Pathology of the reward system : long term effects of chronic exposure to nicotine and sucrose

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PATHOLOGIE DU SYSTÈME DE RÉCOMPENSE:

Effets à long terme d'une exposition chronique

à la nicotine et au sucrose

Membres du Jury

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THESIS

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DOCTORAL SCHOOL OF LIFESCIENCES AND HEALTH

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Members of the Jury

Dr Véronique DEROCHE – GAMONET, INSERM U862, Bordeaux, France	President
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ABSTRACT

Learning mechanisms associated with active responding for nicotine enhanced the excitability of the ILCx-BNST pathway. The objective of this project was to better understand the involvement of the ILCx-BNST pathway in nicotine self-administration. Since the endocannabinoid system controls nicotine reinforcement and nicotine-induced synaptic modifications, we examined the role of CB1 receptors in the BNST.

We showed that acquisition of nicotine IVSA was associated with a persistent facilitation of LTP induction at ILCx-BNST synapses. Behaviorally, electrical stimulation temporarily increased excessive responding to nicotine when nicotine was not available. Moreover, using intra-BNST pharmacology, we revealed that stimulation of BNST CB1 receptors enhanced sensitivity to nicotine-paired cue. In contrast, after a prolonged history of nicotine intake, it blocked drug-seeking in a reinstatement model of relapse.

Drug addiction is partly due to the inability to stop using despite negative consequences. The hypothesis that palatable food induces similar uncontrolled consumption is becoming more widespread. As drug addiction is known to increases activity of VTA DA neurons, we aimed to examine whether exposure to sucrose would induce similar neuronal modifications and impair the capacity to respond to an aversive stimulus. We found that sucrose enhanced spontaneous activity of DA VTA neurons. In addition, while a footshock caused a nearly complete inhibition of activity of VTA DA neurons in control rats, sucrose disrupted signaling of an aversive stimulus. These modifications were independent from the caloric state of the rats.

Keywords: Nicotine, intravenous self-administration, infralimbic cortex, bed nucleus of stria terminalis, CB1 receptor, *in vivo* electrophysiology, ventral tegmental area, natural reward, aversive stimulus.

RÉSUME

La prise volontaire de nicotine augmente l'excitabilité de la voie ILCx-BNST, entraînant une hyperactivité des neurones DA de l'ATV. Dans une première partie, l'objectif était d'étudier les neuroadaptations de la voie ILCx-BNST induites par l'auto-administration intraveineuse (AAIV) de nicotine. Les récepteurs cannabinoides CB1 contrôlent les propriétés renforçantes de la nicotine. Par conséquent, nous avons examiné le rôle des récepteurs CB1 du BNST.

Nous montrons que l'acquisition de l'AAIV de nicotine est associée à une facilitation persistante de l'induction d'une potentialisation à long terme (LTP) CB1-dépendantes des synapses ILCx-BNST. La stimulation électrique du ILCx favorise également la persistance du comportement de recherche de nicotine pendant les périodes où la drogue n'est pas disponible. En outre, en utilisant la pharmacologie intra-BNST, nous montrons que la stimulation des récepteurs CB1 du BNST au cours de l'acquisition de lAAIV augmente la sensibilité aux stimuli associés à la nicotine.

L'idée qu'il existe un appétit incontrôlable pour les aliments palatables, en dépit des conséquences négatives. Dans une seconde partie, notre projet a porté sur le rôle des neurones dopaminergiques (DA) de l'ATV dans la perception d'un stimulus aversif chez l'animal exposé au sucrose. Nos résultats indiquent que le sucrose augmente l'activité spontanée des neurones DA de la VTA. En outre, si un choc électrique provoque une inhibition presque complète de l'activité de VTA neurones DA chez les rats témoins, le sucrose perturbe la signalisation d'un stimulus aversif, indépendamment de l'état calorique du rat.

Mots clés : Nicotine, auto-administration intraveineuse, cortex infralimbic, noyau du lit de strie terminale, récepteur CB1, électrophysiologie *in vivo*, aire tegmentale ventrale, récompense naturelle, stimulus aversif.

RÉSUMÉ SUBSTANTIEL

Première partie

L'usage du tabac est la principale cause évitable de décès, causant près de 6 millions de décès chaque année (WHO , 2011). La plupart des fumeurs identifient l'usage du tabac comme nocif et expriment le désir de réduire ou de cesser leur consommation. Toutefois, le nombre de rechutes, même après de longues périodes d'abstinence, reste élevé malgré la disponibilité de plusieurs pharmacothérapies visant à traiter la dépendance au tabac (Hughes et al., 1992). Bien que le tabac contienne de nombreux composés, la nicotine est considérée comme le principal composant psychoactif du tabac et de nombreuses études fournissent la preuve de son implication dans le maintien de la dépendance au tabac. La nicotine exerce ses effets renforçants en agissant sur les récepteurs nicotiniques de l'acetylcholine (nAChRs) dans le cerveau. Lorsque la nicotine se lie à ces récepteurs, ils deviennent perméables aux cations entraînant une dépolarisation de la cellule. La nicotine augmente l'activité des neurones dopaminergiques (DA) de l'aire tegmentale ventrale (VTA) par une balance complexe de l'activation des nAChRs des neurones GABA, DA et glutamate.

Le noyau du lit de la strie terminale (BNST), une structure appartenant au complexe de l'amygdale étendue, projette sur la VTA et exerce une influence excitatrice majeure sur les neurones dopaminergiques (Georges et Aston-Jones, 2001, 2002). Le BNST est impliqué dans les comportements liés à la récompense et la toxicomanie (Aston-Jones et Harris, 2004; Dumont et al., 2005). L'une des principales afférences du BNST vient du cortex prefrontal medioventral (mvPFC), et plus particulièrement du cortex infralimbic (ILCx). Le ILCx est un substrat neuronal important pour le contrôle inhibiteur précédent l'extinction de la prise de drogue (Peters et al. , 2008) et également pour l'élaboration de réponses de type automatique, habituel (Coutureau et Killcross , 2003). En outre, dans notre laboratoire, il a été montré précédemment que l'auto-administration de nicotine augmente l'excitabilité de la voie ILCx - BNST, entraînant une hyperactivité des neurones DA de l'ATV (Caille et al., 2009).

Dans le système endocannabinoïde, les récepteurs CB1 jouent un rôle important dans le contrôle de la prise de nicotine (Maldonado et al, 2006; Simonnet et al, 2012). Dans le BNST, les récepteurs CB1 sont localisés sur 90 % des neurones glutamatergiques provenant de l'ILCx et contrôlent l'excitation corticale des neurones du BNST (Massi et al., 2008).

On ignore si une plasticité synaptique se développe entre l'ILCx et le BNST pendant l'AAIV de nicotine, en réponse à une stimulation du ILCx à une fréquence physiologique (10 Hz). Pour répondre à cette question, des rats ont été soumis à l'auto-administration de nicotine pour différentes durées d'entrainement (1 jour, 8 jours et 60 jours), afin d'étudier la corrélation entre l'émergence d'une

potentialisation synaptique et l'acquisition de l'AAIV de nicotine. Par la suite, nous avons testé l'effet d'une faible stimulation (1 minute 10Hz) sur l'activité in vivo des neurones BNST, 24 heures après la dernière séance d'auto administration. En nous basant sur l'hypothèse qu'une neuroadaptation à long terme des synapses pourrait sous-tendre le comportement persistant de recherche de drogue, nous avons également examiné l'effet de la stimulation de 10Hz après un mois d'abstinence de nicotine. Une autre étape a consisté à examiner si la stimulation électrique de la voie ILCx - BNST modifiait le comportement opérant pour la nicotine. Enfin, étant donné que les récepteurs aux cannabinoïdes de type 1 (CB1) jouent un rôle important dans les comportements liés à la nicotine (Maldonado et al, 2006; Simonnet et al, 2012) et que les récepteurs CB1 dans le BNST contrôlent l'excitation corticale des neurones du BNST (Massi et al, 2008), nous avons testé si les changements de neuroplasticité et les changements comportementaux produits par la stimulation à 10 Hz étaient dépendants des récepteur CB1. Nous avons montré que la stimulation à 10Hz du ILCx induit une potentialisation à long terme (LTP) dans le BNST pour les animaux ayant eu une prise volontaire prolongée de nicotine, mais pas pour ceux ayant reçu la nicotine de façon passive. En outre, cette neuroplasticité persiste après 30 jours d'abstinence forcée de nicotine alors qu'une extinction du comportement opérant provoque une dépression à long terme (LTD). Nous avons démontré que la stimulation électrique du ILCx favorise la persistance du comportement de recherche de nicotine pendant les périodes où la drogue n'est pas disponible. Ces résultats révèlent que la consommation volontaire et prolongée de nicotine facilite de façon persistante la potentialisation des réponses excitatrices dans le BNST en réponse à une stimulation de 10 HZ des afférences ILCx. Cette LTP semble contribuer à un comportement stimulus-réponse inadapté, est contrôlée par les récepteurs CB1 (Marsicano et al., 2002) dans le BNST et pourrait être responsable de la vulnérabilité à la rechute induite par les stimuli associés à la prise de drogue. La caractérisation fonctionnelle de la synapse ILCx - BNST aura donc un impact significatif sur la compréhension du phénomène de recherche de nicotine lors de l' abstinence prolongée.

Nous avons également posé la question de l'implication du BNST et des récepteurs aux CB1 dans chacun des différents processus cognitifs et motivationnels impliqué dans la réponse contingent de l'auto-administration de la nicotine (l'acquisition, le maintien, la motivation, l'extinction et la rechute).

Dans le groupe 1, l'agoniste CB1 WIN55 ,212 -2 (WIN55) a été administré avant chaque session des 6 premiers jours d'acquisition, puis nous avons poursuivi l'AAIV de nicotine afin de tester les éventuels effets à long terme du WIN55. Dans le groupe 2, l'effet de l'injection intra-BNST du WIN55 a été testé sur le maintien de l'AAIV et la motivation pour la prise de nicotine. Dans le groupe 3, une

procédure d'extinction a été réalisée après 5 semaines d'AAIV de nicotine et les effets du WIN55 ont été testés sur la rechute.

Dans le groupe 1, nous avons constaté que la stimulation des récepteurs CB1 du BNST altère de façon transitoire la première étape de l'apprentissage de l'AAIV de nicotine, principalement en raison d'une réduction de l'activité générale des animaux. Cet effet disparaît dès l'arrêt du traitement. Cependant, ultérieurement, les rats prétraités au WIN55 sont incapables de s'adapter aux différents changements de conditions expérimentales : à l'augmentation de la charge de travail pour obtenir la nicotine, à la fois pour l'augmentation d'un ratio fixe et d'un ratio progressif de renforcement. Enfin, le prétraitement au WIN55 n'affecte pas la réinstallation du comportement de recherche de nicotine induite par la nicotine ou par des stimuli précédemment associés à la drogue. Dans le groupe 2, la stimulation des récepteurs CB1 dans le BNST n'affecte pas la réponse opérante pour un ratio fixe ou pour un ratio progressif de renforcement. Dans le groupe 3, après extinction du comportement d'AAIV, l'injection de WIN55 bloque la rechute induite par les indices précédemment associés à la prise de nicotine et par la nicotine elle-même.

Ces données impliquent que la stimulation des récepteurs CB1 dans le BNST, avant l'exposition à long terme à la nicotine, induit des difficultés prolongées pour s'adapter à l'augmentation de la charge de travail et diminue de la motivation pour la nicotine, sans toutefois altérer la prise. En outre, les rats traités avec le WIN55 pendant la phase d'acquisition de l'AAIV de nicotine sont encore sensibles à la nicotine et aux indices sensoriels associés. Cependant, après exposition à la nicotine, alors que l'agoniste CB1 dans le BNST n'affecte pas la motivation et de la prise de nicotine, il bloque les propriétés incitatives de indices sensoriels associés à la nicotine et de la nicotine elle-même.

L'objectif de cette partie de la thèse était d'examiner l'implication de la voie ILCx-BNST dans le développement d'un comportement motivé par la nicotine. Avec les résultats obtenus, nous pouvons dire que cette voie est recrutée pendant l'AAIV prolongée de nicotine, avec une facilitation de la potentialisation persistante des réponses excitatrices dans le BNST en réponse à une stimulation de 10Hz. Par ailleurs, nous avons montré que cette voie est importante et critique pour la bonne acquisition de l'association entre la drogue et les stimuli associés, et qu'elle est contrôlée les récepteurs CB1 du BNST. En revanche, la voie ILCx et les récepteurs CB1 du BNST ne semblent pas impliqués dans le contrôle des effets renforçants directs de la nicotine. Afin de déterminer le rôle central de ces récepteurs dans le développement de l'« incentive salience » qui motive la réponse excessive aux stimuli associés à la nicotine, d'autres recherches sont nécessaires. Ce travail est une première étape dans la caractérisation de la voie d'ILCx - BNST et de son implication dans la dépendance à la nicotine. Si l'activation de cette voie est un substrat neuronal du comportement de

stimulus-réponse, cela pourrait avoir des implications pour la compréhension de la recherche persistante de drogue et représenter une cible possible pour traiter la vulnérabilité à long terme à la rechute. Par conséquent, la (pharmaco)thérapie visant à réduire les afférences excitatrices du ILCx sur le BNST peut aider à réduire la recherche de drogues chez les toxicomanes humains.

Deuxième Partie

La nourriture est essentielle à la survie des animaux. Il a été démontré que les processus de récompense, de la motivation et de l'apprentissage jouent un rôle important dans le comportement d'approche des ressources vitales. Cependant, dans un environnement en constante évolution, les animaux doivent adapter leur comportement en réponse à des stimuli aversifs et s'engager dans un comportement d'évitement. L'aire tegmentale ventrale (VTA) est une structure importante du système de récompense, ensemble de structure mises en jeu dans l'élaboration des comportements liés à l'obtention d'une récompense. Des données électrophysiologiques démontrent que des neurones dopaminergiques de la VTA sont impliqués dans le traitement des stimulus aversifs, et y répondent principalement par une inhibition de leur activité de décharge (Ungless et al., 2004). Ce serait cette adaptation du système DA qui permettrait à l'animal d'établir une réaction d'aversion et d'évitement (Di chiara et al., 1999).

L'addiction aux drogues est en partie liée à l'incapacité d'arrêter la prise en dépit des conséquences négatives. Ce concept a été modélisé chez l'animal dans le modèle de l'auto-administration de cocaïne. En effet, les études montrent que les rats « addicts » à la cocaïne maintiennent leur comportement d'approche pour obtenir la drogue malgré qu'ils reçoivent des chocs électriques (Deroche-Gamonet et al., 2004; Vanderschuren and Everitt, 2004). L'idée qu'il existe un appétit incontrôlable pour les aliments très palatables est de plus en plus répandue. Récemment, plusieurs études se sont attachées à modéliser le concept de l'addiction au sucre chez l'animal. Toutefois, contrairement aux rats exposés à la drogue, les rats exposés au sucrose semblent encore en mesure de présenter un comportement d'évitement quand un stimulus aversif est présenté (Pelloux et al., 2007). Cette dernière donnée pose la question de l'effet d'un état hédonique induit par le sucrose sur l'activité spontanée de base des neurones dopaminergiques du VTA et s'ils sont en mesure de traiter l'information d'un stimulus aversif.

Pour répondre à cette question, nous avons utilisé un paradigme oral d'auto-administration de sucrose 5%. Les rats ont été soumis à au moins 3 semaines d'auto-administration de sucrose ou d'eau. Ensuite, l'activité spontanée des neurones dopaminergiques de la VTA a été enregistrée. Très rapidement, la consommation de liquide des rats « sucrose » est largement supérieure à celle des rats « eau », ce qui est très probablement lié à la forte valeur hédonique du sucrose 5%. En outre, la

motivation pour le sucrose est significativement plus élevée chez les rats s'auto-administrant le sucrose par rapport aux rats contrôles. Les enregistrements électrophysiologiques *in vivo* ont montré une forte augmentation de l'activité spontanée des neurones dopaminergiques de la VTA après auto-administration de sucrose, ceci indiqué par l'augmentation du taux de décharge, une tendance à l'augmentation du taux de burst et une augmentation des décharges moyennes par burst. Ensuite, nous avons examiné l'effet d'un stimulus aversif sur le profil de décharge des neurones dopaminergiques du VTA. Pour ce faire, un choc électrique a été appliqué lors de l'enregistrement électrophysiologique et la réponse (inhibition, d'excitation ou d'absence de réponse) du profil de décharge a été évaluée. Chez les rats « sucrose » tant que chez les rats « eau », les populations de neurones DA ont répondu dans les mêmes proportions : environ 50 % ont présenté une inhibition, moins de 10% ont présenté une excitation et environ 45% n'ont pas répondu. Toutefois, lorsque nous avons analysé dans le détail la réponse des neurones présentant une inhibition, nous avons trouvé une différence significative entre les groupes dans la durée de l'inhibition au cours d'un choc électrique de 4 secondes. Les neurones de rats qui se sont auto-administrés l'eau ont montré une inhibition presque complète de l'activité de décharge, alors que les neurones des rats qui se sont auto-administrés du sucrose ne montrent qu'une inhibition temporaire.

En raison de la valeur calorique du sucrose, nous sommes conscients de l'impact que cela pourrait avoir sur le comportement de la prise alimentaire, et donc sur l'activité VTA DA que ce soit à l'état basal ou en réponse au choc électrique. Par conséquent, nous avons comparé ces constantes électrophysiologiques après d'auto-administration de sucrose chez des rats nourris *ad libitum* avec des rats soumis à un régime restreint. Nous avons constaté que les rats avec un régime alimentaire restreint consomment plus de sucrose que les rats *ad libitum* et sont plus motivés pour obtenir un volume de sucrose. Les enregistrements électrophysiologique n'indiquent pas de différence dans le taux de décharge par rapport à des rats *ad libitum*, mais le taux en burst était significativement plus élevé. En revanche, la durée de l'inhibition en réponse à un choc électrique n'est pas différente.

Dans l'ensemble, nous avons démontré que l'auto-administration de sucrose est capable d'induire un état de type hédonique, avec une prise alimentaire élevé et une forte motivation pour le sucrose. Par la suite, cet état de type hédonique induit des neuroadaptations au niveau des neurones dopaminergiques de la VTA qui sont caractérisés par une augmentation du taux de décharge et dans une moindre mesure du taux de burst. Des données probantes montrent que l'augmentation de l'activité des neurones DA de la VTA est conséquente à l'augmentation de l'activité des afférences glutamatergiques (Marinelli et al., 2006). Nos données sont en accord avec l'étude récente qui a montré que l'auto-administration de sucrose augmente la force excitatrice sur les neurones DA de la VTA, indiqué par une augmentation du rapport AMPA / NMDA (Chen et al., 2008). Cependant,

contrairement aux drogues d'abus, cette augmentation dans la potentialisation synaptique après auto-administration d'une récompense naturelle est transitoire et facilement inversée par une période d'abstinence d'un mois (Chen et al., 2008). Le BNST est un candidat pouvant être responsable de l'excitation accrue des neurones DA de la VTA. Le BNST envoie des projections glutamatergiques directement dans le VTA (Georges et Aston-Jones, 2001, 2002). Il a été montré que la plasticité des synapses excitatrices dans le BNST est associée à l'apprentissage opérant pour la nourriture et l'abus des drogues (Dumont et al. , 2005). En outre, l'hyperactivité des neurones DA VTA après auto-administration volontaire de nicotine est entraînée par des changements des afférences excitatrices du BNST (Caille et al., 2009).

En outre, si un choc électrique provoque une inhibition presque totale de l'activité des neurones dopaminergiques du VTA chez les rats qui travaillent pour un renforçateur neutre, l'exposition à une renforçateur très palatable a provoqué une inhibition transitoire en réponse à un choc électrique. L'habenula latéral (LH) est une zone du cerveau connue pour être impliquée dans le traitement des stimuli aversifs. Le LH envoie des projections glutamatergiques sur les neurones GABAergiques dans le RMTG qui inhibent des neurones DA VTA (Kaufling et al., 2009). En utilisant des outils d'optogénétique, il a été démontré que l'activation des afférences de la LH sur les neurones dopaminergiques du VTA induit une aversion chez la souris et favorise un comportement d'évitement (Lammel et al., 2012; Stamatakis et Stuber, 2012). On peut spéculer que l'état de type hédonique diminue l'influence de ces circuits perturbant les voies de signalisations des mécanismes d'aversion.

L'état calorique des rats n'a pas d'incidence sur le taux de décharge, mais a augmenté le taux de burst des neurones dopaminergiques de la VTA. En outre, la réactivité à un stimulus aversif après l'auto-administration de sucrose n'est pas différente entre les rats nourris *ad libitum* et les rats nourris avec un régime alimentaire restreint. Cela implique que la combinaison de l'exposition à une solution palatable et l'apprentissage d'un comportement opérant perturbe la signalisation aversive, indépendamment de l'état calorique de l'animal. Basé sur ces résultats, nous concluons que dans nos conditions expérimentales l'état de type hédonique modifie le système de récompense et perturbe la signalisation à un stimulus aversif qui pourrait avoir des conséquences négatives pour l'intégration des stimuli de l'environnement et de l'acquisition d'un comportement adéquat.

LIST OF PUBLICATIONS

Anne-Ruth REISIGER, Olivier MANZONI, Martine CADOR, François GEORGES and Stephanie CAILLE
Nicotine seeking depends on the CB1 receptor-dependent potentiation of Infralimbic Cortex – Bed Nucleus Stria Terminalis excitatory synapses.
Submitted

Anne-Ruth REISIGER, Martine CADOR, François GEORGES and Stephanie CAILLE
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In preparation

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A hedonic state induces hyperactivity of ventral tegmental area dopamine neurons and disrupts encoding of an aversive stimulus.
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POSTER PRESENTATIONS

Anne-Ruth REISIGER, Martine CADOR, Olivier MANZONI, François GEORGES and Stéphanie CAILLÉ
Endocannabinoid-dependent long term potentiation in the BNST gates compulsive nicotine seeking

- Scientific day, Bordeaux Doctoral School, Arcachon, France, March 28, 2012
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- Scientific day, Bordeaux Doctoral School, Arcachon, France, April 10, 2013

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LIST OF ABBREVIATIONS

2-AG	2-arachidonoyl glycerol
AC	adenylyl cyclase
AEA	anandamide
BNST	bed nucleus of stria terminalis
CB1	cannabinoid-1
CB2	cannabinoid 2
Cli	caudal linear nucleus
CNS	central nervous system
CPP	conditioned place preference
CRF	corticotrophin-releasing factor
CS	conditioned stimulus
DAG	1,2-diacylglycerol
DAGL	1,2-diacylglycerol lipase
DSI	depolarization-induced suppression of inhibition
DSE	depolarization-induced suppression of excitation
ECs	endocannabinoids
FAAH	fatty acid amide hydrolase
FR	fixed ratio
GPCR	G protein coupled receptor
HPA	hypothalamo–pituitary–adrenocortical
i.c.	intracerebral
IF	interfascicular nucleus
ILCx	infralimbic cortex
i.p.	intraperitoneal
IVSA	intravenous self-administration
KO	knock-out
LH	lateral habenula
LTD	long-term depression
LTP	long-term potentiation
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
NAc	nucleus accumbens
NAcC	nucleus accumbens core

NAcS	nucleus accumbens shell
nAChRs	nicotinic acetylcholine receptors
NAPE	N-arachidonoyl phosphatidylethanolamine
NAPE-PLD	N-acylphosphatidylethanolamine-hydrolyzing phospholipase D
NAT	N-acyltransferase
PBP	parabranchial pigmented nucleu
PFC	prefrontal cortex
PFR	parafascicular retroflexed area
PHAL	Phaseolus vulgaris-leucoagglutinin
PLC	phospholipase C
PN	paranigral nucleus
PR	progressive ratio
PSTH	peristimulus time histogram
PVN	periventricular nucleus
RLi	rostral lineas nucleus
RMTg	rostromedial tegmental nucleus
SA	self-administration
SNc	substantia nigra pars compacta
THC	delta^{9}-tetrahydrocannabinol
US	unconditioned stimulus
VTA	ventral tegmental area
VTT	ventral tegmental tail
WIN55	WIN55,212-2

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PREFACE

This Ph.D. thesis is based on the work carried out in the laboratory of « Neuropsychopharmacology of addiction » CNRS UMR 5287, in collaboration with Dr. François Georges, CNRS UMR 5297. It is based on three experimental studies, one of which has been submitted to a peer-reviewed journal and two of which are in preparation.

Tobacco use is the leading preventable cause of death, causing almost 6 million deaths annually (WHO, 2011). Most smokers identify tobacco use as harmful and express a desire to reduce or stop using it. However, relapsing rates, even after long periods of abstinence, remain high despite the availability of several pharmacotherapies aimed at treating tobacco addiction (Hughes et al., 1992). Although tobacco contains numerous compounds, nicotine is considered the main psychoactive component of tobacco and many studies provide evidence for the role of nicotine is sustaining tobacco addiction (Stolerman and Jarvis, 1995).

Nicotine use, like other drugs of abuse, leads to powerful and long-lasting memories of the drug experience. With repeated exposure, persistent synaptic neuroplastic changes develop, resulting in the expression of addictive behavior such as compulsive drug seeking. It is well accepted that drugs of abuse usurp the neuronal circuitry normally involved in natural reward.

The first part of this thesis starts with an overview of the literature on nicotine addiction, the neurocircuitry involved in nicotine addiction and the endocannabinoid system, which is a strong neuromodulatory system involved in the induction of persistent modifications following drug exposure. Then two articles are presented where we examined the involved of the ILCx – BNST pathway in associative learning of nicotine self-administration in rats. It has been shown previously in our laboratory that this pathway is important for nicotine reinforcement learning, but it is not known whether nicotine exposure facilitates LTP-induction. Moreover, this part of the thesis addresses the involvement of the CB1 receptors of the endocannabinoid system in the motivational processes of nicotine addiction.

In the second part, since natural reinforcers and drugs of abuse share a common neural pathway, we tested the hypothesis that a natural reward induces modifications similar to those caused by drugs of abuse in the VTA of the dopaminergic reward system. As drug addiction is characterized by the inability to stop the drug despite negative consequences, we examined the ability of these neurons to process information related to an aversive stimulus after exposure to a natural reward.

MATERIALS AND METHODS

MATERIALS AND METHODS

The experiments described in Part I and Part II require different experimental design. However, the main techniques and common protocols used throughout the thesis are described in this section.

1. Operant conditioning

1.1. Operant chambers

Operant conditioning for intravenous nicotine and oral saccharin and sucrose was conducted in operant chambers (30 cm height x 40 cm length x 35 cm depth, Imetronic, Pessac, France) located in an experimental room equipped with white noise generators. Each experimental chamber was individually housed in attenuation boxes fitted with ventilation fans and had two Plexiglass walls on the front and back sides and two opaque panels in the right and left sides. The floor consisted of 6 mm diameter steel bars. Two nose-poke devices ('active' and 'inactive') were located on either side of the chamber. In order to record locomotor activity, each chamber was equipped with two pairs of infrared beams (**Figure 1**).

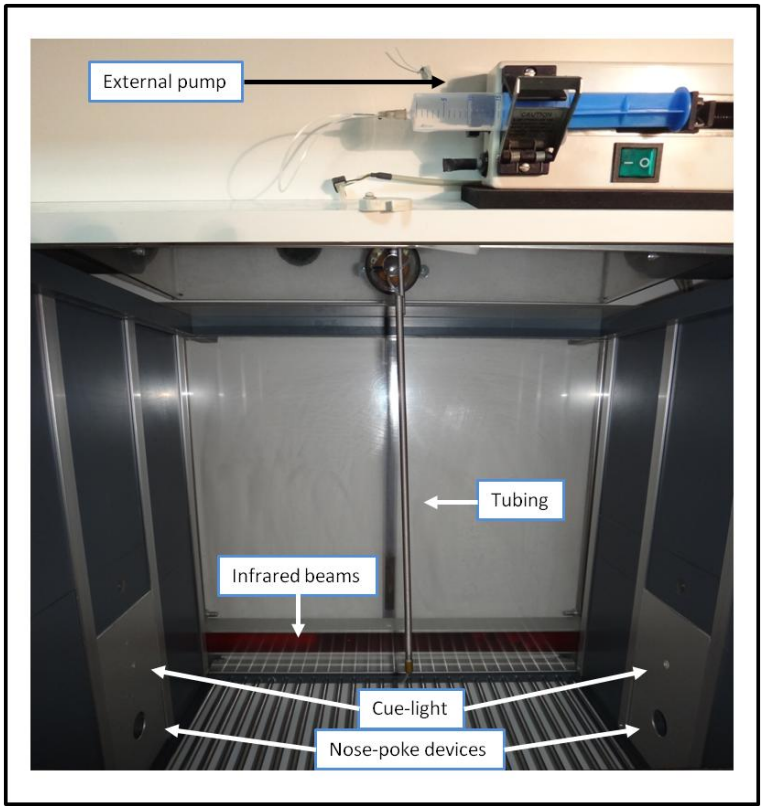


Figure 1 Operant conditioning cage for intravenous nicotine self-administration.

1.2. Intravenous nicotine self-administration

The beginning of the 2 h self-administration session was indicated by illumination of the house light and a single non-contingent infusion of the drug solution. Activation of the active nose-hole resulted in the infusion of 100 µl of nicotine (30 µg/kg/infusion) over 4 s, and was accompanied by the illumination of a white cue-light for 3 s, positioned above the nose-hole (**Figure 1**). A 20 s time-out period followed each infusion whereby activation of the active nose-hole had no consequences. Inactive nose-hole responses were recorded but had no programmed consequences.

1.3. Oral saccharin and sucrose self-administration

The cages described for oral self-administration were the same cages used for nicotine IVSA. However, these cages were equipped with a dipper for solution delivery. Thirty min sessions started with the illumination of the house light. Responses in the active nose-hole resulted in the delivery of 112 µl volume of 5 % sucrose or 0.13 % saccharine over 4 s via a fluid injection assembly into the dipper cup (**Figure 2**).

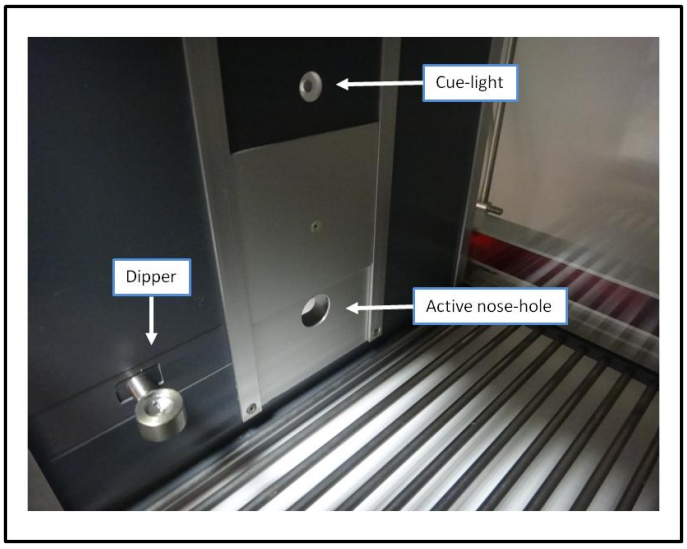


Figure 2 Operant conditioning cage for oral self-administration.

2. Stereotaxic surgery

Stereotaxic surgery was required for implantation of guide cannulae for application of intracerebral injections and for *in vivo* electrophysiological recordings. Surgeries were performed under inhalation anesthesia.

2.1. Guide cannulae implantation

In order to perform intra – BNST infusions, animals were stereotaxically implanted with bilateral 22-gauge, 10-mm stainless steel guide cannulae that terminated 2 mm above the BNST. The stereotaxic coordinates were 0.3 mm posterior to bregma, 3.8 mm lateral to the midsagittal sinus and 6.3 mm ventral to the level of the dura mater with a 19.7° lateral angle in order to prevent perforation of the ventricles (**Figure 3** Paxinos and Watson, 1998). Four anchor screws were placed. Cannulae were fixed using dental cement and obturators were placed.

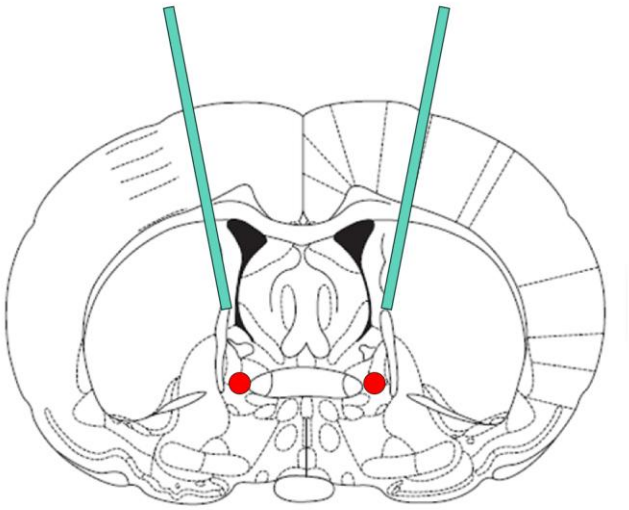


Figure 3 Schematic representation of the guide-cannulae implantation. Coordinates of guide cannula: 0.3 mm posterior to bregma, 3.8 mm lateral to the midsagittal sinus and 6.3 mm ventral to the level of the dura mater with a 19.7° lateral angle (bars). Infusion sites of WIN55,212-2 was 2 mm lower (red circles).

2.2. Recording and electrical stimulation site for *in vivo* electrophysiology

Stimulation electrodes and recording and injection pipettes were inserted into ILCx, BNST, or VTA at the following coordinates (Paxinos and Watson, 1998): ILCx: +3.0 mm from bregma, 0.5 mm from midline and 4.5 mm from dura; BNST: -0.3 mm from bregma, 1.5 mm from midline, 6.0–7.5 mm from dura, VTA: -5.3 mm from bregma, 0.7 mm from midline and 7.5 mm – 8.5 mm from brain surface (**Figure 4**).

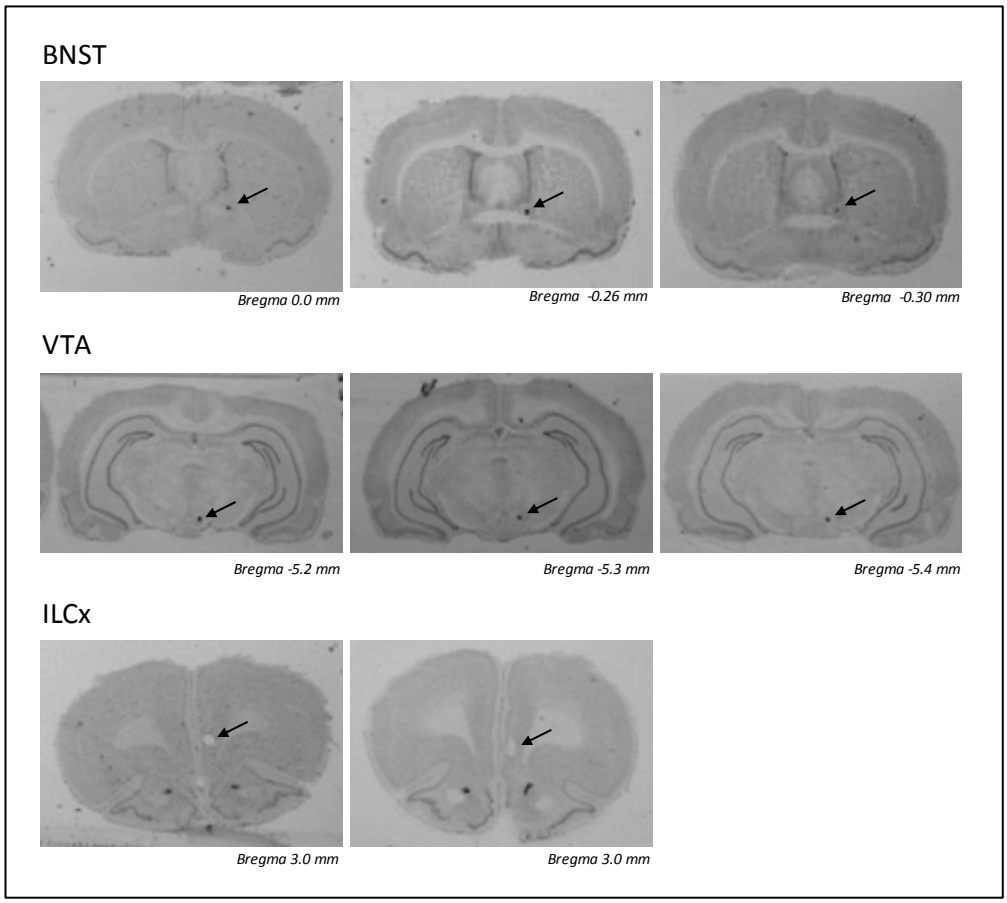


Figure 4 Histological representation of valid placement of recording electrodes in the BNST and VTA marked with a blue spot and stimulation electrodes in the ILCx marked with a lesion (arrows). Paxinos and Watson, 1998.

PART I

The involvement of the ILCx – BNST pathway in nicotine addiction

INTRODUCTION

INTRODUCTION

1. Nicotine addiction

Tobacco smoking is one of the leading causes of premature, preventable death. Annually, tobacco use is associated with more than 5 million deaths (WHO, 2011). Although other constituents may contribute to the addictive properties of tobacco (Fowler et al., 1998), nicotine is assumed to be the main addictive component of tobacco (Stolerman and Jarvis, 1995). Nicotine is one of the most heavily used addictive drugs; 35 to 40 percent of the persons who try smoking develop nicotine addiction (USDHHS, 1994). Moreover, ninety percent of smokers who attempt to quit fall in into relapse within 12 months (Garvey et al., 1992). Therefore, it is clear that a better understanding of the neurological mechanisms of nicotine addiction could help us develop a therapeutic strategy.

1.1. Definition of addiction

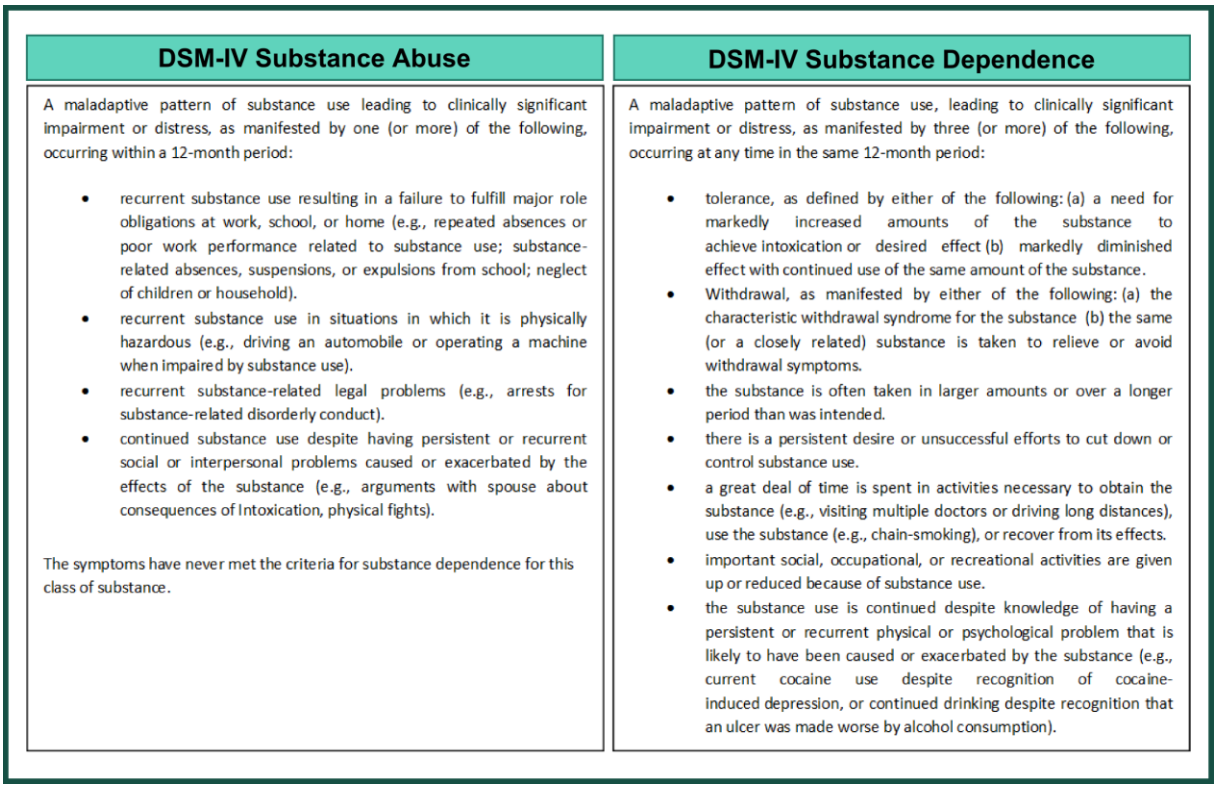
Most people try a potentially addictive drug at least once in their life. This use is occasional and controlled. However, with repeated exposure, a small percentage (20 – 40 %, depending on the type of drug) of those using drugs develops drug abuse and ultimately drug addiction (Nutt et al., 2007) which manifests as an intense desire for the drug with an inability to control intake, despite negative consequences (Volkow and Li, 2004).

The Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) (APA, 1994) used three distinct levels of substance or drug use:

1) occasional, controlled use;
2) substance abuse or harmful use, and
3) substance dependence or drug addiction.

Figure 5 gives an overview of the criteria used to differentiate between substance abuse and substance dependence according to DSM-IV. In 2013, DSM-5 (American Psychiatric Association, 2013) was released, which has revised the terminology of substance abuse and substance dependence. In DSM-5, the DSM-IV criteria for substance abuse and substance dependence have been combined into **substance use disorder**, specific to each substance. Each substance use disorder is divided in to mild, moderate and severe subtypes. A combined list of DSM-IV and DSM-5 criteria[1] allows clinicians to specify how severe the substance use disorder is, depending on how many symptoms are identified; two or three symptoms indicate a *mild* substance use disorder, four or five symptoms indicate a *moderate* substance use disorder, and six or more are required to diagnose *severe*

[1] Of note, there are two major changes in the new DSM-5 criteria for substance use disorder: 1) «Recurrent legal problems» criterion for substance abuse has been deleted from DSM5 and 2) a new criterion has been added: «Craving or a strong desire or urge to use a substance.



DSM-IV Substance Abuse	DSM-IV Substance Dependence
A maladaptive pattern of substance use leading to clinically significant impairment or distress, as manifested by one (or more) of the following, occurring within a 12-month period: • recurrent substance use resulting in a failure to fulfill major role obligations at work, school, or home (e.g., repeated absences or poor work performance related to substance use; substance-related absences, suspensions, or expulsions from school; neglect of children or household). • recurrent substance use in situations in which it is physically hazardous (e.g., driving an automobile or operating a machine when impaired by substance use). • recurrent substance-related legal problems (e.g., arrests for substance-related disorderly conduct). • continued substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance (e.g., arguments with spouse about consequences of Intoxication, physical fights). The symptoms have never met the criteria for substance dependence for this class of substance.	A maladaptive pattern of substance use, leading to clinically significant impairment or distress, as manifested by three (or more) of the following, occurring at any time in the same 12-month period: • tolerance, as defined by either of the following: (a) a need for markedly increased amounts of the substance to achieve intoxication or desired effect (b) markedly diminished effect with continued use of the same amount of the substance. • Withdrawal, as manifested by either of the following: (a) the characteristic withdrawal syndrome for the substance (b) the same (or a closely related) substance is taken to relieve or avoid withdrawal symptoms. • the substance is often taken in larger amounts or over a longer period than was intended. • there is a persistent desire or unsuccessful efforts to cut down or control substance use. • a great deal of time is spent in activities necessary to obtain the substance (e.g., visiting multiple doctors or driving long distances), use the substance (e.g., chain-smoking), or recover from its effects. • important social, occupational, or recreational activities are given up or reduced because of substance use. • the substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance (e.g., current cocaine use despite recognition of cocaine-induced depression, or continued drinking despite recognition that an ulcer was made worse by alcohol consumption).

Figure 5 Official criteria used to diagnose substance abuse and substance dependence according to DSM-IV.

substance use disorder. The DSM-5 revision aims to clarify the definition of *dependence*, which is often misinterpreted as implying addiction. However, features of physical dependence, such as tolerance and withdrawal, can be normal responses to medications that affect the central nervous system. Therefore, the new DSM-5 criteria recognize that mental and behavioral aspects of substance use disorders are more specific to substance use disorders than the physical domains of tolerance and withdrawal.

Drug addiction, including nicotine addiction, is a complex phenomenon that starts with molecular interaction of the drug with its target. This alters the activity and metabolism of the drug-sensitive neurons. Over time, this will change the properties of individual neurons and consequently complete circuits, which leads to behaviors such as dependence, tolerance, sensitization and craving (Benowitz, 2008). Therefore, pharmacotherapies to aid smoking cessation should ideally reduce nicotine withdrawal symptoms (*e.g.* cravings, irritability and anxiety) without causing excessive adverse effects. So far, available therapies have shown to be marginally successful (Mitrouska et al., 2007).

1.2. History of nicotine use

Tobacco is the only known natural source of nicotine. Nicotine is derived mainly from the tobacco plant *Nicotiana tabacum* (**Figure 6A**). There is no evidence for tobacco use before the 15th century, but it is generally accepted that indigenous groups in the North and South of America were the first and only users of tobacco. In 1492, Christopher Columbus was the first to bring some tobacco leaves and seeds to Europe (Hajdu and Vadmal, 2010). Tobacco was fast spread as a cure for many diseases; it was claimed as a remedy for colds, headache, tooth problems, ingestion problems and many other illnesses. However, over time, it was recognized that tobacco use was not so harmless as initially thought. Medical use of tobacco gradually decreased from the 18th century, but was replaced by smoking for pleasure. In 1931, Hoffman published the first data on the relationship between smoking and lung cancer (Hoffman, 1931). From then on, evidence accumulated for the negative consequences of chronic tobacco use (Auerbach et al., 1957; Doll and Hill, 1950; Hammond and Horn, 1958).

Tobacco products are generally divided into two types: smoked tobacco and smokeless tobacco. Examples of smoked tobacco are cigarettes, cigars and hookah (waterpipe). Smokeless tobacco refers to a number of tobacco products that are used by means other than smoking. These include chewing, sniffing and application to the skin (**Figure 6B**).

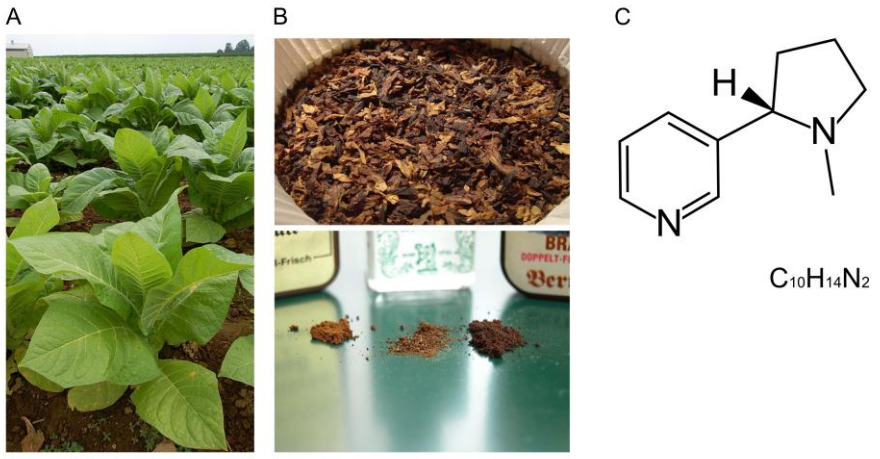


Figure 6. Nicotine is considered the main addictive component of tobacco. (A) tobacco plant **(B)** different forms of tobacco use. Upper image: loose tobacco used to role cigarettes. Lower image: fine-ground tobacco, insufflated through the nose. **(C)** molecular structure and chemical formula of nicotine.

1.3. Nicotinic acetylcholine receptors

Nicotine, an alkaloid (**Figure 6C**), acts as an agonist at nicotinic acetylcholine receptors (nAChRs). nAChRs are members of a family of pentameric ligand-gated transmembrane ion channels, consisting of various heteromeric or homomeric combinations of α ($\alpha_2 - \alpha_{10}$) and β ($\beta_2 - \beta_4$) subunits (Gotti et al., 2006). Although the biophysical and pharmacological properties of nAChRs are highly dependent on the subunit composition and location of the receptors (Wooltorton et al., 2003), they share basic features. Brief exposure to high concentration of agonist results in opening the cation-selective pore, causing a depolarization. After a couple of milliseconds, the receptor closes to a non-conducting state. In contrast, prolonged exposure to low concentrations of an agonist, as is the case in tobacco use, produces desensitization of nAChRs, which stabilizes the receptor in a closed state that is insensitive to agonist (Wooltorton et al., 2003).

Nicotinic receptors are ubiquitously present in the central and peripheral nervous system (Dani and Bertrand, 2007; Gotti and Clementi, 2004). Within the central nervous system, the majority of nicotinic receptors are localized presynaptically, where they are able to modulate the release of almost all neurotransmitters (Wonnacott, 1997). They can also be found at the level of the preterminal, axon, dendrite and soma (Zarei et al., 1999). nACRs containing β_2 subunits (β_2* nAChRs, of which $\alpha_4\beta_2$ is the most abundant) and α_7* are the two main subtypes of nAChRs, and are widely expressed in the brain (**Figure 7**) (Gotti et al., 2009), whereas others have a more restricted distribution pattern. The α_7 receptor is characterized by fast activation, low affinity and high permeability to Ca^{2+}. In contrast, $\alpha_4\beta_2$ has a high affinity and slow desensitization (Marks et al., 2006). The regions with highest density of high affinity nAChRs include areas of the mesolimbic dopaminergic pathway, in particular the ventral tegmental area (VTA) and nucleus accumbens (NAc) (Gotti et al., 2006). The homopentamer α_7 is expressed in the cortex, hippocampus, amygdala and olfactory bulb (Seguela et al., 1993).

1.3.1. Role of different subunits in nicotine reinforcement

The development of knock-out (KO) mice with targeted deletion of specific subunits of nAChRs and the re-expression of a deleted gene (Maskos et al., 2005) has led to the identification of complex subtypes and allowed the study of individual subtypes in cells and neurobiological systems. There is considerable diversity in sensitivity and affinity of different subtypes to nicotine. This leads to differences in channel activation and subsequent desensitization in chronic presence of nicotine.

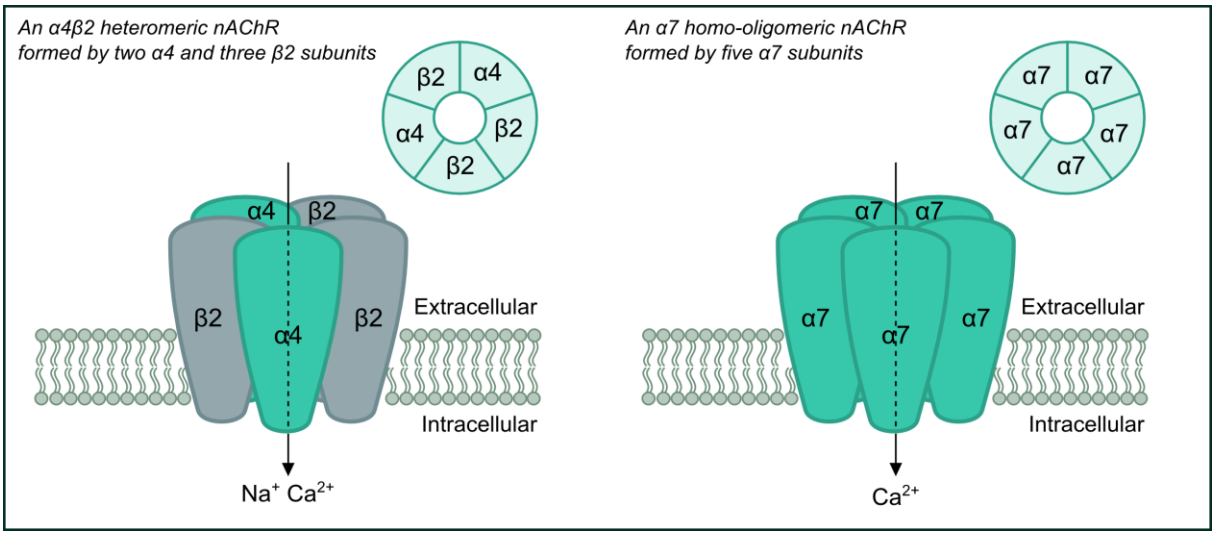


Figure 7. Schematic representation of the two most common subtypes in nAChRs. Both receptors consist of five subunits. The α4β2 nAChR is composed of two α4 subunits and three β2 subunits and called an α4β2 heteromeric nAChR (left). The α7 nAChR consists of five α7 subunits and is called an α7 homo-oligomeric nAChR (right). In both nAChRs, the subunits are arranged around a central pore or channel that opens when ligands such as acetylcholine or nicotine bind, allowing positively charged ions to flow through the channel into the cell. The α4β2 nAChR allows passage of both calcium (Ca2+) and sodium (Na+), whereas the α7 nAChR principally allows passage of Ca2+. From (Davis and de Fiebre, 2006).

An important line of evidence reveals the importance of β_2*-containing subtypes in reinforcing effects of nicotine in a conditioned place preference paradigm (CPP) (Walters et al., 2006). Moreover, mice with a specific deletion in β_2*-containing subtypes do not self-administer nicotine (Lena and Changeux, 1999). In addition, mice lacking α_4 and α_6 subunits also did not self-administer nicotine intravenously. This behavior was rescued by re-expressing the subunits in the VTA (Pons et al., 2008). The involvement of the α_4 subunit in the reinforcing effects of nicotine was confirmed by others, using self-administration of nicotine directly into the VTA (Exley et al., 2011). Deletion of α_5 (Fowler et al., 2011) or overexpression of β_4 subunits (Frahm et al., 2011) enhance self-administration of nicotine in mice, suggesting a modulatory effect of these receptors on the rewarding effects of nicotine (Changeux, 2010).

In contrast, the involvement of the α_7 receptor in nicotine addiction is less well established and there are discrepancies between the results (Grottick et al., 2000; Markou and Paterson, 2001). Studies performed on α_7 KO mice did not show a difference in preference for nicotine compared to wild-type animals (Walters et al., 2006), and nicotine self-administration is reduced with a low dose of nicotine, but is no longer different from wildtype mice when the dose is increased (Besson et al., 2012). Neuronal α_7 nAChRs are expressed on presynaptic terminals where they can modulate glutamate release (Mansvelder and McGehee, 2000). While they have a minimal effect on the excitability of dopaminergic neurons in adult tissue slices (Mao et al., 2011), the presynaptic effects are larger in younger animals (Placzek et al., 2009). Varenicline, a full agonist of α_7 receptors (Mihalak et al., 2006),

has been shown to decrease nicotine self-administration in rats (George et al., 2011). However, as varenicline also acts as a partial $\alpha_4\beta_2$ agonist, the involvement of these subunits in decreasing nicotine intake cannot be ruled out.

Overall, these results provide evidence that nAChRs containing β_2 and α4, and to some extent α5, α6 and β_4 mediate the rewarding effects of nicotine. Although some controversy exists on the behavioral involvement of α_7, it seems clear that this subunit has a role in nicotine-induced synaptic plasticity.

1.3.2. Nicotine-induced upregulation of nicotinic receptors

Chronic nicotine use is associated with an upregulation of nAChRs in the brain. This might be a consequence of the rapid desensitization of nAChRs (Govind et al., 2009). The loss of receptor function would promote up-regulation in order to compensate for the reduced signaling (Fenster et al., 1999). Physiological concentrations of nicotine exposure (100-200 nM) result in an increase of high-affinity nAChRs $\alpha_4\beta_2$ (Buisson and Bertrand, 2001). Higher concentration of nicotine can induce up-regulation of other subtypes, such as α_7 (Molinari et al., 1998). The nicotine-induced compensation in receptor numbers also depends on the way nicotine is administered. For example, the number of α_7 receptors is increased by self-administration of nicotine (Parker et al., 2004), while a decrease was found after exposure to nicotine by an osmotic minipump or drinking water (Lai et al., 2005; Mugnaini et al., 2006). Most likely, several mechanisms for nicotine-induced upregulation of nAChRs are involved, including alteration in receptor assembly (Nashmi et al., 2003), trafficking (Harkness and Millar, 2002) and decreased turnover (Wang et al., 1998).

1.3.3. The effects of nicotine on neural activity

Nicotine, like all drugs of abuse, hijacks the brain's reward system by modulating dopamine release in the NAc. It is demonstrated that nicotine mediates its reinforcing properties by increasing firing activity of dopaminergic neurons in the VTA (Marti et al., 2011). The VTA is the origin of the mesocorticolimbic dopamine pathway and projects mainly to the NAc and prefrontal cortex (PFC). The VTA receives both excitatory and inhibitory input from different brain areas. The principal glutamatergic input includes projections from the PFC (Carr and Sesack., 2000). GABAergic afferents arise mainly from the NAc and ventral pallidum, as well as interneurons (Kalivas et al., 1993). A balance in the activity of these inhibitory and excitatory influences determines the firing of VTA DA neurons. Nicotine stimulates bursting activity in VTA neurons, resulting in enhanced dopamine release in the NAc (Pontieri et al., 1996). Indeed, electrophysiological data show that acute and chronic nicotine exposure increases the spontaneous activity of DA neurons in the VTA (Caille et al.,

2009; Pidoplichko et al., 1997). Accordingly, both acute and chronic exposure to nicotine elevates dopamine levels in the nucleus accumbens (Nisell et al., 1997; Pontieri et al., 1996).

1.4. Animal models

Studies on the neuronal mechanisms of addiction, including nicotine addiction, depend mainly on the availability of suitable animal models. Because of the complexity of drug addiction, it is not possible to fully mimic the human condition. However, animal models do permit investigation of specific elements of the drug addiction process, including tolerance, withdrawal, loss of control over intake and relapse.

There are several animal models available to study nicotine dependence. The most commonly used ones are: conditioned place preference (CPP), non-contingent exposure and intravenous self-administration (IVSA). The CPP paradigm evaluates the rewarding effects of a drug by the development of conditioned preference for distinct drug-paired environments through Pavlovian conditioning (Carr et al., 1989). In rodents, achieving nicotine-induced CPP appears to be challenging compared to other drugs of abuse, and contrary results have been reported (Jorenby et al., 1990; Le Foll and Goldberg, 2005) possibly due to the weaker rewarding properties of nicotine.

Non-contingent exposure to nicotine is a simple and efficient way to induce dependence in animals. Experimenter-administered subcutaneous (s.c.) injections or s.c. minipumps of nicotine have been very useful in identifying the acute and chronic effects of nicotine on a wide range of drug-related behaviors, such as locomotor activity (Clarke and Kumar, 1983), withdrawal (O'Dell et al., 2004) and tolerance (Morgan and Ellison, 1987).

Nevertheless, IVSA of drugs is generally considered the most direct measure of the reinforcing properties of drugs of abuse in animals and emerge to be the animal model with the highest validity to study addiction. The drug self-administration paradigm was developed to examine the behavioral and neurological mechanisms of drug reinforcement (Schuster and Thompson, 1969). Nicotine IVSA is demonstrated in numerous species (Stolerman, 1999), including rats (Corrigall and Coen, 1989; Shoaib et al., 1997), mice (Fowler and Kenny, 2011; Stolerman et al., 1999), non-human primates (Goldberg et al., 1981) and humans (Sofuoglu et al., 2008).

The next sections will focus on the IVSA paradigm as it is the animal model for nicotine addiction used for the experiments throughout this thesis.

1.4.1. Validation of the IVSA model of nicotine addiction

The nicotine IVSA animal model has been proven to be a powerful tool to study behavioral and neurobiological aspects of nicotine reinforcement. However, some debate exists concerning the voluntary aspect of the IVSA paradigm (Ahmed, 2010) due to lack of choice (Carroll et al., 1989). Usually, to evaluate the validity of an animal model, three main criteria are used: face, construct, and predictive validity (O'Dell and Khroyan, 2009). The IVSA paradigm is based on the principles of rewards that involves strengthening of a behavioral response by presentation of nicotine after execution of an operant response.

1.4.1.1. Face validity

« Are the overt behavioral qualities seen in the human condition measured in the animal model? »

The face validity is commonly the start point of the development of an animal model. The IVSA model is seen as the model with the highest face validity of voluntary tobacco use. (O'Dell and Khroyan, 2009). Drug IVSA is commonly used to study addiction and aims at unraveling the underlying mechanisms. However, voluntary drug taking, measured in a drug IVSA paradigm, is not the only criterion of addiction (DSM-IV, DSM-5) and does not necessarily imply that the subject is addicted to the drug. Therefore, Deroche-Gamonet and colleagues (2004) developed a behavioral model that differentiates rats that control their drug use from rats undergoing transition to addiction. They used three criteria that are also used to diagnose substance use disorder in humans: 1) the subject has difficulty stopping drug use or limiting drug intake; 2) the subject has an extremely high motivation to take the drug and 3) substance use is continued despite harmful consequences. In this study 17 % of the rats met the three criteria and thus was considered to show addiction-like behavior. This percentage is similar to that of human cocaine users diagnosed as addicts (Nutt et al., 2007). Although this model was developed for cocaine self-administration, one may predict that similar criteria could account for nicotine administration.

1.4.1.2. Construct validity

« Is the theoretical principle underlying human nicotine addiction similar to that in the animal model? »

The construct validity is probably the most important, but most difficult criterion for the validation of an animal model. It is difficult to determine the level of construct validity because the underlying mechanisms are not fully understood. The underlying substrates mediating nicotine addiction are complex and involve behavioral, cognitive and physiological aspects, of which some of them are hard to measure in animals.

1.4.1.3. Predictive validity

« Are pharmacotherapies used in clinical settings effective in the animal model? »

Animal models with a high predictive validity are able to evaluate whether a novel drug possesses abuse liability in humans. The IVSA paradigm has been proven to have a high level of predictive validity; it has been shown that non-human animals self-administer nearly every drug that is abused by humans, but not hallucinogens (Collins et al., 1984).

In terms of pharmacotherapy, there are several medications available, such as nicotine replacement therapies (NRT), bupropion and varenicline. However, the results are limited, as at best about a fifth of smokers are able to maintain abstinence for 12 months with these approaches (Schnoll and Lerman, 2006). The results obtained from animal models on the effects of bupropion on nicotine IVSA are not consistent. Some studies show an increase in nicotine IVSA, while others report a decrease (Bruijnzeel and Markou, 2003; Shoaib et al., 2003). However, many compounds that reduce nicotine dependence in a laboratory setting, have failed in clinical trials. A possible explanation for the limitations in the use of the current animal model is the absence of other chemicals found in tobacco that might contribute to the reinforcing effects of smoking (Belluzzi et al., 2005; Villegier et al., 2006).

1.4.2. Procedural parameters

In contrast to other drugs of abuse like cocaine and heroin, stable rates of nicotine IVSA are difficult to establish and therefore, careful control of several experimental parameters, such as diet conditions, operant devices and availability of secondary cues is required (Chaudhri et al., 2006; Le Foll and Goldberg, 2005; Stolerman and Jarvis, 1995).

Concentration of drug

The dose-response curve for nicotine IVSA is an inverted U-curve, like for other drugs of abuse. Yet, nicotine IVSA compensation in responding with dose is restricted to ends of the curve. This partial compensation appears to be characteristic to nicotine IVSA across species (Corrigall and Coen, 1989). It appears that the optimal dose lies between 0.03 and 0.06 mg /kg, while a dose of 0.1 mg /kg is aversive and has been reported to cause seizures (Corrigall and Coen, 1989). On the other hand, a dose as low as 0.003 mg /kg does not reliably maintain IVSA, due to lack of reinforcing effects (Donny et al., 1995).

Response operandum

For most drugs, IVSA involves lever pressing but this operant behavior appears to be more difficult for nicotine IVSA. Therefore, for both mice and rats, operant cages are often equipped with nose-poke devices. It has been demonstrated that nicotine IVSA by a nose-poke device both facilitates and

sustain responding (Clemens et al., 2010). It is suggested that this type of responding correlates better with the natural exploratory behavior of rodents.

Pre-training with food and diet control

Because of difficulties with establishing nicotine IVSA, some laboratories use instrumental training with a natural reinforcer prior to the start of nicotine IVSA to facilitate training (Liu et al., 2007; Shram et al., 2008). Yet, it does not seem to affect nicotine intake across subsequent sessions (Clemens et al., 2010). Moreover, pre-training with food may bias the learning process and it has been shown to influence later susceptibility of rats to reinstatement (Clemens et al., 2010). Therefore, more often, to facilitate responding, rats are subjected to a restricted diet (20 g /day). This is sufficient to maintain growth and body weight (Donny et al., 1998). Food restriction enhances the number of infusions during IVSA sessions (Donny et al., 1998).

Nonpharmacological stimuli

Nicotine IVSA in rats is usually performed with the presence of a nonpharmacological stimulus (auditory or visual cue), associated with the delivery of nicotine. Converging evidence from several studies suggest that they considerably contribute to the acquisition and maintenance of nicotine self-administration (Caggiula et al., 2002a; Caggiula et al., 2002b). Progressively, the cue will become established as conditioned stimuli because of repeated association with nicotine, and will acquire motivational value. This is consistent with finding that nicotine intake can be reinstated by presentation of these conditioned stimuli (Goldberg et al., 1983).

Access to nicotine

Typically, nicotine IVSA is performed under limited-access conditions of daily 1- or 2-h sessions. This results in stable nicotine intake for weeks (Caille et al., 2009). However, in order to better mimic the human condition of smoking, some studies have used an extended access protocol of 6 to 23 h a day (Kenny and Markou, 2006; LeSage et al., 2002; O'Dell et al., 2007). Yet, unlike other drugs of abuse (Ahmed and Koob, 1999; Greenwell et al., 2009), extended nicotine IVSA does not show escalation in drug intake, but rather a reduction intake followed by stabilization (Kenny and Markou, 2006; Valentine et al., 1997). Interestingly, extended (21 h /day), but intermittent (every 24 – 48 h) access to nicotine IVSA did result in escalation profile, in combination with enhanced motivation to obtain nicotine in a progressive ratio schedule of reinforcement (Cohen et al., 2012). It is suggested that the appearance of a negative withdrawal state is required for the development of escalation to nicotine (Gilpin et al., 2012).

The various access conditions to nicotine used for studying nicotine addiction raise questions about the validity of each protocol. Dependent smokers maintain relatively stable nicotine blood levels

during waking hours (Benowitz and Jacob, 1984), which would point to a preferential access to nicotine for ~12h daily. However, 25-33 % of the human smokers population are light smokers (one to five cigarettes/ day) or non-daily, intermittent smokers (Nasim et al., 2012). This group is thought to use nicotine occasionally and to not be dependent on tobacco. Animal studies show that 1-2 h daily access to nicotine results in stable nicotine intake levels, motivation for nicotine and high relapse rates after abstinence, which are all important characteristics of nicotine addiction. Therefore, 1-2h daily access seems to be a valid protocol to study nicotine addiction in rodents.

1.4.3. Different stages of IVSA paradigm

The IVSA paradigm allows for longitudinal studies and performing several behavioral tests within subjects.

1.4.3.1. Acquisition

Acquisition of drug IVSA refers to the initial use of a drug, a transition from occasional use to an increase over a period of hours, days or weeks to a stable rate of intake (Campbell and Carroll, 2000). During the acquisition phase, rats progressively acquire to self-administer nicotine by learning the instrumental contingency between the response and reward. Usually, the protocol starts with fixed-ratio (FR) 1 schedule of reinforcement and over days increase to FR2 and FR5. With the increase of FR schedule, rats are able to adapt the responses in order to obtain a constant level of nicotine.

1.4.3.2. Maintenance

After having acquired IVSA, nicotine IVSA can be maintained for weeks or months (Caille et al., 2009). During the maintenance phase of IVSA, the reward or the reward-paired cue develops incentive value causing compulsive motivation to take the drug. Using intracranial self-administration, is has been shown that voluntary nicotine intake increases the sensitivity of the reward system, which last at least 36 days after nicotine self-administration had stopped (Kenny and Markou, 2006). In contrast to other drugs of abuse, such as cocaine (Ahmed and Koob, 1998), prolonged exposure to nicotine does not result in an escalation of intake, (Paterson and Markou, 2004), which is defined as a progressive increase in drug consumption over time that becomes excessive, overwhelming, and difficult to control.

1.4.3.3. Progressive ratio

The cost-benefit aspect of drug addiction is not fully addressed by the IVSA paradigm. However, studies try to tackle this aspect by using a progressive ratio (PR) schedule of reinforcement. In this procedure, the response requirement increases for each successive drug delivery, and the breakpoint (the point at which the animal will to longer respond to obtain a drug delivery) is determined (Hodos,

1961). This paradigm evaluates the reinforcing efficacy of the drug and the motivation of the animal to work for it.

1.4.3.4. Abstinence and extinction

Withdrawal can be induced by forced abstinence or extinction training. During abstinence, the animal is confined to the homecage for a prescribed amount of time. Under these conditions, at the time of relapse testing, the drug-taking behavior and drug-associated cues in the drug-taking environment are thought to be preserved (Reichel and Bevins, 2009). In contrast, for rats that are subjected to learn that the environment that supplied them with drug does not do so anymore, the drug seeking behavior will be suppressed after several sessions of extinction training. Therefore, extinction is conceptualized as inhibitory learning and/or a new learning in which associations between the drug and the paired-cue are weakened and new associations being developed (Myers and Carlezon, 2010). The abstinence model might better simulate the human condition and thus allow for the study of potential treatment to prevent relapse (Reichel and Bevins, 2009).

1.4.3.5. Reinstatement

Relapse is defined as the reinitiation of drug seeking and drug-taking after a period of abstinence (Stewart, 2008). The majority (90 %) of smokers who try to quit, relapse within 1 year of abstinence (Garvey et al., 1992). In animals, relapse can be studied after a period of forced abstinence or after extinction training in the reinstatement model of relapse. Drug seeking can be triggered by stress, drug-associated (conditioned) cues and re-exposure to the previously experienced drug (Fattore et al., 2009; Fuchs et al., 2004; Shalev et al., 2000). These three conditions are also known to cause relapse in humans (Childress et al., 1988; Sinha et al., 1999). Reinstatement is then evaluated by the number of responses in the former active nose-hole or lever, yet with the absence of further drug.

It has been shown that the level of reinforcement increases over the time of abstinence, which is called the incubation effect (Grimm et al., 2001). This has been observed after a history of several drug, including nicotine (Abdolahi et al., 2010), cocaine (Grimm et al., 2001), heroin (Shalev et al., 2001), alcohol (Bienkowski et al., 2004) and sucrose (Grimm et al., 2002).

1.5. Nicotine-paired stimuli

As stated earlier, nicotine IVSA in animals was initially difficult to establish. Therefore, nicotine IVSA had to be performed under certain experimental conditions. For example, rats maintained on a free-feeding diet did not acquire nicotine IVSA, whereas rats maintained at a reduced bodyweight by restricted food access gradually acquired low rates of nicotine IVSA (Lang et al., 1977). Based on this, and other results, it was suggested that nicotine is a weak primary reinforcer, which led to the question:

« *How can nicotine, an apparently weak primary reinforcer, support the establishment of smoking, one of the most addictive behaviors worldwide* » (Chaudhri et al., 2006).

The observation that environmental stimuli frequently associated with drugs, including nicotine, can induce craving and relapse after prolonged abstinence in humans (Rose and Levin, 1991), resulted in the introduction of nonpharmacological stimuli (*e.g.* light or tone) in the drug self-administration paradigm. Indeed, the acquisition of nicotine IVSA is facilitated by combining drug delivery with a nonpharmacological stimulus, compared to responding for nicotine alone (Caggiula et al., 2002a; Caggiula et al., 2002b) or the stimulus alone (Caggiula et al., 2002b; Cohen et al., 2005b). The efficacy of the nonpharmacological stimulus to enhance nicotine IVSA depends on the contingency with the delivery of the drug or the response to obtain the drug (*e.g.* lever-pressing). It has been shown that noncontingent presentation of the stimulus in a nicotine IVSA paradigm does not enhance responding for nicotine (Caggiula et al., 2002b). This also accounts for other drugs such as cocaine and heroin (Di Ciano and Everitt, 2003). Interestingly, when nicotine is substituted with saline after establishment of stable nicotine IVSA, rats sustain reduced but stable responding for the nicotine-associated stimulus (Caggiula et al., 2001). Then, removing the stimulus after animals have reached stable responding for it during saline substitution, causes a further decrease in responding (Caggiula et al., 2001; Cohen et al., 2005b). This result implies that resistance to extinction and occurrence of relapse after prolonged periods of abstinence could be attributed to the reinforcing properties of the nicotine-associated stimulus. Together, these results suggest that smoking is maintained by the primary (although weak) reinforcing effect of nicotine and by environmental stimuli that become established as conditioned stimuli because of repeated association with nicotine (Caggiula et al., 2001; Rose and Levin, 1991).

1.5.1. Incentive salience

In a Pavlovian set-up, the approach behavior towards a reward-associated stimulus is referred to as sign-tracking behavior. Sign-tracking has long been associated with maladaptive behavioral patterns and compulsive responding for a drug (Tomie et al., 2008). In a Pavlovian paradigm, the presentation

of a stimulus (conditioned stimulus, CS) is followed by presentation of a reward (unconditioned stimulus, US), independent of the response of the animals. Repeated CS-US pairings result in the development of sign-tracking behavior (Tomie et al., 2008). Recently, it has been shown that nicotine persistently increases sign-tracking behavior for liquid sucrose, suggesting that nicotine enhances incentive motivation to a reinforcer-associated cue (Palmatier et al., 2013). In a nicotine IVSA paradigm, the nicotine-paired stimulus acquires reinforcing properties due to the reinforcement-enhancing effect of nicotine as a consequence of repeated conditioned responding. Berridge and Robinson referred to the motivational aspect of reward as « incentive salience » to the drug-paired stimulus. The stimulus becomes highly attractive and desired. Therefore, it has been proposed that sign-tracking behavior may be the behavioral expression of the attribution of incentive salience to a reward-related stimulus (Uslaner et al., 2006).

1.5.2. Dopamine signaling

It is well known that mesocorticolimbic dopamine signaling is important for the reinforcing properties of nicotine. For example, nicotine self-administration is attenuated by lesions of midbrain DA neurons (Corrigall et al., 1992), systemic injections of dopamine antagonist (Corrigall and Coen, 1991) or local infusion of GABA agonist (Corrigall et al., 2000). While these, and other studies, have had a great impact on the understanding of the rewarding properties of nicotine, they have used a nicotine IVSA paradigm in which the nicotine delivery was combined with a paired stimulus. Therefore, the decrease in responding for nicotine can be due to a decrease in primary reinforcement of nicotine, the reinforcement-enhancing properties of nicotine, or a combination of both. Preferentially, incentive salience is studied using Pavlovian conditioning. These studies show that the NAcc is important for sign-tracking behavior, as lesions in the NAc disrupt acquisition of sign-tracking performance (Cardinal et al., 2002; Parkinson et al., 2000). In a self-administration paradigm, conditioned incentive properties of nicotine-associated cues are usually tested in a cue-induced reinstatement protocol. It has been shown that cue-induced reinstatement is dependent on NAc activity (Gipson et al., 2013) and DA signaling (Liu et al., 2010), confirming the involvement of the mesocorticolimbic system in nicotine reinforcement. However, few data exist on the implication of upstream regulatory brain structures.

2. Neurobiology of addiction

The key question in addiction research is why some susceptible individuals undergo a transition from occasional drug use to compulsive patterns of drug use and why relapse rates are so high even after long periods of abstinence. Much research has been done to better understand the neuronal underpinnings of this transition and characterize the brain systems that mediate the rewarding effects of drugs of abuse. However, most available treatments are still relatively ineffective,

indicating that although a lot is known about the neuronal mechanisms underlying addiction, still a lot is unknown in order to understand the plasticity mechanisms that trigger relapse and therefore, to develop a suitable therapeutic strategy.

It is generally accepted that repeated exposure to drugs of abuse results in persistent neuronal modifications that strengthen the desire to obtain the drug and enhances the processing of drug-conditioned stimuli (Shaham and Hope, 2005). Initially, the main focus has been on the mesolimbic dopamine system. Even though drugs of abuse are chemically different with very different molecular targets, they all converge in a common circuitry in the brain; the mesolimbic dopamine pathway (Koob and Le Moal, 2001; Wise, 2004), which includes dopaminergic neurons in the VTA of the midbrain and their target area in the limbic forebrain, the NAc. Accordingly, it has been shown that drugs of abuse share the ability to enhance dopaminergic transmission in this pathway, either by increasing the firing rate of VTA DA neurons or by enhancing extracellular DA levels in the NAc (Bassareo and Di Chiara, 1997; Di Chiara and Imperato, 1988). It is widely accepted that enhanced VTA DA neurons following drug exposure is a result of increased glutamatergic input and reduced GABAergic-mediated inhibition (Overton and Clark, 1997). However, a recent study shows that activation of both GABA neurons in the VTA is necessary for the enhanced VTA DA activity that mediates nicotine reinforcement (Tolu et al., 2013). Together, this suggests that the mesolimbic pathway is a likely substrate for drug-induced neuroadaptations that mediate addiction-related behaviors. However, the development of the self-administration paradigm has led to the assumption that other processes are involved in drug addiction, such as learning and memory. Now it is well accepted that addictive drugs usurp the neuronal circuitry normally involved in motivation and reward of natural reinforcers. Hence, drug addiction is a maladaptive form of learning and memory leading to aberrant engagement in drug seeking (Hyman, 2005; Kauer and Malenka, 2007). This view shifted the focus to brain areas involved in learning and memory and their possible role in the development of addictive-like behavior (Kalivas, 2004).

2.1. Medial prefrontal cortex

Substantial evidence indicates the involvement of the medial prefrontal cortex (mPFC) in reinforcement learning and the acquisition of drug self-administration (Tzschentke, 2000).

2.1.1. Anatomy

The mPFC is located in the limbic forebrain and part of the mesocorticolimbic dopaminergic system. It receives dense dopaminergic input from the VTA and, via the mediodorsal thalamus, inputs from other subcortical basal ganglia structures (Groenewegen et al., 1990). In turn, it projects back to the VTA and the NAc, which are considered as main components of the brain reward system. The mPFC

in the rat is commonly divided in the dorsal and ventral mPFC. The dorsal mPFC consists of the anterior cingulate (AC) and dorsal prelimbic cortex (dPLCx), while the ventral mPFC includes the ventral PLCx and the infralimbic cortex (ILCx) (**Figure 8**). The subdivisions of the mPFC appear to be involved in separate aspects of drug-related behaviors (Van den Oever et al., 2010).

2.1.2. Involvement of the mPFC in reinstatement

Using human imaging studies, it has been shown that the PLCx and ILCx mediate the rewarding effects of conditioned stimuli in an opposing way. For example, positron emission tomography (PET), showed that drug-craving elicited by drug-associated stimuli is accompanied by hyperfunction of PLCx and hypofunction of ILCx (Bonson et al., 2002). The involvement of the PLCx in reinstatement is confirmed in rats, showing that reinstatement increases expression of immediate early genes, a marker of neuronal activity (Koya et al., 2006). Moreover, inactivation of dorsal mPFC (AC and PL) blocks reinstatement to drug, stress and conditioned cue (Fuchs et al., 2005). Although the dorsal mPFC is critically involved in the reinstatement to drug-seeking following extinction, inactivation of the dorsal mPFC after a period of forced abstinence has no effect on cue-induced reinstatement (Koya et al., 2009). This suggests that the neuronal substrates controlling reinstatement following extinction training and abstinence are different, and that the dorsal mPFC is recruited during extinction training, and not during abstinence. In contrast, inactivation of the ventral mPFC results in resumption of cocaine seeking following extinction training (Peters et al., 2008). Accordingly, stimulation of glutamatergic transmission in the ILCx attenuates reinstatement (Peters et al., 2008). Therefore, it is proposed that the ILCx exerts inhibitory control over drug-seeking. The ILCx is specifically recruited during extinction training, as inactivation of the ILCx after abstinence attenuates conditioned cocaine seeking during reinstatement test (Koya et al., 2009).

It is unknown whether the opposing role of the two subdivisions of the mPFC generalizes to other drugs of abuse. For example, studies on cue-induced reinstatement to heroin seeking after extinction have reported that activation of the ventral mPFC promotes heroin seeking (Bossert et al., 2011), while others show that inactivation of the ventral mPFC promotes cue-induced heroin seeking (Schmidt et al., 2005).

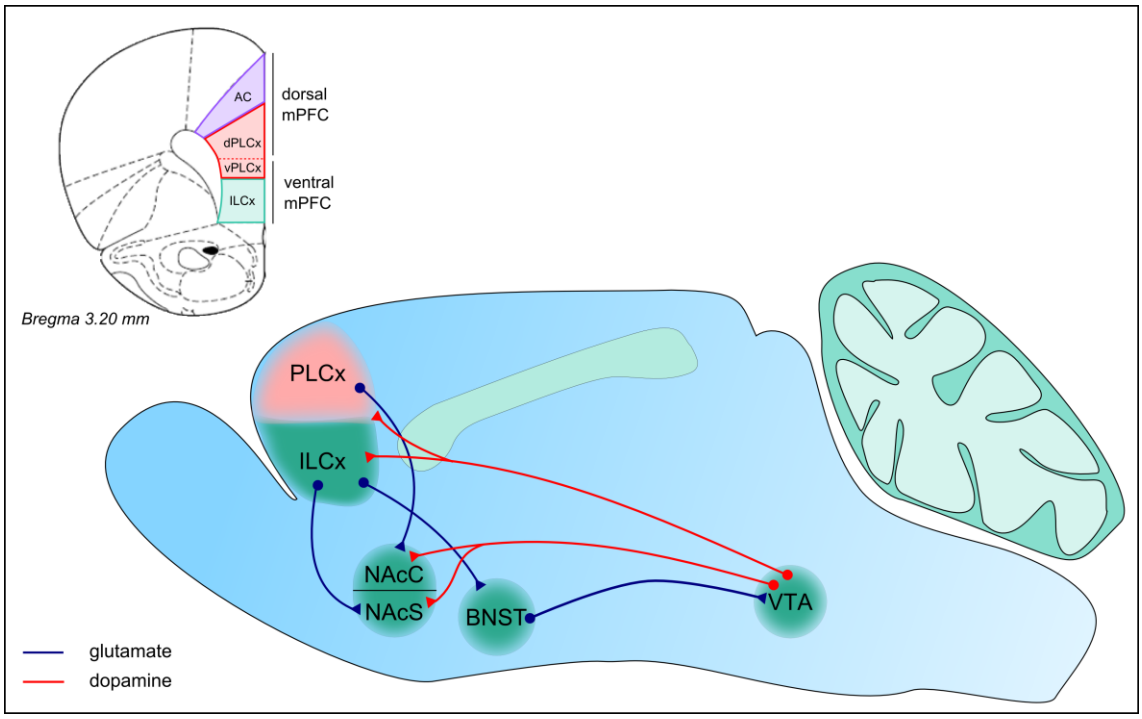


Figure 8 The mPFC is implicated in relapse to drug-associated cues. Upper figure: coronal section of the rodent prefrontal cortex at 3.20 mm from Bregma. Subdivisions of the mPFC are depicted in different colors. Based on functional and anatomical characteristics, the mPFC can be divided into a dorsal region, composed of the anterior cingulated (AC) and dorsal prelimbic cortex (dPLCx) and a ventral region that includes the ventral prelimbic cortex (vPLCx) and infralimbic cortex (ILCx). Lower figure: Activation of glutamatergic projections from the PLCx to the NAc core (NAcC) initiate drug seeking responses. The ILCx can suppress reinstatement through a glutamatergic projection to the NAc shell (NAcS), a function that may be impaired after re-exposure to drugs or drug-associated stimuli. Dopaminergic projections from the VTA to both the mPFC and NAc drive drug seeking. Adapted from (Van den Oever et al., 2010).

2.1.3. Neurocircuitry

Imaging studies in humans have revealed that the projection from the PFC to the NAc is involved in drug seeking. Basal activity in the PFC is reduced in addicts during a period of abstinence. However, following presentation of drug-associated cues, the PFC and NAc show large increases in activity that are associated with increased self-reports of 'drug-craving' (Goldstein and Volkow, 2002). In non-addicts, such increased activity is seen after presentation of stimuli associated with biological reinforcers (Garavan et al., 2000).

Anatomically, the subdivisions of the mPFC target different parts of the NAc (**Figure 8**). The dorsal mPFC projects mainly to the NAc core (NAcC), while the ventral mPFC projects to the NAc shell (NAcS). In line with the involvement of the ventral mPFC in reinstatement, reinstatement to nicotine cocaine and heroin, but not sucrose, is associated with an increase in extracellular glutamate levels in the NAcC (Gipson et al., 2013; LaLumiere and Kalivas, 2008; McFarland et al., 2003). In contrast, glutamatergic projections from the ventral mPFC to the NAcS are thought to suppress conditioned

drug seeking following extinction training, as disconnection of this pathway or inactivation of the NAcS leads to resumption of cocaine seeking (Peters et al., 2008).

The mPFC is also densely interconnected with the VTA that is critically involved in different aspects of drug-related behavior, such as reward salience and predictive value of drug-paired stimuli (Heidbreder and Groenewegen, 2003; Schultz, 1998). It has been shown that the specific projection of VTA dopaminergic efferents to the dorsal mPFC is involved in the initiation of drug seeking. For example, reinstatement of drug seeking was blocked by infusion of dopamine antagonist in the dorsal mPFC and dopamine administration into the dorsal PFC was sufficient to induce reinstatement (McFarland and Kalivas, 2001). Although the ventral mPFC is innervated by VTA dopaminergic neurons (Heidbreder and Groenewegen, 2003), little is known about the role of dopaminergic transmission from the VTA to the ventral mPFC in drug seeking.

Together, these results show that the mPFC is involved in drug-seeking behavior and in particular to drug-paired stimuli. The above discussed studies investigating the role of the mPFC in drug-seeking are using cocaine as a drug. It is not known whether the implication if the mPFC can be generalized to other drugs. Considering nicotine's reinforcement enhancing properties to drug-paired stimuli, it is likely that the mPFC also plays in important for in nicotine addiction. However, little attention has been paid to the issue whether the mPFC plays a role in mediation of the conditioned incentive properties of nicotine-associated cues. Yet, in our laboratory we showed that the glutamatergic projections from the ILCx to the bed nucleus of the stria terminalis (BNST) are involved in the association learning of voluntary nicotine intake and nicotine-paired cues (Caille et al., 2009).

2.2. Bed nucleus of stria terminalis

The name of the BNST is derived from its location in the brain, which is at one extremity of the stria terminalis. The stria terminalis is a bundle of axons that connects the BNST with the amygdala. Therefore, the BNST is also known as a part of the extended amygdala (Alheid, 2003). The BNST is a heterogeneous structure that is composed of several distinct nuclei, surrounding the caudal part of the anterior commissure. Depending on the criteria, the number of nuclei composing the BNST can vary. Currently, the BNST is divided in different nuclei based on anatomical location.

2.2.1. Composition of different nuclei

The complexity of the BNST is reflected in its composition of several nuclei. Based on chemical, electrophysiological, and behavioral findings, the BNST is divided in 15 subdivisions. The most clear division separates the BNST in an anterior and posterior part. Then, the anterior part can be divided in the anterodorsal, anteroventral (who are together the anteromedial BNST) and anterolateral BNST (**Figure 9**). Embedded within these three areas various cell groups or nuclei have been identified. The

anteroventral part is comprised of the dorsomedial dorsolateral magnocellular and ventral nuclei. The anterolateral BNST consists of the fusiform, oval, juxtacapsular and rhomboid nuclei and a subcommisural zone. Finally, the posterior part of the BNST has five distinguishable regions: the principal, intrafascicular, transverse, premedullary and dorsal nuclei (Dong et al., 2001; Ju and Swanson, 1989). In my thesis, I will focus on the anteromedial part of the BNST because of its connectivity with the reward system and the functional implication in drug addiction.

The majority of the neurons found in the BNST are GABA-ergic (75 – 90%), as measured using *in situ* hybridization (Cullinan et al., 1993). Also, some vesicular glutamate transporter (VGlut)-expressing neurons are found in the BNST in the medial anterior part (Herzog et al., 2004) and posterior part (Poulin et al., 2009).

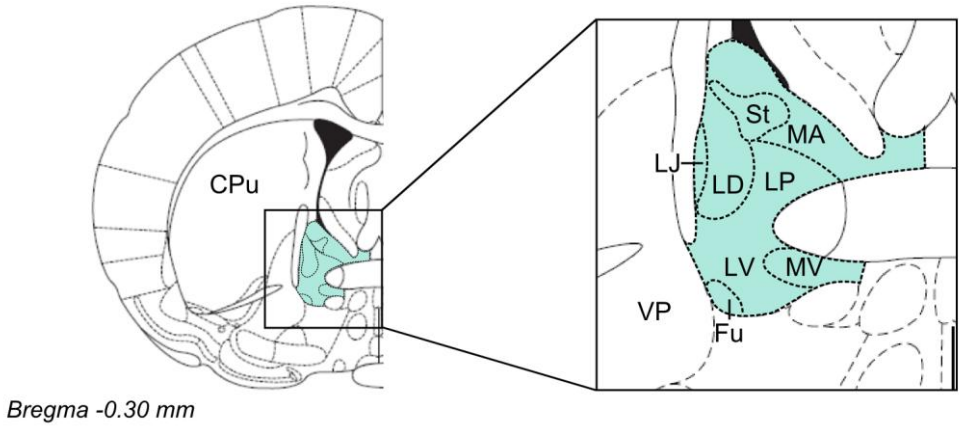


Figure 9 Structural anatomy of the BNST. *Left*: Coronal section at -0.30 mm posterior from Bregma presenting the localization of the BNST. *Right*: representation of different subnuclei of the BNST. CPu: caudate putamen, Fu: fusiform part of BNST, LD: lateral division, dorsal part of BNST, LJ: lateral division, juxtacapsular part of BNST, LP: lateral division, posterior part of BNST, LV: lateral division, ventral part of BNST; MA: medial division, anterior part of BNST, MV: medial division, ventral part of BNST, St: stria terminalis, VP: ventral pallidum. Paxinos and Watson, 1998.

2.2.2. Neurocircuitry

The BNST is connected with widespread brain regions. Numerous brain areas of both the reward system as well as the stress circuit send afferents to different nuclei of the BNST. For example, the BNST receives input from the VTA, PFC, nucleus of the solitary tract, dorsal raphe nucleus, central amygdala (CeA), basolateral amygdala (BLA), and hippocampus (Dong et al., 2001; Massi et al., 2008; McDonald, 1998; Stamatakis et al., 2013). Conversely, the BNST sends projections to the VTA, paraventricular nucleus of the hypothalamus (PVN) and lateral hypothalamus (Dong and Swanson, 2004, 2006; Georges and Aston-Jones, 2001).

2.2.2.1. Infralimbic cortex

The BNST receives major excitatory input from the PFC, including the ILCx. Using anterograde tracing with the tracer Phaseolus vulgaris-leucoagglutinin (PHAL), Dong et al. found the highest density of PHAL in the caudal region of anteroventral part of the BNST and more specifically in the dorsomedial, dorsolateral, magnocellular and ventral nuclei. In contrast, the anterodorsal part and the posterior region showed little to no connection with the ILCx. These results were in general in agreement with findings by others. However, some studies also found ILCx projections to the posterior division (McDonald, 1998; Vertes, 2004). Moreover, electrophysiological data has demonstrated an excitatory projection of the ILCx to the BNST (Massi et al., 2008). The prelimbic cortex sends minimal projections to the BNST (Massi et al., 2008).

2.2.2.2. VTA

Using retrograde tracing and electrophysiological studies, is has been demonstrated that the BNST sends both a GABAergic and glutamatergic projection to the VTA (Cullinan et al., 1993; Georges and Aston-Jones, 2001, 2002). Specifically, the anterolateral and anteroventral regions of the BNST send monosynaptic excitatory output to VTA DA neurons (Dumont and Williams, 2004; Georges and Aston-Jones, 2002). In contrast, a recent study showed that the input from the BNST to the VTA is mainly GABAergic (90%), whereas only a few percent were VGlu2 and VGlut3-expressing neurons (4.3% and 3.7%, respectively) (Kudo et al., 2012). With a combination of retrograde tracing and fluorescence *in situ* hybridization, it is shown that both GABAergic and glutamatergic BNST neurons projecting to the VTA target putative VTA GABA neurons (Kudo et al., 2012). This was shortly after confirmed using optogenetic identification and recordings (Jennings et al., 2013). GABAergic neurons in the VTA are mainly interneurons that inhibit dopaminergic neurons (van Zessen et al., 2012). Therefore, GABAergic and glutamatergic projections from the BNST are likely exciting or inhibiting VTA DA neurons, respectively (Stamatakis et al., 2013).

Using local microinfusions, it has been shown that the BNST has a strong influence on the bursting activity of VTA DA neurons; microinfusion of glutamate into the BNST enhanced bursting activity of

VTA DA neurons, whereas microinfusion of GABA decreased bursting activity in DA cells (Georges and Aston-Jones, 2002). Since the bursting activity of VTA DA neurons required and sufficient for the development of associative conditioning (Tsai et al., 2009), it is likely that the BNST plays a major role in this process.

2.2.3. Role of the BNST in behavior

The BNST has been implicated in both stress- and reward-related behaviors, and is thought to act as a mediator between these two systems (Harris and Aston-Jones, 2007; Walker et al., 2003).

2.2.3.1. Implication in reward

Several studies have shown that the BNST is involved in reward-related behavior and drug addiction. From an anatomical point of view, the BNST is connected to different brain areas related to reward-directed behavior, including the ILCx and VTA (Dong and Swanson, 2004; Vertes, 2002). Besides GABAergic and glutamatergic afferents, the BNST (in particular the dorsolateral part) receives dopaminergic input from the VTA (Meloni et al., 2006). Using microdialysis, is has been shown that drugs of abuse, including nicotine, cocaine, morphine and ethanol increase effectively and dose-dependently extracellular dopamine in the BNST (Carboni et al., 2000). Also, dopamine is released in response to natural rewards and reward-associated cues (Park et al., 2013; Park et al., 2012). On a behavioral level, it has been shown that the BNST, and in particular the ILCx-BNST-VTA pathway, is involved in cue-associated self-administration of nicotine (Caille et al., 2009). This study demonstrated that extended nicotine IVSA increases the excitability of excitatory input from the ILCx to the BNST. Interestingly, this augmentation of excitability was not seen after passive or non-contingent administration of nicotine, indicating the importance of the BNST in the associative learning of reward and reward-associated cues. Similar results were found with an *in vitro* approach, showing that cocaine self-administration, but not passive exposure, increases excitatory synaptic transmission in the BNST (Dumont et al., 2005). Moreover, a recent study shows that motivation to self-administer cocaine induces long-term potentiation at GABA synapses in the oval BNST, mediated by D1-like dopamine receptors (Krawczyk et al., 2013). This potentiation was absent in rats that received cocaine passively, supporting the hypothesis that the BNST is involved in associative learning.

2.2.3.2. Implication in stress

The BNST receives increased attention in the field of addiction, due to its role as an integrator of motivated behaviors associated with drug addiction and the implication in stress and anxiety. The BNST connects brain regions involved in stress. For example, the BNST receives input from limbic regions such as the BLA and the hippocampus and sends projections to the periventricular nucleus (PVN). The PVN of the hypothalamus is involved in the maintenance of homeostasis and is a critical

component of the hypothalamo–pituitary–adrenocortical (HPA) axis. Projections from the BNST to the PVN are both GABAergic and corticotrophin-releasing factor (CRF) ergic. CRF appears to play a major role in fear and stress-related behaviors (Hauger et al., 2009), including stress-induced reinstatement to drugs of abuse (Erb et al., 2001; Shalev et al., 2010). Several nuclei of the BNST express CRF. In the anterior part of the BNST, CRF is expressed in the dorsomedial, fusiform and oval which project to the PVN (Dabrowska et al., 2011; Dong et al., 2001b). The posterior part of the BNST also projects to the PVN. Interestingly, it has been shown that the anterior and posterior part exert opposite effects on the HPA-axis: while the anterior BNST activated the HPA-axis, the posterior part has a inhibitory influence (Dabrowska et al., 2011).

Together, an increasing amount of evidence points to the involvement of the BNST in addiction-related behavior. In particular, nicotine IVSA has been shown to enhance excitability of excitatory projections from the ILCx to the BNST (Caille et al., 2009). This was absent in rats exposed to passive nicotine exposure, indicating that the BNST is implicated in the associative learning of reward and reward-paired cue. The neuroadaptations induced by drugs may cause long-lasting memories related to the drug experience, resulting in high rates of relapse even after prolonged periods of abstinence. Therefore, the BNST may be a neuronal substrate for excessive drug-seeking, and merits further investigation.

3. Endocannabinoid system

The endocannabinoid system is a neuromodulatory system that is shown to be involved in a variety of physiological processes including appetite, pain-sensation, mood, memory and addiction (Mechoulam and Parker, 2013). Also, it mediates the psychoactive effects of cannabis.

Increasing amount of evidence suggest a functional interaction between nicotine addiction and the endocannabinoid system. For example, it is well established that cannabinoid type 1 (CB1) receptors have a role in the reinforcing effects of nicotine, demonstrated by a reduction in nicotine IVSA following peripheral administration if CB1 antagonist (Cohen et al., 2002). Also, nicotine facilitates the discriminative effects of THC (Solinas et al., 2007b). In contrast, prior exposure to THC enhances both the acquisition of nicotine IVSA and motivation to obtain nicotine (Panlilio et al., 2013). In the BNST, (CB1) receptors are detected on both excitatory axon terminals as well as on inhibitory inputs (Massi et al., 2008). These receptors have been shown to regulate the firing of dopamine cells in the VTA. However, the role of BNST CB1 receptors in nicotine addiction is unknown.

3.1. Endocannabinoid receptors

CB1 and cannabinoid 2 (CB2,) receptors are the two major cannabinoid receptors. Both cannabinoid receptors exhibit 48 % amino acid sequence identity. Additional cannabinoid receptors have been

suggested to exist in the brain by pharmacological and genetic studies (Begg et al., 2005). The discovery and cloning of these receptors allowed the development of mice with a selective deletion of either receptor. These transgenic mice, in particular CB1 knockout mice, are now widely used to explore the physiological and pathological functions of cannabinoid receptors (Howlett et al., 2002).

3.1.1. CB1 receptor

In 1990, the CB1 receptor was cloned and characterized for the first time (Matsuda et al., 1990). The CB1 receptor is G protein-coupled receptor (GPCR) composed of 473-amino acids in rats. Later, a human 472-amino acids homolog has been reported (Gerard et al., 1990). The CB1 receptor possesses seven transmembrane domains connected by three extracellular and three intracellular loops, an extracellular N-terminal and an intracellular C-terminal. CB1 receptors are able to form heteromeric complexes with other GPCRs, which may induce different pharmacological profiles (Pertwee et al., 2010).

It was originally thought that CB1 receptors were expressed mainly in the central nervous system (CNS), but CB1 receptors have been found also in peripheral organs, though levels are often low (Pertwee, 1997). Using *in situ* hybridization and immunohistochemistry, the CB1 receptors are ubiquitous found in the brain in areas like the basal ganglia, substantia nigra, globus pallidus, cerebellum hippocampus and BNST (Massi et al., 2008; Matsuda et al., 1993; Tsou et al., 1998). The heterogeneous distribution of CB1 receptors accounts for its role in numerous functions, including cognition, memory, ingestive behavior and motor control (Katona and Freund, 2012). CB1 receptors are mainly localized on presynaptic terminals, but are also found at the level of the soma and on dendrites (Tsou et al., 1998).

3.1.2. CB2 receptor

The CB2 receptor was cloned in 1992, and initially assumed to be present only in cells of the immune system. Today however, CB2 receptors have been identified throughout the CNS (Ashton et al., 2006; Van Sickle et al., 2005), in particular on microglial cells (Nunez et al., 2004), though at lower levels than those of CB1 receptors. Like the CB1 receptor, the CB2 receptor is a GPCR with seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus. Yet, with 360 amino acids, the CB2 receptor is somewhat shorter than the CB1 receptor. Despite the low homology between the two cannabinoid receptors, they share pharmacological characteristics and the majority of ligands binding to CB1 receptors, also binds to CB2 receptors (Di Marzo et al., 2004).

Initial research has focused on the implication of CB2 receptors in the immunological activity of leukocytes (Kaminski, 1998). It has been shown that CB2 receptors have a role in immune suppression, induction of apoptosis and cell migration (Basu and Dittel, 2011). CB2 receptors found in the brain are thought to be involved in the effects of drug abuse (Onaivi et al., 2008).

3.2. Endogenous cannabinoids

The discovery of the cannabinoid receptors led to the assumption that endogenous ligands would be present in the mammalian body. Indeed, two main compounds were isolated and identified; anandamide (AEA) (Devane et al., 1992) and 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1995). Other compounds are also identified that might act as endocannabinoids (ECs); dihomo-γ-linolenoyl ethanolamide, docosatetraenoyl ethanolamide (Hanus et al., 1993), 2-arachidonyl glycerol ether (Hanus et al., 2001), O-arachidonoylethanolamide (Porter et al., 2002) and N-arachidonoyldopamine (Huang et al., 2002). In this section, I will focus on the two main ECs, AEA and 2-AG.

AEA act as a partial agonist to both CB1 and CB2 receptors (Sugiura et al., 2002). Besides activating the endocannabinoid system, AEA can also activate the endovanilloid system by binding to the vanilloid 1 (TRPV1) receptor (Starowicz et al., 2007). 2-AG acts as a full agonist to both CB1 and CB2 receptors, and does not bind to any other receptors (Sugiura et al., 2006).

3.2.1. Metabolism

ECs act as retrograde messengers and are produced in the postsynaptic membrane. They are synthesized « on demand » to be immediately released from the cell, without being stored in vesicles. Both AEA and 2-AG are produced following the enhancement of intracellular Ca^{2+} concentrations. This can be caused either by Ca^{2+} influx following cell depolarization or by mobilization of intracellular Ca^{2+} stores as a result of GPCRs.

3.2.1.1. Synthesis and degradation

The production of AEA starts with the transfer of an arachidonate group from glycerophospholipid to obtain N-arachidonoyl phosphatidylethanolamine (NAPE). This reaction is catalyzed by N-acyltransferase (NAT). Then, NAPE is hydrolyzed by N-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) to form AEA and phophatidic acid. NAT activity is highly dependent on Ca^{2+} and cyclic AMP, and generally thought to be the rate-limiting step in the production of AEA (Cadas et al., 1997) (**Figure 10**).

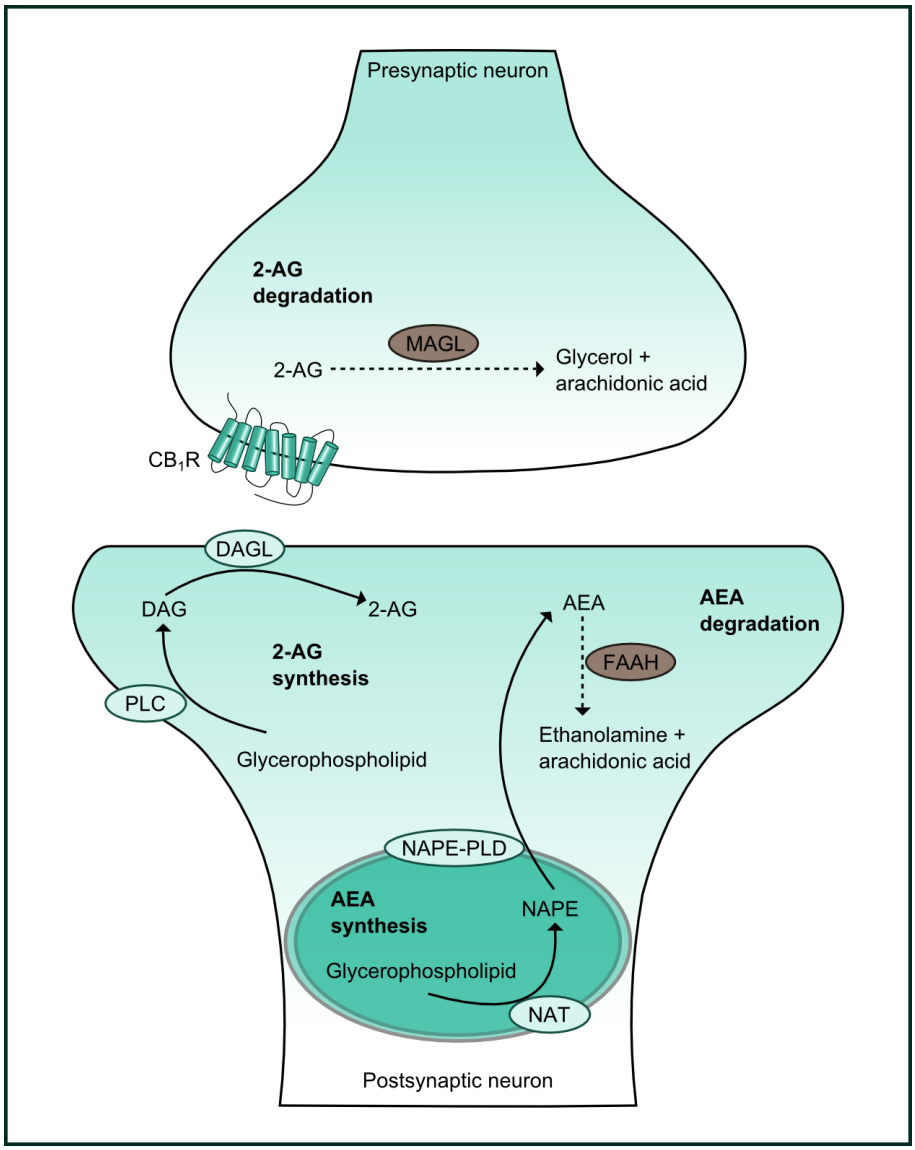


Figure 10. Synthesis and degradation pathways of endocannabinoids and their likely subcellular localization. The enzymes for 2-arachidonoylglycerol (2-AG) synthesis, phospholipase C (PLC) and diacylglycerol lipase (DAGL) seem to be mostly localized on the plasma membrane of the postsynaptic terminal, whereas monoacylglycerol lipase (MAGL) for 2-AG degradation is localized in the presynaptic terminal. The anandamide (AEA) synthesis enzymes N-acyltransferase (NAT) and N-acylphosphatidyl-ethanolamine phospholipase (NAPE-PLD), and the inactivating enzyme fatty acid amide hydrolase (FAAH) are all located on intracellular membranes. FAAH seems to be most abundant in postsynaptic neurons. However, it is not known whether NAT and NAPE-PLD are pre- or postsynaptic.

Several pathways are revealed for the synthesis of 2-AG. The main pathway includes the enzymes phospholipase C (PLC) and 1,2-diacylglycerol lipase (DAGL), which both seem to be mainly localized on the postsynaptic plasma membrane. First, PLC hydrolyses glycerophospholipid, producing 1,2-diacylglycerol (DAG). Then, 2-AG is produced from DAG by the action of DAGL (Hashimotodani et al., 2013; Stella et al., 1997) (**Figure 10**). PLC can be activated either directly by increased levels of Ca^{2+} or by activation of postsynaptic group I metabotropic glutamate receptors (I mGluRs) (Hashimotodani et al., 2007; Varma et al., 2001). PLC is thought to act as a coincidence detector to integrate I mGluRs

signaling at intracellular CA^{2+} levels (Hashimotodani et al., 2005). However, it has been shown that I mGluRs activation is sufficient to mobilize ECs and induce short- and long-term plasticity (Chevaleyre et al., 2006). Another pathway for the production of 2-AG include hydrolysis of phosphatidylinositol phospholipase A1 to 2-arachidonoyl-lysophospholipid, followed by the action of lyso-PLC to produce 2-AG (Higgs and Glomset, 1994). The different pathway to synthesize 2-AG might be used in different tissues and cells. They might also be dependent on different conditions of stimulation (Kano et al., 2009).

ECs can be degraded by hydrolysis. This includes two enzymes; fatty acid amide hydrolase (FAAH) for AEA and monoacylglycerol lipase (MAGL) for 2AG. ECs cross the postsynaptic membrane via a yet uncharacterized mechanism (Fowler, 2013). Once inside the cell, AEA is degraded by FAAH into ethanolamine and arachidonic acid (Di Marzo et al., 1994). 2-AG is degraded in presynaptic terminals expressing CB1 receptors by being transformed to glycerol and arachidonic acid (Dinh et al., 2002) (**Figure 10**).

3.2.2. Signaling

Neural activity is a potent stimulus for the release of ECs from the post-synaptic terminal. The principal mechanism by which ECs act is through retrograde signaling, resulting in inhibition of neurotransmitter release via presynaptic CB1 receptors. There is also evidence that ECs can act in an autocrine way, in which they can bind to TRPV1 receptors and CB1 receptors located on the postsynaptic cell (Di Marzo et al., 2002). Since 2001, ECs have been identified as triggers for short- and long-term plasticity. In agreement with the ubiquitous expression of CB1 receptors (Tsou et al., 1998), synaptic plasticity induced by ECs is seen throughout the brain (Chevaleyre et al., 2006). In the following section I will describe how retrograde signaling can modulate synaptic transmission.

3.2.2.1. Regulation of short-term synaptic plasticity

Changes in the strength and number of synaptic connections between neurons are believed to be the major mechanism underlying learning and memory and mediating other physiological function of the CNS.

The classical example of short-term modulation of synaptic transmission by ECs is called depolarization-induced suppression of inhibition (DSI). This phenomenon was first described in 1992, in the cerebellum (Llano et al., 1991) and hippocampus (Pitler and Alger, 1992). In these studies it was reported that brief activation of Purkinje cells and pyramidal cells in the cerebellum and hippocampus, respectively, caused a reduction in the amplitude of GABAergic inhibitory postsynaptic currents (IPSCs). DSI is initiated postsynaptically by the influx of Ca^{2+}, but is expressed presynaptically through inhibition of transmitter release from axon terminal. This suggested that a messenger

generated during depolarization of the neurons must have travelled backwards across to synapse to inhibit neurotransmitter release and this induce DSI. This retrograde messenger in DSI remained unknown until 2001, when it was indicated that DSI in the hippocampus was likely to be mediated by an endocannabinoid (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). The year after, cerebellar DSI was also reported to be mediated by an endocannabinoid (Kreitzer and Regehr, 2001a). Several lines of evidence support the hypothesis that DSI is mediated by ECs: 1) CB1 receptor agonists enhance DSI, whereas CB1 receptor antagonist block it (Ohno-Shosaku et al., 2001), 2) DSI is completely absent in CB1 KO mice (Wilson et al., 2001) and 3) The GABA interneurons that are implicated in DSI express high levels of CB1 receptors, which are localized to their axon terminals (Katona et al., 1999). Similar mechanisms have now been described for excitatory neurons and referred to as depolarization-induced suppression of excitation (DSE). Like DSI, DSE can be mimicked and blocked by CB1 receptor agonists and antagonists, respectively (Kreitzer and Regehr, 2001b; Maejima et al., 2001) and is absent in CB1 KO mice (Ohno-Shosaku et al., 2002). Existence of DSE has been shown in the cerebellum (Kreitzer and Regehr, 2001b), VTA (Melis et al., 2004), lateral

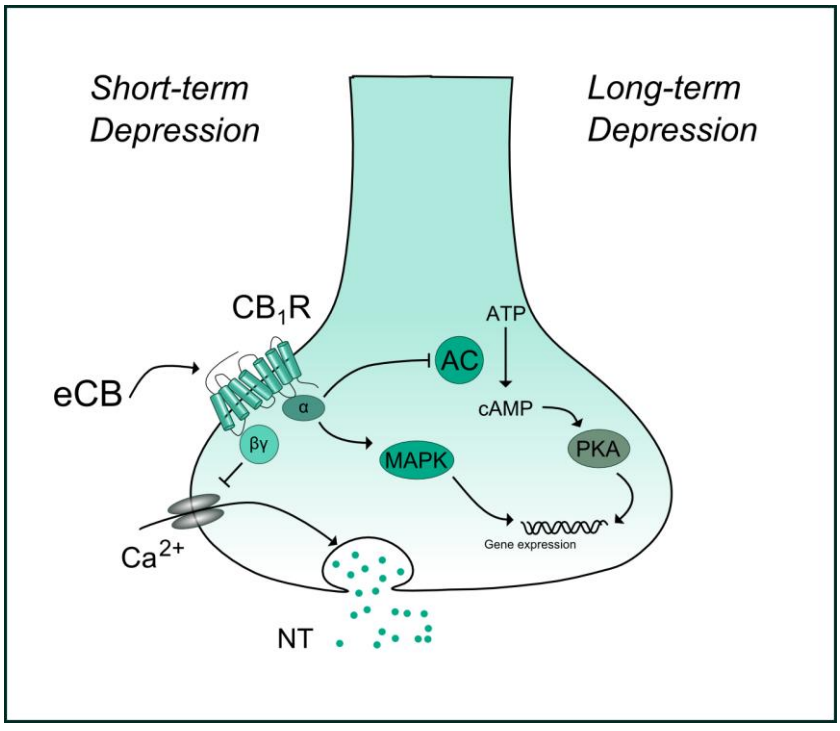


Figure 11. Molecular mechanisms underlying endocannabinoid-mediated short- and long-term synaptic plasticity. *Left*: during EC-mediated short-term depression, brief activation of CB1 receptors reversibly depresses neurotransmitter release, mainly by blocking presynaptic voltage-gated Ca2+ channels (VGCC), presumably via Gβ/γ subunits. *Right*: During the induction of EC-mediated long-term depression, a longer-lasting activation of CB1 receptors inhibits adenylyl cyclase (AC) activity and activated MAPK via Gα subunits, thereby reducing presynaptic cAMP lvels and PKA activity. Both pathways ultimately leads to regulation of gene expression.

amygdala (Kodirov et al., 2010) and less prominent in the hippocampus (Ohno-Shosaku et al., 2002).

Synthesis and mobilization of ECs from postsynaptic neurons is triggered by depolarization and subsequent elevation of intracellular Ca^{2+} concentrations (described in section 3.2.1.1). Upon release, ECs travel backwards across the synapse to target presynaptic CB1 receptors. The mechanism by which a lipid like ECs can cross an aqueous environment is still unresolved. Brief presynaptic binding of ECs at the CB1 receptor results presumably in the activation of $G_{\beta/\gamma}$ subunits (Herlitze et al., 1996), which in turn blocks Ca^{2+} influx through presynaptic VGCC. This puts the presynaptic terminal in a hyperpolarized state and thereby transiently inhibiting neurotransmitter release (Chevaleyre et al., 2006) (**Figure 11**).

3.2.2.2. Regulation of long-term synaptic plasticity

Besides short-term plasticity, ECs are also able to trigger long-term plasticity, though through a different signaling pathway. In the striatum and hippocampus, it was observed that long-term depression (LTD) was absent in CB1 KO mice, reduced or blocked by CB1 antagonist and enhanced by CB1 receptor agonist (Gerdeman et al., 2002; Robbe et al., 2002). However, once LTD was induced, administration of an antagonist did not affect it, demonstrating that the LTD was not maintained by a continual release of endocannabinoids, but rather represented a persistent effect of transient CB1 receptor activation. In the hippocampus, it has been shown CB1 receptor activation inhibits both long term potentiation (LTP) and LTD (Sullivan, 2000). Seemingly in contrast to this, one study showed that, by mediating DSI, endocannabinoids facilitate the induction of LTP in the hippocampus (Carlson et al., 2002). They state that this result does not contradict previous reports, because in these reposts exogenous cannabinoids used to impair LTP globally affect all cells, while, in their set-up, LTP induction should be enabled only in those neurons undergoing DSI (Carlson et al., 2002).

CB1 receptors need to be longer activated to be able to induce this type of plasticity (*i.e.* few minutes). Through the activation of G_{α} subunits, adenylyl cyclase (AC) activity is inhibited. This will lead to inhibition of the cAMP/PKA signaling pathway, and stimulation of mitogen-activated protein kinase (MAPK) pathways, ultimately resulting in regulation of expression of several genes. The type of signaling pathway affected by CB1 receptor activation depends on the type of agonist used in the study and the brain area and type of neuron involved (Howlett et al., 2004) (**Figure 11**).

3.3. Exogenous cannabinoids

The discovery of the endocannabinoid system including its receptors and endocannabinoids prompted the development of CB1 and CB2 (non)selective agonists and antagonists (Howlett et al., 2002). Synthetic cannabinoids have often stronger effects of longer duration, compared to

endogenous cannabinoids (Crawley et al., 1993). Here I will describe the two cannabinoid antagonists and agonist that I used in the experiments of my thesis.

3.3.1. AM251

AM251 is a selective CB1 receptor antagonist. The K_i value at CB1 receptors is 7.49 nM, and is more than 300-fold selective over CB2 receptors. AM251 is structurally very close to SR141716A (Rimonabant) (**Figure 12**). However, AM251 is about 2-fold more selective for the CB1 receptors than Rimonabant. There is evidence that AM251 does not act as a neutral antagonist, but rather as an inverse agonist. Thus, besides attenuating effects of CB1 receptors agonists, they may by themselves elicit responses that are opposite from those induced by CB1 agonists (Pertwee, 2005).

The use of synthetic CB1 antagonist like AM251 have been proven very useful in understanding the involvement of CB1 receptors in a wide range of behavior, including addiction (Corbille et al., 2007; Xi et al., 2006).

3.3.2. O-2050

O-2050 is a silent or neutral antagonist for cannabinoid receptors. It is a structural analog of Δ^8-tetrahydrocannabinol, in which the pentyl side chain at position C3 is replaced by an acetylene moiety with a terminal sulfonamide group (**Figure 12**). The K_i value at CB1 receptors is 2.5 ± 0.4 nM. Its affinity for CB2 receptors (0.2 ± 0.1 nM) complicates its use as a tool to evaluate the unique contribution of cannabinoid CB1 receptor mediation of specific pharmacological effects. However, *in vivo* O-2050 has been proven to act as a neutral CB1 antagonist (Dubreucq et al., 2013). *In vitro*, O-2050 antagonized the effects of cannabinoid agonists (Wiley et al., 2011).

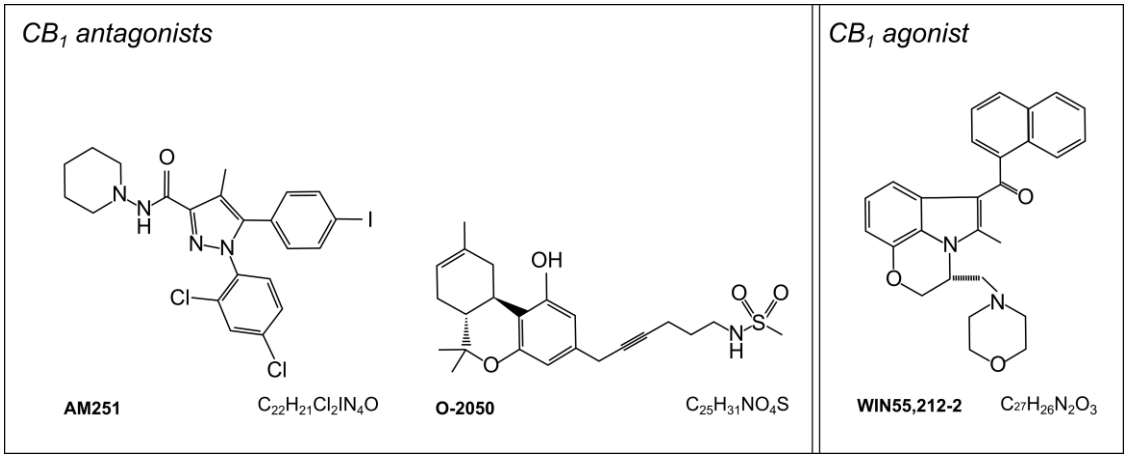


Figure 12 Molecular structures of exogenous endocannabinoids using in this project. Left: two CB1 antagonist; AM251 and O-2050. Right: CB1 agonist WIN55,212-2.

3.3.3. WIN55,212-2

WIN55,212-2 (WIN55) is a full agonist at the CB1 receptor (K_i = 1.9 nM) and has much higher affinity than the natural agonist THC (K_i = 41 nM) for CB1 receptors (**Figure 12**). WIN55 also binds to CB2 receptors. However, while it induced cataplexy in wildtype mice, it did not have any effect in CB1 KO mice (Ibrahim et al., 2003). Recent data indicate that WIN55 can also bind to and inhibit TRPV1 receptors (Patwardhan et al., 2006).

WIN55 is a commonly used agonist to study the involvement of the endocannabinoid system in addiction (Fattore et al., 2011; Malinen and Hyytia, 2008; Solinas et al., 2005). *In vivo* microdialysis showed that acute systemic WIN55 administration increases AEA and 2-AG levels in the brain of rats, which is blocked by selective CB1 antagonist Rimonabant (Bequet et al., 2007). This indicates that the effects of WIN55 in the brain are mainly conducted through CB1 receptors.

3.4. Involvement of the endocannabinoid system in nicotine addiction

Considering the co-localization of CB1 receptors and nAChRs in many brain structures such as the hippocampus and amygdala it is suggested that these two systems may have a close interaction (Herkenham et al., 1990; Picciotto et al., 2000). Indeed, it has been shown that nicotine enhances the THC-induced tetrad (*i.e*. hypothermia, analgesia, hypolocomotion et catalepsy) (Pryor et al., 1978; (Valjent et al., 2002). Inversely, delta9-THC decreases the somatic and motivational signs of nicotine withdrawal (Balerio et al., 2004). Moreover, blockade of α_7 nAChRs reverses the discriminative effects of THC and WIN55 and reduces self-administration of WIN55 in rats (Solinas et al., 2007a). This clearly demonstrates the existence of a functional interaction between the endocannabinoid system and nicotine.

3.4.1. Behavioral level

Numerous studies have explored the involvement of the endocannabinoid system, in particular the influence of CB1 receptor signaling on drug-induced behaviors, including the rewarding effects of nicotine. For example, it has been demonstrated that while nicotine induces place preference in a CPP paradigm in wildtype mice, it fails to do so in CB KO mice (Castane et al., 2002; Cossu et al., 2001), indicating the importance of CB1 signaling in the reinforcing effects of nicotine. Accordingly, CB1 antagonist Rimonabant blocks CPP to nicotine in rats (Le Foll and Goldberg, 2004). Also, CB1 antagonist dose-dependently decreases nicotine self-administration in rats (Cohen et al., 2002; Shoaib, 2008), and CB1 KO mice self-administer less nicotine than their wildtype littermates (Cossu et al., 2001). CB1 receptors have also been shown to be involved in reinstatement of nicotine-seeking, as Rimonabant decreases cue-induced reinstatement in rats (Cohen et al., 2005b; De Vries and Schoffelmeer, 2005), likely via the PFC, NAc and BLA (Kodas et al., 2007). Consistent with this result, CB1-agonist reinstates extinguished place preference to nicotine (Biala and Budzynska, 2008).

3.4.2. Brain level

Chronic exposure to nicotine increases AEA et 2-AG in limbic areas, and brain stem, and decreases AEA and 2-AG in the hippocampus, striatum and cerebral cortex (Gonzalez et al., 2002). Using microdialysis, it has been shown that CB1 antagonist blocks DA levels in the NAc induced by a single nicotine injection (Cohen et al., 2002).

3.4.3. Drug-induced disruption of EC-mediated plasticity

Synaptic plasticity is one of the main mechanisms underlying learning and memory processes. It is generally accepted that it enables experience-dependent modifications in neural function that is important for behavioral flexibility. Long-term forms of synaptic plasticity include LTP and LTD, and cause a long-lasting strengthening and weakening of synaptic strength, respectively and can persist for hours to weeks.

It is thought that drugs of abuse usurp this mechanism in circuits normally responsible for reinforcement of non-drug rewards (*e.g.* food) (Hyman, 2005). Therefore, drug addiction is considered a powerful but pathological form of learning and memory (Kalivas, 2009; Kauer and Malenka, 2007). A substantial amount of research has focused on the precise mechanisms of this process and has studied the potential brain areas involved. Despite the complexity of the brain circuits involved in addiction, it is clear that the mesocorticolimbic dopaminergic system, including the VTA, NAc and PFC, is a critical substrate for the neural adaptations that underlie addiction (Kalivas and O'Brien, 2008). Synaptic modifications in several brain areas have been shown to contribute to different aspects of reward-related behaviors, such as craving, withdrawal and relapse (Kauer and Malenka, 2007).

As described before, the endocannabinoid system is a powerful neuromodulator, as it suppresses neurotransmitter release at both inhibitory and excitatory terminals. The effect of several types of drugs of abuse has been studied and it is shown that they can modify the functioning of the endocannabinoid system. For example, a single exposure to cocaine abolishes EC-mediated LTD of excitatory transmission, resulting in enhanced excitatory signaling in the NAc (Fourgeaud et al., 2004). Accordingly, repeated cocaine exposure reduces the inhibition of VTA DA neurons through enhancing EC-mediated LTD of GABA-ergic cells (Pan et al., 2008). Drug-induced disruption of EC-mediated LTD of excitatory transmission had also been observed in the BNST. This was seen after repeated, but not single exposure to cocaine (Grueter et al., 2008).

Thus, it may be clear that interference of drugs in EC-LTD contributes to synaptic modification in several brain areas which might contribute to maladaptive behavior associated with addiction.

PROBLEM STATEMENT

PROBLEM STATEMENT

Nicotine, like other drugs of abuse, enhances dopaminergic transmission in the mesocorticolimbic pathway, resulting in enhanced dopamine levels in the NAc (Di Chiara and Imperato, 1988). With repeated exposure, long-lasting synaptic changes in the brain will develop that strengthen the desire to obtain the drug. A considerable amount of evidence indicates that the VTA is critically involved in the primary rewarding effects of nicotine. However, other brain areas might be involved in different aspects of nicotine-induced behaviors. Indeed, using the self-administration model of drug addiction, it is now well accepted that the neuronal circuitry normally involved in learning and memory is usurped by drugs of abuse, including nicotine. Therefore, drug addiction in a maladaptive form of learning and memory, leading to compulsive drug use.

The BNST, as part of the extended amygdala, projects to the VTA, where it exerts major influence on the firing properties of dopaminergic neurons (Georges and Aston-Jones, 2001, 2002). The BNST is shown to be involved in reward-related behavior and drug addiction (Aston-Jones and Harris, 2004; Dumont et al., 2005). One of the main inputs of the BNST arise from the mPFC, and more specifically the ILCx of the ventral mPFC. The ILCx is an important neuronal substrate of inhibitory control following extinction training of drug intake (Peters et al., 2008) and has also been involved in the development of habitual responses (Coutureau and Killcross, 2003). Moreover, in our laboratory, it has been shown previously that volitional nicotine self-administration enhances the excitability of the ILCx-BNST pathway, resulting in hyperactivity of VTA DA neurons (Caille et al., 2009).

The endocannabinoid system, in particular CB1 receptors play an important role in nicotine-related behavior (Maldonado et al., 2006; Simonnet et al., 2012). CB1 receptors in the BNST are localized mainly glutamatergic neurons projecting from the ILCx and are shown to control cortical excitation of BNST neurons (Massi et al., 2008). However, it is unknown whether CB1 receptors in the BNST are involved in nicotine-induced neuronal modifications.

AIM

The experiments in this part of the thesis were conducted with the aim of elucidating the involvement of the ILCx-BNST pathway in the maladaptive behaviors induced by nicotine exposure. Moreover, since the endocannabinoid system, and in particular the CB1 receptors, controls nicotine reinforcement and nicotine-induced synaptic modification, we examine the involvement of CB1 receptors in the neural modifications and maladaptive behavior in rats induced by nicotine.

To this end, the following questions will be approached:

Question 1

Does learning of an association between active responding and nicotine reward provoke neuronal modifications in the ILCx-BNST pathway? And if so, is this mediated by CB1 receptors?

In order to identify the contribution of the associative learning process of cue-paired nicotine taking in the synaptic plasticity in this pathway, we will assess synaptic potentiation after various durations of nicotine IVSA training. To examine the persistence of the neuronal modifications, rats will be exposed to either forced abstinence or extinction training.

Question 2

What is the behavioral involvement of the ILCx-BNST pathway in nicotine-related behavior?

To address this question, we examined whether electrical potentiation of the glutamatergic ILCx projection to the BNST modifies operant behavior. If the synaptic potentiation is dependent on CB1 receptors, then blocking these receptors should prevent the expression of this behavior.

Question 3

Are CB1 receptors in the BNST implicated in all stages of the nicotine addiction process?

Following the results obtained with the previous experiments, we hypothesized that the BNST and local CB1 receptors contribute to the associative learning of nicotine and nicotine-associated cues. However, it is unknown whether the stimulation of CB1 receptors is critical for each of the cognitive and motivational processes involved in response contingent nicotine IVSA. Therefore, in the last part of the project we tested the involvement of BNST CB1 receptors at different behavioral stages of nicotine addiction (initiation, maintenance, motivation, extinction and reinstatement).

Question 1 and 2 are included in paper 1; paper 2 addresses question 3.

In vivo electrophysiological recordings are done in collaboration with Dr. François Georges, UMR5297.

RESULTS

ARTICLE 1

ARTICLE 1

Nicotine seeking depends on the CB1 receptor-dependent potentiation of Infralimbic Cortex – Bed Nucleus Stria Terminalis excitatory synapses.

Abbreviated title: CB1 receptors in the BNST control nicotine seeking

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The authors declare no competing financial interests.

ABSTRACT

Nicotine addiction is characterized by repetitive drug-seeking and consumption, but the underlying neural substrates are still unknown. Previous findings indicate that during active, but not passive nicotine taking in rats, excitatory projections from the infralimbic cortex (ILCx) to the bed nucleus of the stria terminalis (BNST) show an enhanced excitability. Thus, we hypothesized that the strengthening of the ILCx – BNST synapses contributes to the development of intravenous self-administration (IVSA) of nicotine. Using a combination of *in vivo* methods (electrical stimulation, extracellular recordings and pharmacological manipulations), we characterized the effects of a 10Hz – stimulation of the ILCx – BNST pathway on nicotine IVSA. In line with the involvement of cannabinoid CB1 receptors in nicotine addiction, we investigated whether the effects of the ILCx stimulation were CB1 receptor-dependent. Voluntary nicotine IVSA was associated with a persistent potentiation of excitatory responses in the BNST in response to 10Hz – stimulation of ILCx efferents. Behaviorally, this stimulation temporarily increased inappropriate responding for nicotine, but did not alter the number of nicotine infusions. This behavior was tightly controlled by CB1 receptors in the BNST. These findings suggest that the synaptic potentiation within the BNST leads to CB1 receptor-dependent functional changes crucial for the perseverative cue-elicited seeking in nicotine IVSA. Further functional characterization of the ILCx – BNST synapse will have significant impact on the understanding of long-term vulnerability to relapse during protracted abstinence.

INTRODUCTION

The ventromedial prefrontal cortex [infralimbic cortex (ILCx) in rodents] is involved in behavioral inhibition, attentional processes and goal-directed behaviors (Smith et al., 2012), suggesting a crucial role in drug-related behavior such as learning and relapse (Luscher and Malenka, 2011; Peters et al., 2009). Interestingly, the ILCx sends major excitatory projections to the bed nucleus of the stria terminalis (BNST) (Massi et al., 2008) which has been implicated in addiction (Carboni et al., 2000; Dumont et al., 2005; Dumont et al., 2008; Krawczyk et al., 2013). In agreement with these findings, we previously showed that the hyperactivity of the ventral tegmental area (VTA) dopamine neurons after volitional nicotine IVSA, and not forced nicotine exposure, is driven by changes in the ILCx – BNST excitatory afferents to the VTA (Caille et al., 2009). However, it is unknown whether ILCx – BNST synapses are more susceptible to a LTP-induction protocol after nicotine IVSA in response to the stimulation of the ILCx at a physiological relevant frequency (10 Hz). To address this issue, we trained rats for nicotine IVSA for several durations, and investigated the correlation between the emergence of synaptic potentiation and the acquisition of nicotine IVSA. Subsequently, a 10Hz – stimulation of the ILCx was applied and *in vivo* recordings of BNST neurons were performed twenty-four hours after the last session. Based on the hypothesis that long-term neuroadaptations of synapses may support persistent drug seeking, we also investigated whether the 10Hz – stimulation – induced neuroplastic adaptation in the BNST is resistant to one-month of nicotine abstinence. A further step was to examine whether the electrical stimulation of the ILCx – BNST pathway contributes to operant behavior for nicotine and nicotine-paired cues. Lastly, since the cannabinoid CB1 receptors play an important role in nicotine-related behaviors (Maldonado et al., 2006; Simonnet et al., 2012) and CB1 receptors in the BNST control cortical excitation of the BNST neurons (Massi et al., 2008), we tested whether the neuroplastic changes and behavioral changes produced by 10 Hz stimulation were CB1 receptor-dependent.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats weighing 175–200 g (Charles River, St Germain sur l'Arbresle, France) were housed collectively at 20–22°C with a reversed 12 hr light/dark cycle (lights off from 9:30 a.m.). One week before experiment start, rats were placed on a restricted diet of 20 g/ day lab chow, sufficient to maintain body weight and growth. Water was available *ad libitum* and food was given daily after the self-administration session. All procedures were conducted in accordance with the European directive 2010-63-EU on the protection of animals used for scientific purposes and with approval of the Bordeaux University Animal Care and Use Committee (# 5012058-A).

Surgery

Intravenous surgery was performed under anesthesia with ketamine (75 mg/ kg) and xylazine (7.5 mg/ kg) intraperitoneal (i.p.). Rats were prepared with chronic indwelling jugular catheters as described previously (Caille et al., 2009). During postoperative recovery, catheters were flushed daily with 0.2 mL ampicillin (0.1 g/ mL; Coophavet, Ancenis, France) in heparinized saline (300 IU heparin per mL 0.9 % NaCl) for 6 days. Subsequently, catheters were flushed daily with heparinized saline.

Stereotaxic surgery for *in vivo* electrophysiology experiments was performed under isoflurane anesthesia as previously described (Georges and Aston-Jones, 2002). Stimulation electrodes and recording and injection pipettes were inserted into ILCx or BNST at the following coordinates (Paxinos and Watson, 1998): ILCx: +3.0 mm from bregma, 0.5 mm from midline and 4.5 mm from dura; BNST: -0.3 mm from bregma, 1.5 mm from midline, 6.0–7.5 mm from dura.

Nicotine intravenous self-administration

Experiments started at the beginning of the dark phase. Rats were tested in standard operant chambers (Imetronic, Pessac, France) equipped with two nose-poke devices ("active" and "inactive"). Daily 2h IVSA sessions started with illumination of the house light and a single non-contingent infusion. Session of NIC1day rats continued till rats received 10 injections of nicotine. "Active" nose-poke responding resulted in the delivery of a nicotine infusion (30 µg base/ kg/ 100µL, and increased to 60 µg base/ kg/ 100µL when stable responding was established; rats trained for 1 and 8 days nicotine IVSA received directly 60 µg base/ kg/ 100µL) or saline over 4 s. Each infusion was paired with a cue-light for 4 s and followed by a 20 s time-out period during which visits of the active nose-poke were recorded but had no consequences. "Inactive" nose-poke responding was recorded but had no programmed consequences. Infusions were earned on a fixed-ratio (FR) schedule of reinforcement (10 days FR1; 2 days FR2; remaining days on FR5). NIC1day and NIC8days rats were trained under FR1 schedule of reinforcement.

Oral saccharin self-administration

Experiments started at the beginning of the dark phase. Rats were tested in standard operant chambers (see above, Imetronic, Pessac, France). Daily 30 min sessions started with illumination of the house light. “Active” nose-poke responding resulted in the delivery of 112µl volume of 0.13 % saccharin (Sigma-Aldrich, Lyon, France) over 4 s via a fluid injection assembly (syringe pump) into a dipper cup. Each delivery was paired with a cue-light above the nose-hole for 4 s. “Inactive” nose-poke responses were recorded but had no programmed consequences. Rats were trained for the acquisition of self-administration under a FR schedule of reinforcement (5 days FR1; 2 days FR2; remaining days FR5). For each rat, training lasted at least 3 weeks and continued until electrophysiological recording.

***In vivo* single-unit electrophysiology**

Electrical stimulation of the ILCx

Electrical pulses were applied to a stimulating electrode to activate excitatory efferents that project to the recorded target cells in the BNST. Bipolar electrical stimulation of the ILCx was conducted with a concentric electrode (Phymep, Paris, France) and a stimulus isolator (500 µs, 0.2–1 mA, DS3; Digitimer, Welwyn Garden City, UK). First, a 10 min baseline was established. This consisted of application of a 100 pulse train (0.5 Hz) twice, while evoked spiking in the BNST was recorded. Then, tetanic stimulation was performed (1 min, 10Hz). To measure the effect of tetanic stimulation on magnitude of evoked responses, we applied at least four 100 pulse trains (0.5 Hz),while recording evoked responses in the BNST.

BNST recordings and pharmacological micro-infusion

Evoked response magnitude was measured in the BNST. A glass micropipette (tip diameter, 1–2 µm; 10–15 MΩ) filled with a 2% pontamine sky blue solution in 0.5 M sodium acetate was lowered into the BNST. The extracellular potential was recorded with an Axoclamp2B amplifier and filtered (300 Hz/ 0.5 kHz) via a differential AC amplifier (Georges and Aston-Jones, 2002). Single neuron spikes were discriminated and collected online (CED 1401, SPIKE 2; Cambridge Electronic Design, UK).

Double-barreled pipettes (Georges and Aston-Jones, 2002) were used to record BNST activity while microinjecting drugs. A total volume of 60 nL was infused into the BNST, using pneumatic pressure (Picospritzer; General Valve, Fairfield, NJ, USA). One min after micro-infusion into the BNST, the ILCx was electrically stimulated for 1 min at 10Hz. Post-tetanic evoked responses were recorded for at least 20 min while stimulating the ILCx (100 pulses; 0.5 Hz).

Experimental design

a) Nicotine-dependent long term potentiation in the BNST

The first question was to determine the conditions under which a 10Hz – stimulation of the ILCx would induce a LTP in the BNST. The stimulation protocol was run at several time points in order to examine whether LTP was correlated with the acquisition of cue-paired nicotine self-administration (Figure 1A). We used 5 groups: i) rats with a single nicotine IVSA session (NIC-1day, n=5);ii) rats with 8 nicotine IVSA sessions (NIC-8days, n=9) iii) rats with extended training for nicotine (NIC-60days, n=8); iv) nicotine yoked rats (n=5) which received nicotine infusions matched to those of NIC-60days rats, so yoked rats were not able to associate the nicotine delivery with the visual stimuli; and v) rats with extended training for saline (SAL-60days, n=7). A last control group, trained for saccharin 0.13 % (n=5), was added to examine if LTP in the BNST, induced by electrical stimulation of ILCx, was specific to nicotine IVSA. Electrophysiological recordings were performed 24 h after the last access to the operant chambers.

b) Persistence of the long term potentiation in the BNST

We examined whether the ILCx – BNST LTP is sensitive to passive abstinence or to active suppression of acquired responses (extinction training). A group of animals was trained for nicotine IVSA. Part of the group (Abst, n=7) was confined to home cages for 30 days, until electrophysiological recordings were performed. The other part (Ext, n=6) performed extinction training where active nose-poke responding had no programmed consequences: no drug delivery and no cue presentation. For each rat, extinction training continued until the active responding level was 30% of baseline (average of 3 consecutive days), then electrophysiological recordings were performed 24 h after the last extinction session.

c) Characterization of the long term potentiation in the BNST

NMDA-dependent plasticity. Rats were trained for nicotine IVSA. Twenty-four hours after the last session, each rat was subjected to *in vivo* electrophysiology. One minute before tetanic stimulation, either the NMDA receptor antagonist AP5 (100 µM; NIC-60days + AP5, n=5) or saline vehicle (NIC-60days + Veh; n=8) was micro-infused into the BNST.

CB1 receptor-dependent plasticity. Rats were trained for nicotine IVSA and recorded 24 h after the last access to the IVSA chambers. Two groups were tested with intravenous injection of two CB1 antagonists: AM251 (2 mg/kg; NIC-60days + AM251, n=5) or O-2050 (0.5 mg/kg i.v., n= 5) or its vehicle (Ringer solution containing 0.25% DMSO and 0.25% cremophor; NIC-60days + Veh, n=3), 15 min (AM251) and 60 min (O-2050) before tetanic stimulation.

Two additional groups were tested with intra-BNST infusion of AM251 (400 µM; NIC-60days + AM251, n=5) or vehicle (NIC-60days + Veh; n=5).

d) Effect of electrical stimulation of the ILCx on nicotine IVSA

Rats were trained for nicotine IVSA for 2 months. Twenty-four hours after the last IVSA session, electrodes were bilaterally inserted into the ILCx as described above. Electrical stimulation (1 min, 10Hz) was administered once using a square pulse stimulator and stimulus isolator (DS3; Digitimer). One minute before tetanic stimulation, rats received intra-BNST infusion of either AM251 (400 µM, n=13) or vehicle (n=13). After a 48 h recovery period, rats were placed back in the operant cages and nicotine IVSA was monitored for 4 consecutive sessions.

Data analysis

Behavioral data. Nicotine self-administration data were subjected to ANOVAs, with nicotine exposure (NIC-1day, NIC-60days, yoked, saline) as the between-subject factor and IVSA session as the within-subject factor. Whenever main factor effects were found, *post hoc* comparisons were performed using the Fisher PLSD test. The average (5 days prior to electrophysiological recordings) discrimination rate [(active nose-pokes / total nose-pokes) × 100] was compared to chance (50%) with a Student's *t* test. For statistical analysis of locomotor activity, 2 rats in Veh group and 1 rat in AM251 group were excluded because we were not able to record activity of these rats during IVSA sessions. In all cases, differences with $p < 0.05$ were considered significant.

Electrophysiological recordings. During electrical stimulation of the ILCx, cumulative peristimulus time histograms (PSTHs, 5 ms bin width) of BNST activity were generated for each neuron recorded. PSTHs were analyzed to determine excitatory epochs as previously described (Georges and Aston-Jones, 2002). Excitatory magnitudes (R_{mag} values) were normalized for different levels of baseline impulse activity, allowing comparison of effects of stimulus intensity on evoked responses independent of those on baseline activity. R_{mag} values for excitation were calculated with the following equation: Excitation R_{mag} = (counts in excitatory epoch) – (mean counts per baseline bin × number of bins in excitatory epoch). For statistical analysis, average of excitatory response magnitude of 30-40 min post-tetanus was used. For multiple comparison, One-way ANOVA was used. Where two means were compared or comparison with baseline (100%), the two-tailed paired Student's *t* test was used.

RESULTS

A 10 Hz-stimulation of the ILCx onto BNST excitatory synapses induces long-term potentiation in rats with extended nicotine IVSA training

We first examined whether extended nicotine IVSA training strengthens the ILCx – BNST excitatory synapses. Rats had access to nicotine IVSA by nose-poke operant responding for one day (NIC-1day), eight days (NIC-8days) or sixty days (NIC-60days; timeline, **Figure 1A**). Control rats were trained with saline (SAL-60days) and the volitional aspect was examined with a yoked group (Palmatier et al., 2007). NIC-60days and Abst groups showed significantly more responses in the active nose-hole compared to other experimental groups (One-way ANOVA, $F_{(6,40)} = 9.47$; NIC-1day: 19.6 ± 2.3; NIC-8days: 29.1 ± 1.6; SAL-60days: 21.1 ± 4.9; NIC-60days: 70.0 ± 10.4; Yoked: 16.9 ± 6.5; Ext: 15.1 ± 3.8; Abst: 50.8 ± 10.1; **Figure 1B**). We observed differences in discriminations rate between groups (One-way ANOVA, $F_{(4,29)} = 3.90$, $p < 0.01$). NIC-8days and NIC-60days rats acquired nicotine IVSA, indicated by a discrimination rate greater than chance level. (NIC-8days: 60.1 ± 3.9; $t_{(8)} = 2.62$, $p < 0.05$; NIC-60days: 76.9 ± 3.7; $t_{(7)} = 7.33$, $p < 0.001$; **Figure 1C**). Discrimination rate of NIC-1 day, SAL-60days and

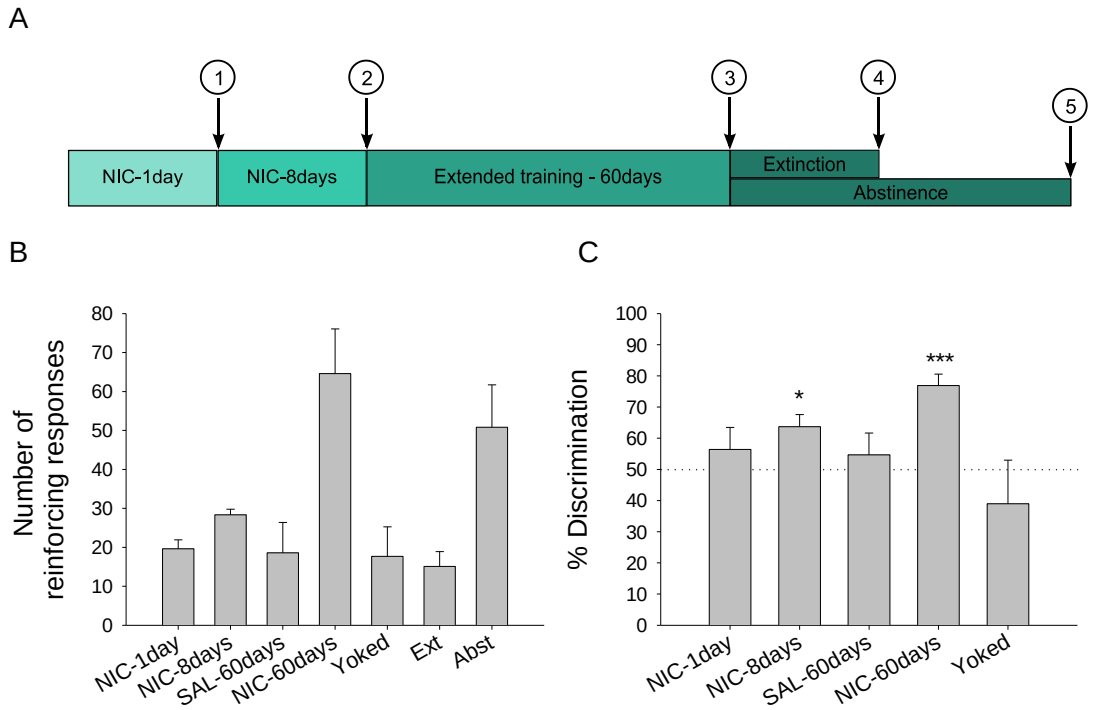

Figure 1 Operant behavior before electrophysiological recordings. **(A)** Timeline of experimental set-up: animals were recorded 24 h after (1) one nicotine self-administration session (NIC-1day, n=5), (2) 8 days of nicotine self-administration (NIC-8days, n=9), (3) extended training (NIC-60days, n=8; SAL-60days, n=7; yoked, n=5), (4) extinction (n=6) or (5) abstinence (n=7). **(B)** Number of reinforcing responses before electrophysiological recording for each experimental group **(C)** NIC-60days and NIC-8days rats showed strong preference for the active nose-hole (discrimination rate vs. chance, *p < 0.05 ***p < 0.001). Data are shown as mean ± SEM.

Yoked group did not differ from chance level, suggesting that they did not acquire the self-administration paradigm (NIC-1day: 56.4 ± 7.1, $t_{(4)} = 0.91$, $p > 0.05$; SAL-60days: 54.7 ± 7.0, $t_{(6)} = 0.67$, $p > 0.05$; Yoked: 39.0 ± 14.0, $t_{(4)} = 0.79$, $p > 0.05$; **Figure 1C**). At the end of the 2-months training, NIC-60days and yoked rats had similar total nicotine intake (NIC-60 days, 31.75 ± 1.63 mg/ kg; yoked, 36.30 ± 4.74 mg/kg; $t_{(11)} = 0.57$, $p > 0.05$). *In vivo* electrophysiological recordings of BNST neurons were performed 24 h after the last IVSA session. Excitatory responses to electrical ILCx stimulation (1 min, 10 Hz; **Figure 2A, B**) in NIC-60days rats were greater than in NIC-1day, SAL-60days and yoked rats (average 30-40 min post-tetanus; NIC-60days: 159 ± 17 %; NIC-1days: 93 ± 7 %; SAL-60days: 92 ± 6 %; yoked: 102 ± 11 %; One-way ANOVA, $F_{(4,23)} = 4.08$, *Post hoc* LSD, $^{*}p < 0.05$, $^{**}p < 0.01$; **Figure 2C, D**) and was significant different from baseline (NIC-60days: $t_{(5)} = 3.47$, $p < 0.05$). Although excitatory responses in NIC-8days were greater than NIC-1day and SAL-60days, it did not differ from baseline (NIC-8days: 137 ± 20 %; $t_{6)} = 1.87$, $p > 0.05$). Excitatory responses of NIC-1day, SAL-60days and yoked did not differ from each other and from baseline (NIC-1day: $t_{(4)} = 1.06$, $p > 0.05$; SAL-60day: $t_{(4)} = 1.46$, $p > 0.05$; Yoked: $t_{(4)} = 0.21$, $p > 0.05$; **Figure 2D**). Increase in excitatory response magnitude seen after 60 days of nicotine IVSA is NMDA-dependent as we blocked LTP with NMDA-receptor antagonist AP5 (average 30-40 min post-tetanus; NIC-60days: 159 ± 17 %; AP5: 81 ± 14 %; $t_{(9)} = 3.46$; $p < 0.05$; **Figure 2E**). Excitatory responses of NIC-60days was greater than baseline, while NIC-60days + AP5 was not (NIC-60days: $t_{(5)} = 3.47$, $p < 0.05$; NIC-60days + AP5: $t_{(4)} = 1.39$; $p > 0.05$).

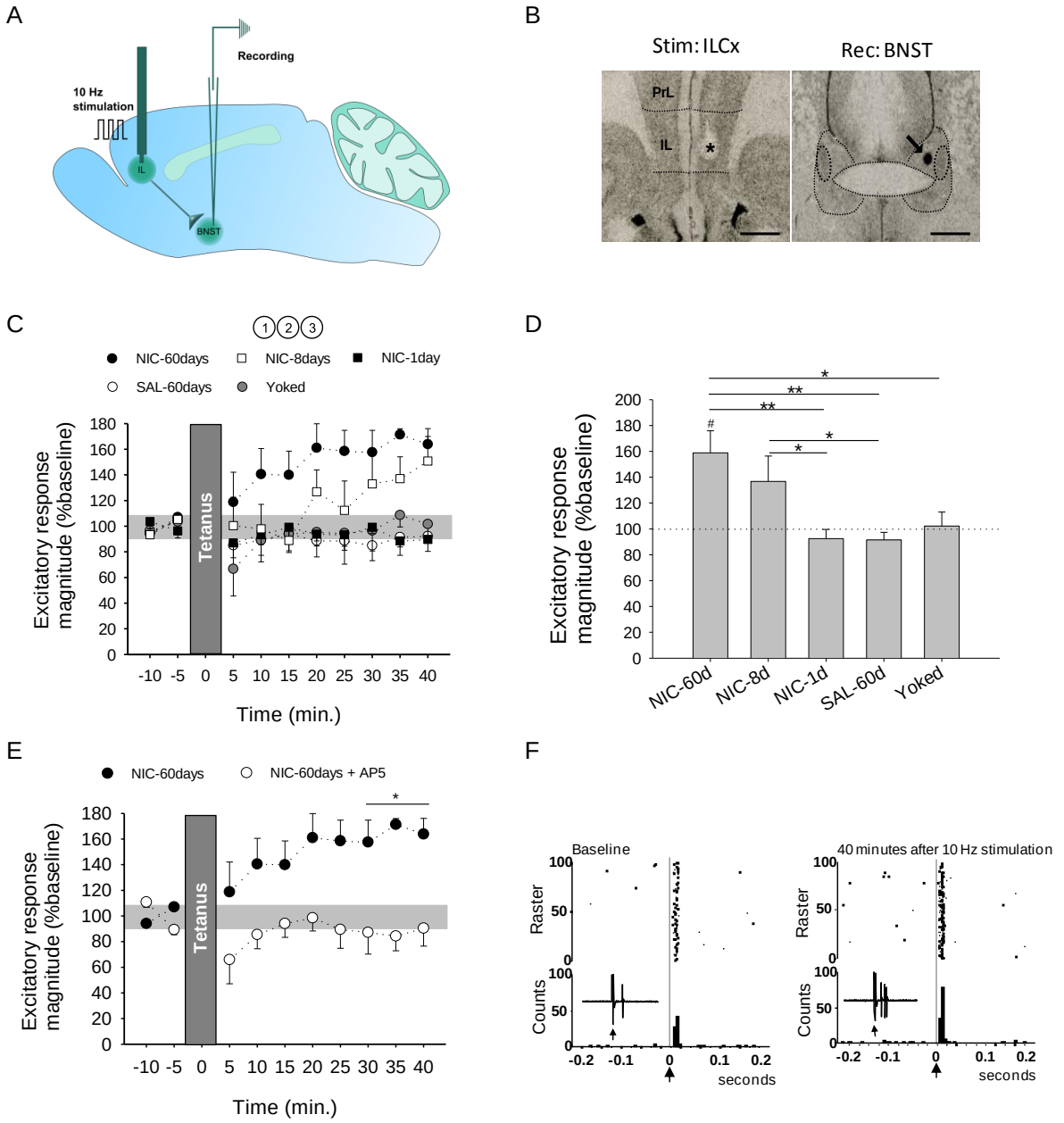

Figure 2 Neuroplastic changes in the BNST after various durations of nicotine self-administration. **(A)** Stimulation and recording protocol. **(B)** Histological control of stimulation (ILCx, asterisk) and recording (BNST, arrow) sites; scale bar, 1 mm. **(C)** LTP seen in NIC-60days but not NIC-1day, NIC-8days, SAL-60days or yoked rats. **(D)** Average excitatory response magnitude of 30-40 min after tetanus. **p* < 0.05, **p < 0.01, #p < 0.05 **(E)** Intra-BNST infusion of AP5 blocks LTP induced after 60 days of nicotine IVSA * p < 0.05 **(F)** Baseline and post-tetanic stimulation responses of ILCx – BNST projection neuron in NIC-60days rats. Stimulus delivered at time 0 (arrow). Each histogram consists of 100 trials individually illustrated in the associated raster. Bin width, 5 ms.

Detailed analysis of individual rats self-administering nicotine indicates a correlation between discrimination rate and the excitatory response magnitude (average of 30 – 40 min after tetanic stimulation) induced in the BNST for NIC-8days, but not NIC-1day and NIC-60days. (Spearman correlation, NIC-1day: $r = 0.30$, $p > 0.05$; NIC-8days: $r = 0.86$, $p < 0.01$; NIC-60days: $r = 0.20$, $p > 0.05$, **Figure 3**).

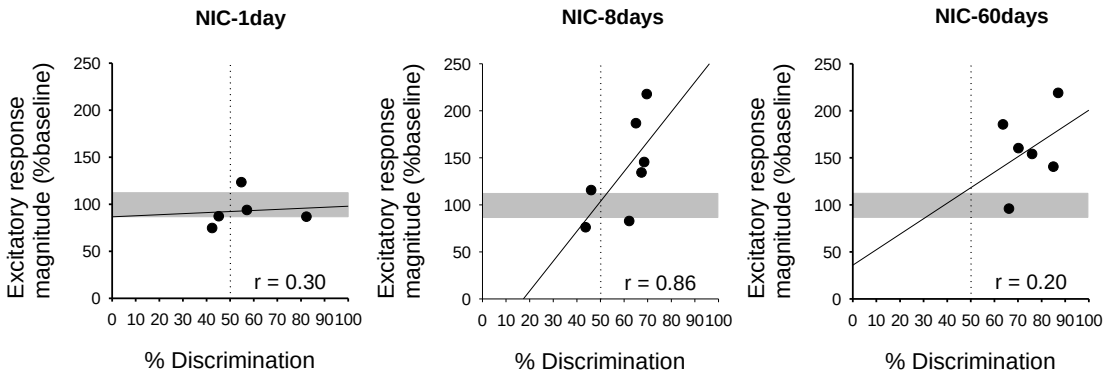

Figure 3 Neuroplastic changes of individual rats after various periods of nicotine IVSA. Correlation between discrimination rate and excitatory response magnitude during 30 – 40 min after tetanic stimulation after various periods of nicotine IVSA.

Figure 4 displays the behavioral and electrophysiological data of oral saccharin self-administration. Responses in the active nose-hole increased across sessions, while responses in the inactive nose-hole remained low (Two-way ANOVA, session X hole interaction, $F_{(17,102)} = 5.07$, $p < 0.001$; **Figure 4A**). Rats acquired the saccharin self-administration paradigm, indicated by a discrimination rate between active and inactive nose-hole greater than chance level (Student's t-test discrimination rate compared to chance level (50 %), $t_{(4)} = 21.62$, $p < 0.001$; **Figure 4B**). Saccharin self-administration did not induce LTP within the BNST (20 – 40 min after tetanus: 134.2 ± 46.7; $t_{(4)} = 1.10$, $p > 0.05$; **Figure 4C**). We did not find a correlation between discrimination rate and the excitatory response magnitude (average 20 - 40 min after tetanic stimulation) (Spearman correlation; $r = 0.00$; $p > 0.05$; **Figure 4D**). These data suggest that the LTP at ILCx – BNST synapses in response to 10 Hz – stimulation of ILCx afferents is specific to acquisition of active nicotine self-administration.

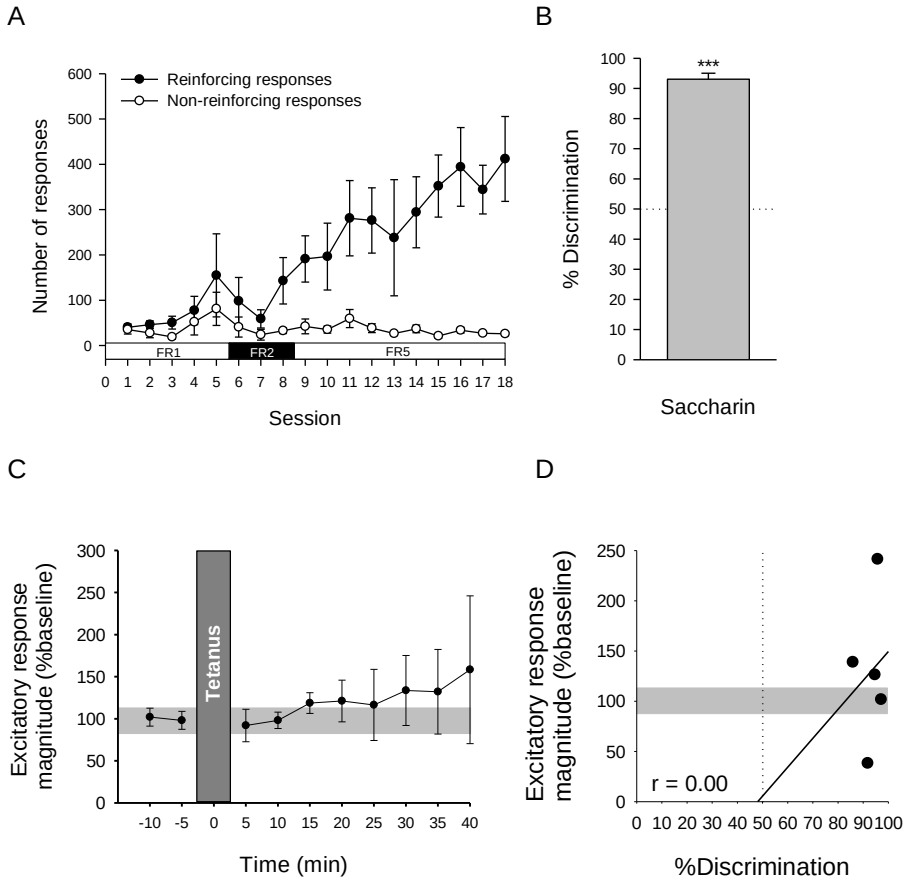

Figure 4 Acquisition of saccharin self-administration and electrophysiological recordings in the BNST (A) Responding during saccharin (0.13 %) self-administration training. Rats were trained under a fixed ratio (FR) schedule of reinforcement (FR1, FR2 and FR5). **(B)** Average of discrimination rate during the last 5 days of saccharin self-administration before electrophysiological recordings. Dotted line represents chance level. **(C)** No LTP seen after saccharin self-administration. **(D)** Correlation between discrimination rate and excitatory response magnitude during 20 – 40 min after tetanic stimulation after saccharin self-administration.

Persistence of long-term potentiation in the BNST

To determine the persistence of LTP, we subjected rats to either forced abstinence, or extinction training (inhibitory learning of previously acquired responding) after 60 days of nicotine IVSA. The number of responses in former active nose-hole declined across sessions (t-test, average of responses during 5 days before extinction compared to average of responses during the last 3 days of extinction training, $t_{(5)} = 3.97$, $p < 0.01$; **Figure 5A**), while responses in the inactive hole remained stable (data not shown). Rats required 17.2 ± 2.7 days (ranging from 11 to 27 days) to reach the extinction criterion. Extinction training, but not abstinence, suppressed LTP and provoked long-term depression (LTD) in the BNST (average 30-40 min post-tetanus; Abst: 156 ± 15 %; Ext: 42 ± 9 %; $t_{(8)} = 6.58$, $p < 0.001$; **Figure 5B, C**). Excitatory responses of Abst was greater than baseline, while it was significant smaller in the Ext group (Abst: $t_{(4)} = 3.82$, $p < 0.05$; Ext: $t_{(4)} = 6.27$, $p < 0.01$; **Figure 5C**). This indicates that the LTP is resistant to a passive drug-free period, but sensitive to the acquisition of inhibitory learning.

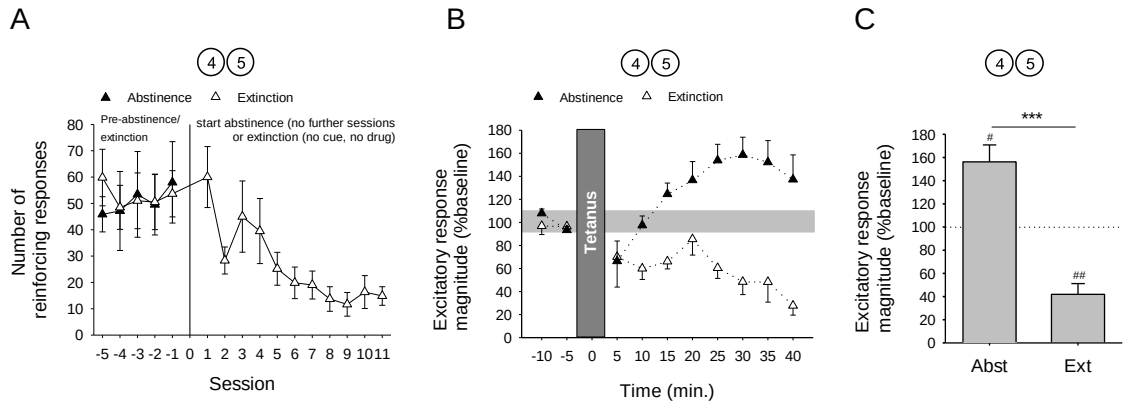

Figure 5 Persistence of neuroplastic changes in the BNST. (A) Decreasing active responses during extinction training. Rats in the Abst group do not have any further sessions. **(B)** Extinction (Ext, n=6) provokes LTD while LTP was still present after abstinence (Abst, n=7). **(C)** Average excitatory response magnitude of 30-40 min after tetanus. ***p<0.001, #p < 0.05.

Long-term potentiation in the BNST is CB1 receptor-dependent

Since the endocannabinoid system plays an important role in nicotine reward (Simonnet et al., 2012) and the modulation of synaptic plasticity in the BNST (Massi et al., 2008; Puente et al., 2011), we investigated whether the potentiation of ILCx – BNST excitatory synapses seen after 60days of nicotine IVSA is dependent on CB1 receptors activation. LTP in the BNST of NIC-60days rats was blocked by peripheral injection of the CB1 antagonists (excitatory response magnitude 20-40 min, AM251 2 mg/ kg i.v.: 85 ± 12%, $t_{(4)} = 1.20$, $p > 0.05$; O-2050 0.5 mg/ kg i.v., 129 ± 33 %; $p > 0.5$). More specifically, LTP was blocked after infusion of AM251 within the BNST (60 nL, 400 µM; **Figure 6A, B**). This suggests that volitional nicotine IVSA induces CB1-dependent synaptic strengthening at BNST synapses.

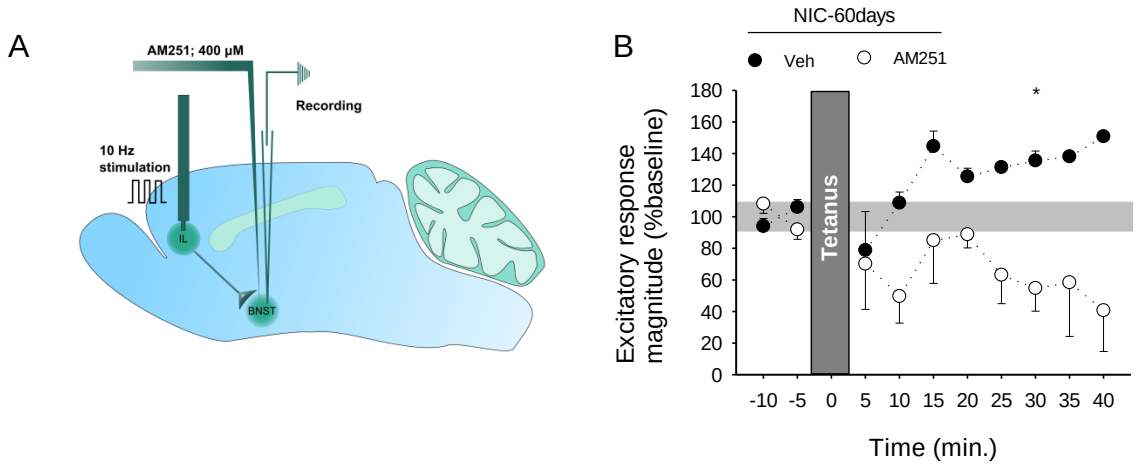

Figure 6 (A) *In vivo* ILCx stimulation in anesthetized rats (1 min, 10 Hz). CB1 antagonist AM251 (60 nL, 400 µM) or vehicle was infused into the BNST before stimulation. **(B)** AM251 blocks LTP induced by extended nicotine IVSA (Veh, n=5; AM251, n=5).

Electrical stimulation of the ILCx temporarily induces aberrant stimulus-response behavior controlled by CB1 receptors in the BNST

Finally, we examined whether electrical stimulation of the ILCx modifies operant behavior for nicotine IVSA (timeline, **Figure 7A**). In line with the finding that local CB1 antagonism blocked LTP, we hypothesized that intra-BNST microinfusion of AM251 prior to ILCx stimulation would prevent any changes in operant behavior induced by ILCx stimulation. Our results show that stimulation of the ILCx after extended training temporarily increases responding in the active nose-hole during the post-injection pause (time-out) (Two-way ANOVA, $F_{(3,72)} = 3.64$, $p < 0.05$, *post hoc* LSD, $^{**}p < 0.01$; t-test, AM251 session 3 compared to 100%, $t_{(12)} = 2.84$, $\#p < 0.05$). This behavior was prevented by micro-infusion of CB1 receptor in the BNST (AM251, 400 µM; **Figure 7B**). Before stimulation of the ILCx, no difference between responding during the time-out period were detected (Veh: 9.4 ± 1.4; AM251: 11.3 ± 4.0: $t_{(24)} = 0.44$, $p > 0.05$). Stimulation of ILCx did not affect the number of nicotine infusions (Veh: 706 ± 54.8; AM251: 692 ± 70.1: $t_{(24)} = 0.16$, $p > 0.05$; **Figure 7C**) and locomotor activity (Veh: 653.3 ± 66.6; AM251: 556.1 ± 86.6: $t_{(21)} = 0.88$, $p > 0.05$), indicating that ILCx stimulation affects specifically nicotine-paired cue seeking behavior while the reinforcing and psychostimulant properties of nicotine were unaffected.

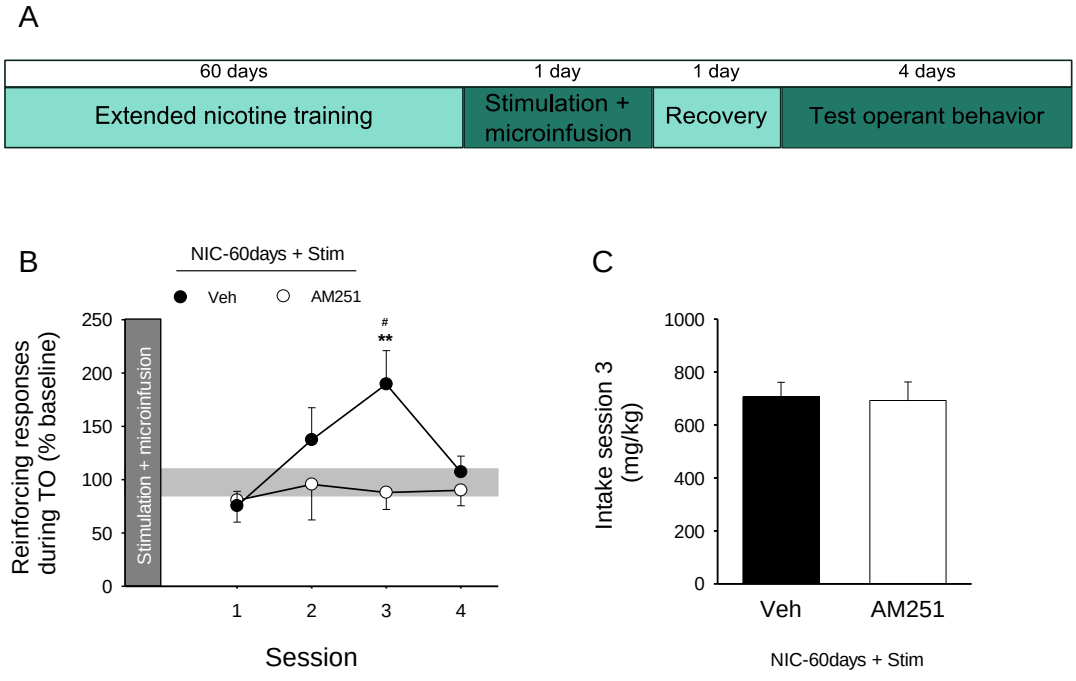

Figure 7 CB1 receptor-mediated LTP in the BNST gates excessive nicotine seeking. (A) Timeline of the experimental set-up. **(B)** ILCx stimulation increases inappropriate responding in the active nose-hole during time-out period, but not after intra-BNST AM251 (Veh n=13, AM251 n=13;$^{**}p<0.01$, $\#p<0.05$). **(C)** Nicotine intake during session 3 does not differ between groups.

DISCUSSION

In this study, we examined whether the strengthening of the ILCx – BNST excitatory synapses contributes to the acquisition of nicotine IVSA. We showed that 10 Hz – stimulation of the ILCx elicits long-term potentiation in the BNST of animals with extended active, but not passive nicotine taking. In addition, this neuroplasticity remains following 30 days of forced nicotine abstinence. We demonstrated that the electrical stimulation of the ILCx promotes perseverative nicotine seeking behavior during periods when the drug is unavailable. Lastly, CB1 receptors in the BNST are necessary for both the nicotine IVSA dependent LTP and excessive nicotine seeking behavior.

Acquisition of the nicotine IVSA paradigm is necessary for the induction of LTP in the BNST. Rats that did not self-administer nicotine (SAL-60days or saccharin groups) or rats that did not meet the learning criterion (yoked and NIC-1day groups) did not show LTP in response to the electrical stimulation of the ILCx. Importantly, NIC-8days rats provided further evidence for the hypothesis that a high discrimination rate is crucial for the induction of LTP, indicated as a correlation between the discrimination rate and the emergence of synaptic plasticity in the ILCx – BNST pathway. Our results are in agreement with other studies showing that addiction-related synaptic changes only fully developed after the transition from controlled intake to compulsive behavior (Kasanetz et al., 2013). Thus, we speculate that 8 days of nicotine IVSA is sufficient to induce LTP at excitatory synapses in the BNST for some rats, while for others this may develop progressively and concomitant with an increase in discrimination rate.

Extinction training is conceptualized as a form of inhibitory learning or new learning that reduces the expression of a previously acquired memory and/or forms memory of the new situation where drug is unavailable. Thus, extinction training is an active learning process that involves reduced drug seeking when the contingency between drug seeking behavior and the delivery of the drug is degraded. It has been shown that the ILCx is necessary for the extinction of drug seeking, as inactivation of the ILCx induced reinstatement following extinction training (Peters et al., 2008). Moreover, this study shows that activation of projections from the ILCx to the NAc shell are important for the inhibitory control of drug seeking (Peters et al., 2008). Here we demonstrate that extinction training inhibits the ILCx – BNST pathway, while the synaptic strengthening remains intact after forced abstinence.

The extinction protocol used in this study resulted in an average of 17.2 days to meet extinction criterion. However, the forced abstinence period was 30 days, which leads to the possibility that LTP, found after abstinence might be due to an incubation effect (Grimm et al., 2001), rather than persistence of synaptic modifications to a drug-free period. However, this is unlikely as the number of days of extinction training ranged from 11 to 27 days, while all rats showed induction

of LTD. Yet, we have not subjected rats to various durations of forced abstinence to rule out the incubation effect of LTP over time.

Our findings suggest that the potentiation of the ILCx – BNST pathway is linked to an aberrant stimulus-response behavior and might contribute to the established habitual behaviors observed in drug addiction (Everitt and Robbins, 2005). The development of drug-taking habits is involved in the transition from drug use to drug abuse. A further step would be to manipulate the habitual learning (Smith et al., 2012; Zapata et al., 2010) and examine the consequences on nicotine seeking and on the induction of LTP within the BNST.

A major disadvantage of *in vivo* electrical stimulation is that not only cell bodies of the neurons projecting to the region of interest (BNST) are activated by the stimulation applied, but also neurons projecting to other areas. For example, projections of the ILCx to the NAc are shown to be involved in drug-seeking (Peters et al., 2008). We have overcome this limitation by showing that the stimulation-induced excessive responding for nicotine is blocked by CB1 antagonist in the BNST, indicating that the ILCx – BNST pathway is specifically mediates this behavior.

Related to the cue-associated IVSA model used in this study, smoking-related cues are of increased significance in animal models of nicotine addiction (Chaudhri et al., 2006) and in human populations of smokers (Rose and Corrigall, 1997). We show here that LTP induction and suppression are both directly linked to the learning of a specific relationship between motor actions and cue-associated reward rather than to passive administration of nicotine. Thus, while nicotine enhances the reinforcing properties of contextual stimuli (Palmatier et al., 2007), re-exposing rats with a long history of nicotine-taking may reactivate the strengthened projections of the ILCx (Bossert et al., 2011) and subsequently enhance nicotine seeking. In accordance with this idea, we show here that the 10Hz – stimulation of the ILCx gradually and temporarily promotes excessive visits of the nicotine- and cue-paired nose-poke hole. The return of the time-out responses to baseline level at post-stimulation day 4 may be explained by re-acquisition of contingency learning.

In rats with extended access to nicotine, the synaptic modifications in the ILCx – BNST pathway are CB1 receptor-dependent. Notably, this effect is unlikely due to the inverse agonist properties of AM251 as LTP was also blocked by the administration of O-2050, a CB1 receptor neutral antagonist (Dubreucq et al., 2013). In naïve rats, *ex vivo* electrophysiological studies have already supported the existence of endocannabinoid-dependent forms of synaptic plasticity in the BNST (Puente et al., 2011). Interestingly, nicotine self-administration increases 2-AG as well as anandamide (AEA) levels in the VTA of behaving animals (Buczynski et al., 2013). While the increased VTA AEA levels correlate with the volitional nature of drug exposure, the specific blockade of CB1 receptors in the VTA strongly decreases nicotine intravenous self-administration in rats (Simonnet et al., 2012). Thus, one can propose that similar to the VTA mechanisms, chronic nicotine consumption might lead

to changes in the endocannabinoid tone within the BNST and contribute to the potentiation of excitatory ILCx-BNXT synapses. However, the nature of the endocannabinoid involved here remains unknown, leaving the examination of extracellular endocannabinoid levels in the BNST to future investigations in rats taking nicotine.

Peripheral administration of a CB1 antagonist strongly decreases nicotine self-administration (Cohen et al., 2002; Shoaib, 2008; Simonnet et al., 2012) suggesting that stimulation of CB1 receptors are necessary for nicotine reinforcement. In the present study, AM251 injected within the BNST selectively blocks excessive responding during time-out but does not change nicotine intake. In line with this observation, previous studies have indicated that the BNST is involved in cue-induced drug seeking rather than in the control of drug taking. For instance, increased activity of neurons in the BNST is associated with increased drug seeking (in a model of opiate-induced conditioned place preference) during protracted withdrawal (Harris and Aston-Jones, 2003). Moreover, inactivation of the BNST specifically blocks cocaine seeking elicited by drug-paired cues (Buffalari and See, 2011). These findings support the hypothesis that extended access to nicotine induces CB1 receptor-dependent synaptic plasticity in the BNST that might enhance the stimulus-response behavior paired with nicotine taking (Palmatier et al., 2013; Paolone et al., 2013).

In conclusion, our findings reveal that extended voluntary nicotine intake leads to a persistent potentiation of excitatory responses in the BNST in response to 10Hz – stimulation of ILCx afferents. This LTP seems to contribute to a maladaptive stimulus-response behavior controlled by CB1 receptors (Marsicano et al., 2002) in the BNST and might be responsible for vulnerability to cue-induced relapse. Further functional characterization of the ILCx – BNST synapse will have a significant impact on the understanding of nicotine seeking during protracted abstinence.

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ARTICLE 2

ARTICLE 2

Behavioral effects of cannabinoid-1 receptor agonist in the bed nucleus of the stria terminalis depends on the stage of voluntary nicotine self-administration in rats.

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ABSTRACT

We have previously shown that the synaptic potentiation of the network including the infralimbic cortex (ILCx), bed nucleus of the stria terminals (BNST) and ventral tegmental area (VTA) is important for the associative learning process underlying nicotine intravenous self-administration (IVSA) in rats and depends on activation of cannabinoid-1 (CB1) receptors in the BNST. However, it is unknown whether the role of CB1 receptors depends on the level of learning and/or level of nicotine intake. Therefore, we examined the behavioral implication of BNST CB1 receptors at different stages of the nicotine IVSA paradigm.

In group 1, the CB1 agonist WIN55,212-2 (WIN55) was administered before each session of the first 6 days of acquisition after which IVSA was continued to test for any long-term effects of WIN55. In group 2, the effect of intra-BNST WIN55 was tested after 5 weeks of nicotine IVSA on maintenance and motivation to nicotine. In group 3, extinction training was performed after 5 weeks of nicotine IVSA and WIN55 effects were tested on reinstatement.

In group 1, we found that BNST CB1 receptors stimulation transiently impairs the first stage of nicotine IVSA learning mainly due to acute reduced locomotion. This effect disappeared as soon as the treatment ceased. However, later on, WIN55 pretreated rats were unable to adapt to increasing workload to obtain nicotine, both during increased fixed ratio and during progressive ratio schedule of reinforcement. Finally, WIN55 pre-treatment does not affect nicotine- and cue-induced reinstatement. In group 2, we observed that stimulation of CB1 receptors in the BNST after 5 weeks of nicotine IVSA does not affect operant responding during fixed ratio and progressive ratio schedule of reinforcement. In group 3, WIN55 injection blocks both cue-induced and nicotine-induced reinstatement following extinction training.

These data imply that CB1 receptor stimulation in the BNST before long-term exposure to nicotine induces protracted difficulty to adapt to increasing workload and decreased motivation, without altering nicotine intake. Moreover, rats treated with WIN55 during acquisition phase of nicotine IVSA are still sensitive to nicotine and nicotine-associated cue. However, after a history of nicotine, while CB1 agonist in the BNST does not affect motivation to nicotine and nicotine-taking, it blocks the incentive properties of both nicotine and nicotine-associated cue.

INTRODUCTION

The bed nucleus of the stria terminalis (BNST), a component of the extended amygdala, is involved in addiction-related behavior (Dumont et al., 2005; Dumont et al., 2008; Grueter et al., 2006; Harris and Aston-Jones, 2007), including addiction to nicotine (Caille et al., 2009). The BNST is well located in order to efficiently relay information from the prefrontal cortex, including the infralimbic cortex (ILCx), to the midbrain, including the VTA (Jennings et al., 2013; Massi et al., 2008). In our laboratory, we showed that the pathway of the ILCx, BNST and VTA is involved in voluntary nicotine intake, as extended nicotine IVSA enhances excitability at excitatory neurons projecting from the ILCx to the BNST (Caille et al., 2009). Also, extended voluntary nicotine IVSA induces persistent potentiation of excitatory responses in the BNST in response to stimulation of ILCx afferents (Reisiger et al., submitted). The IVSA learning process appears to be an important determinant for the induction of synaptic plasticity, as rats that did not acquire the IVSA paradigm (passive nicotine administration and rats exposed to 1 day of nicotine IVSA) did not show an enhanced excitatory transmission. Interestingly, rats that were subjected to 8 days of nicotine IVSA showed variability in acquisition, measured as discrimination between active and inactive nose-hole. Accordingly, we found a correlation between discrimination rate and induction of LTP, further confirming the hypothesis that the ILCx-BNST pathway is involved in associative learning.

Anatomical and electrophysiological evidence reveal that CB1 receptors in the BNST are localized on both glutamatergic and GABAergic terminals and have been shown to play an important role in the induction of synaptic plasticity (Puente et al., 2011; Puente et al., 2010). We showed that nicotine-induced potentiation of excitatory responses in the BNST in response to stimulation of ILCx afferents is dependent on CB1 activation, as intra-BNST infusion of CB1 antagonist prevents induction of LTP (Reisiger et al., submitted). Moreover, electrical stimulation of the ILCx-BNST pathway promotes aberrant nicotine-seeking, indicated by excessive nose-hole responses without increasing nicotine intake. According to the involvement of BNST CB1 receptors in LTP induction, this behavior was blocked by CB1 antagonist (Reisiger et al., submitted). These results suggest that the BNST and local CB1 receptors might be a substrate for cue-paired nicotine associative learning. The learning process of response-contingent drug IVSA involves acquisition and maintenance of drug-seeking. However, it unknown whether each stage of nicotine addiction is controlled by BNST CB1 receptors. Therefore, we want to examine the effect of stimulation of BNST CB1 receptors on operant behavior before and after nicotine IVSA has been acquired. First, we examine the effect of intra-BNST CB1 agonist WIN55 on the acquisition of nicotine IVSA. During acquisition of drug IVSA, associations between the drug and drug-associated cue are established which are critical for the development of addiction. Therefore, we hypothesize that, if BNST CB1 receptors are involved in associative learning

of nicotine and nicotine-paired cue, WIN55 would enhance the development of incentive salience to the nicotine-paired cue and thus facilitate acquisition of the IVSA paradigm. We will continue nicotine IVSA to examine long-term effects of BNST CB1 receptor stimulation during the acquisition period. Motivation to nicotine will be tested using a progressive ratio schedule of reinforcement. Using different doses of nicotine, we examine the primary reinforcing effects of nicotine. Finally, drug- and cue-seeking is examined in a reinstatement model of relapse following extinction training. In another group of rats we examine the involvement of BNST CB1 receptors after acquisition of nicotine IVSA in drug-taking and drug-seeking. We hypothesize that BNST CB1 stimulation would enhances nicotine taking and seeking, by increasing incentive value to nicotine and the nicotine-paired cue.

MATERIAL AND METHODS

Animals

Male Sprague Dawley (n = 33) rats (Charles River) were used, weighing 175-200 g at arrival. They were housed collectively (3/ cage) and maintained in rooms at 20-22°C with a 12 h reversed light/dark cycle (light off at 8:00 A.M.). Four days before the start of self-administration training, rats were placed on a restricted diet of 20 g/ day lab chow, sufficient to maintain body weight and growth throughout the experiment. Water was available *ad libitum*, and food was given daily after the session.

Surgery

Animals were sedated using an isoflurane-O_2/air inhalation anesthesia and prepared with chronic indwelling SILASTIC jugular catheters as described previously (Caille et al., 2009). During the same surgery, animals were stereotaxically implanted with bilateral 22-gauge, 10-mm stainless steel guide cannulae that terminated 2 mm above the BNST. The stereotaxic coordinates were 0.3 mm posterior to bregma, 3.8 mm lateral to the midsagittal sinus and 6.3 mm ventral to the level of the dura mater with a 19.7° lateral angle in order to prevent perforation of the ventricles (Paxinos and Watson, 2007). Four anchor screws were placed. Cannulae were fixed using dental cement and obturators were placed.

After surgery, catheters were flushed daily with 0.2 ml of a solution of ampicillin (0.1 g/ml; PANPHARMA Laboratories) in heparinized saline (300 IU heparin per ml 0.9% NaCl) for a minimum of 7 postoperative recovery days and during the first 6 days of self-administration when rats received intracerebral (i.c.) infusions. During the whole experiment, catheters were flushed daily with 0.2 ml heparinized saline.

Intravenous nicotine self-administration

Experiments were conducted at the beginning of the dark phase. Rats were tested in operant chambers (30 x 40 x 37 cm; Imetronic, France) equipped with two nose-poke devices (‘active’ and ‘inactive’). The beginning of the 2 h self-administration session was indicated by illumination of the house light and a single non-contingent infusion of the drug solution. Activation of the active nose-hole resulted in the infusion of 100 µl of nicotine (30 µg/kg/infusion) over 4 s, and was accompanied by the illumination of a white cue-light for 3 s, positioned above the nose-hole. A 20 s time-out followed each infusion whereby activation of the active nose-hole had no consequences. Inactive nose-hole responses were recorded but had no programmed consequences. Rats were trained for the acquisition of self-administration under a fixed-ratio (FR) schedule of reinforcement (10 days FR1; 3 days FR2; remaining days FR5).

Dose-response

Sensitivity to nicotine was tested in a dose-response paradigm. Doses of 60, 30 15 and 0 µg/ kg/ infusion were tested on consecutive sessions in a descending order to prevent confounding effects by a lower dose. Between the dose of 15 and 0 µg/ kg/ infusion and after the last dose, rats were subjected to one or two sessions of 30 µg/ kg/ infusion to re-stabilize nicotine intake.

Progressive ratio

Motivation to nicotine was tested in a progressive ratio (PR) schedule wherein the response requirement increased with each successive infusion. The response requirement progression was adapted from Roberts and Bennett (1993). The breakpoint was defined as the highest ratio completed before the end of the session (Roberts and Bennett, 1993). A period of 30 minutes without infusion resulted in ending of the session. The session lasted a maximum of 3 hours.

Extinction training and reinstatement

Extinction training was conducted in 2 h daily sessions in the absence of nicotine and cue-light reinforcement. Extinction training continued until the rats responded on average fewer than 30 % responses in the former active nose hole over the last 3 sessions compared to baseline responses. Baseline was defined as the average of reinforcing responses of the last 3 days of nicotine self-administration under FR5 schedule of reinforcement.

Reinstatement testing was done over 2 days. Day 1 started with an extinction session of 45 min. Then, rats were subcutaneously (s.c.) injected with saline (0.5 ml) and responses were recorded in the absence of nicotine and cue-light for 1 hour. The second testing day started with a re-extinction session of 45 min with the absence of nicotine and cue-light. Then, to test nicotine-induced reinstatement, rats were injected with nicotine (0.3 mg/ kg; s.c.) prior to the test session of 1 h without cue-light. The second reinstatement test started right after the end of the previous test, but included the illumination of the cue-light following responding.

Intra-BNST microinfusion

For each test performed, micro-infusions in the BNST were done immediately before the start of the IVSA session. Rats were mildly restrained by the experimenter and obturators were removed from the guide cannulae. Sterile micro-injectors were bilateral inserted and connected to two 100 µl micro-syringes (Hamilton) mounted on a micro-infusion pump (Harvard). Micro-injectors were inserted to a depth 2 mm beyond the tip of the guide cannulae. Solutions were infused in a volume of 0.5 µl/ side over a period of 2 min. Micro-injectors were left in place for an additional 1 min to avoid reflux into the guide cannulae and to allow drug diffusion. Subsequently, obturators were replaced and rats were allowed immediate access to the operant chambers.

Experimental design

Experiment 1

A total of 18 rats were used in this experiment in which we examined the effect of intra-BNST infusion of CB1 agonist WIN55,212-2 (WIN55) on acquisition of nicotine IVSA and its long-lasting effects on operant behavior. Acquisition of nicotine IVSA was tested under FR1 schedule of reinforcement. During the first 6 days of self-administration under FR1 schedule, rats received an intra-BNST infusion of either WIN55 (5 µg/ 0,5 µl/ side; n = 9) or its vehicle (n = 9) before the start of the session.

During the remaining of the experiment rats received no i.c. infusions. The effect on responding and nicotine intake was measured under increasing FR schedules of reinforcement (FR1 – FR2 – FR5). Four additional sessions under FR1 ratio were done before to continue with FR2 and FR5. After at least 9 FR5 sessions, sensitivity to the primary reinforcing effects of nicotine was tested in a dose-response paradigm. After the last dose, rats were subjected to 2 FR5 sessions to re-stabilize intake. Then, motivation to obtain nicotine was tested under a PR schedule of reinforcement. Intake was re-stabilized before to start extinction training. When rats reached the criterion of extinction, rats were subjected to a reinstatement test (See **Figure 1** for timeline).

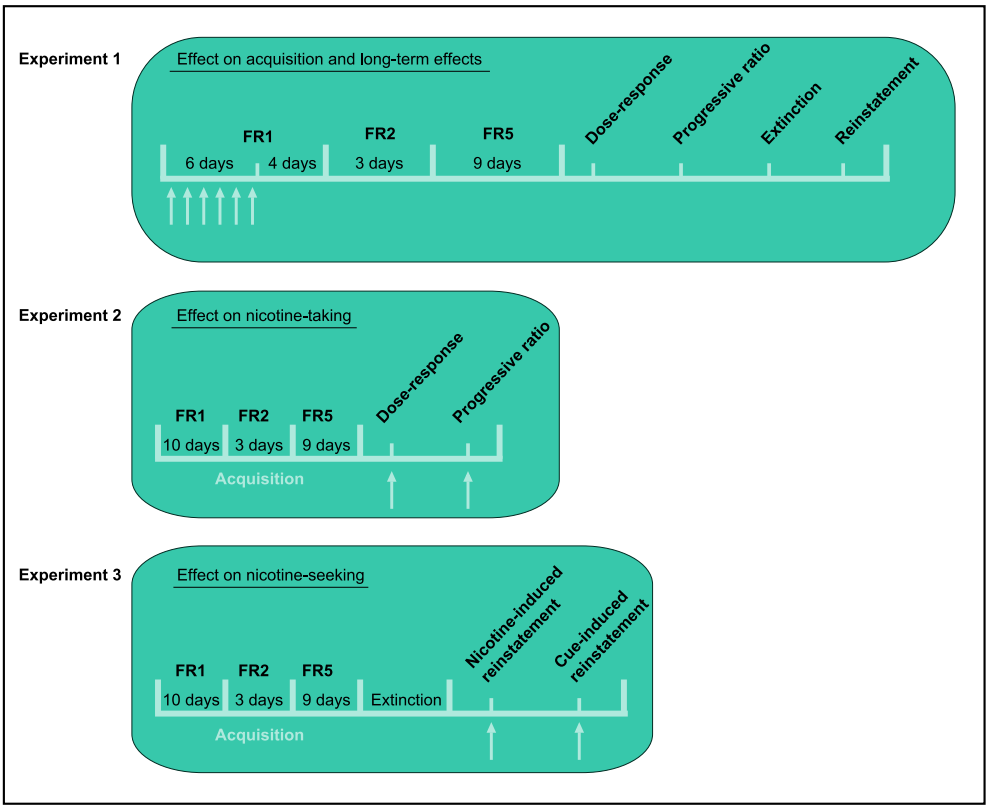

Figure 1 Timeline for the experimental set-up. Three cohorts of rats were used to examine the effects of CB1 agonist WIN55,212-2 on nicotine taking and seeking before and after acquisition of nicotine IVSA. Arrows indicate the timing of intra-BNST infusion of WIN55,212-2.

Experiment 2

A total of 10 rats were used to examine the effects of intra-BNST infusion of CB1 agonist WIN55 on nicotine taking and motivation to nicotine after prolonged exposure to nicotine IVSA. Therefore, rats were first trained to self-administer nicotine under a FR schedule of reinforcement (10 days FR1; 3 days FR2; remaining days FR5). After stable intake (5 weeks), the effect of WIN55 was tested on nicotine taking under FR5 schedule of reinforcement and motivation in a PR schedule. Before the session, rats received an intra-BNST micro-infusion of WIN55 at the following doses; 0, 2.5 or 5.0 µg/ 0,5 µl/ side. Rats received all doses in increasing order. Before the start of the PR paradigm, 2 FR5 sessions without micro-infusion were done to re-stabilize nicotine intake.

Experiment 3

A total of 5 rats were used to examine the effects of intra-BNST infusion of WIN55 on nicotine seeking after prolonged exposure to nicotine IVSA. Therefore, rats were first trained to self-administer nicotine under a FR schedule of reinforcement (10 days FR1; 3 days FR2; remaining days FR5). After stable intake (5 weeks), rats were subjected to extinction training followed by reinstatement test. The reinstatement protocol was performed as follows; during the first test, rats received intra-BNST infusion of vehicle before both saline- and nicotine-induced reinstatement. The reinstatement protocol was repeated with intra-BNST micro-infusion of WIN55 (5.0 µg/ 0,5 µl/ side).

Drugs

Nicotine hydrogen tartrate salt (Sigma-Aldrich, N5260) was dissolved in sterile 0.9% saline and stored at room temperature. WIN55,212-2 mesylate salt (Sigma-Aldrich, W102) was dissolved in a vehicle of ethanol: cremophor: saline (1: 1: 18) and stored at 4°C.

Histology

At the end of the experiment, rats were sacrificed. Brains were removed, snap-frozen in isopentane and stored at -20°C until use. Brains were sectioned at 30 µm thickness on a cryostat, mounted on gelatin-coated slides and counterstained with thionine. Brain sections were examined under a light microscope to verify the location of the guide cannulae using a brain atlas (Paxinos and Watson, 2007). In total, 6 rats had to be excluded after examination of the guide cannulae placement. Three rats of the « WIN55 » group were excluded because of ventricle perforation, and 1 rat because of misplacement of the cannulae. In the « Veh » group, 1 rat was excluded because of ventricle perforation, and 1 rat because of inflammation of brain tissue around the cannulae.

Statistical analysis

Due to malfunctioning of the catheter, we had to exclude one rat of the « WIN55 » and « Veh » group before the dose-response and PR test, respectively, they were immediately subjected to

extinction training. Before the start of the reinstatement test, we had to exclude one rat from the « Veh » group, because he never reached the criterion of extinction.

Results are expressed throughout as mean ± SEM. Multiple comparisons were analyzed by performing two-way ANOVA test with session X treatment interaction. *Post hoc* comparison was performed using Fisher LSD test. Acquisition rate was analyzed using two sample t-test between percentage on day 6. Comparison to baseline (100 %) or chance level (50 %) was performed using Student's t-test. For statistical analysis of acquisition of nicotine IVSA, rats in experiment 2 and experiment 3 were considered one group. When two means are compared, statistical significance of their difference was assessed using the paired Student's *t* test. In all cases, differences with $p \leq 0.05$ were considered significant.

RESULTS

Effect of intra-BNST microinfusion of WIN55 on acquisition of nicotine IVSA

Intra-BNST microinfusion of CB1 agonist WIN55 during the first 6 days of nicotine IVSA reduced the number of reinforcing responses during 2h sessions compared to rats that received intra-BNST microinfusions of vehicle (**Figure 2A;** « Veh »: n = 9, « WIN55 »: n = 9; treatment effect, $F_{(1,16)} = 5.48$, $p < 0.05$; no session X treatment interaction, $F_{(5,80)} = 1.38$, $p > 0.05$). Similar curves were found for nicotine (30 µg/ kg/ 0.1 ml) intake (**Figure 2B**; treatment effect, $F_{(1,16)} = 7.50$, $p < 0.01$; no session X treatment interaction, $F_{(5,80)} = 1.74$, $p > 0.05$), and locomotor activity (**Figure 2C**; treatment effect, $F_{(1,16)} = 9.26$, $p < 0.01$; no session X treatment interaction, $F_{(5,80)} = 1.31$, $p > 0.05$). These data imply that the decrease in reinforcing responses and intake might be due to a non-specific changes in locomotor activity. Average discrimination rate over the 6 first days of IVSA was not different between groups (**Figure 2D**; « Veh »: 58.9±3.1 « WIN55 »: 55.9±4.2; $t_{(16)} = 0.57$, $p > 0.05$). However, discrimination rate of « Veh » is significantly greater than chance level (**Figure 2D**; $t_{(8)} = 2.84$, $p < 0.05$),

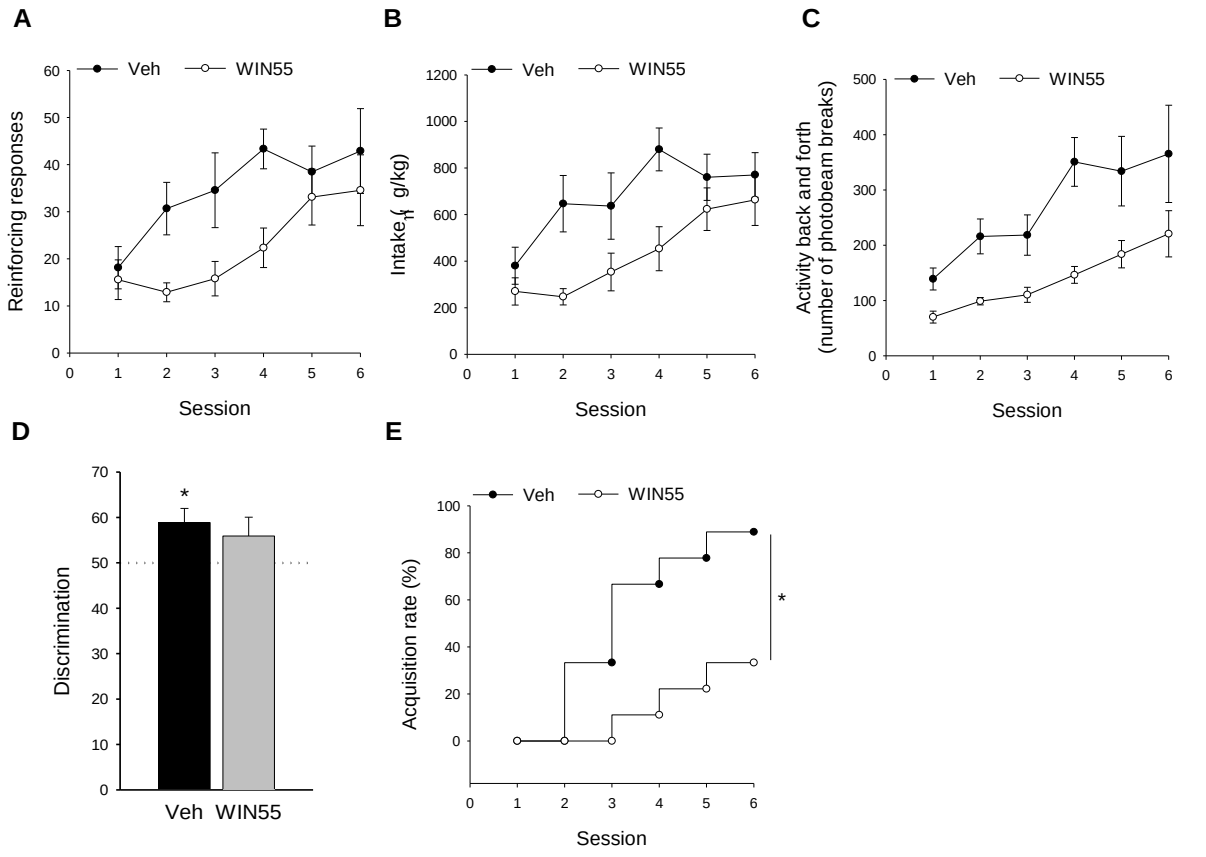

Figure 2. Effect of bilateral intra-BNST microinfusion of CB1 agonist WIN55,212-2 on acquisition of nicotine IVSA. **(A)** Number of reinforcing responses **(B)** intake and **(C)** activity during the first 6 days of nicotine (30 µg/ kg/ infusion) self-administration. WIN55,212-2 (5 µg/ 0.5 µl/ side) was infused before the start of the sessions under fixed-ratio (FR) 1 schedule of reinforcement. Veh n=9, WIN55 n=9 **(D)** Average discrimination rate during first 6 days of nicotine IVSA when rats received either WIN55,212-2 or its vehicle. Discrimination rate of Veh is higher than chance level (t-test, * $p \leq 0.05$). **(E)** Percentage of rats reaching the acquisition criterion (≥ 15 injections for 3 consecutive days) for nicotine self-administration. Two-sample t-test, * $p \leq 0.05$.

whereas discrimination rate of « WIN55 » is not ($t_{(8)} = 1.42$, $p > 0.05$). This suggests that « Veh » learned to self-administer nicotine while « WIN55 » did not. Accordingly, intra-BNST WIN55 caused a slower acquisition rate of nicotine IVSA (**Figure 2E**). Nicotine IVSA was considered acquired when rats obtained 15 or more injections on 3 consecutive days. After 6 days, 88.9 % of « Veh » met this criterion while 33.3% of « WIN55 » did (**Figure 2E**; $t_{(16)} = 2.42$, $p < 0.05$). Before we proceeded to FR2 reinforcement schedule, both groups met the criterion of acquisition (« Veh »: 88.9 %, « WIN55 »: 66.7 %; $t_{(16)} = 1.13$, $p > 0.05$).

Long-term effect of intra-BNST WIN55 during acquisition on maintenance, sensitivity and motivation to nicotine.

As soon as the treatment ceased, the number of reinforcing responses of « WIN55 » turned to levels similar to those of « Veh » and increased gradually across sessions and across FR reinforcement schedule. No differences were found between groups (**Figure 3A**; no FR phase X treatment interaction, $F_{(2,32)} = 1.82$, $p > 0.05$). Also, no significant differences were found for the number of non-reinforcing responses across FR reinforcement schedule (**Figure 3B**; no FR phase X treatment interaction, $F_{(2,32)} = 0.89$, $p > 0.05$). Number of injections decreased across FR reinforcement schedule, but no differences were found between groups (**Figure 3C**, FR phase effect, $F_{(2,32)} = 20.44$, $p < 0.001$; no FR phase X treatment interaction, $F_{(2,32)} = 1.38$, $p > 0.05$). While we did not find a FR phase x treatment interaction between groups for discrimination rate, we found a treatment effect (treatment effect, $F_{(1,16)} = 7.56$, $p < 0.01$; no FR phase X treatment interaction, $F_{(2,32)} = 1.12$, $p > 0.05$). Accordingly, « Veh » gradually learned nicotine IVSA and showed a discrimination rate between active and inactive nose-hole greater than chance level (**Figure 3D**; FR1: 57.2 ± 4.0, $t_{(8)} = 1.81$, $p > 0.05$; FR2: 60.1 ± 3.5, $t_{(8)} = 2.88$, $p < 0.05$; FR5: 66.7 ± 2.5, $t_{(8)} = 6.67$, $p < 0.001$). Discrimination rate of « WIN55 » never differed from chance level (**Figure 3D**; FR1: 48.9 ± 4.3, $t_{(8)} = 0.27$, $p > 0.05$; FR2: 43.7 ± 4.5, $t_{(8)} = 1.41$, $p > 0.05$; FR5: 51.9 ± 5.3, $t_{(8)} = 0.36$, $p > 0.05$). After at least 8 days of nicotine IVSA training under FR5 reinforcement schedule, rats were tested for sensitivity to nicotine using a dose-response protocol. Before the start of the test, there was no difference in number of reinforcing responses between groups (« Veh »: 144 ± 18, « WIN55 »: 149 ± 31; $t_{(15)} = 0.16$, $p > 0.05$). Different doses of nicotine were used (0, 15, 30 and 60 µg kg/ 0.1 ml). All rats (« Veh »: n = 9, « WIN55 »: n = 8) received all doses in a descending dosage schedule, in order to prevent possible confounding effects by a lower dose. We did not find differences in the number of reinforcing responses during 2h session of IVSA with different doses of nicotine (**Figure 3E**; no dose X treatment interaction $F_{(3,45)} = 1.56$, $p > 0.05$). This implies that multiple intra-BNST WIN55 infusions do not have a carryover effect on the sensitivity to nicotine. After completing the dose-response schedule, rats were exposed to 2 sessions of FR5 reinforcement schedule of nicotine (30 µg/ kg/ 0.1 ml) to

restabilize intake. Then, rats (« Veh »: n = 8, « WIN55 »: n = 8) were tested for motivation to nicotine (30 µg/ kg/ 0.1 ml) in a PR reinforcement schedule. We found that the reached breakpoint of « Veh » was higher than « WIN55 » (**Figure 3F**; « Veh »: 29.9±5.4, « WIN55 »: 15.4±2.8; $t_{(14)} = 2.40$, $p < 0.05$). The time before the end of the session did not reach significance between groups, but there was a trend (« Veh »: 83.6 min ± 12.5, « WIN55 »: 57.0±5.2; $t_{(14)} = 1.96$, $p < 0.07$). These data imply that intra-BNST WIN55 produces a carryover effect on the motivation to obtain nicotine later on.

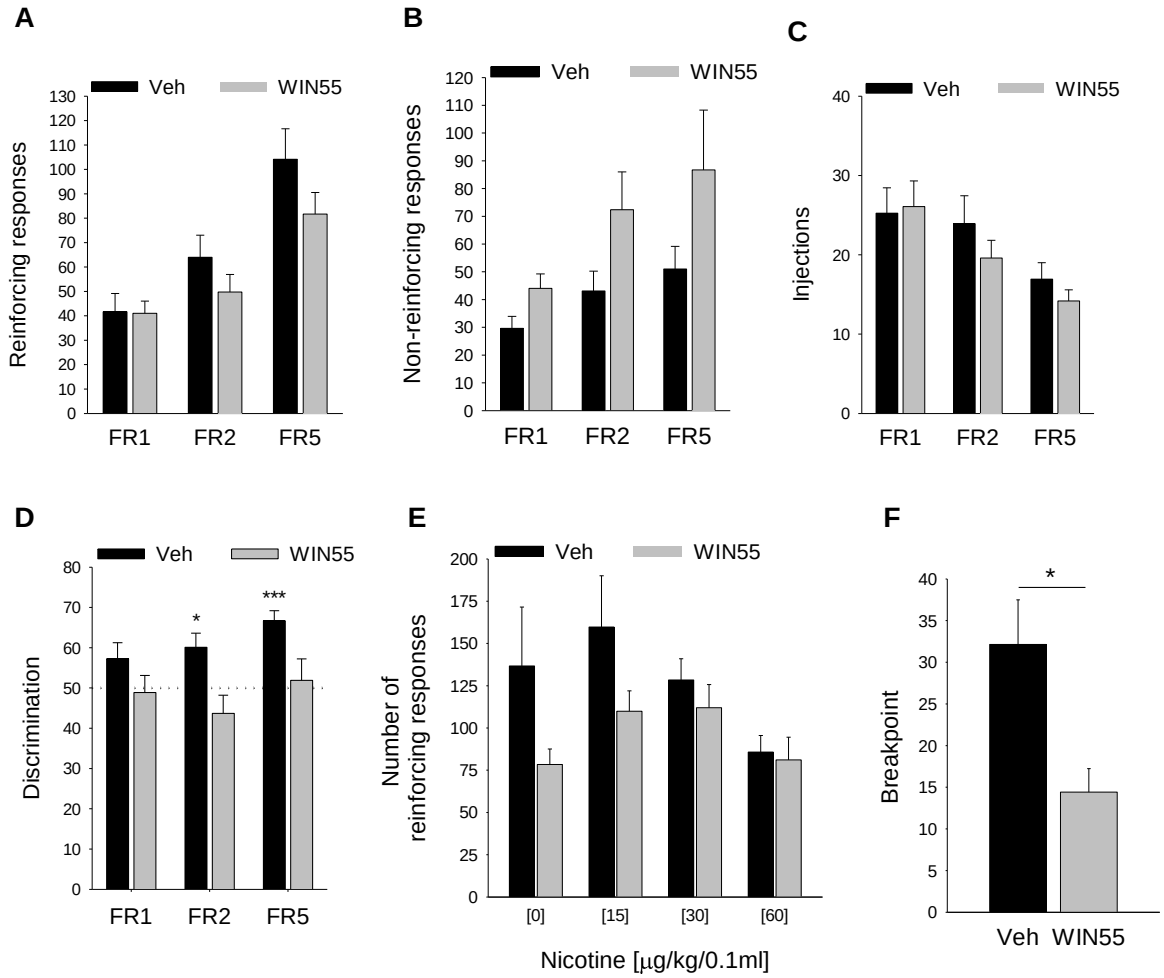

Figure 3 Long-term effect of bilateral intra-BNST WIN55-212,2 on the maintenance, sensitivity and motivation to nicotine. Average of **(A)** reinforcing responses **(B)** non-reinforcing responses **(C)** injections of nicotine and **(D)** discrimination rate during different fixed-ratio schedules of nicotine (30 µg/ kg/ 0.1 ml) IVSA when rats received either WIN55,212-2 or its vehicle. **(E)** Number of reinforcing responses during dose-response test of nicotine (0, 15, 30 and 60 µg/ kg/ 0.1 ml) self-administration under FR5 reinforcement schedule. **(F)** Breakpoint reached under progressive ratio schedule of reinforcement of nicotine (30 µg/ kg/ 0.1 ml) self-administration. Repeated measures treatment effect $p < 0.01$. * $p < 0.05$ and *** $p < 0.001$ discrimination rate versus chance level, t-test. * $p < 0.05$ Veh versus WIN55 pre-treatment, t-test. Data are shown as mean ± SEM.

Long-term effect of intra-BNST WIN55 on extinction and reinstatement.

Then, rats were subjected to extinction training (« Veh »: n = 9, « WIN55 »: n = 9). Before the start of extinction training, there was no difference in the average number of reinforcing responses during the last 3 days of nicotine IVSA between groups (**Figure 4A;** « Veh »: 120 ± 13.2, « WIN55 »: 96.6 ± 11.2, $t_{(16)} = 1.35$, $p > 0.05$). Extinction training continued until responses in the former active hole were fewer than 30% of the baseline. Rats needed at least 8 days to reach this criterion (**Figure 4A**). Analysis of the first session of extinction training reveals that the number of responses of « WIN55 » was smaller compared to « Veh » (**Figure 4A, inset**; « Veh »: 96.0 ± 13.0, « WIN55 »: 55.7 ± 8.6; $t_{(16)} = 2.59$, $p < 0.05$). We did not find a session X treatment interaction across extinction sessions (**Figure 4A**; $F_{(1,16)} = 1.47$, $p > 0.05$). After both groups had reached the criterion for extinction training, rats were tested in a reinstatement paradigm (« Veh »: n = 8, « WIN55 »: n = 9). Both groups showed an increase in former reinforcing responses during reinstatement to nicotine compared to saline (drug effect $F_{(1,14)} = 35.14$, $p < 0.001$). We did not find any differences between groups (**Figure 4B**; no treatment effect $F_{(1,14)} = 0.80952$, $p > 0.05$). Similarly, we found that both group reinstated equally to the nicotine-paired cue compared to an extinction session (cue effect $F_{(1,15)}=10.47$, $p < 0.01$). We did not find any differences between groups (**Fig. 4C**; no treatment effect $F_{(1,15)}=0.36$, $p > 0.05$).

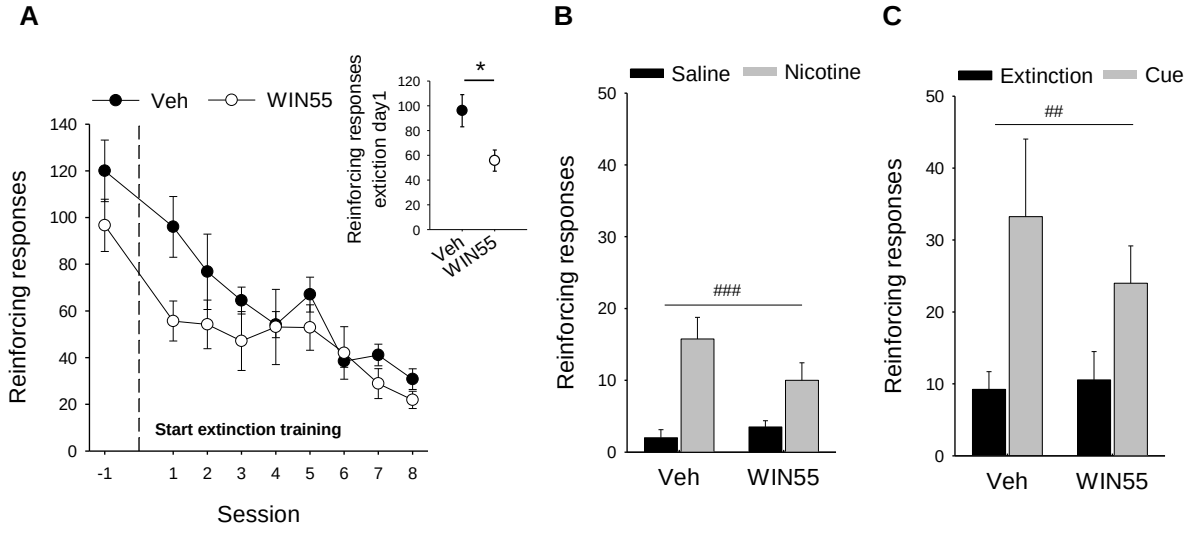

Figure 4. Long-term effect of bilateral intra-BNST WIN55-212,2 on extinction and reinstatement. **(A)** Effect of pre-treatment with WIN55,212-2 on number of responses in former active nose hole during extinction training. Inset shows number of responses in former active nose hole during the first day of extinction training. t-test, * p≤0.05. **(B)** Number of responses in former active nose whole during nicotine- (0.3mg/kg, s.c.), and **(C)** cue-induced reinstatement. Nicotine-induced reinstatement is compared to saline-induced reinstatement (0.5 ml, s.c.); Two-way ANOVA, no interaction, *Post hoc* LSD, drug-effect ### p<0.001. Cue-induced reinstatement is compared to extinction; Two-way ANOVA, no interaction *Post hoc* LSD cue-effect, ## p<0.01. Data are shown as mean ± SEM.

The effect of acute intra-BNST WIN55 on nicotine intake and motivation to nicotine.

Rats were trained to self-administer intravenous nicotine (30 µg/ kg/ infusion) in 2 h daily sessions for 1 month under fixed-ratio schedule (10 days FR1, 3 days FR2, remaining FR5). When responding was stable and discrimination rate greater than chance level (65.2 ± 2.7, $t_{(14)} = 5.54$, $p < 0.001$), effect of WIN55 on nicotine taking and motivation was tested under FR5 and PR schedule of reinforcement, respectively (n = 10; **Figure 5**). Different doses of WIN55 do not affect number of reinforcing responses measured under FR5 reinforcement schedule (**Figure 5A**; [0]: 70.8 ± 8.3, [2.5]: 59.5 ± 6.9, [5.0]: 63.1 ± 3.9; $F_{(2,18)} = 0.77$; $p > 0.05$). Locomotor activity was not affected ([0]: 105.9 ± 10.4, [2.5]: 108.6 ± 16.0, [5.0]: 103.4 ± 10.6; $F_{(2,18)}=0.12$; $p > 0.05$). Moreover, different doses of WIN55 (0, 2.5 and 5.0 µg/ 0.5µl/ side) did not change motivation to obtain an infusion of nicotine, reflected in a similar breakpoint (**Figure 5B**; [0]: 13.9 ± 1.9, [2.5]: 15.4 ± 2.9, [5.0]: 14.9 ± 2.8; $F_{(2.18)} = 0.07$; $p > 0.05$) and similar time required to reach the breakpoint ([0]: 76.3 ± 9.9, [2.5]: 75.1 ± 10.0, [5.0]: 77.3 ± 9.1; $F_{(2,18)} = 0.02$; $p > 0.05$). Locomotor activity was not affected ([0]: 257.8 ± 54.4, [2.5]: 277.2 ± 46.0, [5.0]:279.1 ± 57.7; $F_{(2,18)} = 0.08$; $p > 0.05$).

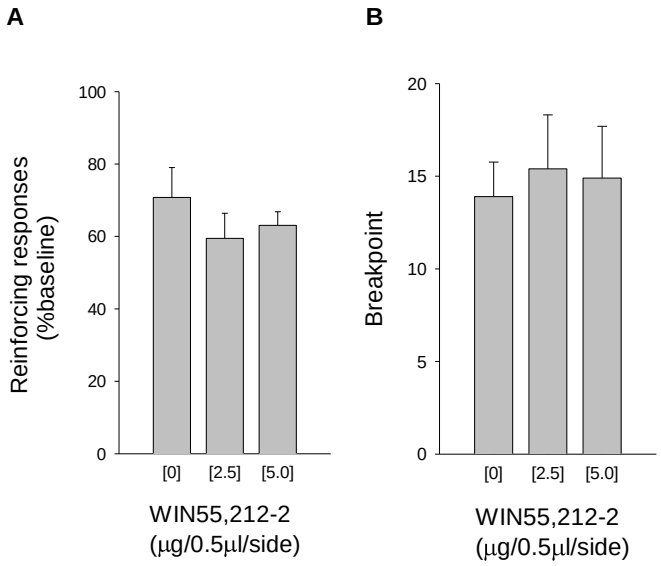

Figure 5. Effect of intra-BNST WIN55,212-2 on maintenance and motivation after prolonged exposure to nicotine. **(A)** Number of reinforcing responses (percentage of baseline) under FR5 schedule of reinforcement after microinfusion of WIN55,212-2 (0, 2.5 and 5.0 µg/ 0.5µl/ side). **(B)** Breakpoint reached under progressive ratio schedule of reinforcement of nicotine (30µg/ kg/ 0.1ml) self-administration after microinfusion of WIN55,212-2 (0, 2.5 and 5.0 µg/ 0.5µl/ side). Data are shown as mean ± SEM.

Intra-BNST injection of WIN55 blocks nicotine- and cue-induced reinstatement.

Rats were trained to self-administer intravenous nicotine (30 µg /kg /infusion) in 2 h daily sessions for 1 month under a fixed-ratio schedule (10 days FR1, 3 days FR2, remaining FR5). When responding was stable and discrimination rate greater than chance level (65.2 ± 2.7, $t_{(14)} = 5.54$, $p < 0.001$), rats were subjected to extinction training. Rats required 4.6 ± 1.3 days to reach the criterion of 30% of responses in former active nose hole. Then, the effect of WIN55 on nicotine- and cue-induced reinstatement was tested. Analysis of the number of responses in the former active nose-hole indicates that intra-BNST injection of vehicle induces a marked reinstatement to nicotine (« Veh »: saline: 4.6 ± 2.2 nicotine: 20.2 ± 4.0;) and nicotine-associated cue (« Veh »: extinction: 13.4 ± 4.5 cue: 39.2 ± 7.0;). In contrast, WIN55 blocked reinstatement to both nicotine (**Figure 6A;** « WIN55 »: saline: 6.8 ± 1.5, nicotine: 9.6 ± 2.9; treatment X nicotine interaction: $F_{(1,4)} = 22.63$, $p < 0.01$) and nicotine-associated cue (**Figure 6B**; « WIN55 »: extinction: 5.4 ± 2.1, cue: 7.2 ± 2.6 treatment X cue interaction: $F_{(1,4)} = 16.32$, $p < 0.05$). No change in locomotor activity was observed (nicotine-induced reinstatement: no treatment effect, $F_{(1,4)} = 3.19$, $p > 0.05$; cue-induced reinstatement: no treatment effect, $F_{(1,4)} = 4.84$, $p > 0.05$).

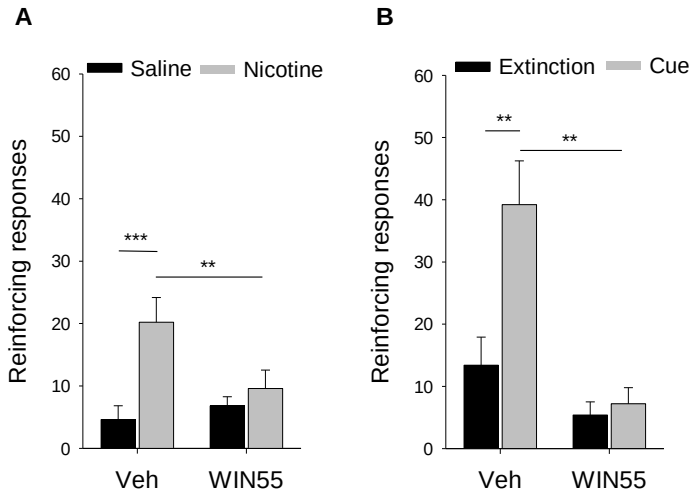

Figure 6 Effect of intra-BNST WIN55,212-2 on reinstatement after prolonged exposure to nicotine. Number of responses in former active nose hole during **(A)** nicotine-induced reinstatement and **(B)** cue-induced reinstatement. First, rats were tested with intra-BNST micro-infusion of vehicle. Then, the protocol was repeated with micro-infusion of WIN55,212-2 (5.0µg/0.5µl/side). Two-way ANOVA, *Post hoc* LSD ** $p < 0.01$, *** $p < 0.001$. Data are shown as mean ± SEM.

DISCUSSION

The present study investigated the involvement of CB1 receptors in the BNST on nicotine-taking and nicotine-seeking before and after prolonged exposure to nicotine. To do so, we first examined whether BNST CB1 receptors are involved in the contingency learning between nicotine and nicotine-associated cues during the acquisition phase of nicotine IVSA. Then, the long-term effects were examined on the sensitivity to nicotine, motivation and drug-seeking behavior in a reinstatement model of relapse following extinction training. In another group of rats, we examined the role of BNST CB1 receptors on nicotine-taking and seeking after acquisition of nicotine IVSA. We found that acquisition of nicotine IVSA is impaired when BNST CB1 receptors are stimulated during the first 6 days of the IVSA paradigm. However, it did not affect nicotine- and cue-induced reinstatement. In contrast, when nicotine IVSA was acquired and stable intake was established, while CB1 receptors in the BNST are not involved in the primary reinforcing effects of nicotine, they seem to be involved in drug seeking as it blocks reinstatement to both nicotine and nicotine-associated stimulus.

Effect of intra BNST WIN55 during acquisition of nicotine IVSA

Effect on acquisition

We tested the involvement of BNST CB1 receptor in nicotine IVSA acquisition by infusion of CB1 agonist WIN55 in the BNST during the first 6 days of the nicotine IVSA paradigm. We found that intra-BNST WIN55 reduces acquisition, responding and nicotine intake. This suggests that BNST CB1 receptors stimulation during the acquisition of cue-paired nicotine IVSA disrupts the process of associative learning. Accordingly, it has been shown that systemic CB1 receptor agonist disrupts acquisition of spatial and associative learning tasks (Da and Takahashi, 2002; Madronal et al., 2012), whereas CB1 antagonists enhance memory (Terranova et al., 1996). The BNST receives dense glutamatergic input from the ILCx (Massi et al., 2008). We have previously shown that extended voluntary nicotine intake leads to a persistent potentiation of excitatory responses in the BNST in response to 10Hz – stimulation of ILCx afferents (Reisiger et al., submitted). We found a correlation between the discrimination rate and the emergence of synaptic plasticity in the ILCx – BNST pathway, indicating that this pathway is involved in the acquisition of associative learning of nicotine IVSA (Reisiger et al., submitted). Therefore, one might assume that disrupting excitatory signaling by WIN55-induced inhibition of neurotransmitter from these structures may impair acquisition of cue-paired nicotine IVSA.

Rats given acute WIN55 exhibited a decrease in locomotor activity. It has been previously reported that systemic CB1/CB2 agonist THC induces hypolocomotion, which is facilitated by nicotine (Valjent et al., 2002). Therefore, the possibility that the concurrent decrease in locomotor activity we

observed during WIN55 treatment contributed to the observed effect on responding and acquisition cannot be ruled out. However, considering the involved of the BNST in stress and anxiety-related behavior (Walker et al., 2003), it is possible that the decreased locomotor activity seen in our study can be related to freezing behavior of the rats. We did not observe a WIN55-induced decrease in locomotor activity in the other experiments of this study.

Long-term effects on nicotine taking and seeking

We continued the nicotine IVSA protocol, in order to asses carryover effects of previous BNST CB1 stimulation on subsequent nicotine taking and seeking.

First of all, we found that rats had difficulties to adapt their behavior to a change in workload imposed by increasing FR schedule from FR1 to FR2 to FR5 and during a PR session when response requirement increased within the session with every successive nicotine delivery. Interestingly, while rats previously treated with WIN55 did not acquire nicotine IVSA across FR reinforcement schedule (reflected in a discrimination rate that did not exceed chance level), nicotine intake did not differ between groups. This indicates that the BNST is not involved in the control of nicotine intake. Rather, BNST CB1 stimulation might interfere with the behavioral flexibility to adapt to increasing workload. This is line with other studies showing that both CB1 agonist as well as upregulation of CB1 receptors are associated with impairment of cognitive flexibility (Hill et al., 2006; Klugmann et al., 2011). For example, in a strategy shifting task rats were required to reverse initial acquired responding, CB1 agonist increased, whereas CB1 antagonist reduced, perseverative errors (Hill et al., 2006). Particularly, upregulation of CB1 receptors in the mPFC impaired reversal learning abilities, indicating reduced behavioral flexibility (Klugmann et al., 2011). When rats were subjected to a PR schedule of reinforcement, WIN55 group had not reached a discrimination rate greater than chance level. However, it is not likely the observed results is due to an early learning deficit rather than behavioral flexibility, because responses in the inactive nose-hole was similar in both groups.

Secondly, we did not find a difference in responding nor nicotine intake during a dose-response test. Commonly, a dose-response paradigm is used to test the sensitivity to a drug. The fact that we did not find a difference between groups, implies that previous exposure to WIN55 does not interfere with the sensitivity and thus primary reinforcing effect of nicotine. This supports the hypothesis that BNST CB1 receptors are not involved in the primary reinforcing effects of nicotine. Indeed, primary reinforcing effects of nicotine appear to be mainly dependent on CB1 receptors located in the VTA (Simonnet et al., 2012).

When we subjected rats to extinction training, we found that rats previously exposed to WIN55 considerably drop responding on the first day of extinction training, in the absence of nicotine and nicotine-associated cue, implying an enhanced sensitivity to the nicotine-associated cue. The

involvement of CB1 receptors has also been shown for extinction of food-reward and aversive memories (Hernandez and Cheer, 2011; Marsicano et al., 2002). Cues associated with rewards acquire incentive salience when they are repeatedly paired and they elicit approach behavior serving as a conditioned reinforcer (Robinson and Berridge, 1993). Therefore, the decreased responding during the first extinction day may be explained by the absence of the nicotine-associated cue, resulting in rapid inhibition of approach behavior.

Finally, after having met the criterion for extinction training, rats were tested for reinstatement. We found that both groups relapsed equally to both nicotine and nicotine-associated cue. The finding that rats reinstate equally to nicotine is in line with the observation in this study that WIN55 treatment during the acquisition phase does not affect the sensitivity to nicotine in a dose-response test. The interoceptive perception of the drug priming was effective to induce reinstatement, supporting the hypothesis that primary reinforcing effect are not mediated in the BNST. Also, we did not find a difference in reinstatement to the nicotine-associated cue, suggesting that the rats are sensitive to the nicotine-associated cue. This corresponds to our finding that rats treated with WIN55 rapidly drop responding during extinction training in the absence of the nicotine-associated cue. This implies that pretreatment with WIN55 developed high incentive salience to the cue. Accordingly, we have previously shown that the ILCx – BNST pathway is involved in the acquisition of associative learning of nicotine and nicotine-associated cue during nicotine IVSA (Reisiger et al., submitted).

Another explanation could be that extinction training and retrieval during reinstatement require different brain systems. Indeed, the infralimbic cortex, with dense glutamatergic projections to the BNST (Massi et al., 2008), is recruited during extinction training to suppress drug-seeking (Peters et al., 2008). In contrast, the prelimbic, which has no to very little connections with the BNST (Vertes, 2004) is involved in reinstatement of drug-seeking behavior (McFarland and Kalivas, 2001). Thus, similar levels of reinstatement may be induced, independent of the BNST.

<u>Effect of WIN55 after acquisition of nicotine IVSA</u>

Effect on maintenance and motivation

We examined the behavioral implication of CB1 receptors in the BNST after a history of nicotine IVSA, *i.e.* after acquisition of nicotine IVSA and establishment of stable nicotine intake. We found that intra-BNST infusion of WIN55 did not affect nicotine taking and motivation measured under FR5 and PR schedule of reinforcement, respectively. This is in agreement with a previous finding in our laboratory showing that intra-BNST CB1 antagonist AM251 did not change responding under FR5 reinforcement schedule and motivation in a PR paradigm (unpublished data). The VTA and its projection to the NAc is considered the main site for processing the primary reinforcing effects.

Indeed, nicotine microinjection into the VTA promotes CPP (Laviolette and van der Kooy, 2003) and rats will self-administer nicotine directly into the VTA (Ikemoto et al., 2006). The reinforcing effects of nicotine are mediated via nAChRs. Using subunit-specific KO mice, pharmacological blockade and lentiviral re-expression, it has been shown that the nAChRs in the VTA, including α7 and α4 subunits are necessary for nicotine IVSA (Besson et al., 2012; Exley et al., 2011). The endocannabinoid system also plays an important role in the VTA-mediated reinforcing effects of nicotine as blockade of CB1 receptors in the VTA considerably decreases nicotine IVSA (Simonnet et al., 2012). Therefore, it seems likely that in our study, WIN55 in the BNST does not alter responding because the primary reinforcing effect of nicotine are mainly achieved in the VTA, indicating that BNST CB1 receptors are not involved in the primary reinforcing properties of nicotine.

Effect on seeking

After extinction training, rats were tested for reinstatement and the involvement of CB1 receptors in the BNST. We showed that BNST CB1 stimulation blocks both nicotine- and cue-induced reinstatement. We have shown previously that extinction training suppresses nicotine-induced LTP and induces LTD at excitatory synapses in the BNST projecting from the ILCx (Reisiger et al., submitted). In control rats, re-exposure to nicotine-associated cue or nicotine priming in a reinstatement model of relapse, might lead to re-activation of the stimulus-response behavior previously acquired. However, when we inhibit neurotransmitter release in the BNST by CB1 agonist, we might block excitatory input to the BNST, ultimately leading to suppression of excitatory potentiation to the VTA (Massi et al., 2008). It has been shown that reinstatement of nicotine seeking is mediated by glutamatergic plasticity in the VTA and NAc (Gipson et al., 2013; Liechti et al., 2007). The inhibition of reinstatement by activation of CB1 receptors suggests that these receptors modulate the glutamate transmission, thereby reducing motivational drive of drug-seeking.

Based on these results, we can conclude that in a reinstatement model of relapse, intra-BNST CB1 agonist WIN55 blocks reinstatement to both nicotine and nicotine-associated cues, supporting the hypothesis that BNST CB1 receptors are involved in development of incentive salience through glutamatergic transmission.

Our behavioral data raise questions about the precise synaptic mechanisms and organization of CB1 receptors in the BNST mediating nicotine-related behavior. Activity of CB1 receptors in reward-related behavior have been proposed to mediate autoregulatory mechanisms compensating for excessive activation of postsynaptic neurons by glutamate (Lupica and Riegel, 2005; Solinas et al., 2007). In line with this idea, it has been shown that, within the BNST, CB1 agonist inhibits the excitatory drive from the ILCx to DA neurons (Massi et al., 2008). Within the BNST, it has been demonstrated that cocaine disrupts endocannabinoid-mediated LTD at excitatory synapses (Grueter

et al., 2008). Therefore, one can postulate that nicotine exposure may lead to a similar effect. In the BNST, CB1 receptors are equally expressed on glutamatergic and GABAergic terminals (Massi et al., 2008; Puente et al., 2010). Consequently, intra-BNST injection of WIN55 would result in a decreased excitatory input, which inevitably will have consequences for nicotine-induced behavior. However, it has also been shown that WIN55 treatment produces cannabinoid receptor desensitization and down-regulation (Sim-Selley and Martin, 2002). Specific regional distributions and/or density of cannabinoid receptors may affect the balance between inhibitory and excitatory control, which may lead to disinhibition of BNST neurons projecting to the VTA. Facilitation of the excitatory pathway from the ILCx to the BNST neurons projecting to the VTA may promote reward-seeking behavior (Caille et al., 2009; Dumont et al., 2005). Future studies, focusing on the pharmacokinetics of nicotine and CB1 agonist/antagonists on CB1 receptors in the BNST would help to better understand the involvement of CB1 receptors in nicotine-related behavior.

Considering its involvement in addiction and efferents to the VTA (Caille et al., 2009; Massi et al., 2008), we aimed at the anteroventral BNST. However, although the target region will receive the highest concentration of the solution when applying i.c. injections, the solution will inevitably diffuse over a wider area. Therefore, stimulation of CB1 receptors in other parts or outside of the BNST might have contributed to the behavioral effects. However, the observed effects are unlikely due to the diffusion of CB1 agonist outside of the region of interest as the behavioral effect was absent in rats with a misplaced cannula.

Altogether, we can conclude that BNST CB1 receptors stimulation before or after acquisition of nicotine IVSA differently affects nicotine-related behavior. BNST CB1 stimulation during acquisition interferes with the development of incentive salience to the nicotine-associated cues, resulting in an increased sensitivity to the cue, whereas sensitivity to nicotine is not altered. When BNST CB1 receptors are stimulated after acquisition of nicotine IVSA, they are not directly involved in the primary reinforcing effects of nicotine. Rather, BNST CB1 receptors disrupt association between nicotine and nicotine-paired cue and thus blocks stimulus-response behavior, measured in reinstatement model of relapse.

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GENERAL DISCUSSION

GENERAL DISCUSSION

Reminder of the aim

The general aim of the project was to elucidate the involvement of the ILCx – BNST pathway in the maladaptive, aberrant behavior induced by nicotine exposure. Moreover, the endocannabinoid system, and in particular the CB1 receptors, controls nicotine reinforcement and nicotine-induced synaptic modification (Maldonado et al., 2006), and modulates synaptic excitation in BNST neurons directly projecting to the VTA (Massi et al., 2008). Therefore, we examined the involvement of CB1 receptors.

In order to identify the role of the ILCx – BNST pathway and the contribution of CB1 receptors in the development of nicotine addiction, three questions were approached:

1) It has been previously shown that excitatory synapses in the BNST projecting from the ILCx are hyperexcitable after voluntary nicotine IVSA and not passive administration. However, it is unknown whether ILCx – BNST synapses are more susceptible to a LTP-induction protocol after the development of an association between active responding and the reward experience. Thus, as a first step we identified the contribution of the associative learning process of nicotine self-administration in the development of synaptic modifications in this pathway and the involvement of BNST CB1 receptors. As addiction is associated with persistent synaptic modifications, we examined the resistance of the synaptic plasticity.

2) A further step was to identify a correlation between the synaptic plasticity in the ILCx – BNST pathway and the operant behavior. Thus, as a second question, the effect of electrical potentiation of the glutamatergic ILCx projection to the BNST on operant behavior was investigated.

3) Following the results obtained with the previous experiments, we hypothesized that the BNST and local CB1 receptors contribute to the associative learning of nicotine and nicotine-associated cues. However, it is unknown whether the stimulation of CB1 receptors is critical for each of the cognitive and motivational processes involved in response-contingent nicotine IVSA. Therefore, in the last part of the project we tested the involvement of BNST CB1 receptors at different behavioral stages of nicotine addiction (initiation, maintenance, motivation, extinction and reinstatement).

Summary of results

1) In the first step of the project we characterized neuroplastic changes at the ILCx – BNST synapse and showed that cue-paired voluntary nicotine consumption is associated with CB1-dependent potentiation of excitatory responses in the BNST in response to 10Hz – stimulation of ILCx afferents. Extinction training, but not abstinence, provoked long-term depression, indicating that LTP is resistant to a passive drug-free period, but sensitive to new learning.

2) Following the characterization of the nicotine-induced synaptic plasticity, we demonstrated that potentiation of ILCx – BNST pathway induced aberrant stimulus-response behavior, controlled by BNST CB1 receptors.

3) In the final stage of the project, we showed that BNST CB1 receptors are important for the associative learning of nicotine and nicotine-paired cue. Moreover, pretreatment with CB1 agonist within the BNST induces difficulty to adapt to increasing workload but does not alter reinstatement of drug-seeking. Finally, BNST CB1 receptors do not modulate primary rewarding properties of nicotine, but rather are required for expression of incentive salience to nicotine or nicotine-paired cue.

The involvement of the ILCx – BNST pathway in nicotine addiction

Compulsive drug use and persistent vulnerability to relapse are key characteristics of drug addiction. Increasing evidence has suggested that chronic exposure to drugs of abuse is associated with dysfunctioning of the PFC. It has been previously shown in our laboratory that the ILCx – BNST pathway is involved in nicotine addiction, as active, but not passive nicotine IVSA enhanced excitability of excitatory projections from the ILCx to the BNST (Caille et al., 2009). Our current work shows that nicotine IVSA facilitates induction of LTP induced by 10Hz – stimulation of the ILCx. This develops with acquisition of the nicotine IVSA behavior, as we demonstrated a correlation between discrimination rate and excitatory response magnitude in rats exposed to 8 days of nicotine IVSA. Also, self-administration of a natural reward (*i.e.* saccharin) did not induce these modifications in the ILCx – BNST pathway, implying that the synaptic potentiation is specific to a drug of abuse, rather than to the acquisition of an operant task for reinforcers in general. This is in line with a recent study showing that a LTP at GABA synapses in the BNST was specific to cocaine, as self-administration of a natural rewards failed to induce similar synaptic modifications (Krawczyk et al., 2013).

Electrical stimulation of the ILCx results in a temporarily increase in perseverative responding during the post-injection period, when nicotine is not available. Therefore, we speculate that the ILCx –

BNST pathway is involved in habitual responding associated with drug addiction. In line with our observation, it has been suggested by others that a transition from recreational drug use to addiction is a consequence of the development of habitual control over drug-taking and drug-seeking, even after prolonged periods of abstinence (Tiffany, 1990; Vanderschuren and Everitt, 2004).

a. The ILCx is necessary for the transition from goal-directed to habitual responding

Studies of instrumental conditioning have demonstrated that during early learning, behavior is goal-directed (Balleine and Dickinson, 1998). This means that the initiation of the response is under direct control of the value and by the instrumental contingency between response and outcome. However, with extended training, instrumental performance shifts to a stimulus-response process. Actions become habitual and responding is characterized by direct initiation of responding by a stimulus presentation. Habitual responding is insensitive to changes in instrumental contingency and independent of the current value of the reinforcer (Balleine and Dickinson, 1998). Habit learning processes have been implicated in the transition from occasional drug use to compulsive drug seeking that characterizes addiction (Everitt and Robbins, 2005). Previous studies suggest that the mPFC has an important role in the transition from goal-directed behavior to habitual responding. More specifically, whereas the PLCx is associated with goal-directed behavior (Chen et al., 2013), the ILCx is recruited when responding becomes habitual (Coutureau and Killcross, 2003). While other studies show the involvement of the dorsal striatum in habitual responding (Packard et al., 1989; Zapata et al., 2010), we hypothesize that the projection of the ILCx to the BNST is recruited during nicotine IVSA, when contingency responding was acquired and has become habitual. Notably, we found a correlation between discrimination rate and facilitation of LTP induction at ILCx – BNST synapses following 8 days of nicotine IVSA, implying that, for some rats, this short period of IVSA is sufficient to shift to habitual responding. In contrast, others have shown that after 20 – 25 sessions of cocaine IVSA in only 43 % of the rats drug seeking was habitual in nature (Zapata et al., 2010). Even though the reinforcement schedule was different from the one used in our study, these data support the hypothesis that drug-related stimuli, which play a crucial role in habits, may be more important in sustaining nicotine IVSA than is the case for other drugs (Arroyo et al., 1998; Balfour et al., 2000; Caggiula et al., 2001).

b. The involvement of the ILCx – BNST pathway in extinction training

In addition to its contribution to habitual responding, the ILCx also appears to be important in inhibiting drug seeking behavior. Importantly, the involvement of the ILCx habit-driven behavior is associated with extended exposure to drug IVSA, while its role in inhibitory control is demonstrated following extinction training (Van den Oever et al., 2010). Several lines of evidence provide support

for the idea initially suggested by Pavlov (1927) that extinction is a new and active form of learning, rather than the erasure of previously formed associations (Gass and Chandler, 2013). For example, after extinction training, drug-seeking behavior can be reactivated with a single stimulus without the need for additional training (Sinha et al., 2000; Stewart, 2003), even after prolonged periods of extinction training, indicating that the original drug-related memory remains, and has not been erased (Hammersley, 1992). While, in the short-term, the new memory provides inhibitory drive over drug-seeking behavior, the original drug-memory remains. Ultimately, these two memories competes with each other for control of conditioned behavior. This may explain the high rates of relapse following behavioral extinction training (Marlatt, 1990).

There is evidence for opposing influences of the PLCx and ILCx on compulsive drug seeking. While activity of the PLCx is necessary for the initiation of drug seeking, the ILCx seems to play an important role in inhibitory control of drug seeking (**Figure 13**) (Peters et al., 2009; Peters et al., 2008). Evidence suggests that distinct prefrontal regions are able to influence activity reciprocally. For example, optical stimulation of the ILCx inhibits activity of the PLCx, suggesting that ILCx output control PLCx output (Ji and Neugebauer, 2012). This supports the hypothesis that the extinction learning mediated by the ILCx, may involve inhibition of the PLCx output. The activation of ILCx projections to the NAcS is hypothesized to contribute to the inhibitory regulation of cocaine seeking (Peters et al., 2008). Accordingly, extinction training induces an upregulation in the expression of AMPA receptor subunits within in the NAcS, an indicator of synaptic plasticity (Sutton et al., 2003). In contrast, projections from the PLCx to the NAcC regulate the expression of drug-seeking behavior (Gipson et al., 2013; LaLumiere and Kalivas, 2008). We observed that extinction training results in a decreased facilitation of LTP induction and we provoked LTD by electrical stimulation of the ILCx. This may be due to reduced activity of the ILCx efferent to the BNST, or to local neuroadaptations. These results are in agreement with our hypothesis that the ILCx – BNST pathway is involved in aberrant responding for nicotine, extinction training reduced synaptic strength at excitatory synapses in the BNST projecting from the ILCx (**Figure 13**).

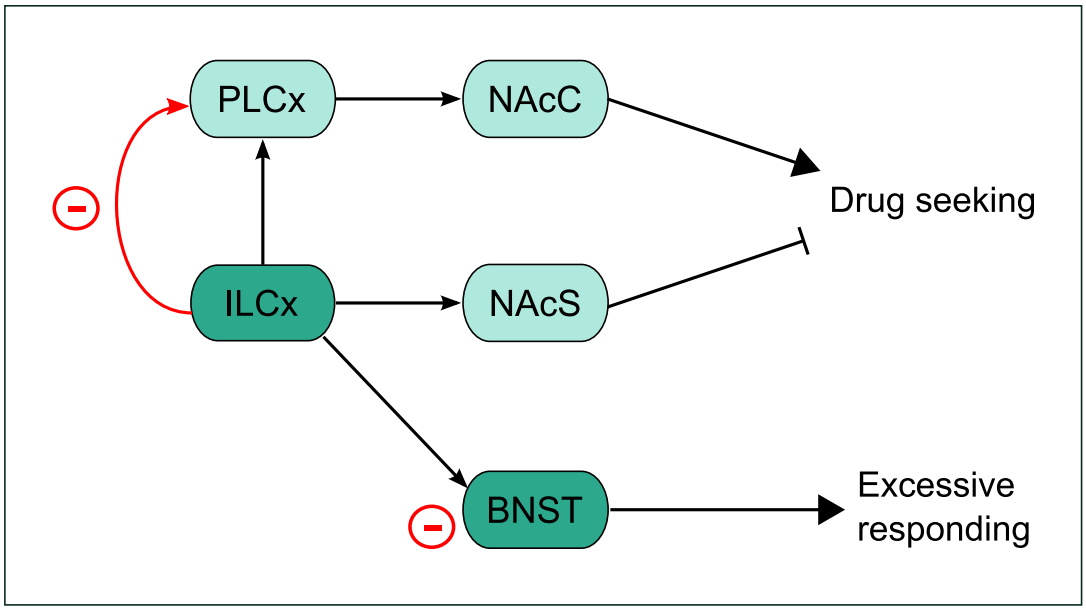

Figure 13 Projection areas of the ILCx implicated in drug seeking. The PLCx and its projection to the NAcC is involved in initiation of drug seeking during reinstatement, while the projection of the ILCx to the NAcS inhibits drug seeking. During extinction training, the ILCx may reduce activity of the PLCx and activity at synapses from the ILCx to the BNST is reduced (in red).

The involvement of BNST CB1 receptors in nicotine addiction

The endocannabinoid system has an important neuromodulatory role in the brain by inhibiting neurotransmitter release through retrograde signaling (Kano et al., 2009). A considerable amount of evidence show that the endocannabinoid system, in particular CB1 receptors, plays a crucial role in drug rewarding properties and drug-seeking behavior (Maldonado et al., 2006). In the BNST, CB1 receptors are expressed on both excitatory and inhibitory synapses (Massi et al., 2008). It is previously shown that CB1 receptors in the BNST mediate $mGluR_{1/5}$-mediated LTD (Grueter et al., 2006). Moreover, this study showed that cocaine self-administration attenuates mGluR1-induced LTP in the BNST, suggesting a reduction on inhibitory control over glutamatergic synaptic transmission, which may play an important role in the neuroadaptations that occur after drug exposure (Grueter et al., 2006). We showed that synaptic potentiation in the ILCx – BNST pathway after nicotine IVSA is dependent on CB1 receptor activation. Anatomical evidence reveals that the majority (~90 %) of the presynaptic terminal projecting from the ILCx to the BNST express CB1 receptors. However, CB1 receptors are also expressed on GABAergic neurons in the BNST and on excitatory terminals from other brain areas, such as the vSub (Massi et al., 2008; Puente et al., 2010). Blocking LTP with CB1 antagonist in the BNST suggests a disinhibition of GABA release. Yet, in the presence of AM251, basal activity of BNST neurons was not affected. Therefore, additional studies are needed to address the underlying mechanisms of the involvement of endocannabinoid in the modulation of synaptic plasticity in the BNST.

The use of CB1 receptor antagonists for the treatment of nicotine addiction has received considerable attention. The selective CB1 receptor antagonist Rimonabant reduces both nicotine self-administration and nicotine seeking (Cohen et al., 2005a). The beneficial effects of Rimonabant on nicotine addiction appear to depend on the attenuation of the nicotine-induced hyperactivity of the dopaminergic reward system (Cohen et al., 2002). Indeed, CB1 receptors in the VTA are shown to mediate the rewarding and motivational effects of nicotine (Gamaleddin et al., 2012; Simonnet et al., 2012). Accordingly, nicotine IVSA induces an increase in anandamide levels in the VTA, which may result in enhanced DA cell activity by CB1-mediated inhibition of GABA release (Buczynski et al., 2013; Lupica et al., 2004). Results of our work indicate that BNST CB1 receptors are not directly involved in the primary reinforcing properties of nicotine, as acute CB1 activation did not modulate operant responding or nicotine intake (Reisiger et al., in preparation). Yet, we showed that activation of CB1 receptors in the BNST prevents drug-seeking following extinction training. This is possibly mediated by reduced of excitatory transmission induced by inhibition of glutamate release in the BNST. As the BNST sends excitatory projections to the VTA, which exert powerful influences over VTA DA neurons controlled by CB1 receptors (Massi et al., 2008), activation of CB1 receptors in the BNST may ultimately lead to decreased activity of VTA DA neurons, inhibiting nicotine seeking.

Tobacco is generally the first drug used by many people. Following tobacco use, it is more likely to proceed the use of other drugs, including cannabis (Kandel et al., 1992). However, in many cases, tobacco and cannabis use develop concomitantly (Agrawal et al., 2012), which may affect following tobacco use. Indeed, it has been shown that pretreatment with THC increases the likelihood of acquiring nicotine IVSA and enhances persistent use (Panlilio et al., 2013). Although cannabis targets both CB1 and CB2 receptors in the brain, we showed that treatment with intra-BNST CB1 agonist at the beginning of nicotine IVSA affects acquisition and also has long-lasting effects on nicotine-related behavior. Considering the involvement of the BNST in cue-associative learning and reinforcement-enhancing effect of nicotine, the co-occurring use of tobacco and cannabis might contribute to the increased susceptibility of cannabis users to nicotine-associated cues.

The involvement of TRPV1 receptors in the BNST

Besides its action on cannabinoid receptors, the endocannabinoid anandamide has also been found to bind to TRPV1 receptors (Zygmunt et al., 1999). TRPV1 receptors are expressed postsynaptically and their activation mediates anandamide-induced LTD in several brain areas, such as the hippocampus, NAc and BNST (Chavez et al., 2010; Grueter et al., 2010; Puente et al., 2011), thereby regulating synaptic strength. Accordingly, anandamide-induced LTD can be completely prevented by TRPV1 antagonists (Chavez et al., 2010; Puente et al., 2011). In the BNST, it has been demonstrated

that a single neuron is able to engage two distinct forms of synaptic plasticity through different signaling pathways: presynaptic activation of CB1 receptors by 2-AG mediates short-term depression, whereas, through activation of mGluR5 receptor, anandamide binds to postsynaptic TRPV1 receptors to induce LTD (Puente et al., 2011). This implies a complementary or synergistic interaction between CB1 and TRPV1 receptors in the induction of synaptic modification.

It has been shown that TRPV1 antagonist blocks cocaine seeking in a reinstatement model of relapse (Adamczyk et al., 2012). However, very little is known about the role of TRPV1 receptors in drug addiction.

Future perspectives

To meet their needs reliably and efficiently, animals and humans learn complex action sequences to the point where behavior becomes habitual, although still flexible enough to respond to unforeseen changes. However, when taken to the extreme, habitual behavior is associated with loss of control and maladaptive behavior. Habit formation is an important feature for the development of nicotine addiction. It has been shown that the ILCx is involved in the formation of habits (Coutureau and Killcross, 2003). Moreover, data point towards a contribution of the endocannabinoid signaling through CB1 receptors in the development of habitual behavior (Hilario et al., 2007). Thus, since our results give strong indications that the ILCx – BNST pathway is necessary for association learning of nicotine and nicotine-paired cues controlled by CB1 receptors, future studies should investigate if this pathway has a role in habit formation, and ultimately to compulsive drug seeking. To determine whether instrumental responding is performed under goal-directed or habitual control, (in)sensitivity to outcome devaluation needs to be assessed (Lopez et al., 1992). In a drug self-administration paradigm, typically a drug seeking-taking chained schedule to obtain a drug is used (Olmstead et al., 2001). Using this schedule of reinforcement, Chen and colleagues (2013) showed that optogenetic activation of the PLCx prevents habitual drug seeking when responding was paired with a noxious footshock. However, the involvement of the ILCx in a shift from goal-directed to habitual control in nicotine IVSA is unknown.

CB1 receptors play an important role in nicotine reward and their expression and function is disrupted by chronic nicotine exposure (Marco et al., 2007; Werling et al., 2009), suggesting nicotine-induced alterations in endocannabinoid signaling. Moreover, voluntary nicotine intake modulates endocannabinoid levels in several brain areas (Buczynski et al., 2013). Since we found differential effects of CB1 stimulation on operant behavior, future work should be done to characterize nicotine-induced neuroanatomical and neurochemical changes in the BNST and their contribution to nicotine-related behavior. One might hypothesize that nicotine induces changes in receptor binding or

expression level of CB1 receptor. This will have implications for the understanding of mechanisms and behavioral outcome of potential therapies targeting CB1 receptors.

Conclusion

The aim of this thesis was to examine the involvement of the ILCx – BNST pathway in the development of nicotine addiction. With the obtained results we can say that this pathway is recruited during extended nicotine IVSA, and induces persistent potentiation of excitatory responses in the BNST in response to 10Hz – stimulation. Moreover, we showed that this pathway is involved in the associative learning of nicotine and nicotine-associated cue, which is mediated by BNST CB1 receptors. In contrast, BNST CB1 receptors do not mediate the primary reinforcing properties of nicotine. In order to actually assign a key role in the development of incentive salience to the nicotine-paired cue excessive responding, further research is needed. This work is a first step in the characterization of the ILCx – BNST pathway and its involvement in nicotine addiction. If the activation of this pathway is a neuronal substrate for habitual drug-seeking, this could have implications for the understanding of aberrant drug-seeking and be a possible target to treat long-term vulnerability to relapse. Therefore, (pharmaco)therapy aimed at reducing the ILCx excitatory output to the BNST at the start of the abstinent period, may help to reduce incentive salience to the drug-associated cue and thereby suppress drug-seeking in human addicts. However, since it is known that a drug-associated cue can reinstate drug-seeking even after long periods of abstinence, this may involve long-term therapy.

PART II

The effect of a hedonic state on the firing properties of VTA DA neurons

INTRODUCTION

INTRODUCTION

Food and reproduction are essential for survival. A main characteristic of these elements is that they are rewarding and motivate organisms to approach them. Learning is an important factor in this process, since it allows the formation of memories and increases the likelihood that the organism will engage in this behavior in the future. However, in a dynamic environment, organisms need to be able to adapt their behavior in case of danger or when food sources get depleted. The brain has evolved to become a flexible organ in order to facilitate adaptation and increase survival.

1. Reward-approaching behavior

1.1. Definition of reward

Typically, a reward is an object or event that elicits approach and is worked for (Wise, 2004), which can be a sensory reward like food, but also a drug of abuse or social interaction (Kringelbach and Berridge, 2009). Sucrose overconsumption is a considerable health problem worldwide, leading to overweight and obesity and ultimately metabolic diseases (Malik et al., 2010). However, the mechanisms by which sucrose regulates synaptic activity to influence behavior is not fully understood. Therefore, in this part of my thesis I will focus on sucrose as a natural reward.

1.2. Processes involved in obtaining natural reward

The central nervous system integrates numerous metabolic signals from the periphery to adjust behavioral responses to changing energy demands, which are mainly processed in hypothalamic and hindbrain areas (Sternson, 2013). However, the metabolic state is not the only aspect that influences feeding behavior. Emotional and cognitive aspects are encoded in the mesocorticolimbic system, assigning salience and motivational value to food (Olsen, 2011). Value representations, together with information about the cues and properties associated with achieving the food are stored as memories, to guide future behaviors (Balleine and Dickinson, 1998; Berridge and Robinson, 2003).

1.2.1. Hedonia

Hedonia is derived from the Greek word « *hedone »* meaning pleasure and is defined as « *the conscious feeling of pleasure* ». It may be clear that hedonia is a rather subjective state, which can make it challenging for scientific research to characterize underlying neurobiological mechanisms. However, the appearance of hedonia seems to be preserved over different mammalian species, including humans, non-human primates, rats and mice (Berridge, 2000; Steiner et al., 2001), and is manifested as measurable affective facial expressions in response to sucrose solution (*e.g.* lip licking and rhythmic tongue protrusions).

Robinson and Berridge referred to this phenomenon as « liking », defined as a pleasure that is directly gained from the consumption of the stimulus (Robinson and Berridge, 1993). It is claimed

that « liking » is independent from dopamine, as dopaminergic lesions and dopamine antagonists do not alter orofacial movements associated with intra-oral sucrose (Berridge et al., 1989; Pecina et al., 1997). This is contrary to the conventional idea that dopamine is the neurotransmitter that mediates immediate hedonic impact. Rather, dopamine is involved in more complex processes of reward-related motivation and learning (Salamone and Correa, 2012). Yet, alternative ideas exist about the involvement of dopamine in reward learning (Wise, 2004). For instance, the *Reinforcement Learning Hypothesis* states that the motivation to seek reward results directly from the release of DA in the NAc following an emitted response. Consequently, discontinued consumption of the reward provokes a decrease in DA levels, which triggers subsequent reward seeking (Wise, 1996).

Several brain structures and other neurochemical systems than dopamine have been shown to be involved in the generation of « liking » reactions (Berridge et al., 2009). One of these structures is a small area in the nucleus accumbens shell (NAcS). Under the influence of both the opioid and endocannabinoid system, it can enhance a « liking » reaction induced by sucrose (Mahler et al., 2007; Pecina and Berridge, 2005). The ventral pallidum (VP) is also shown to be involved in encoding of « liking », as opioid agonist considerable increases « liking » reactions elicited by sucrose (Smith and Berridge, 2005).

1.2.2. Motivation to approach a reward

With repeated exposure to conditioned stimulus paired with the reward, associations are formed and incentive salience will develop. This is called « wanting » (Robinson and Berridge, 1993). « Wanting » can apply to the unconditioned reward or to the stimulus that is associated with the reward. Incentive salience to the associated cue is assigned by mechanisms in the limbic system, which makes the stimulus attractive. Therefore, the motivation for or « wanting » of a reward of conditioned stimulus will ensure that the organism elicit approach behavior towards the food and hence will increase survival.

« Liking » and « wanting » are closely interrelated, but they can be expressed separately and have different neural substrates (Berridge and Robinson, 1998; Kaczmarek and Kiefer, 2000; Wyvell and Berridge, 2000). In contrast to « liking », « wanting » relies on dopamine signaling in the corticolimbic circuit. Activation of the dopamine system amplifies « wanting » without altering « liking » reactions for sucrose (Pecina et al., 2003). Also, amphetamine injections, either systemically or directly in the NAc, increase « wanting » but not « liking » (Robinson and Berridge, 2008). This is in line with the observation that amphetamine increases DA release in the NAc (Di Chiara et al., 1993).

1.2.3. Learning to obtain a reward

Learning is a third component that is important for reward-approaching behavior and plays a role in linking « wanting » and « liking » over time. Associative learning usually refers to either Pavlovian conditioning or instrumental conditioning. In Pavlovian conditioning, conditioned stimuli evoke conditioned responses. In instrumental conditioning, specific instrumental responses (*e.g.* lever press) are strengthened by response-contingent reinforcement.

Reward-related learning has been shown to involve many different brain areas, such as the hippocampus, PFC, amygdala and VTA. All of these areas have overlapping projections to the NAc, where dopamine influences the integration of the inputs to be able to most effectively guide behavior towards the goal of obtaining a reward (Grace et al., 2007). Associative learning is biologically relevant as it facilitates approach behavior and enables the organism to predict and distinguish reinforcing and aversive stimuli (Schultz, 1998).

2. Aversion-avoidance behavior

2.1. Definition of aversion

Behavioral flexibility is required, not only in order to get to the best food resources, but also to able to avoid aversive stimuli and minimize chance of harm. Aversive stimuli are the complete opposite of rewards. They act as negative reinforcers by increasing and maintaining avoidance behavior, thereby reducing the impact of damaging events on the organism (Schultz, 1998).

2.2. Processes involved in avoiding aversive events

The central nervous system integrates numerous interoceptive signals (sickness) and sensory information (pain). This leads to a coordinated set of responses which may include physiological, behavioral and neuronal responses to adjust behavioral output to changing environments and possible danger (Lindquist et al., 2012).

2.2.1. Dislike and fear

A simple example is taste aversions, or the aversion developed to food that has previously resulted in sickness. This behavior is displayed nearly universally in animals since it is a defense against potential poisoning (Bernstein, 1999). Robinson and Berridge observed that bitter tastes elicit negative « disliking » facial expressions such as gapes and nose wrinkling (Robinson and Berridge, 1993). Besides dislike, fear is also a state that promotes avoidance behavior in order to increase survival chance. In an experimental set-up, this is commonly tested using a noxious stimuli (*e.g.* electric shock) in an unconditioned burying test (De Boer and Koolhaas, 2003) or a conditioned avoidance paradigm (Darvas et al., 2011). In normal conditions, subjects will inhibit behavior or redirect it as to escape or avoid an aversive condition, in this case the electric shock.

2.2.2. Motivation to avoid an aversive stimulus

The motivation to avoid food is clearly based on prior experience with tastes that signal poison or sickness. In the case of noxious stimuli, inhibitory or escaping behavior depends on previous exposure to the stimulus. According to the reward theory, aversive stimuli should result in a decrease in DA signaling followed by preventing future engagement. Indeed, it has been reported that when taste of saccharin is made aversive by pairing it with lithium chloride-induced nausea, animals avoid drinking it. Accordingly, DA levels in the NAc drop below baseline (Mark et al., 1991). This conditioned decrease in DA release could be a major factor in inducing avoidance behavior. Yet other studies observe an increase in DA release in response to a stressful stimulus in both the NAc and PFC (Abercrombie et al., 1989; Kalivas and Duffy, 1995), which is most likely mediated by increased firing activity of VTA DA neurons (Brischoux et al., 2009). This implies that the VTA not only encodes rewards but may also be a neuronal substrate for aversive stimuli.

2.2.3. Learning to avoid an aversive stimulus

Akin to approach behavior, learning processes are important in avoidance behavior as it forms memories which food or situations to avoid based on previous experience. Brain structures involved in inhibitory and avoidance learning are similar to approach learning, which includes the hippocampus, PFC, amygdala and VTA (Liang et al., 1996; Yang and Liang, 2013). It has been shown that stimulation of the VTA enhances acquisition of avoidance learning behavior (Shumake et al., 2010), further supporting the hypothesis that the VTA has a central role in association learning of aversion.

3. Inadapted approach and avoidance behavior

It may be clear that approach and avoidance behavior are equally important in order to adapt in a changing environment and therefore, to increase survival. However, a pathophysiological state may alter brain function, which might have consequences for the subsequent integration of external stimuli and adequate behavioral responding. Indeed, an anhedonic state such as depression and chronic stress is related to increased sensitivity to punishment in humans (Padrao et al., 2013; Peri et al., 2000). In contrast, rats chronically exposed to cocaine are resistant to a footshock, as they continue seeking for drugs (Deroche-Gamonet et al., 2004; Pelloux et al., 2007). Evidence suggests that a hedonic state induced by sucrose show addiction-like modifications in the brain. For instance, although less persistent, sucrose self-administration enhances synaptic strength onto VTA DA neurons *ex vivo* as cocaine does (Chen et al., 2008). However, rats exposed to sucrose self-administration show a suppression of sucrose-seeking in response to a footshock (Pelloux et al., 2007). Which indicates that they still have the capacity to respond and integrate environmental stimuli.

4. The involvement of the VTA in reward and aversion signaling

The VTA is a major structure of the reward system, and numerous data show that is has an important role in motivated behaviors (Fields et al., 2007). Specifically, it serves as a neural integrator of metabolic signals and influences from the reward system (Hommel et al., 2006). Also, DA neurons in the VTA are implicated in adaptive brain function related to reward and motivation (Kim et al., 2012). Therefore, I will focus in this study on this brain area, and in particular on DA neurons and their role in aversion signaling.

4.1. Characterization of the VTA

The VTA is located in the midbrain, medial to the substantia nigra, ventral to the red nucleus and dorsal to the interpeduncular nucleus (Oades and Halliday, 1987). It is a heterogeneous area, and composed of three neurochemically different types of neurons: DA neurons (65 %), GABA neurons (30 %) and glutamate (5 %) neurons (Dobi et al., 2010; Nair-Roberts et al., 2008). These types of neurons act in concert to regulate behavior. Numerous studies show the importance of VTA DA neurons in mediating reward-related behavior, in particular its projections to the NAc and PFC (Zellner and Ranaldi, 2010).

Electrophysiological studies showed that *in vivo*, DA neurons display different patterns of activity: a tonic firing pattern, characterized by slow (2 – 10 Hz), irregular, single spiking; and a bursting or phasic mode (Grace and Bunney, 1983). These firing modes are absent *in vitro*, as they are generated by inhibitory and excitatory afferents. Tonic, irregular firing of VTA DA neurons supply a stable baseline level of extrasynaptic DA in target structures of the VTA, including the NAc and PFC (Grace, 1991). A switch from tonic to phasic firing is thought to be dependent on cue-reward association learning (Miller et al., 1981; Schultz, 1998). Using microdialysis, it has been shown that phasic firing transiently increases DA levels in the NAc in response to a reward-associated cue (Wightman et al., 1988). Several brain structures provide excitatory, presumably glutamatergic, projections to the VTA, including the PFC (Sesack and Pickel, 1992), BNST (Georges and Aston-Jones, 2001, 2002) and penduncolopontine tegmental nucleus (PPTg) (Floresco et al., 2003). The VTA receives inhibitory input mainly from the ventral pallidum (VP) (Floresco et al., 2003), the tail of the VTA / rostromedial tegmental nucleus (tVTA/ RMTg) (Jalabert et al., 2011; Lecca et al., 2012) and local interneurons (Omelchenko and Sesack, 2009).

It has been shown in our laboratory that prolonged exposure to nicotine increases both tonic and phasic firing of spontaneous active VTA DA neurons (Caille et al., 2009). Since drugs of abuse hijack the brain's natural reward system, we hypothesize that sucrose self-administration would similarly potentiate spontaneous VTA DA activity.

4.2.The effect of rewarding and aversive stimuli on VTA DA neurons

As described above, animals are able to respond to aversive environmental stimuli in order to decrease damage and increase survival. It is well established that VTA DA neurons play an important role in the integration of sensory information to optimize these behavioral responses. The dopamine learning theory assumes that rewarding stimuli induce an increase in DA neuron activity, while aversive stimuli would induce a decrease in DA transmission. Concerning rewarding stimuli, it is now well established that it causes an transient increase in VTA DA neuron activity (Schultz, 2001). In contrast, using a combination of electrophysiological characterization and single-cell labeling in anesthetized rats, it is shown that VTA DA neurons show an inhibition of firing in response to an aversive stimulus (Ungless et al., 2004), most likely through the activation of VTA GABA interneurons (van Zessen et al., 2012). However, it was demonstrated using the same technique, that a subpopulation of DA neurons ventral area of the VTA are excited (Brischoux et al., 2009). It is suggested that these two distinct responses to an aversive stimulus represent different DA circuits within the VTA (Lammel et al., 2011).

4.3.The effect of sucrose on the reward system

As previously stated, a pathophysiological state may alter sensitivity of the reward system and impair the ability to respond appropriately to environmental stimuli. In our modern society, palatable and energy-dense food is readily available, often resulting in overconsumption and it contributes to obesity (Volkow and Wise, 2005). Sugar is one component of our daily diet and provides us energy. However, sugar-containing food is also consumed for its rewarding and mood-enhancing properties, independent of the caloric state of the organism. Excessive consumption of sugar is thought to induce a hedonic state, which is often compared to drug addiction (DiLeone et al., 2012). Indeed, sucrose can induce similar motivated behaviors seen in rats exposed to drugs of abuse, such as compulsive-like seeking (Latagliata et al., 2010), and might be even more attractive than cocaine (Lenoir et al., 2007). On a neurobiological level, is has been shown that extended access to a highly palatable diet induces deficits in the reward system of rats, reflected in elevated thresholds in a brain-stimulation reward procedure, thus decreasing the sensitivity of the reward system (Johnson and Kenny, 2010). Deficits in reward signaling have also been demonstrated in adults rats that previously had unlimited access to sucrose during their adolescence, indicated by a decreased motivation to natural rewards (Vendruscolo et al., 2010). Together, these date suggest that a hedonic state induced by sucrose leads to modifications in the reward system and alters the sensitivity of the reward system. This might have consequences for the integration of and responding to subsequent stimuli.

PROBLEM STATEMENT

In order for animals to survive, food is required. It has been shown that reward, motivation and learning mechanisms are important in the process of approaching behavior to beneficial resources. However, in a constantly changing environment, animals have to adapt their behavior in response to aversive stimuli and engage in avoidance behavior. The VTA has been shown to be an important area of the reward system that is involved in reward-related behavior. Electrophysiological data demonstrates that VTA DA neurons are implicated in processing of aversive stimuli, and respond by inhibiting or increasing firing activity in anesthetized rats (Brischoux et al., 2009; Ungless et al., 2004). This adaptation may permit the expression of avoidance behavior (Di Chiara et al., 1999).

Addiction is partly due to the inability to stop using the drug, despite negative consequences. This concept has been modeled in rats in the model of self-administration of cocaine. Studies show that « addicted » rats maintain their approach behavior for drugs even though they receive an electric shock (Deroche-Gamonet et al., 2004; Vanderschuren and Everitt, 2004). The hypothesis that highly palatable food induce similar uncontrolled consumption is becoming more widespread. Recently, several studies have attempted to model the concept of addiction to sugar in animals. However, in contrast to rats exposed to drugs of abuse, rats exposed to sucrose seem still able to exhibit avoidance behavior when an aversive stimulus is presented (Pelloux et al., 2007). This raises the question whether a sucrose-induced hedonic state affects spontaneous activity of VTA DA neurons and whether they are still able to process the information of an aversive stimulus.

Aim

The aim of the present study was to examine whether exposure to sucrose exposure would induce neuronal modifications in the brain and impair the capacity to respond to an aversive stimulus.

Therefore, the following questions were addressed:

- **Does a hedonic state alter firing activity of VTA DA neurons?**

  To answer this question, we use an oral sucrose self-administration paradigm. Data from the literature gives us strong indications that exposure to sucrose induces a hedonic state (e.g. facial « liking » expression, reduced sensitivity of the reward system). *In vivo* electrophysiology allows to record activity of single cells in anesthetized rats.

- **If we find a change in spontaneous firing activity in VTA DA neurons induced by sucrose exposure, how would these neurons respond to an aversive stimulus.**

  To determine the effect of an aversive stimulus on the activity of VTA DA neurons, an electric footshock was given during the *in vivo* electrophysiological recording in the anesthetized rat. We used an intensity of 5mA during 4 sec, which is similar to other studies using a footshock

in anesthetized rats (Brischoux et al., 2009). Yet, in freely behaving animals, a lower intensity is commonly used (0.3 – 0.9 mA) (Baldi et al., 2004).

- **Does the caloric state of the animal influences the activity of VTA DA neurons and the way they respond to an aversive stimulus?**

  Because of the caloric content of sucrose, we are aware of the impact it might have on the intake-behavior, and therefore on VTA DA activity, possibly through metabolic influences. Therefore, we compared VTA DA firing properties and responsiveness to a foot shock after sucrose self-administration of rats fed *ad libitum* with rats fed a restricted diet. We hypothesize that rats fed a restricted diet would be more motivated to obtain sucrose, and that this would reflect in higher activity of VTA DA neurons and reduced responsiveness to a footshock.

MATERIALS AND METHODS

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats (Charles River, 175-200 g, n=30) were used. They were housed collectively (3-4 per cage). The room was controlled for temperature (20–22 °C) with a 12 h reversed light/ dark cycle (light off at 8:00 A.M.). One week before the start of the operant training, <sucrose-restricted> rats were placed on a restricted diet of 20 g/d lab chow, sufficient to maintain body weight and growth throughout the experiment. Water was available *ad libitum*. For <sucrose> and <water> rats, both food and water was available *ad libitum*.

Operant training

Experiments were conducted at the beginning of the dark phase. Rats were trained in operant chambers (30 x 40 x 37 cm, Imetronic), equipped with two nose-poke devices (active and inactive), in 30 min daily sessions. The beginning of a session was indicated by illumination of the house light. Activation of the active device resulted in the delivery of 0,11 mL of 5% sucrose (sucrose, n = 7) or tap water (water, n = 11) via a fluid injection assembly (syringe pump) into a dipper cup. The delivery was accompanied by the illumination of a cue-light above the nose-poke hole for 3 s. Inactive nose-poke responses were recorded but had no consequence. Rats were trained for the acquisition of self-administration under a fixed-ratio schedule (5 d FR1; 2 d FR2; remaining days FR5). For each rat, training lasted at least 3 weeks and continued until electrophysiological recording. Part of the rats (sucrose n = 7; water n = 5; sucrose-restricted n = 7) were tested for motivation under a progressive ratio (PR) schedule wherein the response requirement increased with each successive delivery. The breakpoint was defined as the highest ratio completed before the end of the session. A period of 15 minutes without delivery resulted in ending of the session. The session lasted a maximum of 90 minutes.

Stereotaxic surgery and VTA recordings

Stereotaxic surgery for electrophysiological recordings was performed under isoflurane anesthesia. A glass micropipette (tip diameter = 2-3 µm, 4-6 MΩ) filled with 2% pontamine sky blue solution in 0,5 M sodium acetate was lowered into the VTA at the following coordinates: -5.3 mm from bregma, 0.7 mm from midline and 7.5 mm – 8.5 mm from brain surface. DA neurons were identified according to well established electrophysiological features (Grace, 1988; Ungless et al., 2004). These included: 1) a half action potential width ≥ 1.1 ms. 2) slow spontaneous firing rate (<10 Hz). 3) single and burst spontaneous firing patterns. The extracellular potential was recorded with an Axoclamp2B amplifier in the bridge mode. The extracellular potential amplified 10 times by the Axoclamp2B amplifier was further amplified 100 times and filtered (low-pass filter at 300 Hz and high-pass filter at 0,5 kHz) via a differential AC amplifier (model 1700; A-M Systems). Single neuron spikes were discriminated and

digital pulses were collected online using a laboratory interface and software (CED 1401, Spike2; Cambridge Electronic Design). Spontaneous activity was recording for 100 sec.

To examine the effect of an aversive stimulus on activity, two electrical cables were connected to the left hind paw of the rat (**Figure 1**). Footshocks were delivered contralateral to the VTA recording site. Yet, it has been shown that there are no differences in recordings whether footshocks were delivered contralateral or ipsilateral (Brischoux et al., 2009). Electrical shocks (4 s, 5 mA, 20 Hz) were administered via a stimulator (DS3, Digitimer), and activity was measured before (10s) during (4s) and after the footshock (10s). We used a footshock because this is usually used as an acute stressor, and it is demonstrated that it is intense enough to activate nociceptors (Urch et al., 2003) and change firing rate of VTA DA neurons in anesthetized rats (Brischoux et al., 2009).

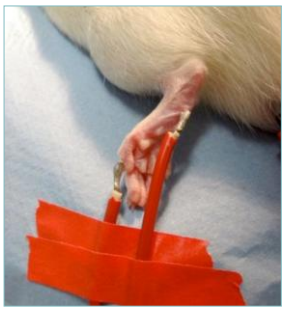

Figure 1 Left hind paw was connected with two electrical cables to deliver a footshock (4 s, 5 mA, 20 Hz).

Histology

At the end of each recording experiment, the electrode placement was marked with an iontophoretic deposit of pontamine sky blue dye (-20 µA, continuous current for 30 min.). Then, rats were sacrificed. Brains were removed, snap-frozen in isopentane of -70°C and stored at -20°C until use. Brains were sectioned at 30 µm thickness on a cryostat, mounted on gelatin-coated slides and counterstained with neutral red. Brains sections were examined under a light microscope to verify the location of the electrode using a brain atlas (Paxinos and Watson, 2007).

Analysis

Data analysis

Each trace was individually inspected to ensure identification of spikes. Two parameters of VTA DA neuron impulse activity were computed over 100 s epochs: 1) the basal firing rate and 2) the bursting rate. The onset of a burst was defined as the occurrence of two spikes with an interspike interval < 80 ms (Grace, 1988). The bursting rate was defined as the number of bursts per second.

Categorization of response to footshock was done arbitrary by two persons blind to the treatment of each animals. Responses were labeled as 'no response', 'excitation' or 'inhibition'. The neurons exhibiting an 'inhibition' were further analyzed, by determining the duration of the inhibition.

Statistical analysis

Results are expressed throughout as mean ± SEM. Multiple comparisons were analyzed by performing analysis of variance with repeated measures. *Post hoc* comparison was performed using Fisher LSD test. When two means are compared, statistical significance of their difference was assessed using the paired Student's *t* test. In all cases, differences with $p < 0.05$ were considered significant.

RESULTS

RESULTS

The behavior of the rats during oral sucrose (5 %) and water self-administration is shown in Figure 2. Rats (water: n = 11; sucrose: n = 7) were subjected to 30 min daily sessions for at least 3 weeks. At the start of the training, there was no difference in numbers of reinforcing responses. However, <sucrose> rapidly increased the visits at the active nose-hole (**Figure 2A**). We found a treatment effect between groups across sessions ($F_{(1,16)} = 29.84$, $p < 0.001$). Accordingly, we found significant differences in intake between groups measured over the last three days before electrophysiological recordings (**Figure 2B**; water: 1.7 ± 0.3; sucrose: 11.0 ± 1.5; $t_{(16)} = 7.52$, $p < 0.001$).

Discrimination rate of <sucrose> was significantly higher than <water> ($t_{(16)} = 4.52$, $p < 0.001$). Nevertheless, discrimination rate of both groups was greater than chance level (water: 68.5 ± 4.5, $t_{(10)} = 4.14$, $p < 0.01$; sucrose: 95.6 ± 2.4, $t_{(6)} = 19.01$, $p < 0.001$), indicating that both groups acquired self-administration (**Figure 2C**).

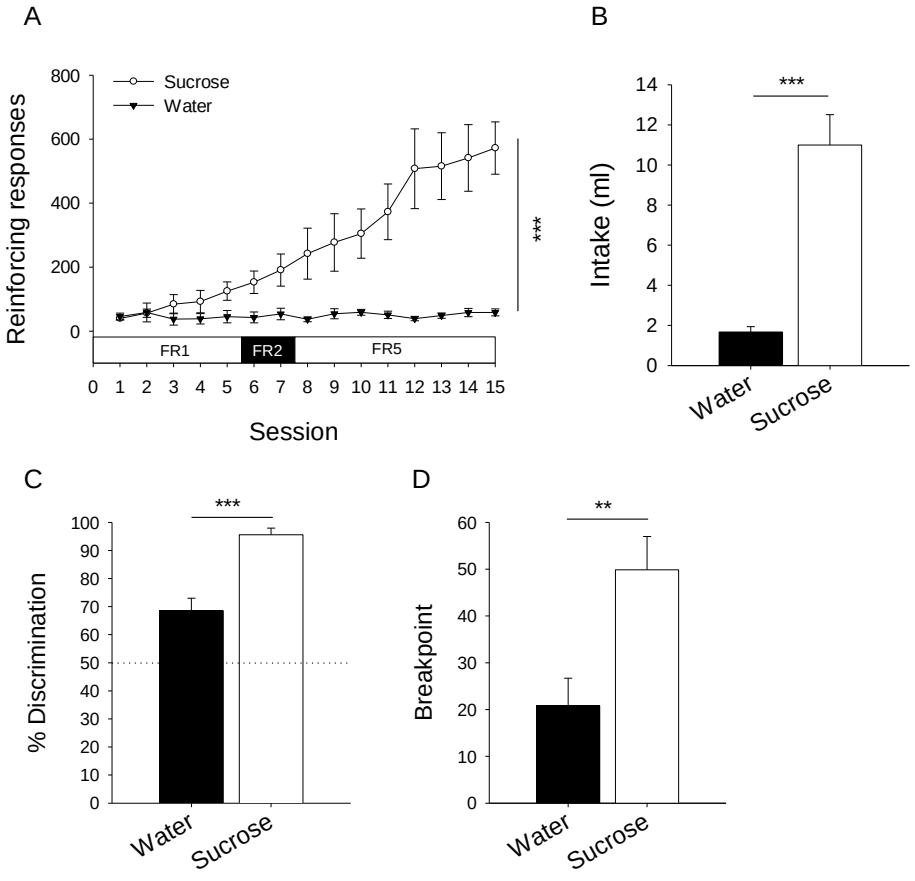

Figure 2. (A) Number of reinforcing responses of sucrose and water self-administration. Rats were trained under fixed-ratio schedules (FR1, FR2 and FR5) for a minimum of 3 weeks. FR5 was continued until electrophysiological recording. Two-way ANOVA; *** $p < 0.001$, treatment effect. Average of **(B)** intake and **(C)** discrimination rate during the last 3 sessions of self-administration before electrophysiological recordings. Dashed line indicates responding under chance level **(D)** Breakpoint of sucrose and water self-administration under a progressive ratio schedule. T-test between groups; ** $p < 0.01$ and *** $p < 0.001$. Data are shown as mean ± SEM.

We tested motivation in a PR schedule of reinforcement (water: n = 5; sucrose: n = 7). We found that breakpoint was significant different between groups (water: 20.8 ± 5.9; sucrose: 49.9 ± 7.1; $t_{(10)} = 2.96$, $p < 0.01$) (**Figure 2D**).

Data obtained during *in vivo* electrophysiological recordings of spontaneous active DA neurons in the VTA (water: n = 90 neurons; sucrose: n = 37 neurons) is summarized in Figure 3. **Figure 3A** shows an example of the location of the recording electrode in the VTA. Whenever recording electrodes were placed outside of the VTA, rats were excluded from the experiment. **Figure 3B** gives typical examples of irregular tonic firing (upper trace) and phasic or bursting firing (lower trace). We found a significant difference between groups in firing rate (**Figure 3C**; water: 3.0 ± 0.25; sucrose: 4.0 ± 0.46; $t_{(124)} = 2.05$; $p < 0.05$). Although bursting rate was not different between groups, we saw a trend (**Figure 3D**; water: 0.4 ± 0.06; sucrose: 0.6 ± 0.09; $t_{(124)} = 1.54$; $p > 0.05$). In contrast, we did find a significant difference between groups in mean spikes per burst (**Figure 3E**; water: 2.2 ± 0.14; sucrose: 2.9 ± 0.25; $t_{(124)} = 2.40$; $p < 0.05$).

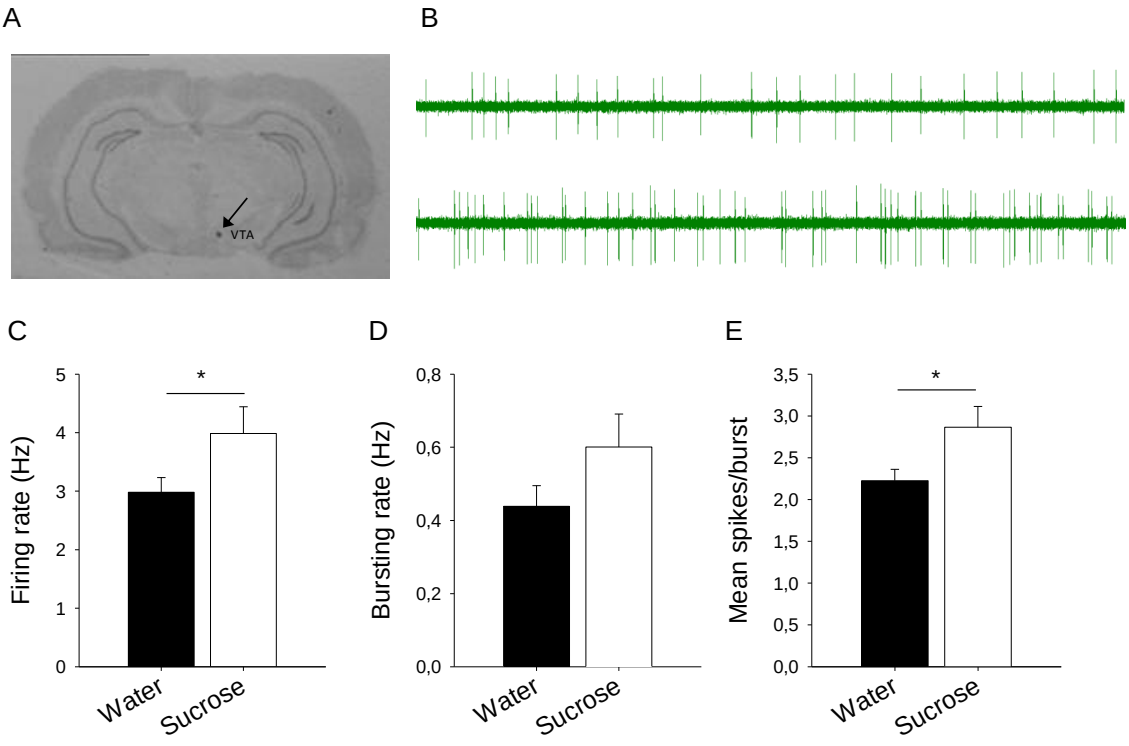

Figure 3 (A) Histological evaluation of recording electrode placement in the VTA **(B)** Examples of VTA DA neurons exhibiting irregular firing (upper trace) and bursting firing (lower trace). Bar plots showing the **(C)** firing rate **(D)** bursting rate and **(E)** mean spikes per burst of VTA DA neurons after sucrose (n=37) and water (n=89) self-administration. T-test between groups, * $p < 0.05$.

The analysis of electrophysiological recordings after administration of a footshock (water: n = 24; sucrose: n = 26) is shown in Figure 4. **Figure 4A** shows examples of measured responses neuronal activity to a footshock (i.e. inhibition, excitation or no response). We did not find a difference in the proportion of recorded neurons exhibiting either response (**Figure 4B**). Figure 4C shows the analysis of only the neurons exhibiting an inhibition to the footshock (sucrose: n = 14; water: n = 13). We found a significant difference between groups in the duration of the inhibition during the 4 sec of the footshock (**Figure 4C**; water: 2.74 ± 0.37; sucrose: 1.47 ± 0.35; $t_{(25)} = 2.51$, $p < 0.05$). Characteristic examples of inhibition of firing seen during administration of a footshock are presented in Figure 4D. The upper trace shows a complete inhibition of firing typically seen in rats exposed to water SA, while the lower trace displays a transient inhibition of firing seen in rats exposed to sucrose SA (**Figure 4D**).

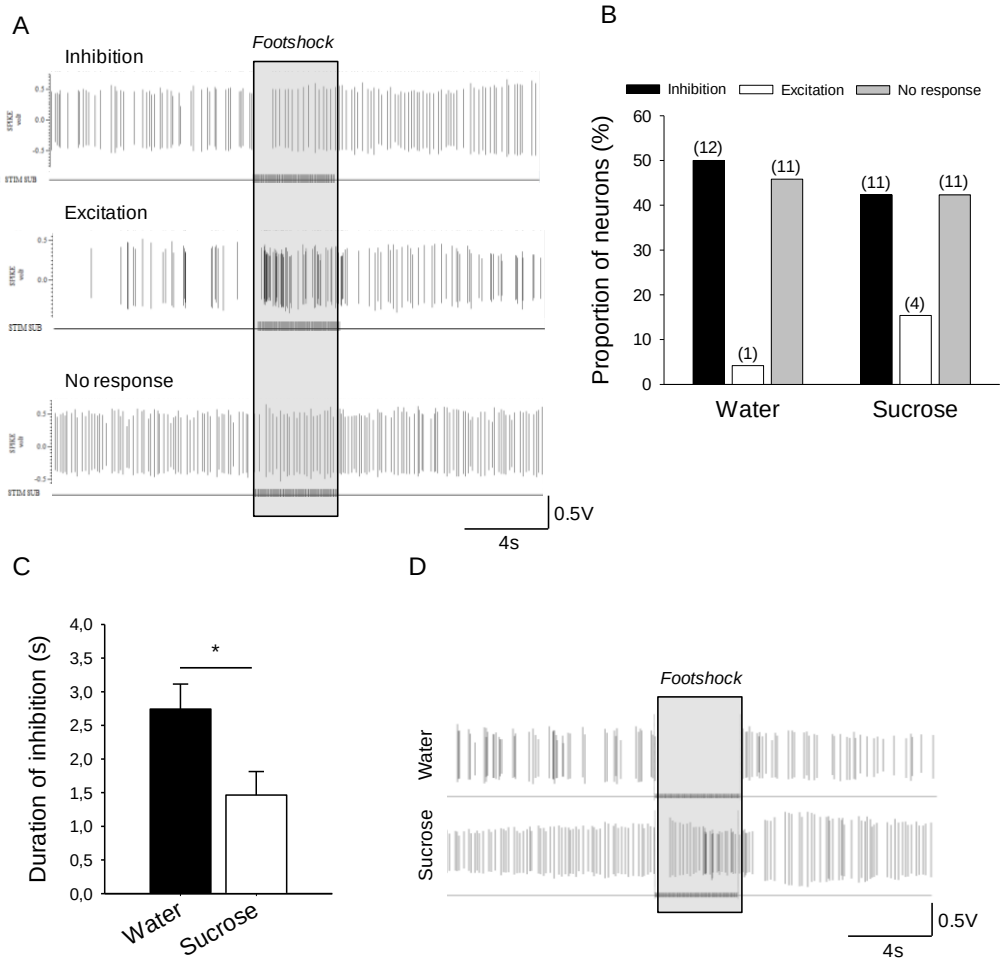

Figure 4 (A) Examples of electrophysiological recordings of VTA DA neurons showing 3 types of responses to a footshock (i.e. inhibition, excitation and no response). Grey bar indicates the duration of the footshock (4 s, 5mA). Activity was recorded 10 s before and 10 s after administration of the footshock **(B)** Bar graph showing the proportions of neurons responding to a footshock with inhibition, excitation or no response. Number in brackets refer to the number of neurons **(C)** Duration of the recorded inhibition during footshock. * $p \leq 0.05$. **(D)** Typical examples of duration of inhibition to footshock of « water » (upper trace) and « sucrose » (lower trace).

To control for the influence of caloric value of sucrose, we used a group of rats that was food restricted (20g / day / rat) during sucrose SA. The weight and intake per groups is shown in **Table 1**. Since rats are housed collectively, we were unable to measure individual chow intake in the home cages, therefore, a hypothetical intake is given per group. As expected, rats on a restricted diet had a significant higher sucrose consumption than rats fed *ad libitum* (**Figure 5A**; ad libitum: 11.0 ± 1.5; restricted: 15.4 ± 0.9; $t_{(18)} = 2.65$, $p < 0.05$). Before the electrophysiological recording, weight was different between groups (ad libitum: 505 ± 17, restricted: 393 ± 18, $t_{(18)}=4.04$, $p < 0.001$).

Table 1. Comparison of weight and intake between groups self-administering sucrose. Weight refers to the weight before the electrophysiological recording. Chow intake refers to the hypothetical average intake. Sucrose intake refers to the average intake over the last 3 sessions before the electrophysiological recording. * $p < 0.05$, *** $p < 0.001$.

		Sucrose SA	
	Diet:	ad libitum	restricted
Weight		505 ± 17	393 ± 18 ***
Chow	Grams	24 - 29	20
	Kcal (3.4 kcal/g)	82 - 99	68
Sucrose 5%	ml	11.0 ± 1.5	15.4 ± 0.9 *
	Kcal (3.9 kcal/g)	2.1 ± 0.29	3.0 ± 0.18

We did not find a difference in breakpoint, as measured in a PR schedule of reinforcement, indicating that motivation to obtain sucrose was similar between groups (**Figure 5B;** ad libitum: 49.9 ± 7.1; restricted: 51.1 ± 6.7; $t_{(12)}$=0.13, $p > 0.05$). To examine more profoundly the PR session, we evaluated the amount of reinforcers obtained in the first minute of the PR session. Even though the difference was not significant, we found a trend (**Figure 5C**; ad libitum: 1.4 ± 0.30; restricted: 2.4 ± 0.36; $t_{(12)} = 2.04$, $p < 0.06$). Moreover, we calculated the cumulative reinforcers. The total amount of reinforcers did not differ between groups, yet <restricted> needed less time to obtain 50 % of them (ad libitum: 10.87 min; restricted: 7.51 min; data not shown). While we did not find a difference in firing rate (**Figure 5D**; ad libitum: 4.0 ± 0.46; restricted: 4.7 ± 0.23; $t_{(134)} = 1.57$, $p > 0.05$) bursting rate was significant different between groups (**Figure 5E**; ad libitum: 0.6 ± 0.09; restricted: 0.8 ± 0.06; $t_{(134)} = 1.98$, $p < 0.05$). Duration of inhibition in response to footshock was not different between groups (**Figure 5F**; ad libitum: 1.5 ± 0.35; restricted: 1.6 ± 0.25; $t_{(37)} = 0.24$, $p > 0.05$).

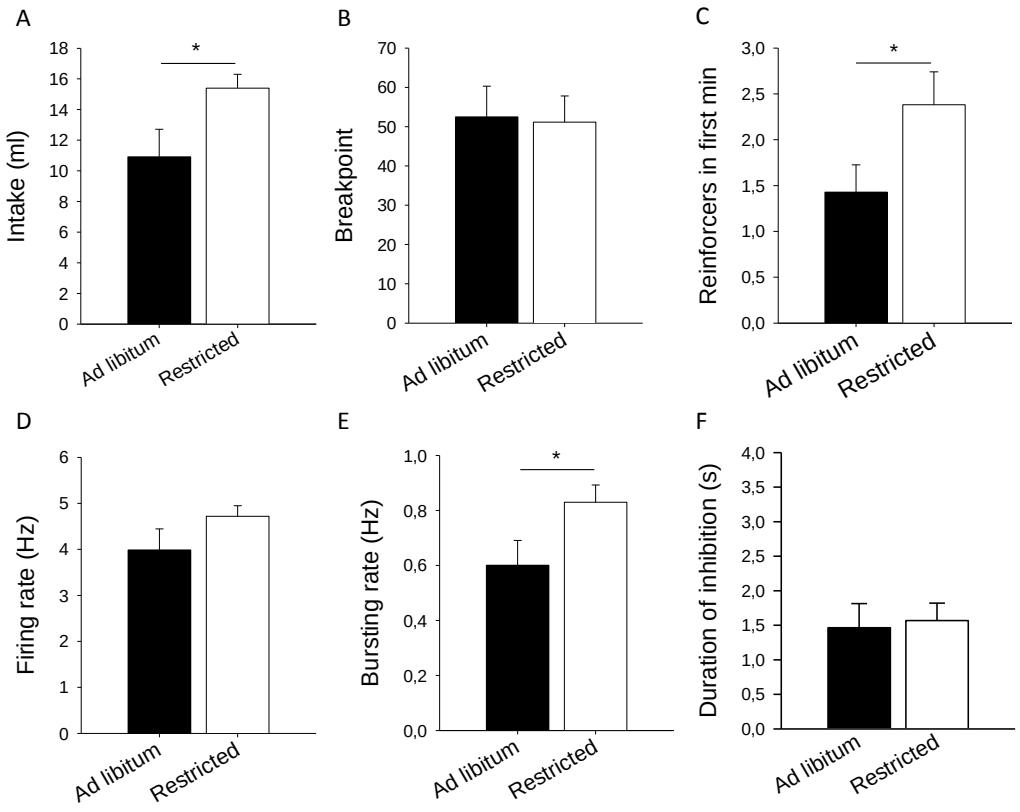

Figure 5 Behavioral and electrophysiological data compared sucrose self-administration on a restricted diet and a *ad libitum* diet. **(A)** Intake (ml) over the last 3 sessions of sucrose self-administration before electrophysiological recordings. **(B)** Sucrose self-administration under a progressive ratio schedule. Breakpoint was defined as the highest ratio completed before the end of the session. **(C)** The numbers of reinforcers obtained during the first minute of the progressive ratio session. Electrophysiological data showing **(D)** firing rate and **(E)** bursting rate of spontaneous activity of VTA DA neurons. **(F)** Duration of inhibition during footshock. T-test between groups, *$p < 0.05$.

DISCUSSION

DISCUSSION

The aim of this part of the project was to examine whether exposure to sucrose would alter spontaneous firing activity of DA neurons in the VTA and impair the ability to respond to aversive environmental stimuli. We showed that a sucrose-induced hedonic-like state enhances spontaneous activity of VTA DA neurons in anesthetized rats. While a footshock causes a nearly complete inhibition of activity of VTA DA neurons in control rats, a hedonic-like state causes a reduced response to a footshock. These observations were independent from the caloric state of the rats, as rats on a restricted diet during sucrose exposure showed the same activity pattern.

Sucrose self-administration induces a hedonic-like state in rats

At a behavioral level, we confirm that sucrose is highly palatable and that rats readily acquire oral self-administration (SA) (Reilly, 1999); in contrast to water SA, responses in the active nose-hole increased rapidly within 3 weeks. Accordingly, at the end of the SA period, <sucrose> rats consumed significantly more sucrose than <water> rats consumed water. In line with our expectations, we found a significant difference in motivation between <water> and <sucrose> tested in a PR schedule of reinforcement. Since <water> rats were not deprived from water during the experiment, their willingness to work for it was low. These results show the high hedonic impact and palatability of sucrose, supposedly resulting in an increased hedonic state. Yet, we do not have data on the facial « liking » expressions and on the sensitivity of the reward system (e.g. electrical brain stimulation), which are crucial measures of a hedonic state (Johnson and Kenny, 2010; Robinson and Berridge, 1993). Therefore, we will refer to this condition, induced by three weeks of sucrose SA, as a hedonic-like state.

Sucrose-induced hedonic-like state alters firing activity of VTA DA neurons

Using *in vivo* electrophysiology, we examined the spontaneous activity of VTA DA neurons in anesthetized rats exposed to sucrose SA. We found that three weeks of sucrose SA resulted in an elevated firing rate and increased bursting activity, indicated as an increase of mean spikes per burst and a trend toward an increase in bursting rate.

As Robinson and Berridge (1993) claimed that « liking » is independent from dopamine signaling, the increase in spontaneous activity induced by sucrose SA is either a result of motivation to sucrose or the learning process of association between sucrose and sucrose-paired cue. Since the rats who self-administered water seem to have acquired the SA paradigm but show a lower firing activity than rats who self-administered sucrose, it is therefore most likely that the increase in firing rate is due to the high motivation for sucrose. Moreover, food restriction induced an increase in motivation, resulting in an enhanced bursting rate of VTA DA neurons, further supporting the observation that the

motivational aspect of the self-administration encodes the increased firing activity of VTA DA neurons.

Accumulating evidence shows that an increase in VTA DA neuron activity results from an enhanced glutamatergic input (Marinelli et al., 2006). In line with our observations, sucrose SA increased excitatory strength in VTA DA neurons, indicated as an increased AMPA/NMDA ratio (Chen et al., 2008). However, in contrast to drugs of abuse, this enhanced synaptic potentiation after SA of a natural reward was considerably less persistent as a period of abstinence readily reversed the AMPA/ NMDA ratio to basal levels (Chen et al., 2008). A likely candidate for the enhanced excitatory input to VTA DA neurons is the BNST. The BNST sends direct glutamatergic projections to the VTA (Georges and Aston-Jones, 2001, 2002). It has been shown that plasticity at excitatory synapses within the BNST is associated with operant learning for both food and drugs of abuse (Dumont et al., 2005). Moreover, hyperactivity of VTA DA neurons after voluntary SA of nicotine is driven by changes in afferent excitatory projections from the BNST (Caille et al., 2009).

These data show that a solution with a high hedonic value (sucrose) induces a motivational state leading to enhanced spontaneous firing activity of VTA DA neurons. Future studies are needed to reveal the underlying mechanism and the contribution of different VTA DA afferents.

Sucrose-induced hedonic-like state alters responsiveness of VTA DA neurons to an aversive stimulus

During *in vivo* electrophysiological recordings, we administered a footshock to determine the response on firing activity of VTA DA neurons. VTA DA neurons responded by either an inhibition, excitation or no response in firing activity following delivery of a footshock. We found a similar number of recorded VTA DA neurons that exhibited an inhibition, excitation or no response in response to a footshock in rats self-administering sucrose compared to control rats. This confirms that VTA DA subpopulations are heterogeneous, and may subserve different functions (Bjorklund and Dunnett, 2007). It is suggested that both the nature of the aversive stimulus (e.g. shock, taste) and the projection area of the neuron determines the response profile to an aversive stimulus (Lammel et al., 2011). We found a small number of neurons that showed an excitatory response to the footshock. According to the literature, these VTA DA neurons are located in the ventral VTA (Brischoux et al., 2009), likely projecting to the mPFC, as *ex vivo* electrophysiology reveals that an aversive stimulus (formalin-induced irritation) causes an increase in the AMPA/NMDA ratio at VTA DA synapses projecting to the mPFC (Lammel et al., 2011).

When we had a closer look at the neurons responding with an inhibition to the footshock, we found that the duration of the inhibition of the firing pattern in response to a footshock was significantly shorter in rats exposed to sucrose SA compared to water SA. This strongly suggests that a hedonic-

like state alters the sensitivity of the reward system and impairs the ability to respond to aversive stimuli. Modulation of the responsiveness of VTA DA neurons might be due to a change in intrinsic properties of VTA DA neurons, synaptic plasticity mechanisms or a combination of both (Morikawa and Paladini, 2011). A brain area that has been shown to be involved in the processing of aversive stimuli, is the lateral habenula (LH). The LH sends glutamatergic projections to GABAergic neurons in the RMTg that inhibit VTA DA neurons (Kaufling et al., 2009). Using optogenetics, it has been demonstrated that activation of inputs to VTA DA neurons from the LH induce aversion in mice and promote avoidance behavior (Lammel et al., 2012; Stamatakis and Stuber, 2012). One might speculate that a hedonic-like state induces synaptic weakening in this circuitry leading disrupted aversion signaling.

A low caloric state increases the bursting activity of VTA DA neurons but does not influence response to an aversive stimulus

In order to determine the effect of a caloric state, we compared a group of rats that was food-restricted during the period of sucrose SA with a group that was fed *ad libitum*. In line with our hypothesis, we found that food restriction increased the intake of sucrose during SA sessions. However, this was not sufficient to increase body weight to the weight of rats fed *ad libitum.* Even though we did not find a direct difference in breakpoint, data show that rats fed a restricted diet were more motivated for sucrose, reflected in higher sucrose consumption in the first minute of the PR session and the shorter time required to obtaining half of the total amount of the reinforcers.

Although the firing rate was not increased in rats on a restricted diet compared to rats fed *ad libitum*, it did differ from rats self-administering water. Moreover, bursting rate was increased in rats on a restricted diet compared to rats fed *ad libitum*, possibly due to the enhanced motivation for sucrose. The increased activity is in line with a previous study showing that food SA enhances excitatory strength in VTA DA neurons (Chen et al., 2008). It is demonstrated that food intake in non-food deprived rats is mainly mediated by the palatability encoded in the reward system, including the PFC and NAc (Wise, 2006), whereas caloric content mediates food intake in food-deprived rats mainly through the hypothalamus (Scheggi et al., 2013). Both circuits pass the VTA where it may influence neuronal activity of DA neurons (Kempadoo et al., 2013). Therefore, it is possible that the sucrose-induced effects on VTA DA neurons under different caloric conditions is provoked through different pathways.

Another possible explanation for the increased bursting activity in rats on a restricted diet is the influence of ingestive hormones. For example, electrophysiological data show that ghrelin, a gut-derived hormone that promotes ingestive behavior, increases the activity of VTA DA neurons in both

rats and mice (Abizaid et al., 2006; Skibicka et al., 2011). Therefore, it is possible that rats on a restricted diet, who have higher levels of orexigenic hormones (Kirchner et al., 2012), would show differences in spontaneous activity either through direct influence of ingestive hormones or through a higher motivation for sucrose (Skibicka et al., 2012). Insulin has also shown to affect firing properties of VTA DA neurons by inducing LTD at excitatory synapses into VTA DA neurons (Labouebe et al., 2013). The lateral hypothalamus seems to be a likely candidate since it is involved in energy homeostasis and reward (Kelley et al., 2005) and is shown to control VTA DA neurons (Kempadoo et al., 2013).

A low caloric state did not alter the response to an aversive stimulus compared to rats fed *ad libitum*. This implies that a combination of exposure to a palatable solution and the learning of operant behavior disrupts aversive signaling, independent of the caloric state of the animal.

Conclusion

Taken together, we demonstrated that sucrose SA is able to induce a hedonic-like state, with high intake and high motivation for sucrose. Subsequently, this hedonic-like state induces neuroadaptations at the level of the VTA DA neurons reflected in an increased firing rate and to a lesser extent bursting activity. Furthermore, while a footshock causes a nearly complete inhibition of activity of VTA DA neurons in control rats, sucrose SA caused a transient inhibition in response to a footshock. These observations were independent from the caloric state of the rats (food restricted or *ad libitum* diet). Based on these results, we conclude that a hedonic-like state modifies the reward system and disrupts signaling of an aversive stimulus that might have negative consequences for the integration of environmental stimuli and subsequent behavioral adaptation.

This study is only a first step in the characterization of the reward circuitry involved in feeding behavior of palatable food and ultimately the effect it has on the ability to integrate and respond to environmental stimuli. This will have a great impact on the understanding of the current overconsumption of sugar-dense food and the underlying mechanisms of adaptive and pathological motivated behaviors.

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

The first part of this thesis focused on the involvement of the ILCx – BNST pathway in nicotine-related behaviors. We found that active nicotine IVSA persistently facilitated the induction of LTP at excitatory synapses in the BNST projecting from the ILCx, controlled by local CB1 receptors. These modifications were associated with the level of acquisition of response-contingent nicotine IVSA, as we found a correlation between discrimination level and the occurrence of facilitation of LTP induction. Electrical stimulation of ILCx efferents temporarily increased excessive responding for nicotine, but did not alter nicotine intake. CB1 receptors in the BNST were necessary for both the facilitation of LTP induction and excessive responding for nicotine.

Moreover, we found that pharmacological stimulation of BNST CB1 receptors before a history of nicotine IVSA increased incentive salience to the nicotine-associated cue, but did not affect sensitivity to nicotine. However, after acquisition of nicotine IVSA, stimulation of BNST CB1 receptors did not modulate primary reinforcing properties of nicotine, but blocks drug seeking induced by nicotine and nicotine-associated cue.

Together, the findings of the first part of the thesis indicates that the ILCx – BNST pathway, with its local CB1 receptors, are important for the acquisition and expression of response-contingent nicotine IVSA when associations are learned between nicotine and the nicotine-paired cue. With the observations of our studies, we conclude that the ILCx – BNST pathway is influential over nicotine-related behavior when nicotine is not actually available. In contrast, when nicotine is « on board », the VTA is more likely to be involved by mediating the primary reinforcing properties of nicotine.

In the second part of this project we aimed to examine the effect of exposure to a natural reward (in our case sucrose) on the firing properties if VTA DA neurons and the responsiveness to an aversive external stimulus. We showed that a sucrose-induced hedonic-like state enhanced spontaneous firing activity of VTA DA neurons. Moreover, while a footshock caused a near complete inhibition of activity of VTA DA neurons in control rats, sucrose SA caused a transient inhibition in response to a footshock. These observations were independent from the caloric state of the rats.

Commonly, synaptic plasticity induced by natural rewards are compared to those induced by drugs of abuse. There seems to be an overlap in neuronal modifications induced by these different types of reinforcers. However, synaptic plasticity induced by drugs of abuse appears to be persistent, in contrast to modifications induced by natural rewards (Chen et al., 2008).

One of the main characteristics of drug-addiction is the inability to stop drug intake despite negative consequences (Deroche-Gamonet et al., 2004). Studies show that « addicted » rats maintain their approach behavior for drugs even though they receive an electric shock (Deroche-Gamonet et al., 2004; Vanderschuren and Everitt, 2004). Following our observation that exposure to a natural reinforcer disrupts encoding of an aversive stimulus, the next important step to understand compulsive drug-seeking would be to examine the effect of nicotine exposure on the responsiveness of VTA DA neurons.

The BNST projects to VTA DA neurons and exerts a strong excitatory influence on their firing properties (Georges and Aston-Jones, 2001, 2002). Moreover, the BNST is necessary for the rely of cortical excitation from the ILCx to VTA DA neurons (Massi et al., 2008). It has been previously shown that the learning process associated with contingency learning of nicotine IVSA increases excitability of excitatory synapses of the ILCx-BNST pathway, resulting in increased spontaneous activity of VTA DA neurons. Therefore, we hypothesize that the nicotine-induced facilitation of LTP induction at excitatory synapses in the BNST projecting from the BNST would also enhance VTA DA activity. However, the facilitation of LTP induction was specific to nicotine, as these modifications were absent after exposure to a natural reward (saccharin). Therefore, input from other brain areas may contribute to the increase of VTA DA activity we observed after sucrose self-administration.

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