased. Devaluation is far more common, as it is easier to reduce the value of an outcome. For instance, giving the animal unlimited exposure to the food reinforcer (i.e. satiation) before a test, or pairing the reward with an unpleasant outcome such as sickness, thus reducing the value of the reward. In such case, if performance is sensitive to manipulations of outcome value (e.g. if the rate of responding emitted in order to obtain the reward decreases after outcome devaluation, relative to the normal-valued reward group), then the behavior is controlled by the

anticipation of the outcome. If performance is insensitive to manipulations (i.e. if there is no difference between devalued and non-devalued groups), the behavior is controlled by antecedent stimuli, and is deemed habitual (Corbit *et al*, 2012, 2014a, 2014b; Hogarth *et al*, 2013; Yin *et al*, 2004, 2005a; Yin and Knowlton, 2006).

Several lines of evidence suggest that the balance between habitual and goal-directed control over behavior is disrupted in addiction. Addictive drugs have been proposed to promote the formation of habits (Corbit *et al*, 2012, 2014a; Everitt and Robbins, 2005; LeBlanc *et al*, 2013; Leong *et al*, 2016; Miles *et al*, 2003; Nelson and Killcross, 2006; Nordquist *et al*, 2007; Zapata *et al*, 2010). Facilitation of habit learning with natural reward has also been observed after chronic exposure to COC (Corbit *et al*, 2014a; LeBlanc *et al*, 2013) and amphetamine (Nelson and Killcross, 2006; Nordquist *et al*, 2007). Also post training administration of COC immediately after instrumental responding for a natural reward was sufficient to induce or accelerate habitual control over performance (Root *et al*, 2009; Schmitzer-Torbert *et al*, 2015).

However, the devaluation component used to demonstrate habitual behavior shows a major complication for its adaptation on drug seeking and consumption: drugs of abuse are extremely hard to devalue. Difficulties inherent in adapting standard devaluation procedures for natural reinforcers to those of intravenously administered drug make it very difficult to adapt self-administration procedures to test for habitual or goal-directedness of behavior. As opposed to natural reinforcers, intravenous drug self-administration is not associated with an obvious consummatory response, and psychostimulants such as COC have unconditioned, behaviorally activating effects that can affect responding (Vandaele and Janak, 2017; Zapata *et al*, 2010).

Second-order schedules of reinforcement have been used to circumvent the constraint that the difficulty of devaluing a drug outcome represents. In this protocol (described below), rather than devaluing the outcome, the extinction procedure permits the devaluation of *the action* of pressing the taking lever, which would eventually deliver the outcome. Interestingly, while on the FR1 schedules of reinforcement the value of outcome must be updated as the animal becomes satiated (in a goal directed frame), in second-order schedules the outcome still has the same value (i.e. the outcome is still desired), but the animals acquire the new learning that the lever no longer delivers the outcome, thus the link (i.e. the lever that delivers the outcome) itself is devalued, not the outcome (Olmstead *et al*, 2001; Thraillkill and Bouton, 2016a, 2017a; Zapata *et al*, 2010).

1.4. Second-order schedule of reinforcement

Drug addiction does not involve only taking drugs, as drug addicts spend most of their time foraging for the drug. During these long periods of drug-seeking behavior, eventually controlled by drug-associated conditioned stimuli (CS) acting as *conditioned reinforcers*⁷, the compulsive feature of addiction is expressed. Most self-administration paradigms combine the consummatory and the appetitive responses in the same lever press or nose poke (Roberts *et al*, 2013). However, in the context of drug addiction this dissociation is very relevant, given that in 'real life' the activities involved in obtaining access to a drug are not the same as those required for taking it. Converging with this line, drug taking and drug seeking responses (i.e. consummatory and appetitive) are dissociable and represent different categories of behavior which are likely regulated by different neural mechanisms, and these behaviors respond differently to neurobiological manipulations. Therefore, different paradigms are necessary to isolate the factors that control these two aspects of behavior (Belin *et al*, 2013; Corbit and Balleine, 2003; Murray *et al*, 2012b; Olmstead *et al*, 2001; Roberts *et al*, 2013; Self, 2004; Zimmer *et al*, 2011).

In humans, the difference between appetitive and consummatory responses is rather obvious; the consummatory response (e.g. smoking, drinking/swallowing, injecting, or snorting) is an ingestive act. On the other hand, appetitive responses involve more varied behaviors that result in gaining access to the drug [acquiring money, searching for suppliers, paying for the drug (Allain *et al*, 2015; Roberts *et al*, 2013)]. Regarding animal models, heterogeneous chained schedules or second-order schedules of reinforcement aim to clearly dissociate drug-seeking from drug-taking behavior, and each step in the chain is reinforced by a conditioned reinforcer that ultimately produces a primary reward. Under these schedules, the presentation of each CS not only reinforces instrumental behavior, but also signals to the animal that a successive step in the chain has been completed, and the next step can be commenced (Di Ciano and Everitt, 2005; Murray *et al*, 2012a; Olmstead *et al*, 2001; Pelloux *et al*, 2007; Schindler *et al*, 2002; Zapata *et al*, 2010). Also, it is important to observe that in drug self-administration, behaviors in a chain are argued to follow the motivational rules that also influence responses in food-reinforced chains (Thrailkill and Bouton, 2016a).

In this procedure animals are initially trained to self-administer drug, with each infusion being paired with a CS, usually a cue-light above one of the manipulanda (henceforth termed

⁷ A *conditioned reinforcer* functions like any other reinforcer in that it increases the probability of any response that precedes its presentation. However, unlike a *primary reinforcer*, it must be previously paired with primary reinforcers to develop reinforcing properties (Schindler *et al*, 2002).

the *taking lever*). During this stage, an association is established between the self-administered intravenous drug infusion and the CS. After repeated pairings, this light stimulus is capable of maintaining responding on its own (i.e. the animal will work for it), and it is this conditioned reinforcing property that ultimately maintains responding under second-order schedules. Thus, once the CS is successfully paired with the drug infusion, the second link of the chained schedule is introduced: the *seeking lever*. The first *seeking lever* press occurring after a random interval is elapsed results in the retraction of the *seeking lever* and presentation of the *taking lever*. A single response on the (previously established) *taking lever* yields a drug infusion. This way responding in an initial, seeking link of the chain is maintained by the conditioned reinforcement provided by access to the terminal, TL rather than by the reinforcer itself. Therefore, the effective outcome of the seeking response is the access to the taking lever (Olmstead *et al*, 2001; Schindler *et al*, 2002). Importantly, the average of the random interval required between both links of the chain is initially short, conferring contiguity for the association of both actions, and the schedule is then gradually incremented such that rats are required to make an increasing number of responses for the presentation of the *taking lever*, with drug being available to reinforce responding [Figure 2.2 (Belin-Rauscent *et al*, 2015; Di Ciano and Everitt, 2005; Everitt and Robbins, 2000; Olmstead *et al*, 2000, 2001)].

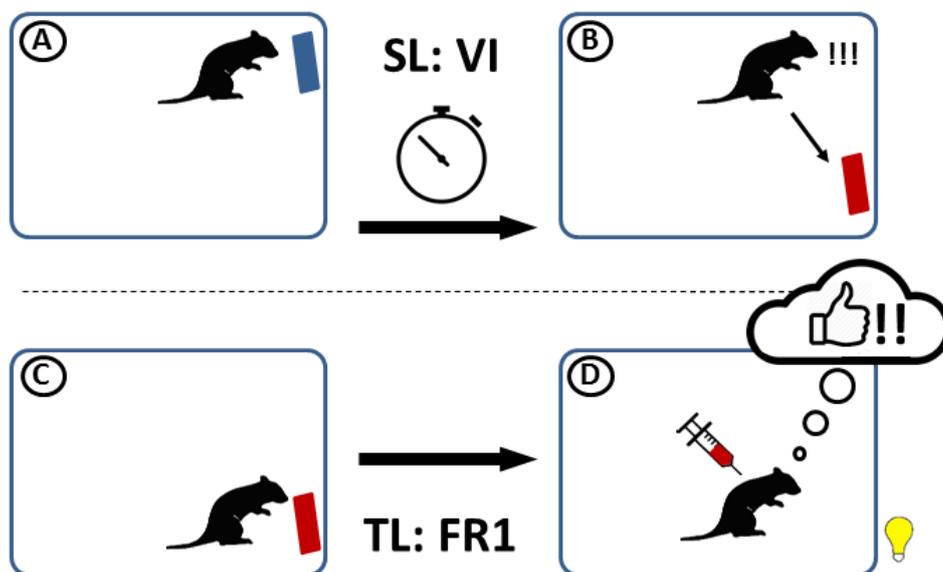


Figure 2.2. Second-order schedule of reinforcement. Once the second-order is established, the first press on the SL (seeking lever) under a variable interval (A) results in the retraction of the SL and presentation of the TL (taking lever; B). One press on the TL results in its retraction (C), followed by an intravenous delivery of the drug and the presentation of a stimulus (e.g. cue light; D). This sequence is usually followed by a period of time out, after which another infusion cycle starts with the presentation of the SL.

1.5. Objectives

The basal ganglia is organized as a series of parallel cortico-striato-pallido-cortical loops, with the more ventral parts of the BG related to emotional learning and the more dorsal parts involved in more cognitive and motor functions. The NAcc has long been suggested to be the interface between emotion, motivation and action, while also having important functions in Pavlovian conditioning and the interaction between Pavlovian and instrumental learning mechanisms. The DS, on the other hand, has been shown to have an important role in cognitive and motor processes, representing a critical substrate for the control of instrumental behavior (Belin *et al*, 2009; Haber *et al*, 2000; Mogenson *et al*, 1980; Parkinson *et al*, 1999).

Indeed it is now well established that all drugs abused by humans have been shown to increase DA transmission within the NAcc (Di Chiara, 1999; Di Chiara and Imperato, 1988; Torregrossa *et al*, 2011). More recent demonstrations suggest that the DS, together with its DA innervation from the SN might also play a role in the effects of addictive drugs (Belin and Everitt, 2008; Everitt and Robbins, 2005, 2016; Takahashi *et al*, 2007; Vanderschuren *et al*, 2005). Therefore, it can be suggested that addiction might not just involve neurobiological adaptations within an incentive mesoaccumbal system, the mesolimbic dopamine pathway that is comprised of projections from the DA neurons in the VTA to the ventral striatum. Rather, it is likely that addiction also involves a complete reorganization of the intra- and inter-striatal mechanisms normally involved in controlling voluntary behavior (Everitt and Robbins, 2013, 2016).

Whereas performance of a food taking response is influenced by Pavlovian conditioning, Corbit and Balleine (2003) found that seeking responses are determined by the current value of the reinforcer. Olmstead *et al* (2001) have shown the goal-directed aspect of COC seeking on a second order schedule by demonstrating that extinguishing the taking response produced an immediate reduction in seeking. Finally, Zapata *et al* (2010) showed that though COC seeking is initially goal-directed it eventually becomes habitual after prolonged training. Therefore, in this chapter is described the attempted validation of a protocol in which rats' performance were intended to shift from goal-directed to habit based behavior, using food as reward. Also, as previously mentioned, given that psychostimulants are believed to accelerate the transition from goal-directed to habitual behavior, we performed a COC sensitization regimen, in order to later be able to investigate the role of dopaminergic receptors within the striatal regions studied in the first chapter of this work.

2. Materials and methods

Experimental protocol:

2.1. *Experimental Design*

Animals acquired the seeking/taking paradigm and were allowed at least 7 sessions on the final VI30/FR1:TO5 (see below) schedule before the first test of outcome devaluation by specific satiety; in which seeking lever presses were assessed (Test 1). After the first test, animals had 3 further training sessions (*reminder* sessions; these were intended to compensate for any extinction effects of the seeking lever, as presses had no effect during test). Then, animals were subjected to 6 sessions of extinction of the taking lever before being again tested 24h later for seeking lever presses, in extinction (Test 2).

As in the second test animals still showed no sign of habitual behavior, they were submitted to a COC sensitization protocol, as the repeated exposure to psychostimulants have been shown to accelerate habit learning with natural reinforcers (Corbit *et al*, 2014a; LeBlanc *et al*, 2013; Nelson and Killcross, 2006; Nordquist *et al*, 2007).

After sensitization regimen, rats were left in their home cages for 10 days then to undergo 6 further daily sessions of training on the chained schedule (under VI30/FR1:TO5). On the day following the 6th session, rats were again tested (Test 3) for seeking lever presses in extinction. Still not displaying habitual behavior, animals underwent further training (26 sessions) in the seeking/taking schedule, but this time with an increased VI of 60s (VI60/FR1:TO5). On the day after the 26th session, animals were again tested for seeking lever presses after outcome devaluation by specific satiety (Test 4) and conditioned taste aversion (CTA; Test 5). With the exception of the last test, all the animals were tested in both conditions (valued and devalued), in a design allowing within subjects comparisons. Also, all tests were interspersed by 2 or 3 *reminder* sessions to recover any potential extinguishing effects exerted by the previous test. A depiction of the protocol is represented in Figure 2.3.

2.2. *Second order schedule of reinforcement with food pellets as rewards*

The training protocol was adapted from Olmstead *et al* (2001). Animals were initially trained to lever press one lever (designated as the food *taking lever*, TL) for delivery of palatable food pellets under a FR1 schedule. Each lever press activated a pellet dispenser

located outside the cage allowing the immediate delivery of one palatable food pellet into the food magazine located midway between the two levers. Delivery of the food pellet was accompanied by retraction of the TL, extinction of the house light and illumination of a cue light above the taking lever for 5s. Sessions ended when animals managed to earn a maximum of 40 pellets or reached a total lapse of 45min, whichever happened first.

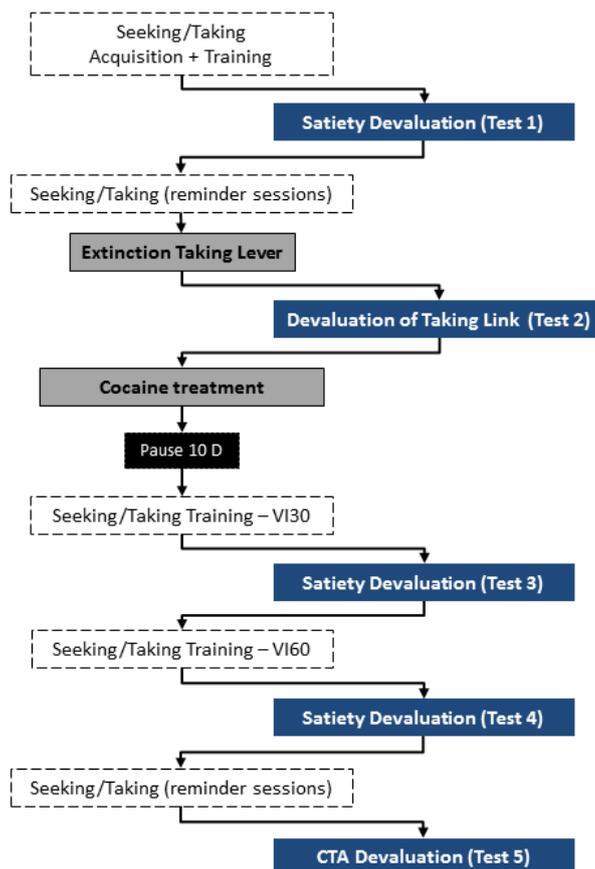


Figure 2.3. Experimental Design. Animals were extensively trained in a chained protocol and were tested in 5 occasions for seeking lever presses, in extinction.

After reliable TL instrumental conditioning was established, the chained schedule was then introduced. Every cycle started with the insertion of a second lever, designated as the food *seeking lever* (SL). The first lever press on the seeking lever after completion of a variable interval of 2 s (VI2; that is, the average of the intervals is 2 seconds, with lapses varying from 1s to 3s) resulted in the retraction of the SL and extension of the TL. The first response on the TL resulted in the pellet delivery sequence (e.g., delivery of one food pellet followed by retraction of the TL, with simultaneous extinction of the house light and illumination of the cue light above the taking lever). A time-out of 5 s (TO5) was imposed after every delivery, after which another delivery cycle started with the presentation of the SL. Thus, animals acquired the food rewards under a VI2/FR1:TO5 chained food seeking/taking schedule (2 daily sessions). Each daily

session lasted 30 min or until 30 pellets were delivered, whichever happened first. The VI component of the chained schedule was then increased: VI5/FR1:TO5 (4 sessions) - VI15/FR1:TO5 (1 session) - VI30/FR1:TO5. Animals were allowed at least 7 sessions on the final VI30/FR1:TO5 schedule before Test 1. Number of presses on each lever, number of food pellets delivered, and number of magazine head-entries were automatically recorded during sessions. All the tests were carried out in extinction conditions, so that behavior was guided by the animals' representation of the outcome.

2.3. *Outcome devaluation by specific satiety*

After stable responding on the final chained schedule was achieved, a protocol of outcome devaluation by specific satiety (Naneix *et al*, 2013) was used in Tests 1; 3 and 4. This test assesses the adaptation to changes in outcome value after initial instrumental training. Rats were divided into two groups and given free access either to the palatable food pellets that served as primary reinforcer during training (devalued; $n= 12$) or to chow (non-devalued; Non-devalued; $n= 12$) in their home cage for 60 min. Immediately after the pre-feeding treatment, rats were placed in the operant chambers for a 15 min test. During the test, the cage was illuminated and the seeking lever was available, but lever presses were without programmed consequence (the TL was never presented). In the following days, animals were retrained in three *reminder* sessions under the same VI schedule for the food reward, in order to compensate for any potential extinction effect from the previous test. A second test was then conducted, identical to the first one, except rats were pre-fed with the alternative reinforcer.

2.4. *Extinction of the pellet seeking/taking link*

After Test 1, animals were divided into 2 groups. The extinguished group ($n= 12$) was exposed to the pellet TL during the entire session, but lever responding had no programmed consequences. The non-extinguished group ($n= 12$), sessions consisted in the exposure to the training environment (conditioning cage) for the same amount of time, without the presentation of levers. After 6 pellet TL extinction sessions, pellet seeking was assessed in a 15 min test (Test 2). During this test, only the pellet SL was present for both groups and responses had no programmed consequences. Number of lever presses and number of magazine head-entries were automatically recorded. This experiment was designed to reproduce results by Olmstead *et al* (2001) in which, instead of devaluing the outcome itself (i.e. the primary reinforcer which in

our case is food pellets), the aim is to devalue the TL by extinction. That is, the value of the TL should decrease once rats learn that pressing it no longer delivers the food outcome.

2.5. Cocaine sensitization

After Test 2, as animals displayed no sign of habitual behavior, animals of the Non-extinguished condition were submitted to a behavioral sensitization procedure. With this treatment, we expected to accelerate the shift of behavioral control to the automatized structure of habitual behavior argued to occur after repeated exposure to psychostimulants (Nelson and Killcross, 2006; Nordquist *et al*, 2007; Schoenbaum and Setlow, 2005; Zapata *et al*, 2010). The day before the start of the sensitization regimen, the rats were placed into the activity chambers for 1h, for habituation purposes and to avoid any neophobia related biasing. The procedure consisted in the habituation of the rats to the activity cages for 1h, and then injection of COC hydrochloride (30 mg/kg) in half of the rats (n=6) which was dissolved in sterile physiological saline (0.9%). The non-sensitized group (n= 6) received similar volume of saline. This dose is similar to that employed by other labs to study molecular and structural brain changes associated with psychomotor sensitization (Li *et al*, 2004; Pierce *et al*, 1995, 1996; Schoenbaum and Setlow, 2005). After injection, rats were returned to the activity cages and had their activity assessed for a period of 2h. Cocaine was administered intraperitoneally and injection volumes were 1 ml/kg of body weight, while the control treatment consisted of an equivalent volume of saline.

2.6. Outcome devaluation by conditioned taste aversion

After the last satiety devaluation test (Test 4), we wondered if the devaluation by CTA would render similar results, since the aversive component of this protocol is argued to engender a more robust devaluation effect in normal animals compared with satiety of the instrumental outcome (Nelson and Killcross, 2006). As for the precedent satiety devaluation test (Test 4), the animals subjected to the sensitization procedure were further divided into two groups, thus yielding four groups (saline non-devalued; SAL-ND n= 3; saline devalued; SAL-D n= 3; cocaine non-devalued; COC-ND, n= 3; cocaine devalued; COC-D, n= 3). Rats were subjected to 7 pairing sessions in which they received 10 min access to a bowl containing 100 palatable food pellets and animals received an injection of either lithium chloride solution (Devalued groups; 0.3 M; LiCl; 5 ml/kg; i.p.) or the equivalent volume of saline (Non-devalued groups). On every second day animals were exposed to the same context, but without the

presentation of the pellets and the injections were counterbalanced. The number of food pellets consumed was weighed after each devaluation session. With this experiment, the devalued animals established an association between the malaises caused by the LiCl injections and the pellets they had eaten just before the injection, while the non-devalued groups did not establish this pairing, even being exposed to the same amount of LiCl throughout the experiment.

Data analyses:

Statistical analyses were performed using *Student's t* test for devaluation experiments, repeated measures analysis of variance (ANOVA) was used for the assessment of the tests and the kinetics of the tests. In the latter, Newman-Keuls was used for *post hoc* comparisons. Statistical significance was considered when $p < 0.05$. Values of devaluation tests are expressed as percentage of baseline; baseline was established as a rate of lever presses or magazine entries performed during the same amount of time as the test in the preceding training session.

3. Results

3.1. Second order schedule with food pellets as reward

3.1.1. Second-order schedule acquisition

Initially, the animals have been trained on an FR1 (data not shown) regimen, and manage to earn all the rewards available (40) by the end of the third session. Thus, as the association between lever presses and outcome delivery has been established, the second (*seeking*) lever has been introduced into the schedule, with increasing intervals along the sessions. Repeated measures one way ANOVA showed an effect of day, conveying that animals increased SL presses according to the gradual increase of VI duration. Post hoc analyses show that on D10 (VI15), followed by D11 (VI30), animals increased significantly SL presses following the increases in VI, eventually stabilizing lever presses under VI30 [$F_{(13,322)} = 59.96$; $p < 0.001$; NK: D10 vs. all days $p < 0.001$; D11-17 vs. D04-09 $p < 0.001$ Figure 2.4].

3.1.2. Outcome devaluation by specific satiety

In this experiment, the group satiated with the outcome immediately before the test showed a marked devaluation effect, which is manifested by a decrease in seeking lever

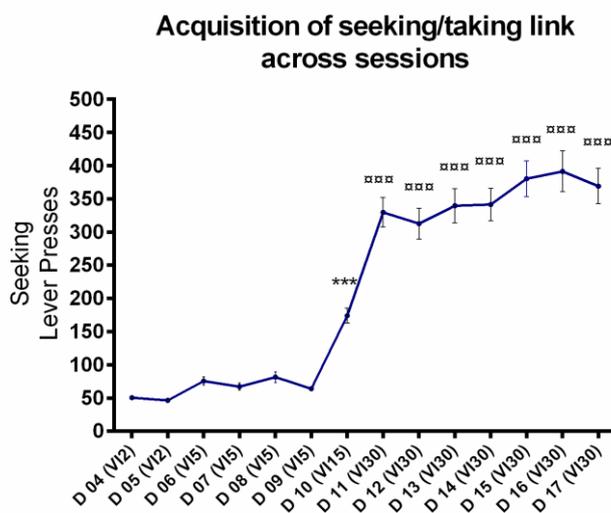


Figure 2.4. Acquisition of the seeking/taking link. Animals learned to press the seeking lever under increasing variable intervals across sessions in order to obtain the taking lever, which was reinforced. Different from D04-09 and D11-17 *** $p < 0.001$; Difference from D04-10 □□□ $p < 0.001$. In each session, the available number of pellets (40) was usually earned before the total time of the session (45 min) had elapsed.

presses, as compared to the non-devalued control group [*student's T test*, $t_{(22)} = 4.99$; $p < 0.001$; Figure 2.5A]. These results suggest that the behavior of the animals was guided by the value of the outcome (i.e. the food pellets). Similar results are observed with magazine entries [*student's T test*, $t_{(22)} = 2.84$; $p < 0.01$; Figure 2.5B], which shows that the animals in the devalued group visited less the magazine conveying that the outcome has been successfully devalued. . Though the kinetics of the session shows no time bins x group interaction [two-way ANOVA $F_{(2,44)} = 1.26$; *n.s.*], it still shows condition [$F_{(1,22)} = 16.09$; $p = 0.001$] and time effects [$F_{(2,44)} = 6.22$; $p < 0.01$], conveying that, [i] in general, the group in the Devalued condition displayed less SL presses when compared to the Non-Devalued condition, showing sensitivity to the value of the outcome, and [ii] groups in both conditions display an extinction effect, decreasing SL presses along time (as SL presses had no programmed results) Figure 2.6.

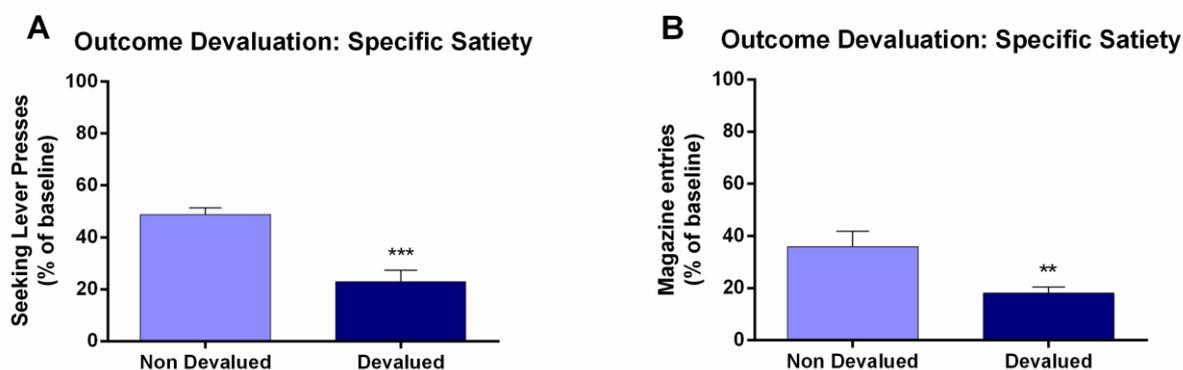


Figure 2.5. Outcome devaluation by specific satiety. Average number of SL presses (A) and magazine entries (B) expressed as percentage of baseline, during 15 min test session. *** $p < 0.001$; ** $p < 0.01$. Baseline was established as a rate of lever presses or magazine entries performed during the same amount of time in the session that preceded the test.

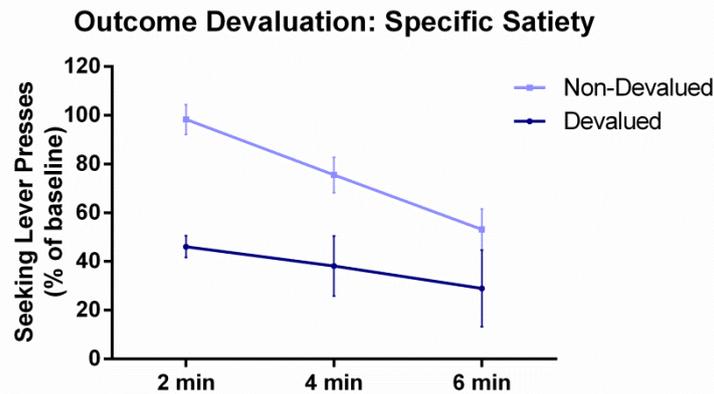


Figure 2.6. Outcome devaluation by specific satiety. Kinetics of seeking lever presses during the first 6 min of test session carried out in extinction. Overall, the group in the Devalued condition decreased SL presses, showing sensitivity to the value of the outcome. Both groups decrease SL presses throughout the session.

3.1.3. Extinction of the pellet seeking/taking link

After the first outcome devaluation test, both groups underwent 6 daily sessions of 2 hours, during which the animals of the extinguished group were exposed only to the TL, and lever presses had no effect (extinction). The extinguished group gradually decreased TL presses across sessions, extinguishing the link between action of pressing the TL and outcome. This extinction eventually stabilized from Session 3 on [*one way ANOVA*; $F_{(5,66)}=16.88$; $p < 0.001$; NK Session 1 vs all sessions $p < 0.001$; Session 2 vs. Sessions 5-6 $p < 0.05$ Figure 2.7]. By extinguishing the taking lever we access whether the animals process this new learning as a devaluation of the TL and, being the case, if acting in a goal-directed fashion animals are expected to decrease SL presses. During test, in which only the SL was presented, the group exposed to the non-reinforced TL (Extinguished) displayed a significantly lower rate of SL presses, when compared to the control (Non-extinguished) group [*student's T test*, $t_{(22)}=3.43$; $p < 0.01$; Figure 2.8A]. Interestingly, the animals displayed similar averages of total magazine head-entries [*student's T test*, $t_{(22)}=0.78$; *n.s.*; Figure 2.8B]. The fact that the magazine entries were similar between groups conveys that the value of the outcome for the Extinguished group was the same as that for the Non-extinguished group. Given that the Extinguished group decreased SL presses but not magazine entries when compared to the Non-extinguished group, one can assume that indeed, the extinction training decreased the value specifically of the TL (see §4 for further discussion). The kinetics of the test session shows a time x condition interaction [*two-way ANOVA* $F_{(2,44)} = 9.38$; $p < 0.001$], and post hoc analysis conveys that initially the Extinguished group displayed less lever presses, but this difference faded across time [NK; Non-extinguished 2 min vs all $p < 0.001$; within comparisons: Extinguished 2 min vs 4 and 6 min bins $p < 0.01$; Figure 2.9]. The low average of SL presses by the Non-extinguished group was possibly caused by an extinction effect, due to repeated exposure to the conditioning environment without any manipulandum available.

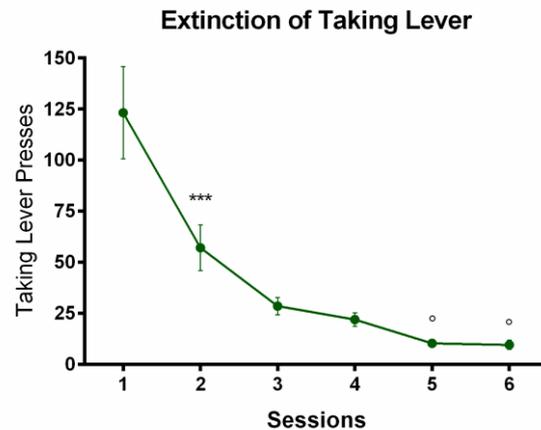


Figure 2.7. Extinction effect on the TL. Taking Lever presses decrease across 6 sessions of 2 h. Different from session 1 *** $p < 0.001$; different from session 2 ° $p < 0.05$.

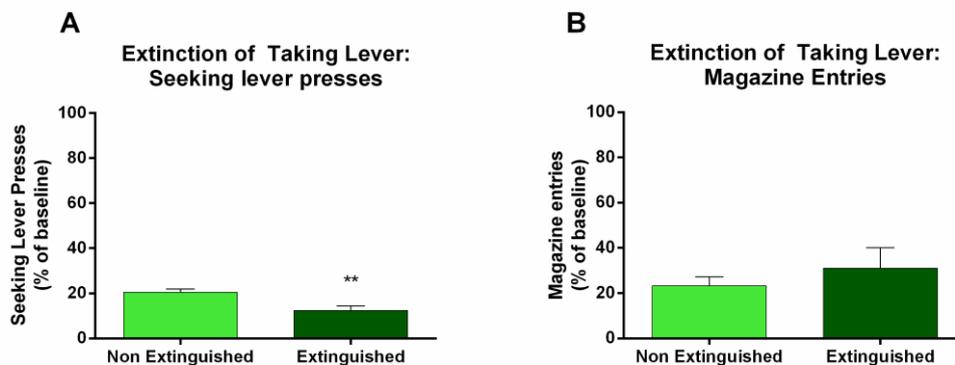


Figure 2.8. Seeking lever presses on extinction test. Average number of SL presses (A) and magazine entries (B) expressed as percentage of baseline, during 15 min test. While EXT group showed less SL lever presses than Non-extinguished group, no differences were observed between averages of magazine entries. Note that in this test only SL was present, on which lever presses had no programmed consequence. ** $p < 0.01$. Baseline was established as a rate of lever presses or magazine entries performed during the same amount of time in the session that preceded the test.

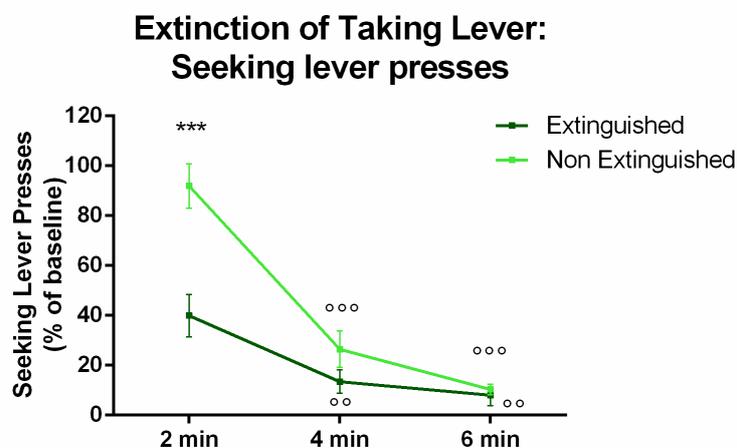


Figure 2.9. Extinction of Taking lever. Kinetics of seeking lever presses during the first 6 min of test session carried out in extinction. Time per group interaction was observed conveying that initially the Extinguished group displayed less SL presses, but this difference faded across time. Between group effect *** $p < 0.001$; within group effect, in comparison to 2 min bin °°° $p < 0.001$; °° $p < 0.01$;

3.1.4. Behavioral Sensitization

For this procedure, animals have been repeatedly treated with cocaine (30 mg/kg) for nine consecutive days in order to induce behavioral sensitization. Stereotypies might be involved in the high variation on the behavior of the rats and the ‘non-escalating’ effect on their response curve throughout sessions (see §4 for discussion), evidenced by no sessions x treatment interaction [*two way ANOVA*, $F_{(8,176)}=1.24$; *n.s.*] in spite of the obviously enhanced average of line crossings showed by the group treated with COC, as compared to the control saline group [group effect: $F_{(1,22)}=20.41$; $p<0.001$; Figure 2.10]. This treatment was intended to accelerate the establishment of habitual behavior in the rats following our chained schedule protocol, as previously mentioned.

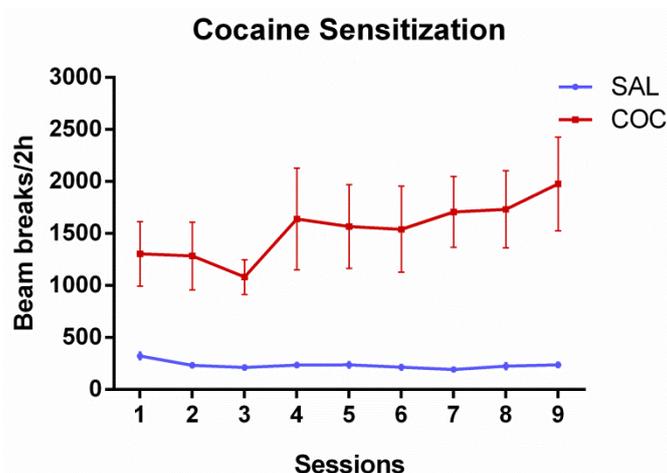


Figure 2.10. Sensitization treatment. Kinetics across days showing effect of COC treatment on locomotor activity. A significant group effect shows the evident enhancement in locomotor activity on the group treated with COC.

3.1.5. Outcome devaluation by specific satiety: limited training [post-sensitization – VI30]

After 10 days of withdrawal, animals have been trained in 6 further sessions of the second order schedule, with a VI of 30s. Here, the animals were divided into 4 groups, which were determined by two treatments and two conditions: sensitized animals were separated in devalued and non-devalued conditions (COC-D; COC-ND, respectively); non-sensitized animals were also separated into devalued/non-devalued conditions (SAL-D; SAL-ND). For the devalued condition, the devaluation procedure was performed in the same manner as in Test 1.

Both devalued groups (SAL-D; COC-D) showed similar levels of lever presses throughout the test session whereas both non-devalued groups performed similarly, with higher lever presses when compared with both devalued groups (SAL-ND; COC-ND). Figure 2.11 depicts the average of seeking lever presses (A) and magazine entries (B) through the whole session,

represented as percentage of baseline, evidencing the devaluation effect [SL presses: $F_{(1,22)}=46.14$; $p<0.001$; magazine entries: $F_{(1,22)}=8.58$; $p<0.01$] as both groups decreased lever pressings and magazine entries when satiated with the outcome before the test, suggesting both groups responded according to the current value of the outcome. However, no interaction between sensitization treatment and devaluation condition was observed [SL presses: $F_{(1,22)}=0.02$; *n.s.*; magazine entries: $F_{(1,22)}=0.70$; *n.s.*], conveying that both groups behaved equally, responding less in the seeking lever, and with less magazine entries when previously exposed to the devalued reinforcer.

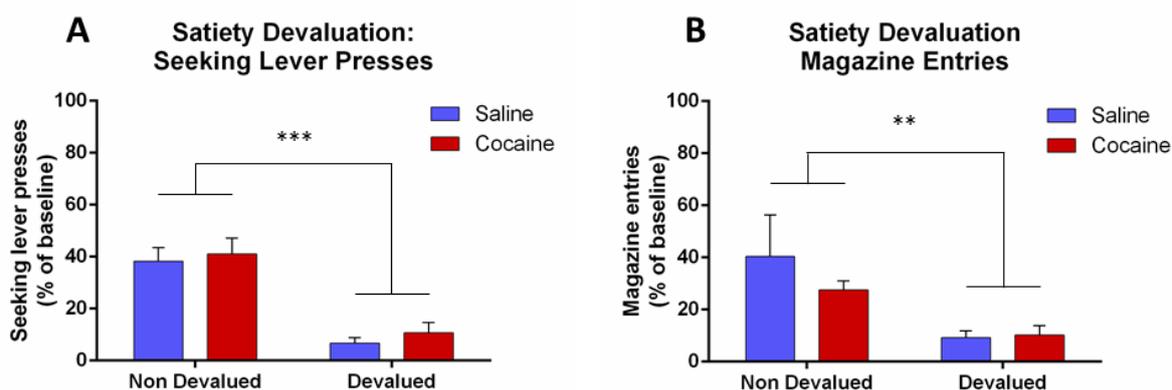


Figure 2.11. First test of outcome devaluation by specific satiety after COC sensitization. Average number of SL presses (A) and magazine entries (B), expressed as percentage of baseline, during 15 min test session. Both groups decreased responding when the reinforcer was devalued, suggesting behavior was driven by the value of the reinforcer. Baseline was established as a rate of lever presses or magazine entries performed during the same amount of time in the session that preceded the test.

Regarding the kinetics of the test, a three way ANOVA with sensitization, time (2 minute bins), and condition (devalued vs. non-devalued) as factors showed no interaction [$F_{(2,88)}=0.91$; *n.s.*], conveying the COC treatment did not accelerate the shift of the sensitized group towards an automatized responding, as both groups were deemed to have responded in a goal-directed manner, according the current value of the reinforcer. However, a devaluation x time interaction was observed [two way ANOVA, $F_{(2,88)}=9.27$; $p<0.001$; Figure 2.12], meaning that [i] the devaluation treatment elicited a decrease in the total number of lever presses of both (sensitized and non-sensitized) groups in the devalued condition, when compared with the non-devalued condition; and [ii] the extinction effect is more pronounced in non-devalued groups. That is, as both groups in the devalued condition showed low SL presses since the beginning of the session, the extinction effect (the slope their lever presses curve) is less pronounced.

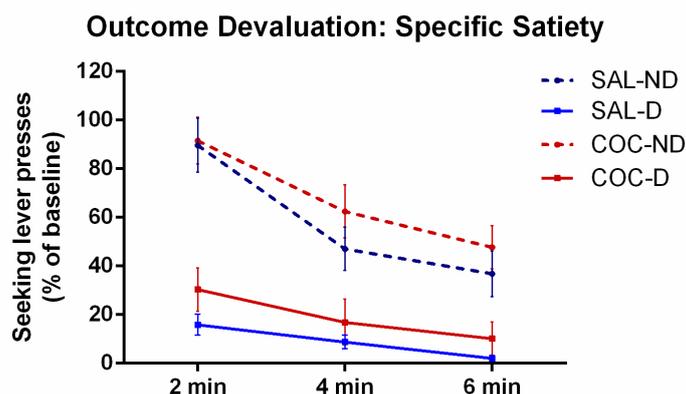


Figure 2.12. Outcome devaluation by specific satiety. Kinetics of SL presses during the first 6 min of test session. Animals in the devalued condition showed significantly less SL responses than animals in the ND condition, regardless of sensitization treatment. Both groups decreased lever presses throughout the session. SAL-D, SAL-ND: Saline group, devalued and non-devalued conditions, respectively; COC-D, COC-ND Cocaine group, devalued and non-devalued conditions, respectively.

3.1.6. Outcome devaluation by specific satiety: extended training [post sensitization; VI60]

As the animals that have previously undergone the sensitization treatment and then trained in 6 sessions on a VI30 regimen did not display habitual behavior, we performed 26 further sessions with an enhanced average of VI (of 60 s), expecting the behavioral control of the rats then to become habit based. After extensive training, animals were once again submitted to the outcome devaluation test by specific satiety, as Tests 1 and 3.

Alike the previous test, two way ANOVA showed a strong effect of devaluation [SL presses: $F_{(1,22)}=52.36$; $p<0.001$; magazine entries: $F_{(1,22)}=40.62$; $p<0.001$] but no sensitization x devaluation interaction [SL presses: $F_{(1,22)}=0.001$; $n.s.$; magazine entries: $F_{(1,22)}=1.67$; $n.s.$; Figure 2.13A and B, respectively], again suggesting that, regardless of sensitization treatment, animals in the devalued condition responded less on the SL than when the outcome was not devalued. Again, analysis of the kinetics of the test showed no group x condition x time interaction [$F_{(2,88)}=0.91$; $n.s.$], conveying both groups decreased lever presses when in the devalued condition, as compared to their behavior in the non-devalued condition (Figure 2.14).

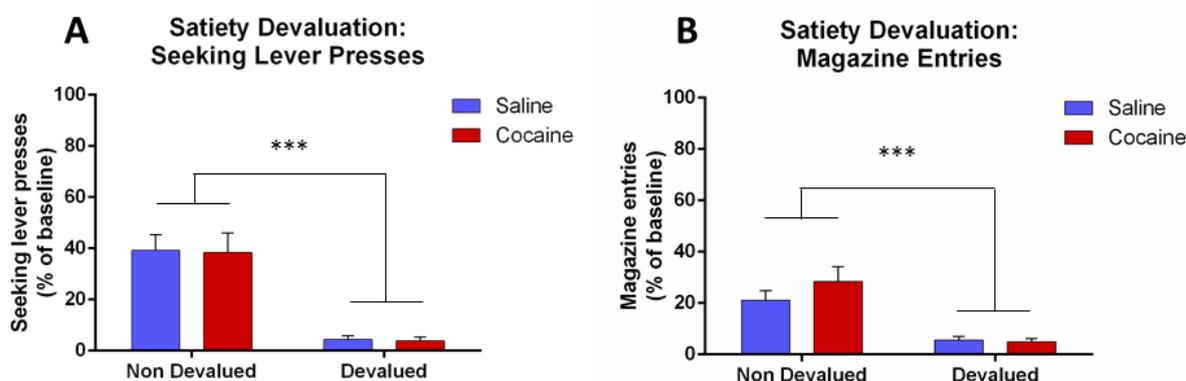


Figure 2.13. Outcome devaluation by specific satiety after COC sensitization. Average number of SL presses (A) and magazine entries (A), expressed as percentage of baseline, during 15 min test session after COC sensitization and training on VI60/FR1:TO5. No condition x devaluation interaction was observed.

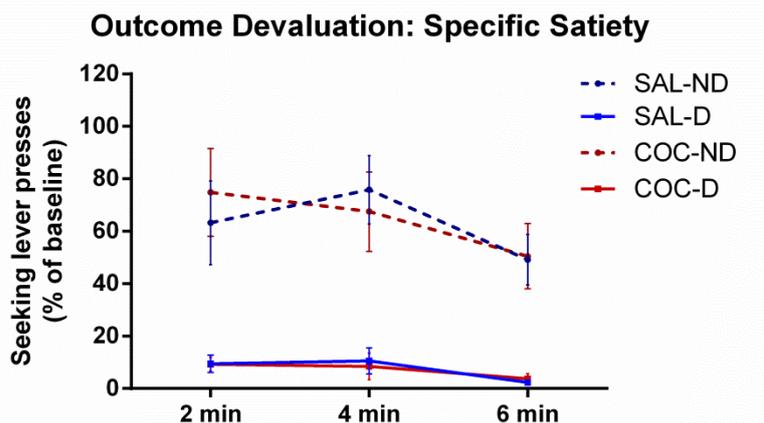


Figure 2.14. Outcome devaluation by specific satiety. Kinetics of SL presses during the first 6 min of test session. Animals in the devalued condition showed significantly less SL responses than animals in the non-devalued condition, regardless of sensitization treatment.

3.1.7. Outcome devaluation by conditioned taste aversion

Finally, the animals have been subjected to the outcome devaluation by the CTA procedure. Two way ANOVA showed a condition x session x devaluation interaction, conveying the devalued animals decreased the amount of pellets eaten across devaluation sessions [three way ANOVA $F_{(6,24)}=7.72$; $p=0.001$; SAL-D NK: D1-2 vs. D4-7 $p=0.001$; COC-D NK: D1-5 vs. D6-7 $p=0.001$]. This interaction suggests that rats subjected to both devalued and non-devalued conditions acted differently in relation to the LiCl pairings. Whereas the SAL-D group decreased amount of eaten pellets after 3 sessions, the COC-D group needed 5 sessions to significantly decrease the amount of pellets eaten across sessions (Figure 2.15).

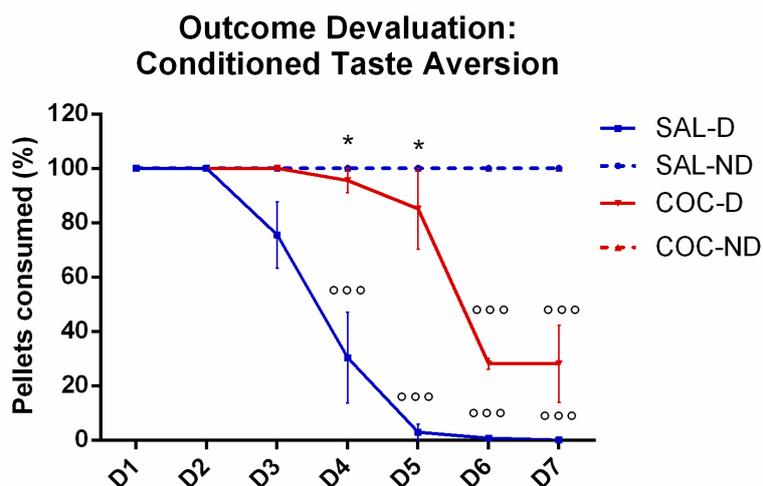


Figure 2.15. Outcome devaluation by conditioned taste aversion. Progression of outcome devaluation by conditioned taste aversion. COC-D group start decreasing the amount of pellets eaten after 5 sessions, whereas SAL-D group ate less after 3 sessions. Within group effects: °°° $p < 0.001$; versus D1-2. Between group effects: * $p < 0.05$.

After the final devaluation session, animals have been submitted to a test identical to the one performed for the specific satiety devaluation, in which animals are placed in the conditioning chambers for 15 min with only the seeking lever available and lever presses did not have any effect. Regarding the average of seeking lever presses during the test session, represented as percentage of baseline, the two way ANOVA showed no treatment x condition interaction for SL presses [$F_{(1,4)}=4.44$; *n.s.*; Figure 2.16A] or magazine entries [$F_{(1,4)}=3.02$; *n.s.*; Figure 2.16B]. These results suggest animals in the COC group still were able to guide their behavior according to the value of the reinforcer, and no habitual behavior has been observed. Finally, three way ANOVA analyzing the kinetics of the test showed no group x condition x time interaction [$F_{(2,16)}=1.31$; *n.s.*; Figure 2.17] regarding lever presses, conveying the behavior of the COC sensitized group was sensitive to the value of the reinforcer, thus characterizing goal-directed behavior. It should be noted that in this last test the number of animals per group was considerably reduced (3), and this might explain the high variation in number of lever presses as well as the low number of seeking lever presses by the non-sensitized group in the non-devalued condition.

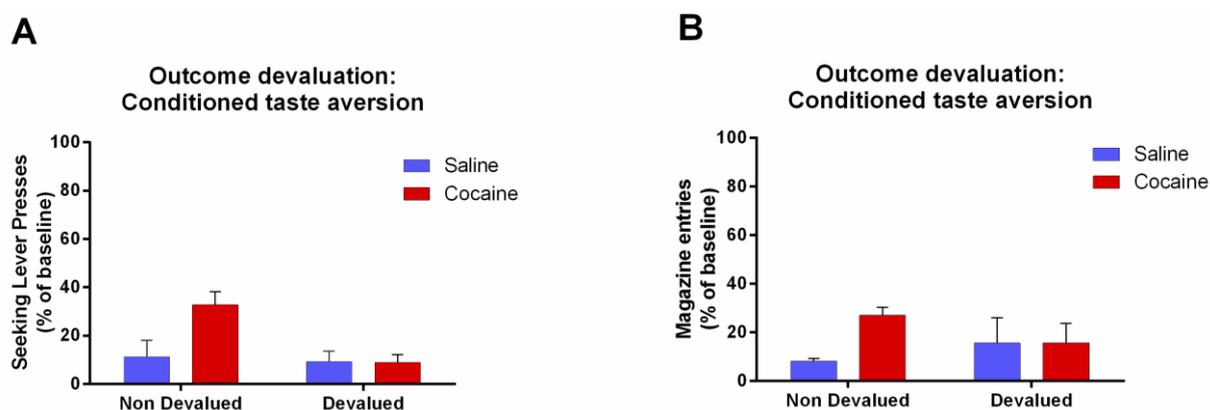


Figure 2.16. Outcome devaluation by conditioned taste aversion test. Average number of SL presses (A) and magazine entries (B), expressed as percentage of baseline, during the 15 min test. After the final test, the behavior of the animals of the COC group was still guided by the value of the outcome, as the devalued group decreased lever presses after pairings between the food reinforcer and LiCl injection. Baseline was established as a rate of lever presses or magazine entries performed during the same amount of time in the session that preceded the test.

4. Discussion

The present work confirms the possibility of the establishment of second-order schedules with palatable food pellets as reinforcers. We also show here that rats subjected to an extended training under this schedule do not adopt habit-based behavior. Furthermore, the repeated

exposure to a COC regimen was also not enough to induce habit based behavior in rats submitted to this kind of protocol.

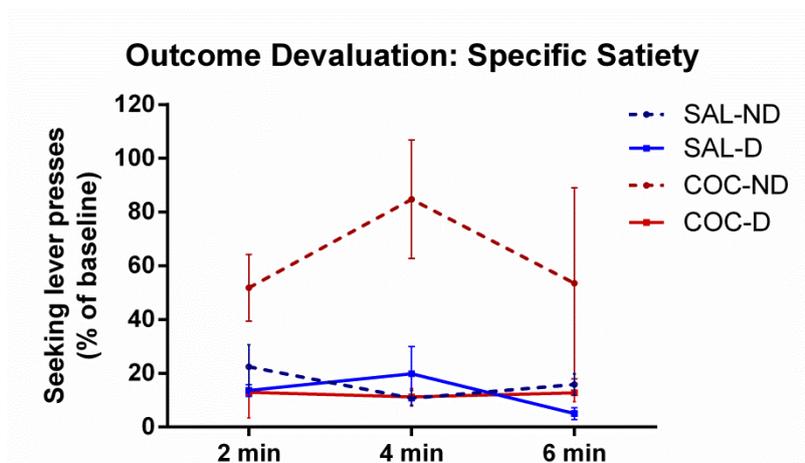


Figure 2.17. Outcome devaluation by CTA. Kinetics of SL presses during the first 6 min of test session. Both groups decreased lever presses throughout the session. Animals in the devalued condition showed significantly less SL responses than animals in the non-devalued condition, regardless of sensitization treatment (in all groups $n = 3$)

4.1. The goal of seeking actions and taking responses

A recent elegant set of studies Thraikill and Bouton (2015, 2016b, 2017b), has extended findings by Olmstead *et al* (2001) and Zapata *et al* (2010), on the role of extinction in chained-schedules. The authors showed that not only the extinction of the taking link weakens seeking responses, but the extinction of the seeking link also weakens taking responding. The authors argue that these effects depend critically on the animals learning to inhibit the response in extinction, by actually emitting the response during instrumental extinction (i.e. the sole exposure to the discriminative stimuli associated with the corresponding seeking or taking links in extinction conditions did not suffice to suppress responding on the other link). Also, the authors showed that after the training of two separate chained schedules, the extinction of one of the links (being it seeking or taking) of one schedule weakens the other link associated with it (Thraikill and Bouton, 2015, 2016b). This conveys that the animals had learned to associate the two responses, in a process where the extinction of one link is specific to the other link of the same schedule, and decreases in responding are not due to generalization between responses or frustration for not obtaining the following element of the chain; a conclusion that we can also assume for our case (Thraikill and Bouton, 2016a, 2017a).

Furthermore, in the classical outcome devaluation procedure by CTA, rats initially acquire instrumental conditioning with food reinforcers to then receive multiple pairings of these

reinforcers with LiCl injections. These pairings yield an association between the two stimuli in which the representation of outcome (i.e. the food) becomes bound to the representation of the malaise induced by LiCl injections. The outcome is, thus, 'devalued'. Next, the animals are allowed to emit responses in extinction. Testing in extinction conditions is important, as it limits any effects of devaluation on operant responding to the rat's representation of the response-outcome relation. In such case, if the response is suppressed it is deemed goal-directed, having the outcome serving as 'goal'. Thus, we deemed it necessary to have reminder sessions between tests, so that animals reestablish the value of the SL before being submitted to the following test (Adams, 1982; Dickinson, 1985).

Once rats learn to associate the two responses (seeking and taking), and the response association makes the SL sensitive to the current status of the TL, then the extinction of the TL seems to weaken the SL in a manner akin to how decreasing the value of the reinforcer (i.e. outcome) for a simple operant response can decrease the performance of this response. These assumptions might raise the question as to what exactly is the 'goal' of the animals pressing the seeking link. One interesting possibility might be that the decrease of the seeking responses after extinction of the TL conveys that the taking link itself, rather than the primary reinforcer (i.e. the food pellet), might be the 'goal' of the seeking response (Adams, 1982; Thraillkill and Bouton, 2017a, 2017b). This might be of relevance if we assume that [i] the association between the distal action (i.e. SL) and the outcome is not the same as that between the proximal action (i.e. TL) and the outcome; and [ii] the sensitivity of instrumental actions to the effects of devaluation demonstrably depends on the position of the action in a chain relative to reward delivery (Balleine *et al*, 1995; Balleine and Dickinson, 2005).

Our results regarding the devaluation of the TL might concur with the hypothesis that the goal of the SL is the TL, rather the reinforcer. Being the TL itself indeed seen as the goal of the seeking response, it would not be surprising that responses on the SL would be susceptible to the devaluation of the TL, as shown by our results. Also, we observed that magazine entries were not affected by the extinction of the TL, which adds to the assumption that the outcome had the same value for Extinguished and Non-extinguished groups, and indeed it was the value of the TL that was decreased after extinction. This idea would also be supported by the assertion that the closer the behavior is to the goal (and in this case the 'goal' of the seeking link would be the taking link), the more likely it is to be sensitive to outcome devaluation (Balleine and Dickinson, 2005; Corbit and Balleine, 2003; Thraillkill and Bouton, 2017a).

This hypothesis, on the other hand, seems to be at odds with our outcome devaluation results. Also in contrast with our results, Thraillkill and Bouton (2017a) have recently shown that, though an outcome devaluation procedure (by CTA with LiCl) yielded a complete rejection of the food pellets in the devalued group, no evidence was found that outcome devaluation

depressed either seeking or taking responses. Nevertheless, as in our own work, magazine entries were also suppressed, reflecting the decreased value of the outcome (Thrailkill and Bouton, 2017a). Regarding lever presses, the authors discard the possibility that the behavior of the rats might have become habitual, since the training was limited to 6 sessions. Instead, it is again argued that the 'goal' representation for the seeking response may be the taking response itself, rather than the representation of the reinforcer. Therefore, in such case rats would not be motivated by a representation of the reinforcer when they perform seeking responses; instead, the seeking response depends on the representation of the next (i.e. taking) response (Olmstead *et al*, 2001; Thrailkill and Bouton, 2016b). In our case, however, it is likely that the value of the primary reinforcer was indeed relevant enough for the devalued group to show less SL presses, when compared to the non-devalued group, even representing a distal response in relation to the food reinforcer.

4.2. *The case for incentive salience*

However, regarding the devaluation of the outcome itself, one point could be raised. In our protocol, immediately before being tested for the SL presses in extinction, animals of the devalued group were pre-fed to satiation with the same reward pellets used as reinforcer beforehand during training. This pre-feeding with the outcome pellets might have given the animals the opportunity to learn about the relatively low value of the reward when in the non-deprived state in an incentive learning process. Once rats get experience with the pellet reinforcer in the non-deprived state, they can learn that it was no longer desired as goal, and thus adjust their response on the SL, and this has indeed been shown to reduce subsequent actions on the distal link of chained schedules (Balleine, 1992; Balleine *et al*, 1995). Interestingly, in our work animals exposed to chow food might have eaten to satiety, but they still pressed for pellets. As predicted by incentive learning hypothesis, this might have been due to the fact that they had not yet been exposed to the chamber while sated with the primary reinforcer (i.e. the food pellets). Finally, since in our case this corresponded to the first test of a series, the results of the following tests might have reflected the same reasoning (Balleine *et al*, 1995; Balleine and Dickinson, 2005).

Thus, the assumption of having each element of the sequence represented separately, allowing individual responses to become associated only with the most proximal events, would suggest that the goal for the SL would be the TL. Once the taking link is available, the goal would be the reinforcer. Observing that different manipulations interfere with either the appetitive or the consummatory components of the second-order would also imply that the

processes motivating these responses are different and dissociable (Balleine *et al*, 1995; Balleine and Dickinson, 2005; Corbit and Balleine, 2003; Thraillkill and Bouton, 2016a).

4.3. *Chunking of actions and hierarchical disposition of goal-directed and habitual systems*

An alternative hypothesis for the representation of the goals in the second-order schedule with food reinforcers could consider that the chunking of actions might have been the strategy adopted by the animals (Jin and Costa, 2015; Ostlund *et al*, 2009). The idea of chunking assumes that an entire action sequence can serve as a behavioral unit, allowing subjects to make sequence-level changes in their behavior. In this process, an entire sequence of actions becomes represented as an integrated unit of behavior capable of entering into associations with discriminative cues and/or rewards (Ostlund *et al*, 2009). In turn, two situations could be considered: [i] the SL is associated with the TL in one response unit, in this case the TL being devalued (i.e. extinguished), all the actions preceding outcome delivery would be conflated and thus devalued; or maybe more likely [ii] the actions directed to the SL under the VI were conflated into one 'response unit', rendering the TL as the goal of SL responses; once the TL is extinguished, it had its value decreased (as discussed previously). In the latter case, SL responses would be generalized to temporal 'response unit' structures in which the animal presses the lever for a certain amount of time in the form of 'bouts' of lever pressing (Shull and Grimes, 2003).

In addition, instead of a 'flat' disposition in which goal-directed and habitual systems work in parallel, the absence of habitual behavior we found could be a product of a hierarchical structuring of behavioral control, as proposed by Dezfouli and Balleine (2012, 2013). In this setting, the goal-directed system stands at a higher level, with the role of the habit process limited to efficiently executing decisions made by the goal-directed system. Therefore one might suggest that the habitual aspect of behavior was directed to the SL pressing only, under the control of a goal-directed system that deemed the outcome valuable during training, but not during testing. When tested, satiated animals would thus not start the action sequence in the same state in which the action was learned and performed so far, which may have inhibited animals to engage SL presses in an indiscriminate manner (Dezfouli and Balleine, 2012, 2013).

4.4. *Manipulation of actions distal or proximal to the goal*

After extinguishing the TL, animals were exposed to the SL to test for their appetitive responses and during this test, no differences were observed with regards to magazine entries. This concurs with previous studies showing that magazine approach is sensitive to changes in the value of the outcome (Balleine *et al*, 1995; Balleine and Dickinson, 2005; Nelson and Killcross, 2006). More specifically, magazine approach in an operant procedure has been showed to depend rather on Pavlovian contingencies, in a demonstration that underlines that instrumental and Pavlovian conditioning are subserved by different neural processes (Corbit and Balleine, 2003; Nelson and Killcross, 2006, 2013). In this sense it has been argued that the Pavlovian associations underlying magazine entries correspond to actions that are more proximal to the goal, being thus less likely to be altered by manipulations (Balleine *et al*, 1995; Balleine and Dickinson, 2005). In our case, however, the extinction of the TL did not alter the value of the primary reinforcer, and thus both groups behave in the same manner towards the magazine where they were delivered.

Behaviors in a chained schedule are argued to follow the same motivational rules influencing responses in both food-reinforced and drug SA paradigms. However, one significant difference between drug and food self-administration paradigms might have led to the results we obtained (Thrailkill and Bouton, 2016a). In the case of i.v. drug SA, paradigms under FR1 schedules of reinforcement, the appetitive and consummatory responses are necessarily combined. As drug reinforcement consists in an infusion delivered through a catheter, there is no stimulus to direct an ingestive behavior, and therefore the operant response becomes both an appetitive response and also a consummatory response that controls drug ingestion. In contrast, in second-order schedules of drug reinforcement, pressing the *taking* lever consists in the consummatory behavior, but still no evident ingestive act results from pressing the lever (Allain *et al*, 2015; Roberts *et al*, 2013). In contrast, under food SA protocols, the *taking* lever will give access to the food reward, which, in our case, is delivered through the magazine. Thus, entries into the food magazine might represent an additional element between the initial seeking and the actual ingestion of the reward, changing the relative proximity of the seeking link to the outcome in comparison with drug SA paradigms. An illustration to sustain this possibility has been represented by Balleine *et al* (1995). In this work, one group of animals was trained to first press a **L**ever (distal action) and then to push a panel (proximal action) that covered the **M**agazine to reach for the outcome (Group LM); a second group had the panel element removed, being trained only to push the **L**ever for the outcome that was delivered in the open magazine (Group L). The results showed that when lever pressing was trained without the panel-pushing component, Group L (in which lever pressing was a more proximal action relative to the outcome) was less sensitive to incentive learning than animals in the Group LM (as in this

group incentive learning enabled the animals to differ in their distal, but not proximal, response relative to the outcome). Therefore, it can be argued that removing the magazine-panel response requirement effectively translated lever pressing from a distal to a more proximal relation to the outcome. Regarding our study, this might represent an important factor differing the position of the SL in relation to the outcome in drug SA and food SA under second-order schedules.

4.5. Protocol issues and COC sensitization

We based the development of our protocol on previous work by Olmstead *et al* (2001), in which they show that, under moderate training, COC seeking is a goal-directed action. This is implied by the fact that COC seeking was shown to be mediated by the knowledge of the contingency between the seeking response and the consequent access to the drug-taking response. Interestingly, in parallel to the groups (devalued and non-devalued) trained in a second-order schedule having COC as reward, the work includes two other sucrose SA groups (devalued and non-devalued). The group submitted to the extinction of the TL after being trained with sucrose reinforcers also showed sensitivity to the devaluation of the TL, much alike both, the COC group in that study, and the results presented here, with food-reinforcers. The length of the training, however, as well as the COC sensitization treatment, are both factors that we hypothesized would establish and accelerate the transition to habit based behavior, respectively. First because when actions are overtrained, the control over behavior undergoes a natural process of shifting from this initial goal-directed process, to a more rigid or habitual system; and second because prior repeated psychostimulant administration has been shown to accelerate habitual responding for food reinforcers (Nelson and Killcross, 2006, 2013; Nordquist *et al*, 2007; Schoenbaum and Setlow, 2005; Zapata *et al*, 2010).

Regarding the work by Zapata *et al* (2010), in which rats were trained in a protocol of second-order with COC reinforcers, other differences (besides the nature of the reinforcers) in comparison to our study should be pointed out. In that study, the group submitted to extended training still displayed goal-directed behavior after 20-25 sessions on the chained schedule of COC SA, undergoing at least 11 further sessions, as argued by the authors, to display habitual responding (even though a subset of animals already displayed habitual responding for COC with moderate training consisting of 13 sessions). In another work using a similar protocol of chained schedule, the extended training group underwent around 32 sessions with COC reinforcement (Pelloux *et al*, 2007). Differences in length of training might also be a relevant factor for the differential results we found. In our study our rats have trained for around 17 sessions before COC sensitization then to undergo at least 32 further sessions after the COC

sensitization treatment. Yet this amount of training seems not to have sufficed for the rats to adopt the automatism we expected. Nonetheless, it is worth to mention that works using protocols intended to have rats expressing habitual performance using variable intervals with simple schedules of natural reinforcement succeeded to instigate this kind of behavior after 12 training sessions [(Nordquist *et al*, 2007) in this case, AMPH treated rats already displayed S – R bound performance after 6 training sessions]. Other cases include 16 (Corbit *et al*, 2014a); 8 (Hitchcott *et al*, 2007); or even 6 sessions under gradually increasing (across sessions) variable intervals (Yin *et al*, 2004) for the successful induction of habitual behavior in rats. Also, besides the method of COC intake (in the cases discussed animals self-administered COC, while in our case COC was experimenter-administered) another factor that may have influenced our inability to find habitual behavior in our rats involve the amount of cocaine consumed, since our rats have been submitted to 9 daily injections of COC (30mg/kg) and in the works by (Pelloux *et al*, 2007) and (Zapata *et al*, 2010) rats received more than 30 sessions of COC SA with 0.25mg/kg and 0.75mg/kg per injection, with 11 or 12 infusions per session respectively. However, Schoenbaum and Setlow (2005) performed a COC sensitizing regimen with 14 daily injections of COC (30mg/kg; in a regimen thus more similar to ours), after which rats maintained responding for a conditioned stimulus associated with an outcome that had been devalued, in a persistent behavior argued by the authors to resemble the automatism of habitual performance. Also, Corbit *et al* (2014a) managed to induce habitual behavior in rats after a COC sensitizing regimen that consisted of 6 daily injections of COC (also 30mg/kg).

However, as a final test, we subjected our animals to outcome devaluation by conditioned taste aversion. During pairing sessions, COC sensitized animals showed an apparent resistance in the acquisition of CTA, evidenced by a delay to inhibit consumption of pellets. This delayed acquisition of CTA, as animals consumed the pellets even after initial pairings with malaise, may somehow suggest [i] compulsive behavior; or [ii] a higher attribution of motivational value to the reinforcer. This is interesting when one takes into consideration that even though our protocol did not succeed in the task of having animals to shift their behavioral control towards automatized responses, they actually presented signs of compulsion or enhanced attribution of incentive value which, represent key characteristic features of addiction, also arguing for a sensitized state (Everitt *et al*, 2008; Pelloux *et al*, 2007; Robinson and Berridge, 1993; Vanderschuren and Everitt, 2004). In any case, we should take into consideration that the number of animals used in this study was low and we cannot rule out the possibility that results might have been different if we had a higher number of animals tested.

Furthermore, in a study using simple schedules with natural reinforcers, Nelson and Killcross (2006, 2013) argue for a dissociation between the effects of pretraining and post-training sensitization on sensitivity to outcome devaluation. In these works, authors sustain that

the sensitizing regimen (in their case with AMPH) disrupts the acquisition, rather than the expression of goal-directed actions, as animals submitted to sensitization after acquiring the devalued group were sensitive to changes in the value of the outcome. In support of this idea, (Nordquist *et al*, 2007) also found rats adopted habitual behavior when AMPH sensitization regimen preceded acquisition of instrumental training. Thus, in both cases the sensitizing treatment was induced before the beginning of instrumental training, under a VI schedule (Nelson and Killcross, 2006, 2013; Nordquist *et al*, 2007). Conversely, in our protocol acquisition of the chained schedule preceded the COC sensitizing regimen, and it might be that the performance during the tests was not habitual (and thus no acceleration by the sensitizing treatment) likely because by the time animals were submitted to COC sensitization, initial learning had already occurred.

Regarding the COC sensitization treatment we performed, it should be pointed out that the establishment of stereotypic behavior is not uncommon under this treatment. Stereotypies (highly repetitive and idiosyncratic motor behaviors), as well as enhanced locomotor activity, are elicited in humans and animals under the influence of psychomotor stimulants such as COC and AMPH. At high doses or with repeated treatments, these psychomotor stimulants suppress locomotion in favor of a limited number of spatially confined stereotypies. These include perseverative movements such as head bobbing, repetitive sniffing, grooming, or oral (licking, nibbling) movements, and this effect may have 'masked' the actual locomotor sensitizing effects of the drug when locomotor responses were assessed (Canales and Graybiel, 2000; Murray *et al*, 2015).

4.6. *Final considerations*

It has been shown that the shift from goal-directed to habit based behavior can be accelerated in animals pre-treated with psychostimulants such as AMPH (Nelson and Killcross, 2006; Nordquist *et al*, 2007) and COC (Schoenbaum and Setlow, 2005; Takahashi *et al*, 2007), using instrumental conditioning under random ratio or random interval schedules or Pavlovian reinforcement. Indeed, these works have used consummatory rewards. Our intent, however, was to try to adapt the second order schedule to this kind of protocol using food (instead of using drugs, as Zapata *et al*. (2010) as reward, aiming to elicit habit based behavior in the sensitized animals. However the second order schedule with food as reward does not seem to elicit habit based behavior, even when the animals have previously been submitted to sensitization and later treated with an extensive training with a high average of variable interval.

Once established, a chained schedule with food reinforcers could be invaluable in the search for the role of DLS and NAcc in the appetitive and consummatory aspects of the transition to an automatized behavior in food-seeking behavior. The understanding of this process with natural reinforcers is important, given that habit formation is a natural process that allows cognitive resources to be liberated for the maintenance of more complex actions (Di Chiara, 2002; Yin *et al*, 2004, 2005b; Yin and Knowlton, 2006). Additionally, the comprehension of habit formation is of especial relevance given that repeated exposure to psychostimulants are argued to accelerate this very same process in the establishment of the compulsive drug consumption (Everitt and Robbins, 2005; Vanderschuren and Everitt, 2004, 2005). Also, the inability of our protocol to elicit automatized behavior with extended training, even after a COC sensitizing regimen, raises several possibilities as to which mechanisms are responsible for the apparent delay in the shift in behavioral control, if this shift is ever to occur under this kind of training. Since it is hypothesized that addicts form habits related to drug use more rapidly than non-addicted individuals and in comparison to habits formed for non-drug reinforcers, the apparent resistance in the transition to habitual behavior in our protocol would also provide an interesting target for further studies (Everitt and Robbins, 2005).

Section III

Pharmacogenetics: Preliminary study on novel approaches to investigate striatal output pathways in drug induced relapse

Section III – Pharmacogenetics: Preliminary study on novel approaches to investigate striatal output pathways in drug induced relapse

1. Introduction

Following the study performed in the first section, we started working on the establishment of new methodologies to tackle the functions of D1R and D2R in the striatum on COC-induced neuroadaptations and reinstatement. Taking into account the segregation of DA receptor subtypes in both projections from the NAcc and DS, which are believed to be either D1R or D2R expressing MSNs, this approach is based on pioneering pharmacogenetic techniques. The technique involves the use of receptor proteins derived from mutagenesis of endogenous G-protein coupled receptor DNA, yielding synthetic receptors (DREADDs; see below) devoid of endogenous ligand. A few weeks after intra-cerebral viral infusion, these receptors are readily expressed in neuronal membranes and are sensitive to an otherwise inert drug which can be delivered systemically. As a result, this technique allows for the manipulation of specific neuronal projections (explained in further detail in section 1.4 – Pharmacogenetic manipulations).

One of the greatest advantages of using DREADDs consists in its ability to provide a 'remote' access to widely distributed neuronal populations, which enables the experimenter to increase or decrease neuronal activity in specific pathways through a non-invasive approach. In this sense, the modulation of neural activity can be induced repeatedly and transiently over days. This would for instance enable us to circumvent the invasiveness as well as the stress undergone by animals submitted to intra-cranial administration of pharmacological agents, still with considerable specificity. Also, this procedure does not necessarily require any intra-cranial cannulation which represents an advantage in comparison to pharmacological approaches. For instance, significant drawbacks of intra-cranial microinjections are [i] that repeated infusions elicit damage at the target site; [ii] the cannulae may become unusable over time because of clogging; and [iii] the target represented by one specific region, that has its target limited by the

volume of the component injected. These limitations are circumvented with DREADDs, which instead only require the peripheral administration of CNO that can be performed repeatedly over a longer period of time, while targeting specific pathways that may extend over longer projections. This can be useful for instance over extensive COC SA training required in studies regarding habitual aspects of drug seeking behavior (Rogan and Roth, 2011; Smith *et al*, 2016; Zapata *et al*, 2010).

Thus, in our case, the techniques developed in this section represent a useful tool to manipulate striatal pathways through systemic injection of the DREADDs ligand, as a means of establishing a relation between the role of D1R and D2R receptors of ventral and dorsal striatal regions, and that of the neuronal pathways in which these receptors are expressed. Through the investigation of the output signal arising from the striatal regions, this approach enables us to examine the different contributions of striatal pathways in future experiments of COC SA and relapse.

1.1. Accumbal D1R and D2R cell populations – Anatomical investigation

As previously described, the striatum is divided into ventral and dorsal portions. Arising from the dorsal striatum are two pathways comprised of GABA MSNs that are segregated into two well established populations. These pathways are defined by their projection targets, peptide co-transmitters and DA receptor expression: the direct pathway is comprised of D1R-expressing MSNs project to structures at the interface between the BG and outer structures; conversely, D2R-MSNs compose the indirect pathway and reach the interface nuclei via an indirect circuit (Gerfen and Surmeier, 2011; Yager *et al*, 2015; Yin *et al*, 2008; Yin and Knowlton, 2006).

In spite of being also segregated into two populations of D1R- and D2R-MSNs, the efferent targets of the MSNs arising from the NAcc are not the same as those in the DS. More importantly, contrasting with this clear segregation observed in the DS, the targets of these two populations have been shown not to be entirely different. Indeed, the D1-MSNs alone project to ventral mesencephalon output structures (SNr and VTA), composing the direct pathway. However, both D1R-MSNs and D2R-MSNs in the NAcc project to VP (which is not an output nucleus of the BG), comprising the indirect pathway (Kupchik *et al*, 2015; Smith *et al*, 2013; Yager *et al*, 2015).

Given the complexity and importance of the striatal projections in addictive processes and, more specifically, taking into account the results presented on Section I regarding the participation of the D1R and D2R within striatal subregions on reinstatement of COC seeking,

our aim was to develop another approach to investigate the participation of D1R and D2R striatal populations on reinstatement. With this purpose, the work described in this section regards the establishment of a pharmacogenetic technique termed DREADD (**D**esigner **R**eceptor **E**xclusively **A**ctivated by **D**esigner **D**rug; described below). Taking into account the aforementioned neuroanatomical differences between the pathways projecting from ventral and dorsal striatum, we considered starting our investigation with those emerging from the NAcc and targeting both the VP and VTA. The clarification of whether the pharmacological activation would coincide with the activation or inhibition of the pathways represents a useful investigation, especially within the NAcc, since the pathways arising from this region are not entirely segregated, as opposed to the DS.

1.2. Pharmacogenetic manipulation – DREADDs

In addition to regulating motivation and reward, the mesocorticolimbic system is involved in cognitive and motor processes. Also, along with addiction, dysfunction of processing within this circuitry has been implicated in several other neuropsychiatric disorders, including ADHD, obsessive compulsive disorder and Parkinson's disease (Crittenden and Graybiel, 2011). Thus it is important that any new interventions to prevent relapse to drug-seeking not interfere with critical brain functions involved in motivation, decision making and motor function (Ferguson and Neumaier, 2015). With that end, a promising methodology has recently emerged and is now allowing for the isolation of the function of targeted neurons. Initially developed in 2007 (Armbruster *et al*, 2007), DREADDs are a powerful approach for reversible and specific modulation of neuronal activity. It represents a synthetic pharmacogenetic methodology whereby designer GPCRs are created and can be activated solely by inert and extrinsic ligands, have minimal constitutive activity *in vitro* and *in vivo*, even when expressed at high levels in neurons; and cannot be activated by any known endogenous ligands (Roth, 2016; Urban and Roth, 2015).

DREADDs were developed by the directed molecular evolution of mutant muscarinic acetylcholine receptors (mAChR₃) in yeast for activity in response to the otherwise pharmacologically inert ligand clozapine-*N*-oxide CNO (Dong *et al*, 2010; Urban and Roth, 2015). Subsequently, a family of mutant muscarinic receptors for the three G protein-dependent signaling pathways (G_i, G_s, and G_q) was developed, and these are activated only by CNO (in the nM range), but not by acetylcholine (Lee *et al*, 2014). Currently, three DREADDs have been most commonly used in the neurosciences: *hM₃D_q*, which is coupled to G_q signaling and induces firing of neurons; *hM₄D_i*, which is coupled to G_i signaling and mediates neuronal and synaptic silencing; and *rM₃D_s*, which is coupled to G_s signaling and which modulates neuronal

activity (Whissell *et al*, 2016; Zhu and Roth, 2015). In this sense, DREADDs have been successfully demonstrated to control individual GPCR signaling in the central nervous system, acting in the control of neuronal activities in a variety of neurons, including glutamatergic, dopaminergic, and GABAergic neurons (Zhu and Roth, 2015).

In brief, in wild type rats the technique consists in the utilization of the flip-excision (FLEX)-switch approach [also known as double-inverted open reading frame (DIO) viral vectors], adapted for use with adeno-associated virus (AAV) delivery systems. The selective modulation of neurons on the basis of their projection paths is achieved by utilizing the DIO DREADD AAVs in combination with the canine adenovirus (CAV) expressing Cre-recombinase⁸ enzyme [CAV-Cre (Atasoy *et al*, 2008; Smith *et al*, 2016)]. More specifically, in our case the technique involves the infusion of both viral vectors (CAV-Cre and AAV-DIO-*hM₃D(G_q)-mCherry*⁹) into two sites that are connected through direct neuronal projections and represent a neuronal pathway (Figure 3.1.A). The AAV-DIO-*hM₃D(G_q)-mCherry* is infused in the site where the cell bodies are located (in our case, the NAcc), infecting the cells regardless of where they project to (Figure 3.1.B; C), while CAV-Cre is infused in the target area of interest, that is innervated by the corresponding axons (in our case, either the VP or the VTA; Figure 3.1.D). After infection of axonal terminals, CAV-Cre viral particles are retrogradely transported back along the axon to the soma and expresses Cre-recombinase. The AAV-DIO-*hM₃D(G_q)-mCherry* contains the floxed inverted sequence of *hM₃D(G_q)-mCherry*, which is reoriented in the presence of the Cre-recombinase enzyme, prompting the expression of *hM₃D(G_q)-mCherry*. The association of both viruses ensures that *hM₃D(G_q)-mCherry* is not expressed in all AAV-DIO-*hM₃D(G_q)-mCherry* infected neurons, but exclusively in those that are also infected with CAV-Cre (Boender *et al*, 2014; Smith *et al*, 2016).

In this sense, the neuronal pathway infected with both viruses will express (in our case) the *hM₃D(G_q)*, which is a GPCR that is only activated by the otherwise pharmacologically inert compound CNO. The binding of CNO to *hM₃D(G_q)* initiates several intracellular cascades, which leads to increases in neuronal activity [(Alexander *et al*, 2009; Bull *et al*, 2014; Scofield *et al*, 2015) Figure 3.1.E]. Once DREADD expression is set, CNO can be administered systemically as it crosses the blood-brain barrier, which facilitates its use in behavioral experiments. Also, CNO only activates those neurons in which *hM₃D(G_q)* is expressed, so the combination of

⁸ In neurons expressing Cre linked to a gene of choice, LoxP sites are cleaved and the inverted gene of the DREADD is flipped into its functional orientation, allowing the DREADD gene to be transcribed exclusively when Cre present (Smith *et al*, 2016).

⁹ AAV-DIO-*hM₃D(G_q)-mCherry* stands for the genes contained along with the AAV virus. DIO conveys that the sequences of the DREADD *hM₃D(G_q)* and of the fluorescent protein *mCherry* are inverted – being then flipped back to the correct translatable orientation by the enzyme Cre recombinase, which accompanies the CAV retrograde virus. This conditions the expression of both the DREADD and the mCherry protein only to the cells infected by both viruses. The expression of the *mCherry* protein allows for the posterior visual confirmation that both viruses were recombined (Lee *et al*, 2014; Smith *et al*, 2016).

$hM_3D(G_q)$ with CAV-Cre allows for the activation of specific neural pathways (Roth, 2016; Urban and Roth, 2015; Whissell *et al*, 2016).

Recent studies have provided evidence of the usefulness of DREADDs in investigations concerning addiction-related behaviors. For instance, in rats it has been demonstrated that the chemogenetic activation of the mPFC–NAcc shell pathway using the G_q -DREADDs effectively reduced reinstatement of COC seeking triggered by COC-associated cues (Augur *et al*, 2016). Also using DREADDs, Ferguson *et al* (2011) found that activating G_i -DREADDs in neurons of the indirect pathway MSNs enhances the development of locomotor sensitization to AMPH, whereas increasing $G_{i/o}$ signaling in direct pathway MSNs impairs the persistence of this behavior.

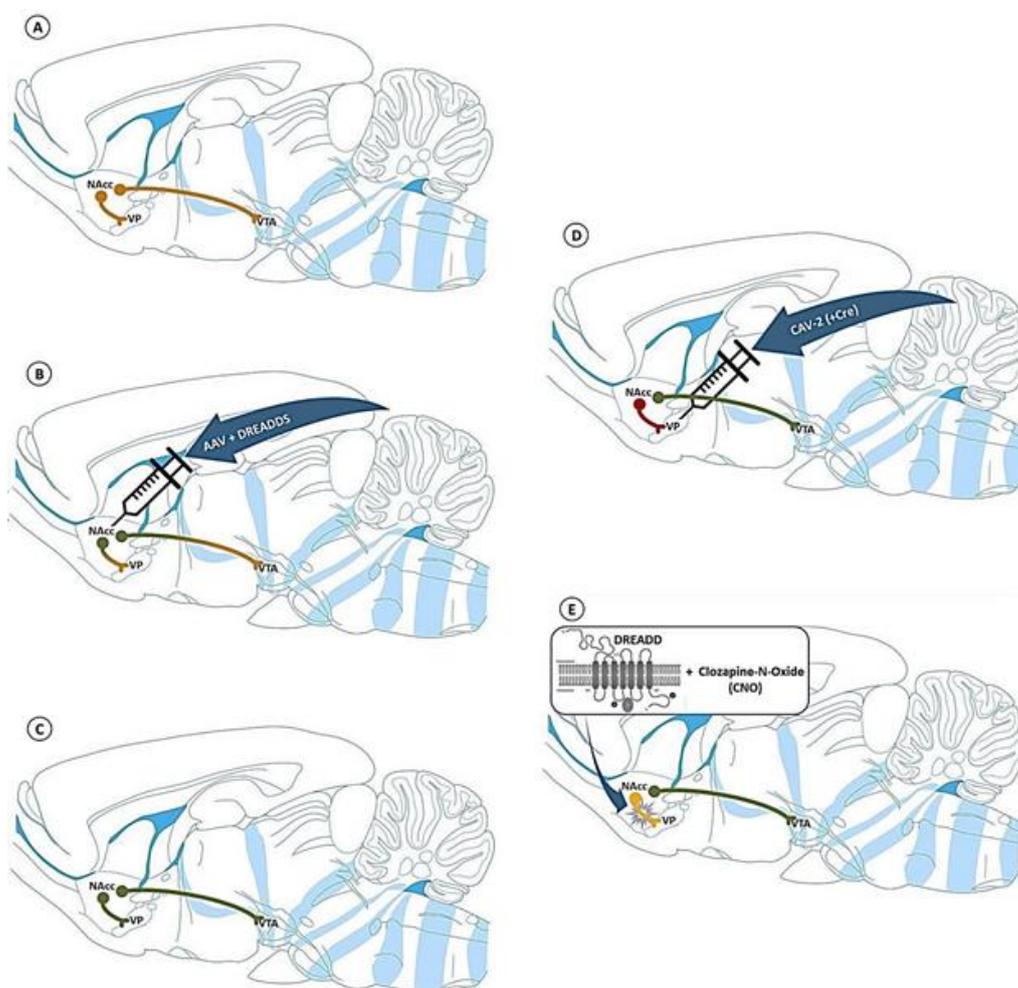


Figure 3.1. Approach for projection-specific modulation of neuronal activity using DREADDs. Representation of both major projections arising from the NAcc, projecting to VP and VTA (A). Injection of AAV vector carrying the inverted DREADDs sequence into the region where the cell bodies are found (i.e. NAcc; B) will lead to the infection of the cells with no regard to the regions they project to (C). The association of the AAV infection within the NAcc with CAV expressing Cre-recombinase (CAV-Cre) transported retrogradely from projection structures (VP in this example), allows for the projection-specific expression of DREADDs (D). This is possible because CAV-Cre is preferentially transported retrogradely to neuronal somas. In the neuronal cell bodies, recombination of AAV-DIO-DREADD constructs can occur to allow expression of DREADDs in a projection-specific fashion. The DREADD $hM_3D(G_q)$ is a G-protein coupled receptor that is activated by the otherwise pharmacologically inert compound CNO. The binding of CNO to $hM_3D(G_q)$ initiates several intracellular cascades which leads to increases in neuronal activity (E).

Furthermore, a number of transgenic mice and rats currently exist in which Cre expression occurs in particular subsets of cells. In these animals, injecting DIO viral vectors that encode DREADDs can give cell type–specific expression of these receptors without the need of infusing both viruses. For instance, the use of genetically engineered rats expressing Cre-recombinase under the control of tyrosine hydroxylase gene promoters can prompt the expression of DREADDs with only the infusion of AAV viruses in the VTA, where the DA cell bodies are found (Mahler *et al*, 2014) . In this case, the DREADDs are to be expressed exclusively in DA, but not on GABA, cells of the VTA. This kind of approach has also been shown with bacterial artificial chromosome transgenic mouse lines that express Cre-recombinase under the control of the D1R or D2R striatal MSN genes. In these mice, it suffices the infusion of AAV DIO DREADDs virus in the striatum for the receptor to be expressed in this region by D1R or D2R receptor-bearing MSNs (Cheng *et al*, 2017; Farrell *et al*, 2013; Gong *et al*, 2007).

Finally, as the work carried out previously dealt with the infusion of agonists of D1Rs and D2Rs into the NAcc and DLS, the combined use of *CAV-Cre* and DREADD methodology was chosen to modulate the specific neural pathways arising from striatal pathways. This possibility could eventually enable us to further investigate the behavioral effects of activating or inhibiting striatal pathways in COC sensitization or COC SA paradigms, in experiments akin to those performed previously with pharmacological experiments.

2. Material and Methods

Given that this section describes the establishment of a new technique in the lab, here are reported the initial experiments performed in order to gather the information and acquire the expertise that are necessary for the development of this technique. These steps consisted, initially, in the establishment of the pathways to be studied, and in the knowledge of the exact regions of the brain where the viruses should be infused. Once gathered, these data enabled us to move on to the investigation of the behavioral effects the DREADDs activation in each region, in pilot behavioral assays. Eventually, we expected to use these manipulations in experiments with COC SA and relapse once establishing the techniques.

For this reason, as a first stage, we worked with neuronal retrograde tracers that once injected in specific brain regions are transported through the axons towards the soma of the neurons. In this sense, the infusion of the conjugate of cholera toxin subunit B (CTB) and Alexa Fluor (AF) fluorescent dye in two of the main target regions of the accumbal direct and indirect pathways, namely the VP or VTA, permitted us to visually analyze these pathways. The knowledge of the origin and destination of these specific neuronal pathways enables us to know

the specific regions of each structure that should be infected with AAV and CAV-Cre viruses further on (Conte *et al*, 2009).

In this sense, once the coordinates were known, the second stage consisted in trials performed with rats that underwent infusions of AAV G_q-DREADD virus in the NAcc and CAV-Cre virus in either target of the direct or indirect pathways (i.e. the VTA or the VP, respectively). Once the viruses were translated into DREADD proteins, rats were submitted to locomotor activity assays to be tested for the behavioral manifestation of the activation of the DREADDs by CNO. If both viruses were recombined, DREADDs were to be expressed exclusively in the neurons comprising the accumbal direct or indirect pathways. The binding of systemically administered CNO and the G_q-DREADDs expressed in these neurons would activate these cells, potentially culminating in GABA release on the target regions and in the consequent behavioral output. This paradigm was chosen because the stimulation of striatal MSNs of the indirect pathway are believed to exert an inhibitory effect on behavior, decreasing locomotor behavior, whereas the activation of striatal MSNs of the direct pathway on the other hand, is thought to increase locomotion (Farrell *et al*, 2013; Freeze *et al*, 2013; Kravitz *et al*, 2010; Lobo *et al*, 2010).

Finally, after being submitted to behavioral assays, the last step of this section consisted in the verification of the virus expression, through fluorescence microscopy analyses; this verification of fluorescence allows us to compare virus recombination with the behavioral responses observed in the locomotor assessments.

2.1. Subjects

For a description of the subjects, see §Material and Methods.

2.2. Stereotaxic surgery: tracer infusion

As mentioned, the first experiments were aimed to establishing the stereotaxic coordinates that should be followed in order to reach neurons projecting to specific structures (i.e. from the NAcc to the VP and VTA). To this end, fluorescent retrograde tracer AF-CTB was injected in surgical procedure in a number of rats with a picospritzer microinjection pump apparatus in two brain regions that receive most of the afferents from the NAcc (i.e. VP or VTA). The AF-CTB compound is a retrograde anatomical tracer that makes reliable tracing of multiple pathways possible using fluorescent microscopy (Conte *et al*, 2009).

Thus, animals were deeply anesthetized with isoflurane (4–5% induction, 1–2% maintenance) and placed in a stereotaxic frame with atraumatic ear bars (Kopf, Tujunga, CA) in a flat skull position. Anaesthesia is maintained with 1.5-2% Isoflurane and complemented by subcutaneous administration of Buprenorphin (Buprecare®; 0.05 mg/kg). Each rat was injected with AF-CTB (1%), in order to label and define the specific areas of the VP and VTA to which the NAcc projects. Rats were given 7 days of recovery to allow migration of the tracers before being killed and having their brains removed for posterior immunohistochemistry assays. Several trials were performed in different rats, with different AF-CTB volumes injected (0.3 µl or 0.6 µl), to induce optimal staining in the soma of NAcc MSNs of the rats. In our trials we used the following coordinates for both NAcc targets:

- VP: AP -0.3 mm;
ML \pm 2.4 or \pm 2.6 mm;
DV -8.5 mm; and
- VTA: AP -5.4 mm;
ML \pm 0.7 or \pm 0.5 mm;
DV -8.5 mm

2.3. Virus infection for DREADDs expression

After establishing coordinates, pilot experiments have been carried out to assess behavioral alterations in response to CNO and posterior observation of virus recombination through fluorescence microscopy. Two sets of rats were bilaterally injected with 1 µl CAV-Cre virus in the VP (n=07) and 0.7 µl in the VTA (n=09). After CAV-Cre infusion, the first group was bilaterally injected with 0.5 µl (CAV-**VP** rats) and the second 1 µl (for CAV-**VTA** rats) of AAV-*hSyn-DIO-hM₃D(G_q)-mCherry* (UNC Vector Core, USA) in the NAcc. The AAV-*hSyn-DIO-hM₃D(G_q)-mCherry* virus (i.e. DREADDs coupled to the G_q protein) was chosen because the stimulation of the pathways would better fit the continuation of the studies carried out on Section I. Rats were given at least 3 weeks of recovery to allow recombination of both viruses (i.e. AAV and CAV-Cre), and consequent DREADDs expression, before behavioral experiments were carried out. Finally, the rats have been killed and had their brains collected for posterior immunohistochemistry assay and confirmation of virus recombination.

2.4. Locomotor activity assessment

Three weeks after the first group of animals has been infused with DREADDs viruses [AAV-DREADDs in the NAcc; CAV-Cre in the VP (CAV-VP group) or VTA (CAV-VTA group)], we performed a pilot experiment to investigate for any behavioral manifestation elicited by a peripheral injection of CNO, which by activating DREADDs was expected to stimulate neurons of either NAcc-VP or NAcc-VTA pathways. Thus, the animals have been exposed to several sessions (interspersed by 24h) in activity cages first to assess for their locomotor responsiveness to a novel environment, and once habituated, to examine for any potential change in their basal locomotor activity elicited by the activation of either pathways.

Thus, for the first experiment, animals received a CNO injection (1 mg/kg i.p.) 30 min before being placed in the activity cages to have their locomotor activity assessed for 2 h in response to the novel environment (Figure 3.2.A). For the second experiment, the same animals underwent two further habituation sessions in the cages (for 2 h; Figure 3.2.B), and in the next session, they were placed in the cages for 1h, to reach basal activity, and then injected with CNO, so to have an activation of the NAcc-VP (CAV-VP group) or NAcc-VTA (CAV-VTA group) pathways and its potential behavioral output. While in the activity cages, rats had their locomotor activity assessed for 2h30min from the moment of injection (Figure 3.2.C).

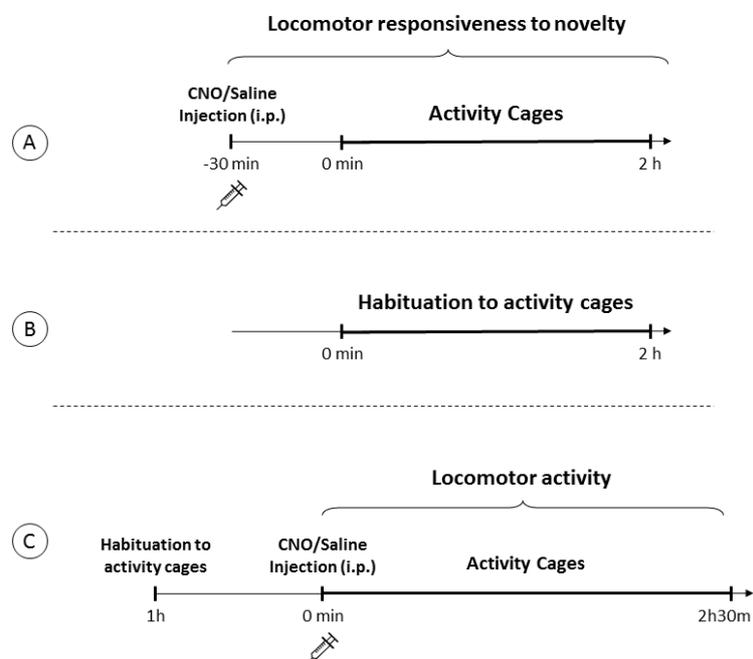


Figure 3.2. Experiment design for the assessment of CNO injections into rats that have undergone injection of DREADDs viruses. Animals have been tested for locomotor responsiveness to novelty, being thus injected with CNO 30 min before the start of the session and having their activity evaluated for 2h (A); In order to assess potential influences of CNO injection on their basal activity, animals were habituated to the activity cages for two sessions (B); In a final session, animals have been placed in the cages and, after stabilizing activity, injected with the same dose of CNO before being replaced in the cages and having their locomotor activity assessed for 2h30min (C).

2.5. *Perfusion, brain slicing and immunohistochemistry*

After behavioral assays rats received a lethal dose of sodium pentobarbital and were perfused transcardially with 150 ml of saline followed by 250 ml of 4% paraformaldehyde (PFA). Following a PFA bath overnight, sections of 45 μm of the NAcc and VP were made using a vibratome. In order to enhance visualization of CTB, immunohistochemistry was performed on the sections. Sections were first rinsed in PBS 0.1 M (3 x 10 minutes), and then incubated in a blocking solution for 1 hour (3% BSA and 0.3% Triton X-100 in PBS 0.1 M). Sections were then incubated with rabbit anti-tetramethylrhodamine (Life technologies, A-6397) primary antibody diluted at 1/600 in the blocking solution for incubation overnight at 4°C. After further rinses in PBS 0.1 M (3 x 10 minutes), slices were placed for 2 hours in a bath containing a goat anti-rabbit secondary antibody coupled to red fluorescent protein (RFP; 1/400 in PBS 0.1M) (Jackson Immuno Research). For the counterstaining, Hoechst solution was added for 15 minutes (1/5000 in PBS 0.1 M), followed by three 10-minute rinses in PBS 0.1 M. Finally, sections were rinsed in PBS 0.1 M (3 x 10 minutes), mounted in PBS 0.05 M onto gelatin-coated slides and coverslipped with the anti-fading reagent Fluoromount G (SouthernBiotech, 0100-01). Slides were analyzed and images were then captured using a MorphoStrider fluorescent microscope (Explora Nova, La Rochelle, France) for coordinates (tracing experiments) and fluorescence (virus experiments) confirmation. For imaging analyses visual criteria were established to discard behavioral data from animals that did not show recombination of viruses.

3. Results

3.1. *Neuronal tracing*

Injections of neuronal tracer AF-CTB were performed aiming at the VP and VTA (examples of the injection sites can be seen on Figures 3.3 and 3.4 for VP and VTA, respectively). After immunohistochemistry assays, labeling yielded by the AF-CTB injection could be observed through fluorescence microscopy in the NAcc of the animals injected (Figures 3.5; 3.6). From these trials, we found that 0.3 μl of AF-CTB was sufficient to induce labeling in the soma of NAcc neurons. Two examples can be seen on Figure 3.5 and 3.6 in close up images of the NAcc, in which labeled cell bodies can be observed. With the images of the fluorescence yielded by the AF-CTB as those exemplified below, we could observe the location (in the NAcc) of the cell bodies that targeted our AF-CTB injection sites in the VP or VTA. In these observations, labeling was not always totally consistent, but in general our

injection sites in the VP and VTA were innervated by neurons arising mainly from the lateral and medial core/shell regions of the NAcc, respectively (Figure 3.5; for NAcc fluorescence after VP injections; Figure 3.6 for NAcc fluorescence after VTA injections). Therefore, for future virus injection of NAcc-VP and NAcc-VTA groups we used the following coordinates:

- [AAV] NAcc: AP +1.8 mm;
ML \pm 1.5;
DV -7.0 mm;
- [CAV-Cre] VP: AP -0.3 mm;
ML \pm 2.4;
DV -8.5 mm; and
- [CAV-Cre] VTA: AP -5.3 mm;
ML \pm 0.5 mm;
DV -8.5 mm

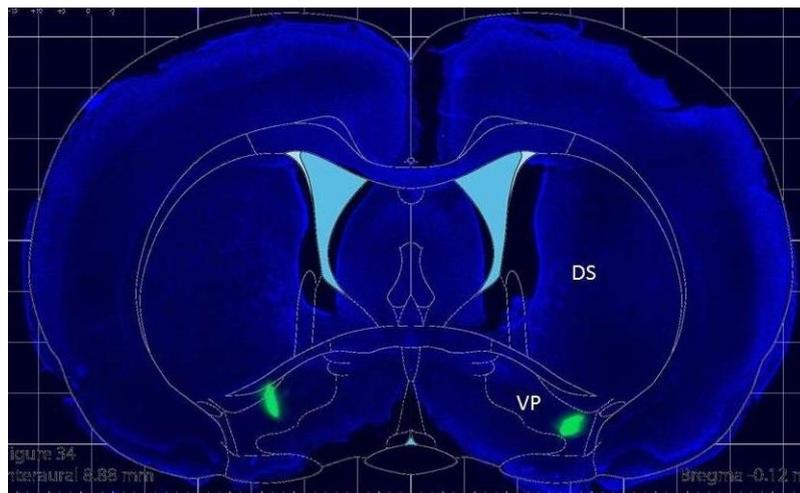


Figure 3.3. CTB injection aimed at the VP. Green spots indicate injection sites at both the medial and lateral regions of the VP (DS: dorsal striatum).

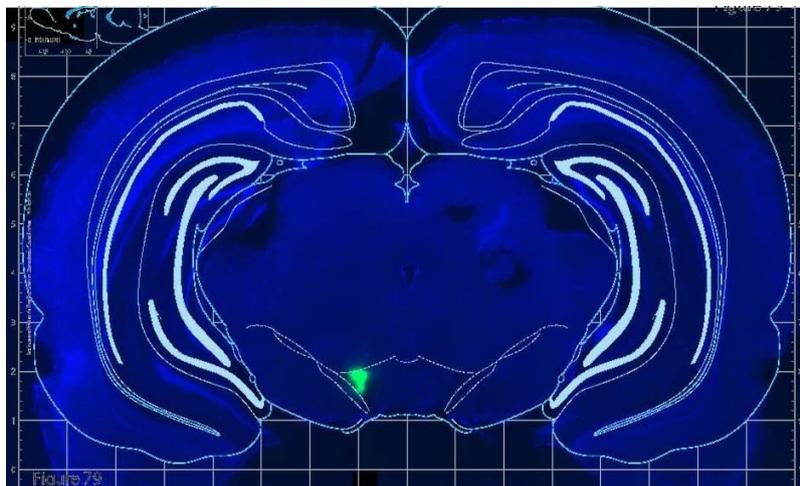


Figure 3.4. CTB injection aimed at the VTA. Green spot corresponds to the injection site in the VTA.

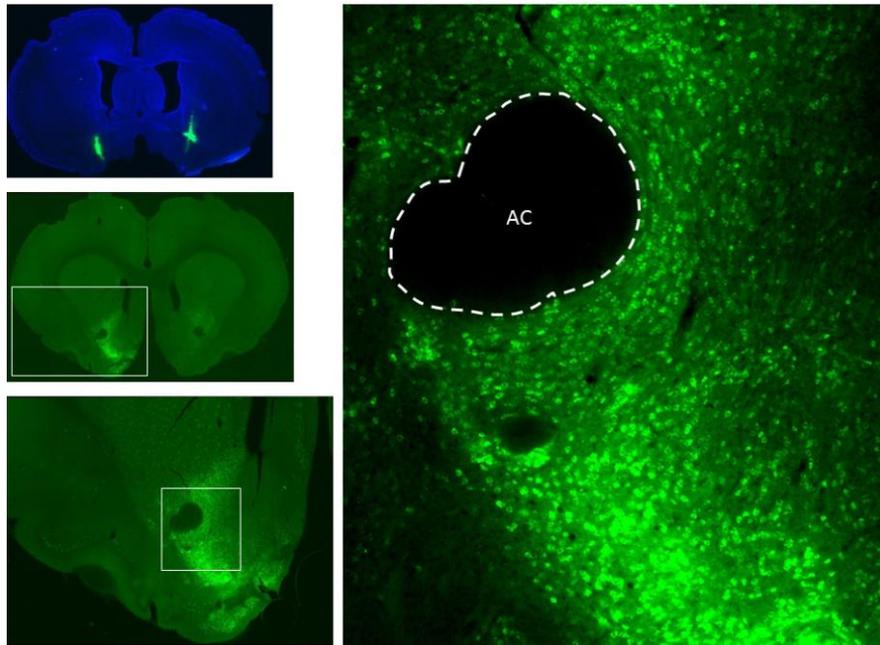


Figure 3.5. Green labeling in the NAcc after AF-CTB injection in the VP. Labeling can be observed predominantly on the lateral core and shell regions of the NAcc (AC: Anterior commissure).

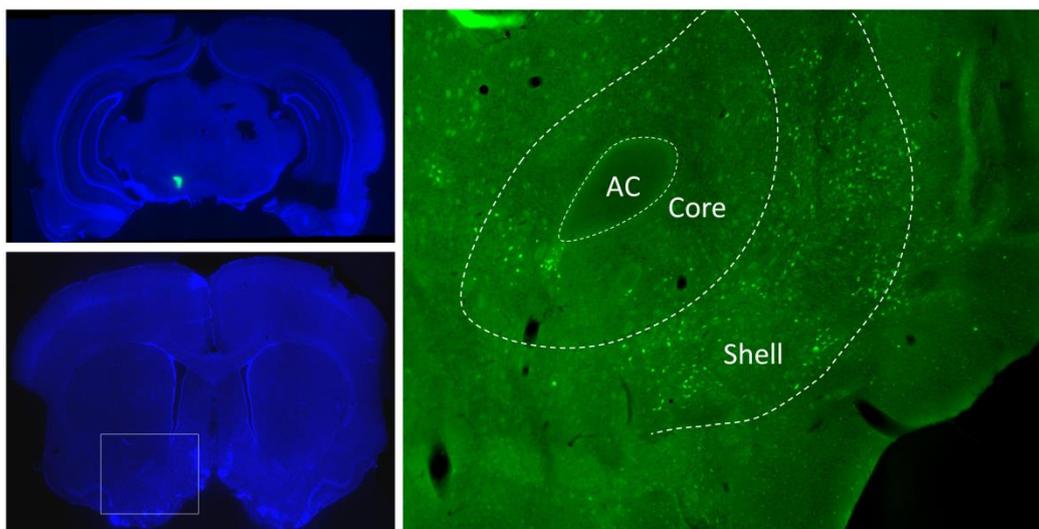


Figure 3.6. Labeling in the NAcc following AF-CTB injection in the VTA. Labeling can be observed predominantly in the medial core and shell regions of the NAcc (AC: anterior commissure).

3.2. Pharmacogenetic manipulation

3.2.1. Behavioral assessment

After establishing the coordinates, we started trials with the infusion of DREADDs viruses targeting accumbal direct (NAcc-VTA) and indirect (NAcc-VP) pathways. Thus, the first set of animals have been infused with *AAV-G_q-DREADDs* in the NAcc and *CAV-Cre* in the VP (CAV-VP group); and a second set of rats underwent the same *AAV-G_q-DREADDs* injection in the

NAcc but received *CAV-Cre* in the VTA (CAV-VTA group). Three weeks after viral infusion (time for the virus to recombine and yield DREADDs expression in the desired pathways) we performed pilot behavioral experiments, on which animals have been tested for potential locomotor alterations caused by the activation of the NAcc-VP or NAcc-VTA pathways through systemic injection of CNO (1 mg/kg). Importantly, the statistical analyses were performed taking into account animals that showed recombination of viruses and consequent DREADDs expression, as analyzed posteriorly through fluorescence microscopy (see §3.2.2 below). In general, we expected that, with the CNO-induced activation of the DREADDs in the NAcc indirect pathway, rats would reduce their locomotor activity. Conversely with the activation of the NAcc direct pathway, we expected to induce an increase in the locomotor response of CNO-treated rats, in comparison with the saline treatment (Farrell *et al*, 2013; Kravitz *et al*, 2010), consistent with results obtained with mice in which DREADDs activation of the ventral striatal D1R-MSNs increased both running and general locomotion while activation of NAcc D2R-MSNs inhibited running and locomotion (Zhu *et al*, 2016).

Thus, in the first experiment the animals received an injection of either saline or CNO and 30 min later placed in the activity cages for the assessment of any locomotor manifestation in response to the novel environment they were being exposed to (i.e. the activity cages). Contrary to our expectations, the comparison of the locomotor activity of rats exposed to a novel environment showed no differences between CNO and SAL treatments in either group (CAV-VP or CAV-VTA) [*student's paired T test*, CAV-VP: $t_{(6)} = 0.30$; *n.s.*, Figure **3.7A**. CAV-VTA: $t_{(8)} = 0.26$; *n.s.*, Figure **3.7C**].

For the second experiment, the animals were habituated for two sessions in the activity cages and in further sessions they were assessed for differences in their basal locomotor activity after CNO injection. Once again, the CAV-VP group showed no differences following the comparison between CNO and SAL treatments [*student's paired T test*, $t_{(6)} = 1.15$; *n.s.*, Figure **3.7B**]. Similar results were observed with the CAV-VTA group, showing a slight increase in response to systemic CNO administration which did not reach significance [*student's paired T test*, $t_{(8)} = 1.95$; $p = 0.08$, Figure **3.7D**]. As the latter results were close to significance, we performed an analysis of the kinetics of this session for both groups. Having time and treatment as factors, two way ANOVA showed no time x group interaction in either case, though a marginal effect can be observed in the CAV-VTA group [two-way ANOVA: CAV-VP $F_{(11,132)} = 1.37$; *n.s.*; Figure **3.8**; CAV-VTA $F_{(11,176)} = 1.73$; $p = 0.07$; Figure **3.9**].

It should be noted that these results were obtained taking into account recombination analyses from fluorescence microscopy data. That is, rats with no labeling were removed from the statistical analysis.

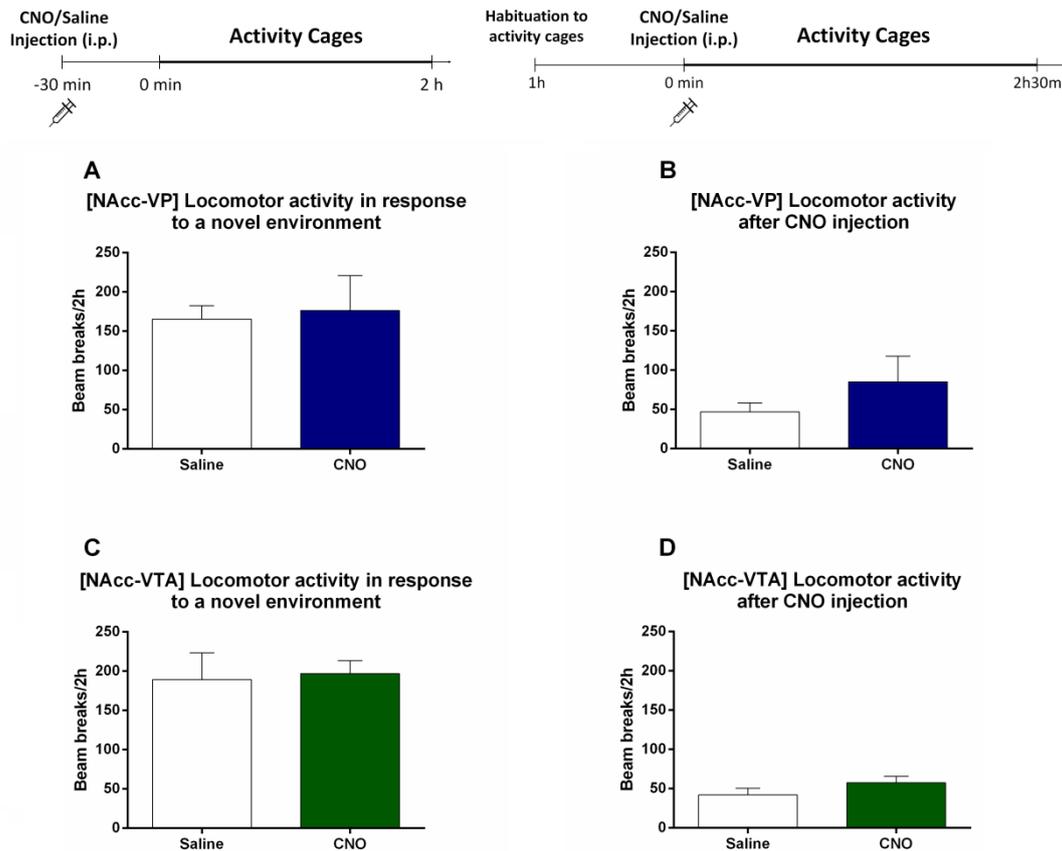


Figure 3.7. Locomotor activity in response to a novel environment and spontaneous locomotor activity after CNO injection. Average locomotor activity from animals treated either with saline or CNO 30 min before being placed for the first time in the activity cages [CAV-VP: **A**; CAV-VTA: **C**]. Average locomotor activity from animals treated either with saline or CNO after being habituated to the activity cages [CAV-VP: **B**; CAV-VTA: **D**]. None of the groups showed behavioral alterations following CNO injection. CAV-VP: $n = 7$; CAV-VTA: $n = 9$.

3.2.2. Confirmation of DREADD recombination

After behavioral assays, animals have been sacrificed and perfused, and had their brains removed for posterior histological analyses, and analysis of the recombination of both viruses. It is worth to notice that the expression of the DREADDs, as well as the *mCherry* red fluorescent protein (RFP) is conditioned by the infection of both viruses in cells within the projection. Example of the expression of red RFP (conveying the expression of the receptor) can be observed on Figure 3.10. Indeed, we could not observe total consistency in the immunoreactivity among the animals in these trials, but fluorescence data conveying the recombination of viruses and DREADDs expression within the NAcc of the rats was collected in order to establish a comparison with the behavioral data from the previous experiment. Akin to the images obtained by CTB tracing, we took into account the behavioral data from animals in whose brains the fluorescence observed in the NAcc for both CAV-VP and CAV-VTA animals. From these samples, fluorescence was observed mainly in the core region of the NAcc.

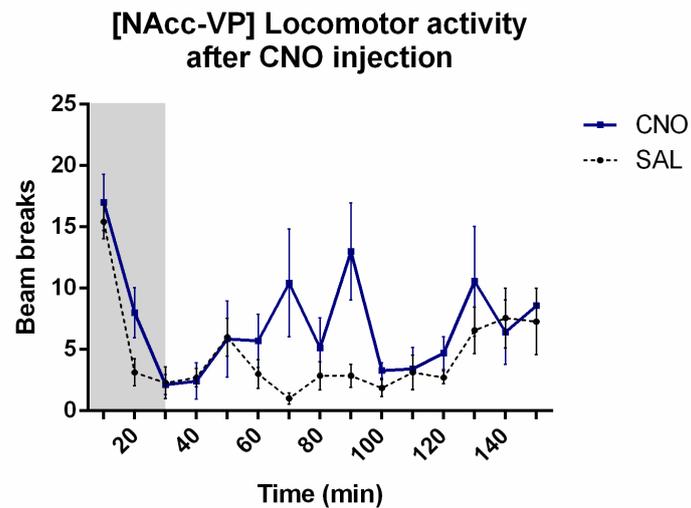


Figure 3.8. Spontaneous locomotor activity after CNO injection of CAV-VP group. Within comparisons of animals treated with either CNO or SAL. Analysis of variance took into account values starting from 30 min. No differences were observed between treatments (n=7).

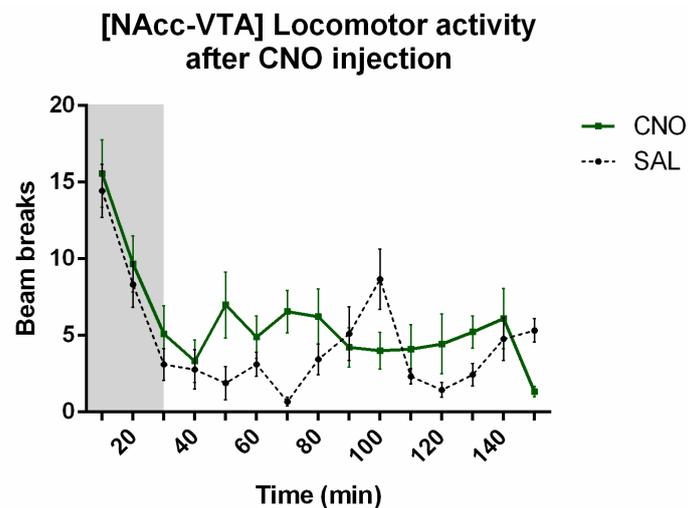


Figure 3.9. Spontaneous locomotor activity after CNO injection of CAV-VTA group. Within comparisons of animals treated with either CNO or SAL. Analysis of variance took into account values starting from 30 min. No differences were observed between treatments (n=9).

4. Discussion

In this section are described a set of preliminary experiments carried out in order to establish a novel methodology in the lab. These experiments consisted in initial trials of neuronal tracing, which eventually gave us the information necessary to perform trials with the infusion of viral vectors that culminated in the expression of DREADD receptors. Our first trials yielded preliminary behavioral data which were later compared with the recombination of DREADDs in the two main projections arising from the NAcc, comprising the direct or indirect pathways.

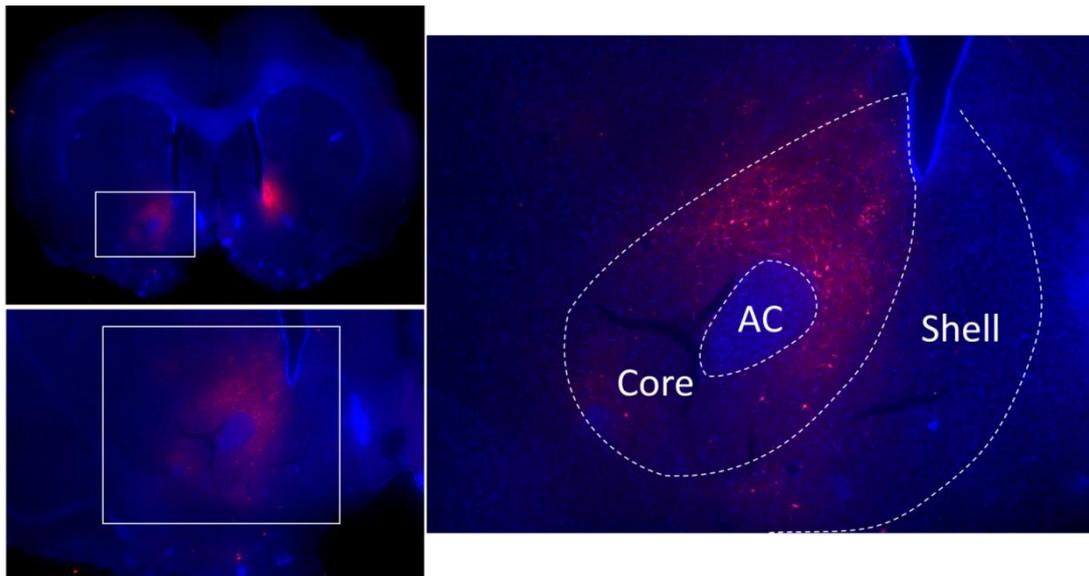


Figure 3.10. Example of *mCherry* labeling in the NAcc. Cell bodies of MSNs stained in the NAcc following recombination of *AAV-DIO-hM₃D(G_q)-mCherry* and *CAV-Cre* viruses injected in the NAcc and in the VP, respectively (AC: anterior commissure).

After establishing the injection sites with data obtained from neuronal tracing, we decided to start with basic behavioral assessments, such as the locomotor response of rats to CNO injections, after animals were injected with AAV-carrying G_qDREADD virus in the NAcc, and CAV-Cre virus in the two principal neuronal targets of the accumbal direct and indirect pathways (i.e. VTA and VP, respectively). This behavioral assessment was chosen in light of the widely accepted role of both pathways in normal locomotor activity (Campbell *et al*, 1997; Zhu *et al*, 2016), as well as their role in response to the administration of DA agents (including psychostimulants) (Campbell *et al*, 1997; Essman *et al*, 1993; Ikemoto, 2002; Kalivas and Stewart, 1991). Indeed, the principal aim of establishing this technique consisted in its application on the study of COC-induced neural and behavioral adaptations, such as those carried out in the two first sections of this work, using COC sensitization and COC SA paradigms. However, we assumed that before carrying out more elaborate experiments with drugs, we should first be able to master this technique under more elementary conditions.

The start of our work dealt with tracing experiments, and with this methodology we managed to confirm the coordinates we should follow to target the same neurons by performing injections two different structures (i.e. the soma in the NAcc and the axons in the target areas, VP and VTA). A great advantage of this tracing technique is precision it offers, as we were able to inject the tracer into a very confined injection site (with 0.3 μ l of AF-CTB), yet still being able to observe considerable labeling patterns in the cell bodies. Although this type of tracing is feasible with other fluorescent conjugates, such as dextran amines (Alcaraz *et al*, 2016),

through observations in initial trials we found AF-CTB to be more stable and precise, showing little to none of the noise we observed with dextran tracing trials.

Also, in these neuronal tracing trials we were able to have an idea of the different regions to which the NAcc projects. Most of our injections targeting the dorsolateral area of the VP yielded labeling in the lateral core of the NAcc, whereas injections that reached more ventromedial portions of the VP were attained by accumbal neurons distributed more medially in the core, in areas close to the shell. Regarding VTA injections, most of our injection sites were reached by neurons in the medial shell. Both of these seem to be in accordance to the literature (Root *et al*, 2013; Zahm and Brog, 1992).

Regarding our behavioral assays, we were not able to observe differences in the activity of animals treated with CNO after having received DREADDs in the NAcc-VP or the NAcc-VTA pathway (Figure 3.7A-D). It should be noted that several rats had to be removed from behavioral analyses, due to absence of observable recombination. Thus, whereas originally the CAV-group VP had 12 rats, and the CAV-VTA had 16, behavioral data from around 40% of rats, had to be removed from behavioral analyses in both groups. Furthermore, we chose to set a margin of 30 min for CNO to bind DREADDs and exert its behavioral effects, after reports that plasma levels of CNO peak within 30 min, declining over the following 2h (Boender *et al*, 2014; Guettier *et al*, 2009; Whissell *et al*, 2016). The behavioral effects of CNO have been reported to be apparent for up to 6 hours after infusion, but a precise assessment of the onset of its behavioral manifestation is a useful parameter, since others have used as little as 10 min (Ferguson *et al*, 2011), or 20 (Farrell *et al*, 2013), 30 (Boender *et al*, 2014), or as much as 45 min after CNO administration as the onset of behavioral assessments (Parkes *et al*, 2017). This parameter would be especially useful in our case, since these were the initial experiments we performed, so the knowledge of the onset of behavioral manifestation of CNO-DREADDs binding would help us define at what moment we should expect to observe differences between treatments in our behavioral data. Indeed, one of our behavioral analyses regarding the CAV-VTA group (second test) was marginally significant if we were to consider the entire 150 min of behavioral assessments. However, once we removed the first 30 min of the behavioral data, the significance was no longer observed (Figure 3.7D). If we take into account the kinetics of the session (Figure 3.9), it is clear that the locomotor activity of the rats was very similar in the first 20 min of either treatment, with a more pronounced (even if still not significant) difference observed in the remainder of the session. On the other hand, differences in the CAV-VP group were overall less close to significance, and despite the CAV-VP group had two peaks of locomotion at 70 min and 90 mins (Figure 3.8), it seems that their locomotion was similar to that of the animals when treated with saline. In general, as both groups (CAV-VP and CAV-VTA)

displayed a low number of crossings in both conditions, the insufficiency of viral recombination cannot be ruled out as a source for the lack of evident behavioral manifestation we observed.

Thus, the lack of significance in our results can be due to the fact that the expression of DREADDs might not have sufficed to exert a behavioral manifestation in our rats. Additionally, the recombination we could observe in the brains was not always anatomically consistent, so that other regions in the vicinity of the NAcc might have been transfected with both DREADDs viruses, being possibly affected by the potential DREADD activation through CNO binding and yielding its influence on behavioral output. In this sense, a factor that might have influenced the recombination (or lack thereof) is the volume of the viruses injected in both regions. As it is difficult to estimate the anatomical spread of the injections, we took care in our NAcc-VP viral transfections to reduce the volume of AAV injected in the NAcc; given its proximity to the VP, we wished to avoid the superposition of the spread of both viruses, which could potentially infect neighbor neurons projecting elsewhere (for instance VP neurons, which may have been also infected by AAV originally aimed at the NAcc) and thus decrease the precision of our study. Similar caution was taken with CAV-Cre in VTA injections: given that the NAcc core also projects to the SNr (Ikemoto, 2007), it is possible that an exceeding amount of CAV-Cre virus injected in the VTA could spread towards SN regions, being taken up by axonal projections of core neurons present in this region and finally also influencing behavioral output once activated by CNO.

Further on, the experiments performed aimed for the manipulation of NAcc pathways. However, akin to our pharmacological experiments described on Section I, our objective was to eventually expand the study to also approach dorsal striatal pathways, so to establish a full comparison of striatal pathways and DA receptor functioning on the reinstatement of COC seeking. In this sense, besides taking into consideration that ventral and dorsal striatal pathways target different regions, through DA receptor-segregated pathways (Gerfen and Surmeier, 2011), another important aspect that should be taken into account regards the downstream elements of DA receptor signaling on striatal MSNs. For instance, whereas D1R activate G_{os} signaling pathway, usually culminating in cell activation and potential GABA release from D1R-bearing MSNs, the activation of D2R engages G_{oi} signaling, which yields opposite modulatory effects on D2-bearing MSNs (Beaulieu and Gainetdinov, 2011). In future studies, as opposed to the hM_3D_q DREADDs we used, the use hM_4D_i DREADDs could more closely relate the activation of D2R in striatal pathways, since their activation recruit downstream targets (through G_{oi}) that eventually yield membrane hyperpolarization and transient neuronal inhibition (Ferguson *et al*, 2011). Thus, as several options of downstream targets are available, the use of DREADDs should consider the neuronal effectors of CNO-DREADDs binding on neuronal

activation or inhibition, if a relation between our pharmacological approach and future DREADD experiments is to be minded (Roth, 2016).

Finally, one of the main obstacles we found in the application of this technique was the assessment of the sufficiency of viral recombination. Indeed, the aim of the fluorescent marker that accompanies the DREADD protein is to give a *post mortem* confirmation that the behavioral effects observed can be credited to the DREADDs, provided its sufficient expression (Lee *et al*, 2014; Smith *et al*, 2016). In this sense, despite our efforts to compare behavioral data to fluorescent images, the definition of a parameter to establish what exactly is *sufficient expression* of DREADDs remains a future challenge in the utilization of this methodology. Therefore, for the sake of validation of behavioral assessments, future experiments with DREADDs demand guidelines for the standardization of quantification analyses that enable experimenters to define criteria for the eventual exclusion of data from animals whose DREADD expression does not suffice to actually elicit a behavioral effect.

Synthesis and Perspectives

Synthesis and conclusions

1. Reminder of the aims

In this thesis, the reinstatement of COC seeking in rats has been studied from different perspectives. The first part dealt with pharmacological interventions to elucidate the role of the dopaminergic receptors of the ventral and dorsal striatal regions using a rat model of self-administration and reinstatement of COC seeking behavior, taking into account previous results from the lab. From that, the next step dealt with a behavioral approach, in the attempt to build model that could shed some light on some of the remaining questions of the pharmacological study. Though we were not able to establish such model, interesting results were obtained, raising interesting questions and possibilities, and enticing for further studies. Finally, the last section of this study had as aim the establishment of pioneering methodologies that, once implemented, could elucidate many questions through a more sophisticated approach.

Thus, the general aims of this thesis were [i] to study the role of the dopamine receptors within the different parts of the striatum in the reinstatement of cocaine seeking behavior; [ii] to develop a behavioral model in which we could study the transition from goal-directed to habitual behavior, also teasing apart the different aspects of appetitive and consummatory behaviors; and [iii] to start the implementation of a novel methodology which allows for the study of drug consumption and relapse from a circuit-based perspective.

2. Summary of results

2.1. Pharmacological investigation: the role of dopamine receptors of the striatum in the relapse of cocaine seeking

In the first part of this work, we found that dopamine receptors act differently within the striatal regions, regarding reinstatement of COC seeking behavior. Previous work in the lab (and by others) has shown that the systemic administration of D2R agonists induces reinstatement of COC seeking, but the stimulation of the D1R does not. Our initial experiments, however, have shown that whereas the pharmacological activation of the D1R subtype within the NAcc induces

reinstatement, the activation of the D2R does not. As these results posed a contrast to the results obtained with the peripheral administration of dopamine receptor agonists, we searched to find where else the D2R could be acting to induce reinstatement. As a second step, we assessed the dorsolateral region of the striatum, with the same pharmacological treatment previously performed in the NAcc. As a result, we observed that the pharmacological activation of D2R in the DLS induces reinstatement, whereas the activation of D1R does not.

From that, we hypothesized that the D2R of the striatum could be major responsible actors in the reinstatement induced by the peripheral stimulation of the D2R. To continue our investigation, we proceeded to assess if the reinstatement triggered by the systemic stimulation of D2R was impeded, or at least diminished, by the pharmacological inhibition of D2R of the DLS. Puzzlingly, the reinstatement was only partially blocked. Further experiments have shown that the pharmacological inhibition of the D1R of the NAcc or the DLS, or the inhibition of the D2R of the NAcc impeded the reinstatement of COC seeking induced by systemic stimulation of D2R.

Thus, it appears that both regions of the striatum act in tandem in the induction of the reinstatement, with the participation of both dopamine receptor subtypes. This might happen in spite of the segregation of the receptors in striatal neurons; one possible mechanism may rely on the collateral connections between D1R- and D2R-MSNs of the striatum, in which D1R and D2R may modulate the activity of each other also mutually modulating the activity of MSNs, finally influencing behavioral output (Philibin *et al*, 2011; Soares-Cunha *et al*, 2016b). Also, we could not discard the influence of one striatal region over the other, in a process that might involve ascending midbrain reaching loops (Belin and Everitt, 2008).

2.2. Behavioral investigation: modelling the transition to the automatization of behavioral control

Following our pharmacological study, several questions remained to be answered. So far, though showing an apparent opposition regarding the results obtained from the systemic activation of D1R and D2R, our results seem to concur with the literature that supports the attribution of the D1R of the NAcc in the processing of rewarding and incentive motivational aspects of drug consumption, as well as the role of D2R of the DLS in the behavioral automatization of responding for drugs (Lobo *et al*, 2010; Soares-Cunha *et al*, 2016b; Vicente *et al*, 2016).

Thus by devising a model that reproduces the transition of behavioral control we could be able to answer several questions that remained from our first study. For instance, if the

behavioral triggers for the reinstatement induced by the D2R in the DLS follow the likely incentive motivational aspects attributed to the activation of the D1R of the NAcc; or whether the relapse triggered by D1R in the NAcc are in some point influenced by any trace of automatism that might be inducing rats to resume COC seeking after the stimulation of the D2R of the DLS.

Thus, we trained rats in a second-order schedule with food reinforcers in order to tease apart the aspects of appetitive responses and consummatory responses and to investigate the transition from goal-directed to automatized behavior through devaluation assays. Once established this model could be applied to COC self-administration studies and help us shed light onto the aforementioned questions.

Rats were trained in the second-order paradigm, in which pressing the seeking lever under a variable interval eventually gave them access to the taking lever. One press on the taking lever delivered the reward. Initially we assessed the effects of devaluation of the outcome by specific satiety in their seeking behavior, in extinction. In this test, animals that have been exposed to the instrumental outcome before the session (and were thus ate it to satiety) showed their actions (SL presses) were driven according to the current value of the outcome (i.e. the food reinforcers): the number of SL presses decreased once the outcome has been devalued by the satiation. In a second test, animals underwent the extinction of TL. The purpose of this assessment was to investigate the influence of their representation of the taking link on their behavior. Importantly, this time the outcome was not devalued; instead, we aimed for the devaluation of the taking link, through its extinction. In this second test, again, animals showed goal-directed behavior, in which animals that underwent extinction of the TL decreased SL presses when tested in extinction. These results convey that, even though the outcome was not devalued, the representation of the value of the TL decreased with the knowledge acquired through the extinction training, that pressing it would not deliver the outcome.

Since we had not observed habitual behavior by then, we decided to perform a COC sensitizing regimen, which according to the literature is believed to accelerate the behavioral shift towards a habitual structure (Nelson and Killcross, 2006; Nordquist *et al*, 2007; Schoenbaum and Setlow, 2005). Following the sensitization procedure, animals were further trained in the second-order paradigm to be then tested for SL after the devaluation of the outcome, again by specific satiety as the first test. Again, animals show sensitivity to the value of the outcome, as the results obtained were similar to those obtained in the first test.

Finally, as the behavior of the rats was still sensitive to the value of the outcome, we decided to train them for 26 further sessions, now increasing the average interval during which they pressed the SL in order to have access to the TL. Then, after further training we performed the same test by devaluing the outcome by specific satiety. As a result, the animals still showed

goal-directed behavior, decreasing lever presses facing a devalued outcome. As a last confirmation, we performed another devaluation paradigm, but this time, through conditioned taste aversion. In this paradigm, the food outcome is repeatedly paired with the malaises induced by LiCl injections, and the aversive component of this protocol is argued to elicit a stronger devaluation effect compared with satiety of the outcome (Nelson and Killcross, 2006). Again, animals displayed sensitivity to the outcome by performing significantly less SL presses when tested in extinction.

As a conclusion, several aspects might have contributed to the apparent resistance of the rats in shifting their behavioral control towards the habitual frame we were expecting, under our protocol. This would include the different goals established by the animals when pressing each link of the chained schedule, as well as the representations that are taken into account when they perform SL presses, whether being the taking link, the outcome itself or, most likely, both. One interesting aspect that should be noted is that the approach to the magazine to collect the food reward might represent an important factor in that the position of the SL in relation to the outcome in drug SA and food SA under second-order schedules is apparently not the same. Also, the fact that preceding the tests, they had been fed to satiety with the outcome they would receive in case they were not tested in extinction might have given the animals the opportunity to learn about the relatively low value of the reward when in the non-deprived state in an incentive learning process.

Regarding the COC treatment, several other studies using normal schedules have trained rats for much shorter periods (Corbit *et al*, 2014a; Nordquist *et al*, 2007), yet successfully inducing habitual behavior in rats, even in the absence of COC treatments (Hitchcott *et al*, 2007; Yin *et al*, 2004). This might raise the question as to whether the behavioral shift is actually feasible under the conditions of our second-order protocol. Still, even in case it is not, an interesting target for future studies would be to search for the reasons why the animals would not acquire habitual behavior even after undergoing COC sensitization treatment and extensive training in the second-order schedule.

2.3. *Pharmacogenetics: preliminary study on novel approaches to investigate striatal output pathways in drug induced relapse*

In this section we worked on the establishment of a new methodology intended for the manipulation of striatal output pathways that would eventually permit the study of these pathways in protocols such as those developed in previous sections of this study. This technique, consisting in the infusion of viral vectors that induce the expression of DREADDs in

specific brain regions permits the activation or inhibition of specific neural pathways through a peripheral infusion of its ligand CNO, in a methodology that is less invasive as well as less stressful for the animals treated.

Logically, as the work carried out in the previous sections dealt with the importance of DA receptors within ventral and dorsal striatum we began this section by working on the striatal pathways that could be related to our pharmacological work and could be aimed in future studies. Indeed, D1R and D2R are not co-expressed in MSNs, but in the NAcc the target of the pathways are less clearly defined: though the D1-MSNs alone project to SN and VTA, composing the direct pathway, both D1R-MSNs and D2R-MSNs in the NAcc form the direct pathway projecting to VP (Kupchik *et al*, 2015; Kupchik and Kalivas, 2017; Smith *et al*, 2013). Taking into consideration this incomplete segregation of both pathways regarding DA receptors, a chemogenetic approach could establish, for instance if the reinstatement observed after stimulation of the D1R of the NAcc was due specifically to the output of the projections targeting the VTA, or somehow influencing the balance of D1R- and D2R-MSNs projecting to the VP, or both. This could be made with DREADDs, once we manage to successfully target either pathway regardless of DA receptor expression, by either eliciting its stimulation or its inhibition.

Thus, through the infusion of retrograde neuronal tracers, we were able to define the regions where the two viruses (AAV DREADDs and CAV-Cre) should be injected in order to have the same cells infected but in two different regions: the NAcc where the cell bodies are found, and the targets of either pathway, namely the VP and the VTA. Once this step has been taken, we started behavioral trials, with viral infusion and expression of excitatory (G_q) DREADDs in either NAcc-VP or NAcc-VTA pathways. To investigate for the behavioral output resulting from the activation of either pathway, we chose to assess the locomotion of animals, given that the stimulation of striatal MSNs of the direct and indirect pathways are believed to exert an inhibitory or excitatory effect on behavior, respectively (Farrell *et al*, 2013; Freeze *et al*, 2013; Kravitz *et al*, 2010; Lobo *et al*, 2010).

Finally the major obstacle we found in the development of the work in this section was the establishment of a reliable method to quantify virus recombination so that we could define a clear threshold to be set for the analysis of behavioral data obtained from DREADDs experiments, which should stand as a priority for future studies using this methodology.

3. Final considerations

3.1. *Why do rats relapse?*

Though several ideas can be put forward, it is no easy task to reason what exactly triggers relapse following pharmacological stimulation of the DA receptors of the NAcc or DLS, especially if we consider the puzzling results obtained from our reversal experiments, in which the reinstatement induced by systemic stimulation of D2R is blocked by local infusion of D1R and D2R antagonists within striatal domains. What is most puzzling in our results is that, though the D1R of the DLS do not induce the reinstatement of COC seeking, they are able to block the reinstatement induced by systemic D2R stimulation. Similarly intriguing, stimulation of the D2R of the striatum by itself does not induce reinstatement, but their inhibition is able to block the reinstatement induced by peripheral D2R stimulation. Further, while the stimulation of D2R of the DLS is able to strongly induce relapse, their inhibition alone is not enough to block the reinstatement induced by the peripheral stimulation of D2R. It is true that one limitation of our work that might have contributed to the ambiguity of our results regards the fact that we did not explicitly target our cannulae implantation to the NAcc core or shell subregions, though cannulae placement was in general localized in the medial core, near its boundaries with medial shell. However, though the observance of these boundaries could have yielded different results, studies that have tried to specify the role of DA receptors in both NAcc subregions still show inconsistent results (e.g. Bachtell *et al*, 2005; Schmidt *et al*, 2006; Schmidt and Pierce, 2006).

Another aspect that should be addressed regards the different possible triggers of relapse. It is possible that the infusion of D1R agonist into the NAcc induces reinstatement through the induction of drug craving, or that the exaggerated attribution of incentive salience to the cues associated with the drug was a decisive aspect. However, the relapse induced by the stimulation of D2R of the DLS might raise questions of the real reasons why rats relapse under these treatments, especially under limited COC SA training. Could it be that the stimulation of the D2R of the NAcc plays with the same incentive motivational attributions? Or is it that the aspects that prompts relapse in this case are already the expression of the automatisms characteristic of habitual behavior?

Usually the stimulation of D1R of the NAcc is associated with the processing of incentive motivational aspects of rewards, whereas the D2R have been associated with the habitual aspects of habitual behavior (Lobo *et al*, 2010; Soares-Cunha *et al*, 2016b; Vicente *et al*, 2016), and these automatisms of habitual behavior are argued to appear with extended instrumental conditioning, when performance is overtrained (Adams, 1982). However, in our experiments of

Section I it cannot be argued that any group of rats (being those who relapsed after stimulation of D1R of the NAcc or those who relapsed after stimulation of the D2R of the DLS) was over trained [as for instance in Zapata *et al* (2010)]. Also, after regular training all animals were tested at similar time points, having had the same history of COC training also with similar amount of total COC consumption.

It is possible that the neural processing that induces reinstatement happens in parallel, with DA signaling in both ventral and dorsolateral striatal domains eliciting reinstatement, but with different motivational or behavioral aspects. The ventral striatal domain would contribute with the incentive motivational features eliciting drug-seeking while the dorsolateral region would simultaneously contribute with the habitual or compulsive component of drug seeking. However, the fact that DA in the DLS has also been shown to be involved in COC reinforcement as much as the NAcc (Veeneman *et al*, 2012) might provide a hint for alternative mechanisms underlying the reinstatement of COC seeking triggered by the stimulation of D2R of the DLS (instead of the suggestion that the habitual behavior might have already ensued with the amount of training our rats have been submitted to). Also in support of this idea, it has been recently shown that the DLS circuitry can enhance the incentive salience of a Pavlovian reward cue, selectively making that cue a stronger motivational magnet (Difeliceantonio and Berridge, 2016). Thus, it could be that, instead of triggering habitual behavior of any sort, the reinstatement of COC seeking observed after stimulation of D2R of the DLS happened by eliciting the representation of the same motivational properties of COC as possibly those energized by stimulation of D1R of the NAcc. That is, the stimulation of both (D1R of the NAcc or D2R of the DLS) might induce the same craving (or maybe less specifically a positive subjective, rewarding feeling), through the same means, during early stages of drug use (before the eventual involvement of habitual behavior), thus eliciting relapse.

Considering this framework, the on-line interactions between both striatal regions observed after extensive training (in a second-order schedule) observed by Belin and Everitt, (2008) could be happening earlier in training, as also suggested by Veeneman *et al* (2015). By infusing the DA antagonist α -flupenthixol unilaterally into the NAcc shell and simultaneously into the contralateral DLS, Veeneman *et al* (2015) managed to show that disrupting DA interconnectivity between NAcc and DLS induced decreased responding for COC under FR1, in rats with limited COC experience. It should be noted, however, that in this case the on-line interactions would not be based on habitual grounds, but on the reinforcing and motivational underpinnings of drug-related behaviors taking place during initial drug use. Therefore, one might consider the possibility that the DA signaling in the DLS or the interconnections between DLS and NAcc that mediate COC reinforcement (or both) might also incite the reinstatement of COC seeking, as observed in our study. Interestingly, if the reinforcing properties of COC are

also processed by the DLS as previously mentioned, this could also suggest that incentive motivational (Difeliceantonio and Berridge, 2016; Veeneman *et al*, 2012) processes cannot be discarded as actors in drug-related behavioral processes later with extended training, given that the DLS is widely argued to be progressively recruited after extended training (Belin and Everitt, 2008; Zapata *et al*, 2010). The latter hypothesis is supported by previous work showing that repeated COC exposure (in this case a COC sensitizing regimen) was enough to produce broad alterations in behavioral control, encompassing both incentive motivational features, in which sensitized rats showed increased Pavlovian to instrumental transfer, and action selection processes, with sensitization inducing an earlier onset for habitual performance (LeBlanc *et al*, 2013). On this matter, it is worth mentioning that increased DA activity in the dorsal striatum is observed during cue-induced COC craving in human addicts (Volkow *et al*, 2006; Wong *et al*, 2006). Obviously, we cannot rule out that later on, in a hypothetical extended training, any habitual aspects might exert a more pronounced influence under the control of the DA activity within the DLS on the induction of COC seeking that we have observed with the stimulation of D2R of this region, but it appears that the DA signaling DLS is already a decisive player in the reinstatement of COC seeking behavior under limited COC SA training.

3.2. *The behavioral control under second-order schedules*

All the aforementioned elucubrations about the driving force triggering the reinstatement we observed in Section I are phenomena the second-order schedule of reinforcement used in Section II could have helped clarify, had we succeeded to model the shift of behavioral control towards an automatized, habitual system. Indeed, we sought to put in practice in our protocol several different features of instrumental conditioning that have been shown to favor (or accelerate) the development of habits. These include the use of extensive training (Adams, 1982) using a variable interval component in our schedule (Dickinson *et al*, 1983) in which high response rates can be maintained, as well as the induction of behavioral sensitization with COC, as the repeated exposure to psychostimulants have been shown to accelerate habit learning with natural reinforcers (Corbit *et al*, 2014a; LeBlanc *et al*, 2013; Nelson and Killcross, 2006; Nordquist *et al*, 2007). To our expectations, this should be enough to weaken the contingency between responses and the delivery of their outcomes, eventually rendering performance of actions insensitive to changes in its value (Balleine *et al*, 2009; Dickinson, 1985; Dickinson *et al*, 1983; Everitt *et al*, 2008; Vandaele and Janak, 2017).

However, the fact that we were not able to induce habitual behavior in rats under our protocol tempts us to put into question the very possibility of inducing habitual behavior under this kind of protocol (specifically using food reinforcers). To some extent, it may be that the

second-order schedule is excessively segmented in a sense of having clearly defined roles for each link of the chain and a clear consummatory action to which behavior can be driven (in the case of chained schedules with food reinforcers). Indeed, when comparing second-order schedules having food reinforcers to those having drug-reinforcers [that have been shown to successfully prompt behavioral automatism with extended training, such as in Zapata *et al* (2010)] through a behavioral point of view, the additional action of approaching the magazine to collect the food pellet might represent a limiting feature which may define an impediment to the onset of behavioral autonomy.

Additionally, it could be that this limiting feature might make second-order schedules with food reinforcers somehow resemble procedures having two actions and two outcomes, which are argued to generate goal-directed actions that are sensitive to outcome devaluation (Halbout *et al*, 2016; Kosaki and Dickinson, 2010; Yin and Knowlton, 2006). For instance, in a set of experiments, hungry rats received extensive training to lever press for food outcomes before one outcome was devalued by aversion conditioning and responding tested in extinction. When the rats were trained on a concurrent schedule in which two responses yielded different outcomes, performance during the extinction test was reduced by devaluation of the associated outcome, conveying goal-directed behavior. Conversely, if a single response was trained concurrently with the noncontingent presentations of the other outcome, test performance was insensitive to devaluation of the contingent outcome (Kosaki and Dickinson, 2010). Another interesting study investigated the effects of COC pre-exposure on goal-directed learning and action selection using a protocol with two actions and two outcomes. During a subsequent outcome devaluation test, both COC- and saline-treated rats showed a strong bias in their choice between the two actions towards whichever action that had been associated with the non-devalued reward. Most intriguingly, when assessing the rats' ability to learn about a selective reduction in the predictive relationship between one of the two actions and its outcome, the COC sensitized rats showed enhanced, rather than diminished, sensitivity to the action–outcome contingency degradation (Halbout *et al*, 2016).

Thus, it can be that the tendency for repeated COC exposure to promote habit formation does not extend to more elaborate complex behavioral scenarios, in which the a hypothetical chunking of actions directed to the SL might be represented as one behavioral unit, eventually followed by the TL press in a sequence ended by further magazine approach and finally the actual consummatory behavior (Dezfouli and Balleine, 2012, 2013; Ostlund *et al*, 2009; Shull and Grimes, 2003).

3.3. *Second-order schedule with COC reinforcement*

It is true that the use of second-order schedules of drug-reinforcement has proven invaluable as it circumvents the conflation of actions that are performed in a simple schedule (FR1) of reinforcement, and it helps to tease apart the appetitive and the consummatory aspects of drug-related behaviors. In a sense, it could be argued that it more closely represent a real-life situation in which humans have to forage for the drug before the actual drug-consumption (Roberts *et al*, 2013; Schindler *et al*, 2002), but this is clearly not a simple task (to say the least) to be implemented with food reinforcers. Still, it could be suggested that the use of second-order schedules of reinforcement using COC as reinforcer, which have previously been shown to induce habit-based behavior (Belin and Everitt, 2008; Zapata *et al*, 2010), could represent a promising alternative in the study of the shift of behavioral control towards a habitual system. Besides allowing for assessments on goal-directed behavior on early on training, with well-defined appetitive and consummatory actions (which can be an alternative for initial investigations), it should be expected that further training in this kind of protocol might elicit the habitual control of behavior.

Finally, the additional use of chemogenetic methodologies could aid with the circumvention of several obstacles that would limit the use of pharmacological approaches in extensive protocols, such as second-order schedules with COC reinforcers. In this sense, these techniques do not require intra-cranial cannulation (with brain-region effects limited to volume of injection, avoiding brain tissue damage elicited by repeated infusions, as well as cannulae clogging, which represents an issue especially with protocols that demand extended training) while targeting specific pathways that may extend over longer projections. This can be performed through a systemic treatment that can be performed repeatedly over long periods, in a non-invasive approach, as previously argued (Rogan and Roth, 2011; Smith *et al*, 2016).

3.4. *Conclusion*

Finally, this work was a first step in the elucidation of the mechanisms involving dopamine receptors of the ventral and dorsal striatal regions in the reinstatement of cocaine seeking behavior. The establishment of model that eventually reproduces automatic, habit-based behaviors could shed light into what is the nature of the processes that prompt cocaine seeking and relapse. Therefore, the insights provided by the association of the aforementioned techniques remains promising, having the potential for providing us with meaningful findings for advancing the field of addiction through a better understanding of how select components of neural circuits, govern behavior.

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ANNEX

Annex

1. Quantification of D1R and D2R in the NAcc after COC SA

The experiments performed in the Section I have shown a double dissociation when D1R and D2R agonists are infused directly into the NAcc and DLS, in which they elicit opposite effects on the reinstatement of COC seeking, depending on the region they are administered. That is, intra-accumbal infusion of D1R, but not D2R, agonist induces reinstatement; whereas intra DLS infusion of the D2R agonist, but not D1 agonist, triggers COC seeking. This raised the question as to which would be the underlying mechanisms for such different effects. One possibility could be that the COC SA regimen might have triggered a change in the expression of the D1R and/or D2R in either region, which could lead to the more evident effect of the stimulation of D1R in the NAcc or D2R in the DLS observed on reinstatement. So we decided to test this hypothesis by assessing the amount of D1R and D2R expression in the NAcc and DS at the same time-point the agonists have been administered in the previous experiments. In order to do that, we trained animals with a similar design of COC SA and extinction paradigm as the previous study, making sure that the amount of COC consumed by the animals was also similar. After the last day of extinction, animals have been sacrificed and had their brains removed for analyses of D1R and D2R expression in the NAcc.

2. Material and Methods

2.1. *Subjects, surgery and COC SA*

The methods regarding animals, intravenous catheter implantation, as well as acquisition and extinction of COC SA utilized in these experiments were carried out exactly in the same manner as those used in the first experiments of this section (See Section I, §Material and methods). However, for this experiment animals of both COC SA (n=11), and SAL SA (n=7) groups did not receive intra-cerebral cannulae implantation, and were killed and perfused, having their brains removed, at the end of the extinction phase of COC SA for the analysis of

the expression of D1R and D2R within the NAcc and DS (experimental design shown on Figure An.1).

2.2. Protein quantification: Western blot analyses

Tissues were homogenized in 500 μ l of cold extraction buffer. Protein concentrations were determined using a DC Protein assay (Bio-Rad, CA), with bovine serum albumin as the standard. Prior to loading, sample buffer was added to each sample. Equivalent amounts of protein (10 μ g) for each sample were resolved in 10% acrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were incubated with powder milk blocking buffer for 1 h at room temperature to block non-specific binding. The blots were reacted overnight at 4 °C with primary antibodies (D1 1:5000; D2 1:1000) and β -tubulin (1:5000). After washing in PBS-T, the blots were incubated in fluorescent secondary antibodies (1:5000) in blocking buffer for 1h at RT. Membranes were then washed three times with PBS-T. Immunolabeling detection and densitometry measurements were performed using the BIO-RAD (Bio-Rad Laboratories, Inc. CA). Ratios of D1 or D2 to β -tubulin densities were calculated for each sample and analyzed across conditions.

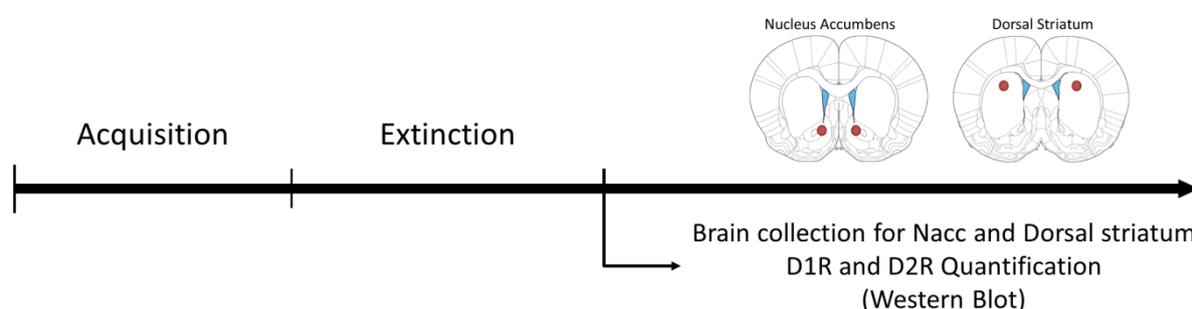


Figure An.1. Experimental timeline. Animals underwent acquisition and extinction in the COC SA paradigm. After extinction, animals were killed and their brains were collected for the quantification of D1R and D2R of the NAcc and dorsal striatum through western blotting.

3. Results

3.1. Acquisition and extinction of cocaine self-administration

Rats of the COC SA group acquired stable responding on the AL [comparison of the last three days of acquisition: $F_{(2,20)} = 1.33$; *n.s.*; averages over the 3 days (\pm SEM): AL 33.36 ± 3.03 ;

IL 1.97 ± 1.59], while the SAL SA group maintained a low level of responding on both levers (AL 8.05 ± 2.20 ; IL 1.97 ± 0.53). During the extinction phase, where COC was replaced by saline, the COC SA group decreased substantially AL presses, eventually stabilizing at a low level of responding [comparison of the last three days of acquisition: $F_{(2,20)} = 0.51$; *n.s.*; averages over the 3 days (\pm SEM): AL 11.70 ± 1.62 ; IL 1.85 ± 0.31 ; SAL SA: AL 9.71 ± 1.57 ; IL 2.57 ± 0.81 ; data not shown. See Figure **An.1** for the outline of experiment].

3.2. COC SA does not alter expression of D1R or D2R of the NAcc or DS

Animals have been sacrificed at the end of the extinction of COC seeking and had their brains removed in order to assess for the expression of D1R and D2R in the NAcc and in the DS. The comparison of D1R and D2R expression within the NAcc after COC SA showed no difference between COC SA and SAL SA groups [Student's *t* test; D1R expression NAcc: $t_{(1,16)} = 0.12$; *n.s.*; D2R expression NAcc: $t_{(1,16)} = 0.13$; *n.s.*; Figures **An.2A** and **An.2B** for D1R and D2R expressions in the NAcc, respectively]. Just as with the NAcc, we also assessed the D1R and D2R expression in the dorsal portion of the striatum of the same rats. Again, the comparison of D1R and D2R expression within the DS after COC SA showed no differences between COC SA and the control SAL SA groups [Student's *t* test; D1R expression DS: $t_{(1,16)} = -0.75$; *n.s.*; D2R expression DS: $t_{(1,16)} = -0.96$; *n.s.*; Figures **An.2C** and **An.2D** for D1R and D2R expressions in the DS, respectively].

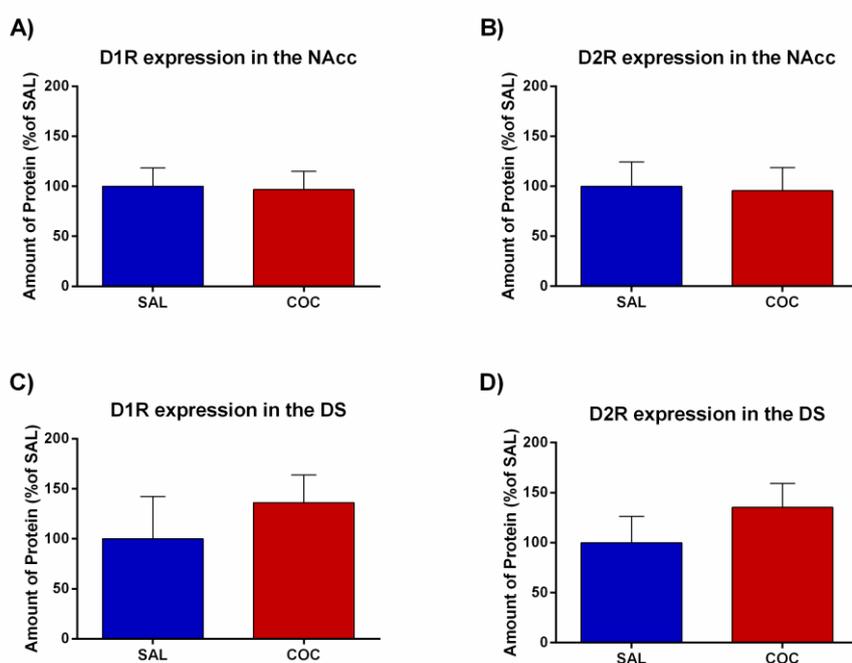


Figure An.2. Expression of D1R and D2R within the NAcc and DS after COC SA. Cocaine self-administration and extinction did not elicit changes in the total expression of D1R and D2R within the striatum (SAL $n=7$; COC $n=11$).