Constitutive cannabinoid 1 and mu opioid receptor activity in the ventral tegmental area:

occurrence, function and therapeutic relevance

COLOFON

The research described in this thesis was performed at the Department of Neuroscience and Pharmacology, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, The Netherlands.

The research was financially supported by *Top Institute Pharma (project D1-105).*

Printing of this thesis was financially supported by Van Leersumfonds.

Cover design & Lay out: *Amy Verspaandonk (www.amyontwerpt.nl)*
Printed by: *Uitgeverij BOXpress*
ISBN: *978-90-8891-455-3*

Constitutive cannabinoid 1 and mu opioid receptor activity in the ventral tegmental area: occurrence, function and therapeutic relevance

Constitutieve cannabinoïd 1 en mu opiaat receptor activiteit in het ventrale tegmentum: *aanwezigheid, functie en therapeutische relevantie*

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 26 september 2012 des ochtends te 10.30 uur

door

Frank Julius Meye

geboren op 19 april 1984 te Utrecht

Promotor: Prof.dr. R.A.H. Adan

Co-promotor: Dr. G.M.J. Ramakers

TABLE OF CONTENTS

Chapter 1

GENERAL INTRODUCTION

INDEX

1. Relevance of constitutive cannabinoid 1 and mu opioid receptor activity

Distributed neural systems detect rewards and their cues in the environment, appraise their desirability, and may eventually initiate goal-directed action to obtain them. Control over this process is lost in some individuals, resulting in aberrations such as drug abuse or overeating[1-3]. In order to treat these conditions, a clear delineation is required of the main neural circuits responsible for maladaptive reward pursuit. An important component of this circuitry is the mesolimbic dopamine system, which is critical in processing (food) rewards and their associated stimuli[4,5], and also contributes to obesity and drug addiction[6,7]. Neuronal activity in the mesolimbic dopamine system is modulated by G-protein-coupled receptors (GPCRs), like the cannabinoid 1 (CB1R) and mu opioid receptor (MOR). Accordingly, these GPCRs are also pivotal in reward-seeking, addiction and obesity[8-12]. CB1Rs and MORs are activated by (endo)cannabinoids and (endogenous) opioids respectively, but an alternative activation mechanism of (agonist-independent) constitutive activity has also been described for these receptors[13-19] (Figure 1A). The constitutive activity of a GPCR population reflects their propensity to spontaneously engage in G protein signaling[20].

The occurrence of GPCR constitutive activity is important from a pharmacological perspective. If a GPCR exhibits constitutive activity, then antagonists for this receptor need to be further subdivided into either neutral antagonists or inverse agonists. Whereas neutral antagonists are capable of preventing agonist-driven activation of the receptor, they do not interfere with constitutive activation. In contrast, inverse agonists interfere with both agonist-driven and constitutive activation (Figure 1B). Tentative evidence is now emerging that CB1R and MOR constitutive activity does not merely play a role in artificial systems, but is also relevant in native brain tissue, regulating behavior[13,21-23]. CB1R and MOR inverse agonists have been put forward as therapeutics for both obesity and addiction, but are often associated with side effects[13,24]. It is therefore important to unravel how constitutive CB1R and MOR activity (which, if it occurs, is suppressed by such therapeutics) contributes to neural activity in therapeutically relevant circuits like the mesolimbic dopamine system.

The aims of this introduction are threefold: (a) Delineate the role of the mesolimbic dopamine system in reward processing, overeating and addiction. (b) Describe the role that the MOR and CB1R play in these processes. (c) Discuss the

extent to which CB1R and MOR constitutive activity can contribute to this role, and how this may be relevant for determining optimal treatment strategies for addiction and obesity.

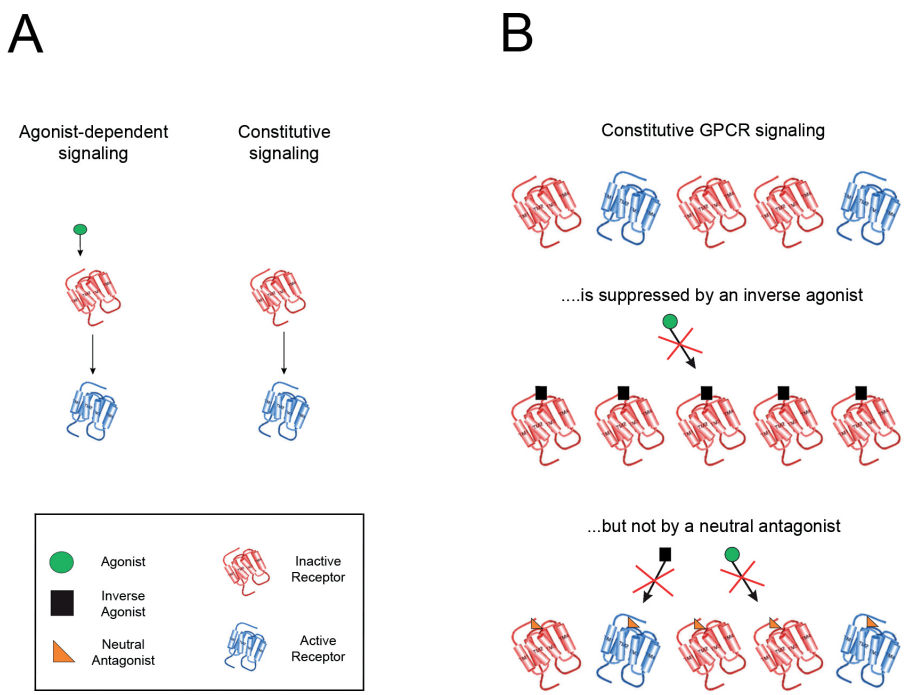

Figure 1

GPCRs exhibit constitutive activity in artificial cell systems. (**A**) Typically agonists drive activation of GPCRs, but even in the absence of a ligand, GPCRs can become constitutively active. (**B**) In case of constitutive activity, antagonists need to be subdivided into inverse agonists and neutral antagonists. Whereas inverse agonists suppress constitutive activity, neutral antagonists do not. Both antagonists prevent agonist-dependent signaling. A neutral antagonist can also prevent inverse agonism.

2. The mesolimbic dopamine system in addiction and obesity

2.1 VTA dopamine signaling and aspects of reward processing

Dopamine neurons in the ventral tegmental area (VTA) in the midbrain (mesencephalon) play a crucial role in the effective pursuit of reward[5,25]. These *mes*encephalic neurons project to *limbic* regions in the forebrain, such as the nucleus accumbens (NAc). This projection makes up the heart of a reward processing network called the *mesolimbic* dopamine reward system. Alternative firing modes of dopamine neurons result in distinct spatiotemporal characteristics of the dopamine signal in target regions, which serve different behavioral functions[5]. Burst firing in dopamine neurons has been implicated in associative (reward) learning[26,27], whereas dopamine signaling with likely alternative spatiotemporal profiles, mediates motivation for reward[26,28-30]. Voltammetric studies with high spatial and temporal resolution have suggested that one dopamine transient in the NAc just precedes active reward pursuit (likely reflecting motivational properties for the reward), whereas another NAc dopamine transient occurs after the reward has been obtained (likely reflecting cue-reward learning[31,32]). In summary, mesolimbic dopamine transmission provides a multi-facetted signal to guide consummatory behavior.

2.2 The mesolimbic dopamine system in addiction

Drugs of abuse have various modes of action, but they generally all share the propensity to increase dopamine levels in target regions[33]. The extent to which a drug leads to a fast transient surge in dopamine in the NAc is also highly related to its reinforcing effect[34]. In animal models for addiction, repeated drug exposure enhances the motivation for the drug and increases dopamine release in the NAc following subsequent drug use[35]. These sensitized responses are probably mediated by changes in the organization and strength of glutamatergic synapses in the mesolimbic dopamine system[36-39]. Drugs of abuse potentiate glutamatergic synapses onto VTA dopamine neurons due to upregulation of AMPA receptors on the membrane of these neurons. They also alter the composition of AMPA receptors to favor the GluR2-subunit lacking forms of this receptor, leading to Ca^{2+}-conducting forms[38,40,41]. This is likely crucial in the development of drug sensitization, since virally-mediated upregulation of the GluR1 (but not GluR2) subunit of the AMPA receptor in the VTA directly enhances the rewarding properties of morphine[36].

Persistent synaptic changes in the VTA can trigger dynamic synaptic changes in the NAc[38,42]: a region considered to be important for the expression rather than the induction of sensitized responses to drugs of abuse[37]. The GABAergic medium-spiny neurons (MSNs) in the NAc can generally be subdivided in two separate classes according to their projection target and dominant type of dopamine receptor. Generally, MSNs from the direct pathway have dopamine 1 receptors (D1Rs) and project directly to regions like the VTA, while MSNs from the indirect class mainly have dopamine 2 receptors (D2Rs) and first make a synapse in the ventral pallidum before also projecting to similar regions[43]. In general, activation of D1R-MSNs is associated with reinforcing behavior, whereas activation of D2R-MSNs has opposite effects[39,44-48]. Notably, due to the differential coupling of D1Rs (to $G\alpha_{s/olf}$-proteins) and D2Rs (to $G\alpha_{i/o}$-proteins), dopamine transmission in the NAc can simultaneously enhance the ability of glutamate to excite D1R-MSNs, and diminish the impact of glutamate transmission on D2R-MSNs[49,50]. As such, elevations in dopamine signaling, like those caused by drugs of abuse, bias activation in the NAc towards the direct

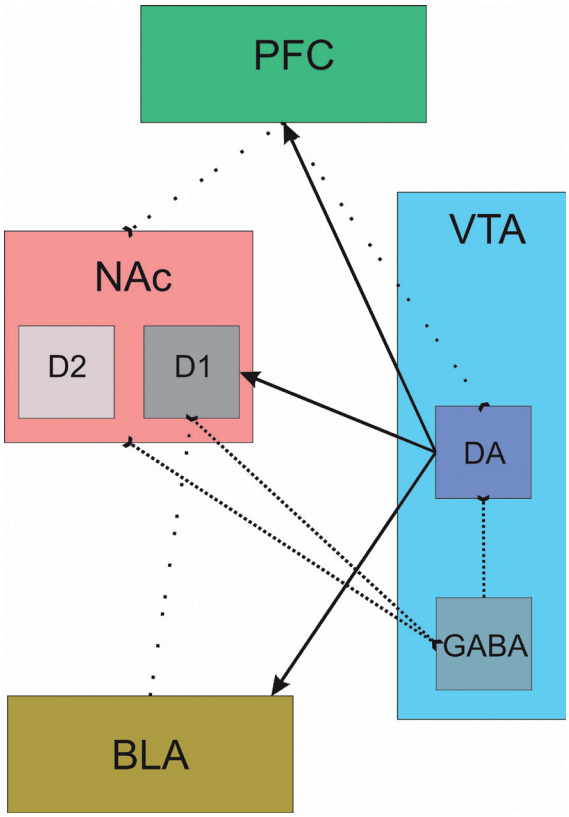

Figure 2
A schematic representation of the mesolimbic dopamine system. For sake of clarity only some of the most prominent connections discussed in this thesis are drawn. Output from the dopamine neurons (DA) of the ventral tegmental area (VTA) innervates important structures such as the prefrontal cortex (PFC), nucleus accumbens (NAc) and basolateral amygdala (BLA). These structures also form reciprocal connections. NAc medium spiny neurons from the direct pathway (D1) directly target VTA GABAergic neurons (GABA), while those from the indirect pathway (D2) innervate the VTA via a disynaptic pathway through the ventral pallidum (not shown). In this schematic, inhibitory inputs are drawn as densely dashed lines, while excitatory inputs are sparsely dashed lines. The modulatory dopaminergic output is drawn as solid black lines with arrowheads.

rather than the indirect pathway by a double action. MSNs belonging to the direct pathway synapse onto GABAergic, but not onto dopaminergic neurons in the VTA[51]. Therefore, the consequence of increased activity of the GABAergic D1R-MSNs may be a further disinhibition of VTA dopamine neurons.

The abovementioned physiological changes occur during the early stages of (drug) reward processing and the potential development of addiction. During later stages, other factors seem to become increasingly relevant. The actual transition from recreational drug use to addiction involves a shift from controlled voluntary to uncontrolled habitual drug use[3,52], potentially due to a hypofunctional prefrontal cortex[34,53]. Overall, the mesolimbic dopamine plays a dynamic and pivotal role throughout multiple stages of addiction. The basic outline of some of the neuronal circuitry involved in reward processing is represented in Figure 2.

2.3 The mesolimbic dopamine system in food reward and obesity

The midbrain dopamine system is also implicated in food reward-related behavior. Imaging studies show that anticipation of a glucose reward activates the dopaminergic midbrain and some of its target areas[54] and rewarding food images elicit activity in the NAc in fasted individuals[6]. In animal studies, food reward raises dopamine levels in VTA-target regions like the NAc[55,56]. Also, phasic activation of VTA dopamine neurons enhances lever pressing for food[57]. Contrarily, pharmacological inactivation of these neurons abolishes conditioned place preference for food[58]. Food reward also heightens motivation for further food reward, analogous to the enhanced motivation for drugs that is associated with drug sensitization[35,59]. The mesolimbic dopamine system is also activated by cues that signal the presence of food-reward, and these cues are particularly salient for obese individuals[2]. Food-reward cues likely trigger burst firing in VTA dopamine neurons, which is sufficient to relapse in food reward-seeking behavior[57].

Food-reward is also capable of altering synaptic plasticity in brain reward circuitry in a manner reminiscent of the effects of drugs of abuse. For instance, when animals voluntarily press levers to obtain natural rewards such as food or sucrose, a transient strong enhancement of glutamatergic signaling onto VTA dopamine neurons occurs[60]. Also, peptides related to food intake, like orexin and ghrelin, are able to potentiate glutamatergic synapses onto VTA dopamine neurons[9,61]. In the long-run, as is the case for addiction, overconsumption and obesity may lead to a hypofunctional prefrontal cortex[62], and compulsive intake[63].

It is noteworthy that contrary to its involvement in active pursuit of food reward, dopamine is not pivotally involved in homeostatic feeding. For instance, rats with a 6-OHDA lesion of the mesolimbic system did not alter their total consumption of a freely available non-palatable food[64]. Moreover, dopamine antagonists infused into the NAc typically do not diminish food intake if the food is freely available[65]. Furthermore, freely available non-palatable food does not lead to increases in NAc dopamine signaling in animals that have not endured serious food deprivation[55]. Finally, optogenetic stimulation of VTA dopamine neurons to produce maximal release of dopamine in the NAc, does not affect the total consumption of food pellets[57]. Therefore, dopamine signaling in the NAc only appears to be important when either the food is desirable (e.g. in the case of food deprivation, or special palatable food), or when effort is required to obtain the food (e.g. lever pressing).

3. CB1Rs and MORs regulate the mesolimbic dopamine system

The many neurobiological similarities between drug addiction and obesity suggest that successful treatments for these disorders may have much in common. The CB1R and MOR are interesting target candidates for such treatment due to their extensive role in reward processing[66-69]. We now review the role of CB1Rs and MORs in reward processing, obesity and addiction. We also take into account how constitutive activity of these receptors may contribute to such behaviors.

3.1.1 Cannabinoids in the mesolimbic dopamine system

In general, endocannabinoids are released from postsynaptic sites in an activity- and Ca^{2+}-dependent manner[70,71]. Upon synthesis, endocannabinoids are released through the membrane into the synaptic cleft potentially by active transport. They then activate CB1Rs located on presynaptic nerve terminals to typically suppress release of neurotransmitter. CB1R activation results in dissociation of the heterotrimeric G protein into the $\alpha_{i/o}$-GTP complex and the $\beta\gamma$-dimer. The $\alpha_{i/o}$-subunit inhibits activity of adenylate cyclase, lowering production of cAMP. The $\beta\gamma$-dimer directly regulates the conductance of ion channels, activating G protein-coupled inwardly rectifying potassium channels (GIRKs), and inhibiting voltage-gated calcium channels (VGCCs) of the N and P/Q types[18,72-74]. Due to this constellation of effects, activation of CB1Rs reduces the likelihood of transmitter release.

Cannabinoids within the mesolimbic dopamine system play a role in the motivational and hedonic properties of rewarding stimuli[10,75]. A likely neural substrate for these effects is the cannabinoid-induced increase in the firing frequency and likelihood of bursting of VTA dopamine neurons[76-80], with a corresponding enhancement of dopamine levels in the NAc[81]. Cannabinoids may exert this effect by interacting with CB1Rs on GABAergic presynaptic nerve terminals in both the VTA and the NAc, which disinhibit output of VTA dopamine neurons[82-85]. The full modulatory scope of (endo)cannabinoids in the mesolimbic dopamine system is broader however, as it also includes the regulation of glutamatergic synapses onto dopamine VTA neurons, and likely the regulation of synapses onto GABAergic VTA neurons[70,86]. Moreover, CB1Rs in the NAc also inhibit both inhibitory and excitatory inputs to MSNs[87-89]. Aside from direct effects on neurotransmission, cannabinoids also play an important role in forms of synaptic plasticity like long-term depression in the mesolimbic dopamine system[90,91].

3.1.2 Cannabinoids in drug use and addiction

Injections of CB1R agonist delta 9-THC, an active component of marijuana, into the posterior VTA and in the NAc shell are rewarding[10]. However, CB1Rs play a role in the reinforcing effects of many other drugs as well, including nicotine, alcohol, morphine and heroine[92]. This is likely because the intake of drugs of abuse typically enhances levels of endocannabinoids within the mesocorticolimbic system[11,93,94], which may be required for its rewarding effect to occur. Indeed, local interference with CB1R signaling in the mesolimbic dopamine system reduces self-administration of several drugs of abuse[11,95-98].

The role of the cannabinoid system is not limited to reward processing, but also extends to addiction, as it contributes to reshaping synaptic strength in key regions such as the VTA[91] and NAc[90]. Cannabinoid signaling also plays an important role in the propensity to relapse into drug-seeking behavior. Indeed, CB1R agonists facilitate reinstatement of seeking and taking of various drugs of abuse, while CB1R antagonism blocks this behavior[69,99-101]. Accordingly, substance abuse has been associated with genetic variation in the gene for fatty acid amide hydrolase (FAAH), limiting its function[102,103]. Since FAAH is an enzyme that terminates the activity of endocannabinoids like anandamide, this suggests that impaired FAAH functionality, leading to prolonged endocannabinoid activity, is associated with risk of drug abuse.

3.1.3 Cannabinoids in food intake and obesity

The cannabinoid system in the brain plays an important role in the regulation of food intake[68]. Endocannabinoids represent important downstream targets of signals that convey information about the status of energy balance. The anorexigenic (feeding-inhibiting) hormone leptin inversely controls endocannabinoid activity in the hypothalamus[104]. Ghrelin exerts its orexigenic (feeding-promoting) effect in the hypothalamus through the cannabinoid system[105]. In healthy individuals, plasma levels of the endocannabinoid 2-arachidonoyl glycerol (2-AG) positively correlate with markers for energy status like body mass index and abdominal adiposity[106]. During periods of fasting, 2-AG levels rise in the hypothalamus and levels of both 2-AG and anandamide rise in the limbic forebrain. Feeding lowers 2-AG levels in the hypothalamus, without affecting endocannabinoids in the limbic forebrain[107].

These findings suggest that CB1R signaling enhances food intake. Indeed, CB1R knockout mice consume less food and are leaner than wild-type litterma-

tes: an effect largely mediated by CB1Rs in the central nervous system[68,104,108,109]. Orexigenic effects are observed after infusions of (endo)cannabinoids in specific brain sites, such as the hypothalamus[110], the NAc shell[107], and the VTA[327]. CB1R agonists also particularly cause overconsumption of palatable foods[111,112]. Peripherally administered CB1R agonists increase responding for palatable food on a progressive ratio task[113], suggesting that the motivation for the food reward is also enhanced. Conversely, CB1R inverse agonists/antagonists suppress food intake[114,115], and reduce motivation for palatable food[113]. Similarly, CB1R knockout mice also exhibit diminished motivation for sucrose reward[116].

The endocannabinoid system is also affected in obesity. Compared to healthy controls, obese patients with high amounts of abdominal fat have elevated 2-AG plasma levels[106]. Similarly, endocannabinoid levels in the hypothalamus are elevated in obese leptin-deficient animals[104]. Moreover, the same missense polymorphism in the endocannabinoid degrading enzyme FAAH (which likely results in enhanced endocannabinoid duration of action) that is associated with drug abuse, is also associated with overweight and obesity[117,118]. Given that elevated endocannabinoid levels are associated with feeding and obesity, it follows that interference with endocannabinoid signaling at CB1Rs may be suitable treatment for obesity. The CB1R antagonist/inverse agonist rimonabant (SR141716A) was marketed to treat obesity in humans. Even though rimonabant made subjects lose weight[119,120], it was ultimately discontinued since it was associated with complaints relating to anxiety and depression in a subgroup of subjects[24,121].

3.1.4 The therapeutic relevance of interfering with endocannabinoid-CB1R signaling

The findings that rimonabant does not only produce therapeutic, but also strong adverse effects, greatly limits the therapeutic use of this and similar compounds. However, these different types of effects can have separate causes. Of particular relevance in this respect are the findings that the CB1R can exhibit agonist-independent constitutive activity. In heterologous expression systems, even in the absence of a ligand, the mere transfection of CB1Rs in the membrane of cells engages downstream signaling such as G protein coupling, cAMP production, MAP kinase activity, and K^+ and Ca^{2+} ion channel conductance[14-16,18,19]. In these artificial systems, compounds like SR141716A behave as inverse CB1R agonists that do not merely act as passive antagonists for the CB1R (like neu-

tral CB1R antagonists), but also suppress constitutive CB1R activity. Inverse CB1R agonists, but not neutral antagonists suppress the downstream effects of constitutive CB1R activation in artificial systems[15,122]. There are also some tentative indications that inverse agonism occurs at endogenously expressed CB1Rs, suggesting that there may also be CB1R constitutive activity in native tissue[17,123]. Therefore, the constellation of *in vivo* effects observed with the CB1R inverse agonist/antagonist rimonabant could potentially be parsed in effects that are solely due to interference with endocannabinoid-CB1R signaling (the antagonistic property of the ligand), and effects that are due to the additional suppression of constitutive CB1R activity (the inverse agonistic property of the ligand).

There is evidence that interference with endocannabinoid-CB1R signaling is sufficient to reduce food intake, as endocannabinoids in regions like the hypothalamus and the mesolimbic dopamine system are important regulators of energy state[104,107]. Accordingly, several reports indicate that both neutral CB1R antagonists and inverse CB1R agonists reduce food intake[114,115,122,124]. Neutral CB1R antagonists and inverse CB1R agonists both interfere with endocannabinoid signaling, while these compounds differ in their capability to suppress constitutive activity. Therefore, these findings suggest that endocannabinoids are important in regulating appetite, and that interference with their signaling at CB1Rs is enough to reduce food intake. Notably, at higher doses, inverse CB1R agonists can cause a degree of malaise and illness, which can contribute to any observed effect of weight loss and food intake. Interestingly, neutral CB1 receptor antagonists appear to lack these effects[122,125].

Many drugs of abuse also increase levels of endocannabinoids in the mesocorticolimbic system[11,93,94]. Also, whereas CB1R agonists cause reinstatement of drug-seeking behavior, interference with CB1R signaling reduces reinstatement of various drugs of abuse[99]. However, since typically the interference is done with inverse CB1R agonists, it remains to be determined to what extent relapse mechanisms depend on endocannabinoids and to what extent they depend on constitutive activity. Additional studies are therefore required to establish this. Notably though, the impulsive behavior associated with use of amphetamine was attenuated equally effectively with a CB1R inverse agonist and a CB1R neutral antagonist[126]. Together such findings tentatively suggest that the beneficial effect of the CB1R inverse agonist SR141716A on weight gain, and potentially on drug use, are to a large extent due to interference with an endocannabinoid tone.

3.1.5 The therapeutic relevance of interfering with constitutive CB1R signaling

The main reason for discontinuation of CB1R inverse agonists was their role in the generation of feelings of depression and anxiety[24]. The neurobiological origin of these pernicious negative effects could partly lie in CB1R signaling within such brain circuitry as the extended mesolimbic dopamine system, which plays a prominent role in both anxiety and depression-associated behavior[127,128].

In animal models, CB1R inverse agonists exert anxiogenic effects that are indeed CB1R-mediated[129]. This raises the possibility that constitutive CB1R activity plays a role in regulating anxiety. In accordance with this, while inverse CB1R agonists cause anxiety and raise c-Fos and Fos levels in the amygdala[130,131], the neutral CB1R antagonist AM4113 does neither[130,132]. This finding suggests that the suppression of constitutive CB1R activity leads to pronounced anxiogenic effects that may not be observed with mere interference with endogenous endocannabinoid-CB1R signaling. This is perhaps surprising since endocannabinoids have been shown to be involved in aspects of fear and anxiety. Endocannabinoid levels in the basolateral amygdala are increased during extinction learning of aversive memories[133]. Also, a general increase in endocannabinoid signaling through hydrolysis inhibitors has anxiolytic properties[134]. Nevertheless, these findings tentatively suggest that, despite their involvement in anxiety and fear, interference with these endocannabinoid processes is itself not necessarily anxiogenic; or considerably less so than the concurrent interference with CB1R constitutive activity. However, additional studies are required to verify this.

Depression is another notable side effect of rimonabant in humans[24]. Depression is a multi-facetted affliction, but a crucial feature is the state of "anhedonia": the impaired motivation for, and enjoyment experienced from pleasurable stimuli[135,136]. Given the role of cannabinoids in reward processing, it is plausible that rimonabant gives rise to depressed symptoms in part by causing anhedonic effects. Such effects are clearly more readily accessible in animal models than the complex disease in its entirety. Impaired motivation for reward is indeed an important readout in animal models for depression. For instance, rats susceptible for learned helplessness (a model for depression) show a strong reduction in motivation for sucrose under a progressive ratio schedule (PR; a schedule in which progressively more effort is required to get the next reward), as compared to rats that are resistant to learned helplessness[137]. Also, in a drug-withdrawal model for depression, rats show diminished willingness to work for sucrose

reward under a PR schedule[138]. The mesolimbic dopamine system, with its large role in reward processing and motivated behavior is a likely candidate substrate for such anhedonia. Indeed, a considerable body of evidence also suggests that depression is associated with alterations in the mesolimbic dopamine system, and impaired dopamine signaling[127,128].

There is ample evidence that interference with cannabinoid signaling (be it with constitutive activity and/or with endocannabinoid signaling) has prominent anhedonic-like effects. Inverse CB1R agonists SR141716A and AM251 decrease motivation for rewards[113,139]. A recent study found that extended use of rimonabant in rats was linked to a depression-like phenotype on a number of parameters, including more immobility in the forced swim test as well as reduced sucrose preference[140]. Also, CB1R knockout animals are more susceptible to anhedonic effects of chronic mild stress[141], which may be particularly mediated by the lack of CB1Rs in target regions of the dopamine system[142]. While these findings illustrate that interference with CB1R signaling has anhedonic effects, these experiments do not give insight in whether this is due to interference with endocannabinoid-CB1R signaling, or due to the additional interference with any CB1R constitutive signaling. Several studies indicate that enhancement of endocannabinoid activity can produce anti-depressant effects in some paradigms[115,143,144]. However, these findings do not address whether, oppositely, interference with endocannabinoid signaling at CB1Rs is sufficient to produce depressant effects. An additional caveat to consider is that indiscriminately enhancing the endocannabinoid tone does not lead to specific effects at the CB1R, since endocannabinoids also act on other targets, such as the CB2R and TRPV1[145]. A more specific way of assessing the contribution of constitutive CB1R activity would be to contrast neutral CB1R antagonists with inverse CB1R agonists on models for depression / anhedonia. To our knowledge no reports have been made on potential differences between neutral CB1R antagonists and inverse CB1R agonists on such measures, like progressive ratio schedules. However, an interesting observation is that the CB1R neutral antagonist AM4113 lowers expended effort for food reward more potently on a low-demanding fixed ratio 1 schedule (FR1; 1 action required for each subsequent reward), than on a somewhat more demanding FR5 schedule[122]. This tentatively suggests that actual motivated behavior, as reflected by a progressive ratio schedule, may be less affected by mere interference with endocannabinoid-CB1R signaling. However, this must be validated further. For instance, by contrasting CB1R neutral

antagonists and inverse agonists on demanding performance schedules that assess motivation such as progressive ratio, to explicitly assess the contribution of CB1R constitutive activity to motivated behavior for reward.

3.2.1 Opioids in the mesolimbic dopamine system

The opioid system consists of several endogenous opioid peptides and receptors. The MOR in particular, plays a prominent role in reward processing and addiction[146]. The main endogenous agonist for the MOR is B-endorphin, which is cleaved from proopiomelanocortin. B-endorphins are mainly synthesized and released from neurons in the arcuate nucleus of the hypothalamus, the pituitary gland and the nucleus tractus solitarius. From there, regions like the VTA and NAc are innervated[147]. Enkephalins, while primarily the endogenous agonist for delta opioid receptors, also have sufficiently high affinity to be physiologically relevant MOR agonists[148].

The MOR and its ligands play an important role upstream of dopamine transmission within the mesocorticolimbic system. Within the VTA, MORs are located both on the somata and the presynaptic nerve terminals of GABA neurons[149-151]. Local administration of opioids inhibits GABAergic neurons in the VTA by activating a K^+ conductance[149]. However, MORs are also present on glutamatergic projections towards VTA dopamine neurons[152]. Despite this potential dual action on inhibitory and excitatory inputs, activation of MORs in the VTA activates dopamine neurons[153,154]. Consequently, MOR agonists increase dopamine levels in target regions like the NAc[155]. Dopamine signaling in the NAc itself also in turn modulates MORs and B-endorphins[156-158].

Like cannabinoids, opioids are involved in both the motivational and hedonic aspects of rewards. Opioid involvement in motivational aspects of reward seems to occur in a neural network including the VTA, NAc shell and ventral pallidum. Opioid involvement in hedonic pleasure may mainly be mediated by a small area in the NAc shell[67,159].

3.2.2 Opioids in drug use and addiction

Intake of drugs of abuse like ethanol, cannabis, amphetamine and cocaine induces the release of endogenous MOR agonist B-endorphin in the mesolimbic dopamine system[156,160-162]. B-endorphin knockout mice exhibit less conditioned place preference for cocaine, and are also less responsive to the locomotor activating effects of cocaine[163]. Also, destruction of B-endorphin producing neurons

in the arcuate nucleus of the hypothalamus reduces cocaine self-administration[164]. In line with these findings, the main target of B-endorphin, the MOR, is critically important for the rewarding effects of many drugs of abuse, including ethanol, nicotine, cocaine and cannabis[66,148,165,166]. VTA dopamine neurons in MOR-knockout mice have a lowered firing frequency and show less burst firing than wild-type animals[66].

Administration of the MOR agonist morphine directly into the VTA is rewarding, but the make-up of the exact neural circuitry involved in this effect depends on the degree of previous exposure to opiates. In opiate-naïve animals intra-VTA morphine is not dependent on dopamine, but requires the integrity of the pedunculopontine tegmental nucleus (PPTg). Conversely, in opiate-dependent animals the rewarding effect of intra-VTA morphine is dopamine-dependent and does not require the PPTg[167]. During morphine dependence, VTA dopamine neurons fire at a higher frequency, and have enhanced burst firing[153]. During withdrawal from morphine, dopamine neurons in the VTA may instead become hypofunctional[154,168], but see[153]. This hypofunctionality may occur due to enhanced GABAergic transmission onto VTA dopamine neurons during withdrawal, likely due to an upregulation of the cAMP cascade in GABAergic terminals[152,169]. Such withdrawal-induced 'cAMP superactivation' is also observed in the NAc after morphine exposure[170].

The opioid system in the mesocorticolimbic system also likely plays a role during relapse into drug taking behavior. Administration of MOR agonists, including the endogenous opioid B-endorphin, within the NAc reinstates cocaine taking[171]. It may in particular be the endogenous opioid system in the indirect pathway in the NAc that is involved in this[172].

3.2.3 Opioids in food intake and obesity

MORs in the mesolimbic dopamine system are also important in food reward. Local infusion of the mu opioid receptor agonist DAMGO in either the VTA or NAc increases non-homeostatic food intake in satiated animals[173,174]. Also, injections of DAMGO in the NAc specifically lead to intake of high-fat food[12,175]. Conversely, local infusion of MOR antagonists B-FNA or CTAP in the NAc shell decreases sucrose intake[176]. The orexigenic effect of MOR activation in the NAc mimics that of pharmacological silencing of the NAc shell[177]. It is not yet clear how these findings fit in with the proposed roles of different NAc MSN subpopulations in reward processing[44], although it could suggest that silencing D2R-MSNs, rather

than activating D1R-MSNs, is sufficient to induce these food reward behaviors.

MORs play a role in both the motivational aspects of food intake and the pleasure associated with consumption of palatable food[67]. MOR knockout mice show diminished motivation for food reward on a PR schedule[178]. Similarly, B-endorphin knockout mice show specific impairment in non-homeostatic feeding[179]. Conversely, stimulation of MORs in the NAc shell leads to enhanced motivation for sucrose pellets[29]. The effects on motivation may stem from MORs that impinge on the dopamine system. Systemic administration of an opioid receptor antagonist inhibited both the intake of palatable food and the associated increase in dopamine signaling in the NAc shell[55]. Aside from their involvement in motivational aspects of food intake, MORs are also crucial in pleasurable responses to food. Indeed, so-called 'hedonic hotspots' have been proposed to be situated in the medial-dorsal shell of the NAc, and in the ventral pallidum. Within these hotspots, stimulation of MOR signaling enhances pleasure obtained from food[67].

3.2.4 The potential relevance of constitutive MOR activity

In heterologous expression systems, transfected MORs exhibit constitutive activity[180-182]. Inverse MOR agonists also reduce constitutive MOR recruitment of G proteins in brain homogenates of mice, but not in MOR knockout mice[183]. The extent of MOR constitutive activity can differ across tissues[183,184], which may be related to the regulatory effects on constitutive signaling of differentially expressed factors like calmodulin and CaM kinase II[13,185,186].

It has been proposed that MORs become increasingly constitutively active after withdrawal from morphine treatment, as an adaptation to the sudden lack of morphine that the system has grown accustomed to[21]. Pretreatment with MOR agonists increases the effect of MOR constitutive activity, as it enhances the effect of MOR inverse agonists, but not of a neutral antagonist, on G protein signaling[180,181,183,187]. In fact, pre-treatment with morphine can fully reveal inverse agonistic function of certain MOR ligands, such as naloxone and naltrexone, which otherwise do not exhibit inverse agonistic properties. This is even true in specific brain regions that are part of the dopamine system such as the midbrain and the striatum. Here, morphine pretreatment reveals an inverse agonistic effect of naloxone, but not of neutral antagonist 6β-naltrexol, on G protein signaling and adenylyl cyclase activity[183,184]. Also within these regions, some inverse MOR agonists, like BNTX, produce inverse agonistic effects even without mor-

phine pretreatment[184]. The time span of constitutive activity of MORs may also depend on the pretreatment regime, as 3 morphine preadministrations led to detectable inverse agonistic effects of naloxone for at least 48 hours, whereas a single dose of morphine only led to detectable inverse agonism for less than 24 hours[183]. Together these findings suggest that MOR constitutive activity occurs in native tissue, and that the extent to which it occurs is dependent on opiate use. It is therefore conceivable that MOR constitutive activity plays a role in aspects of opiate addiction.

Indeed, with regards to physical morphine dependence, it has been shown that certain withdrawal symptoms from morphine treatment, such as tremors, jumping and defecation are precipitated more strongly by inverse MOR agonists than with an equipotent dose of the neutral MOR antagonist 6ß-naltrexol[184]. Moreover, a neutral MOR antagonist attenuates the effects of an inverse MOR agonist on withdrawal symptoms, as well as on G protein signaling, which further suggests that the inverse agonistic effect is indeed MOR-dependent[184]. Chronic morphine-intake, which would enhance constitutive MOR activity also increases the conditioned place aversion induced by naloxone, but not that induced by neutral MOR antagonists[21,22,188].

Hypofunctionality of the mesolimbic dopamine system is a prominent candidate for both the emotional and the physical side effects associated with drug withdrawal[154,168,169,189-194]. Given the large role that withdrawal symptoms play in relapse and maintenance of addiction[189,191], any successful therapeutic strategy for addictive behaviors should aim to limit these negative side effects. Compounds like naltrexone and naloxone are used in the treatment of certain forms of drug addiction and narcotic overdose. However, the downside of these compounds is their propensity to induce strong withdrawal symptoms[13,21,22]. Given the experimental evidence suggesting these compounds act as inverse agonists in an opiate-experienced context, the recent advance of neutral MOR antagonists that are less prone to induce these effects in animal models holds great promise for the future. However, it remains to be determined if constitutive MOR activity, in for instance the mesolimbic dopamine system, indeed regulates neuronal activity; and if MOR inverse agonists may therefore contribute to a hypofunctional dopamine system (with its associated risks) to a greater extent than MOR neutral antagonists.

4. Concluding remarks

The mesolimbic dopamine system plays an extensive role in reward processing and feeding, but also in their aberrant manifestations: addiction and obesity. Activity in the mesolimbic dopamine system is heavily regulated by GPCRs such as CB1Rs and MORs. Antagonists for these receptors have beneficial effects on drug use and overconsumption of food, but are also often accompanied by detrimental side effects. Importantly, MORs and CB1Rs are capable of agonist-independent constitutive activity alongside of the canonical agonist-dependent signaling. Recent pharmacological developments have brought forth compounds that either interfere with both of these activation mechanisms (inverse agonists) or selectively with agonist-dependent activation (neutral antagonists). Recent findings suggest that the beneficial therapeutic effects on aspects of weight control and drug abuse of CB1Rs and MORs may mainly rely on interference with endogenous agonists acting at these receptors. Adverse side effects appear to emerge more readily when the integrity of the constitutive activity of these receptors is also impaired, as likely occurs with the clinically applied MOR and CB1R inverse agonists. In this introduction we have made the case that such effects can occur due to interference with CB1R and MOR constitutive activity in the mesolimbic dopamine system, which is pivotally involved in mood and motivated behavior (Figure 3). Future research needs to address what the exact role of CB1R and MOR constitutive activity is in such regions, as these insights may have great merit for the development of appropriate therapeutic strategies for obesity and addiction.

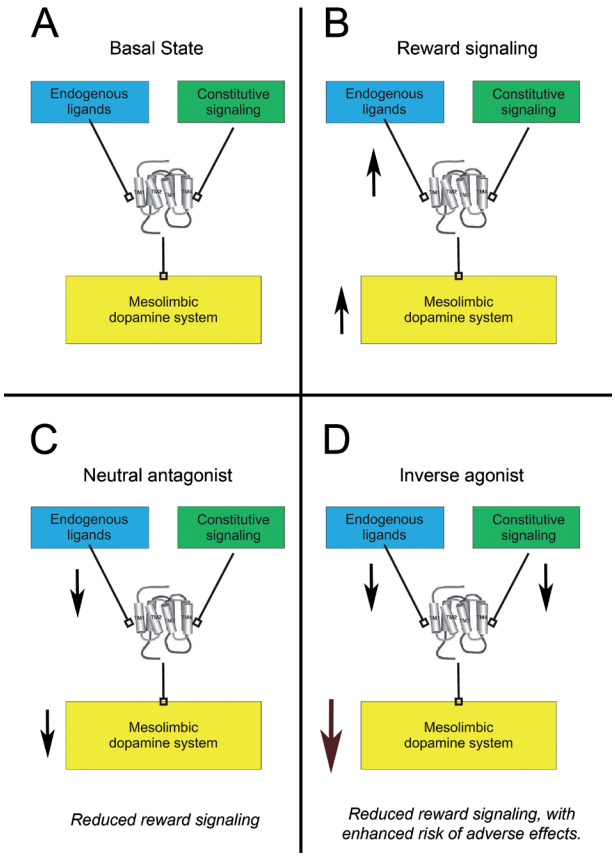

Figure 3

A proposed model for the undesirable side effects of CB1R / MOR inverse agonists. (**A**) A tone of CB1R and MOR activity controls the mesolimbic dopamine system. This tone is the combination of signaling induced by basal levels of endogenous ligands, and by constitutive receptor activity. (**B**) During reward processing, endocannabinoids and endogenous opioids are elevated in the mesolimbic dopamine system resulting in enhanced activity of key elements, such as the VTA dopamine neurons. (**C**) A neutral antagonist for the CB1R or MOR impairs reward signaling, by interfering with signaling of reward-evoked endocannabinoids and endogenous opioids. A neutral antagonist also affects the mesolimbic dopamine system directly by preventing signaling of basal levels of endogenous ligands. (**D**) CB1R or MOR inverse agonists additionally impair constitutive receptor signaling. The resulting effect on the mesolimbic dopamine system is stronger, with concomitant risk of adverse effects associated with its hypofunctionality.

5. Scope of this thesis

5.1 Aims

The successful adjunctive treatment of drug addiction and obesity with pharmaceuticals does not merely hinge on whether these therapeutics are effective, but also on whether or not they are devoid of debilitating side effects. As outlined above, antagonists for the cannabinoid 1 (CB1R) or mu opioid receptor (MOR) have therapeutic effects on weight control and drug addiction. However, their usefulness is greatly hampered by the possibility of severe adverse effects. This thesis is based on two main premises that arise from the conceptual framework outlined in the introduction above:

1. The mesolimbic dopamine system is a neural network pivotally involved in drug addiction and obesity. Although this makes it a prominent therapeutic target, its perturbation can also negatively affect emotional and hedonic processes, resulting in serious side effects. Therefore, modulation of this system must be balanced to minimize negative effects.

2. The CB1R and MOR regulate the mesolimbic dopamine system. However, they each do so via two distinguishable mechanisms. On the one hand, these receptors are activated by (endogenous) agonists. On the other hand, these receptors exhibit agonist-independent constitutive signaling. This distinction is important, as separate classes of antagonists differentially affect these two mechanisms. Neutral antagonists solely block agonist-dependent signaling, whereas inverse agonists also interfere with constitutive activity. Due to their interference with both activation mechanisms, CB1R and MOR inverse agonists are more likely to perturb the mesolimbic dopamine system too much, resulting in negative side effects (see Figure 3).

Currently there is tentative but limited evidence for the role of CB1R and MOR constitutive activity under physiological conditions in general, let alone for the mesolimbic dopamine system in particular. Therefore, the physiological occurrence and relevance of this activation mechanism remains largely hypothetical. The current thesis has chiefly aimed to give insight in whether CB1R and MOR constitutive activity occurs in the mesolimbic dopamine system, by investigating whether it serves a role in the neuronal communication there. Fur-

thermore, we aimed to unravel the relevance that constitutive CB1R and MOR activity may have in the treatment of obesity and drug addiction, respectively.

5.2 Outline

In general our approach to demonstrate constitutive receptor activity has been to contrast neutral antagonists and inverse agonists on particular physiological and behavioral read-outs. These compounds share a capability to interfere with (endogenous) agonist-dependent signaling, whereas they differ in their ability to interfere with any constitutive activity. Consequently, an effect of an inverse agonist that is qualitatively different from that of a neutral antagonist suggests the presence of constitutive activity. However, to be sure that this is indeed the case, care must be taken to ensure that the discrepancy is not due to off-target effects of the inverse agonist or insufficient receptor occupancy by the neutral antagonist. To control for these alternative interpretations we have added a crucial control condition in which we pre-administered a neutral antagonist to attempt to attenuate effects of an inverse agonist. We embedded this pharmacological strategy in a multi-disciplinary approach to investigate the role of constitutive signaling of CB1Rs and MORs in the mesolimbic dopamine system; particularly in the ventral tegmental area which stands at the root of this network. Our findings can be subdivided and summarized as follows:

Chapter 2: Constitutively active CB1 receptors regulate synaptic inputs onto dopamine neurons in the ventral tegmental area and pyramidal neurons in the basolateral amygdala. Behaviorally, interference with CB1R constitutive activity leads to undesirable effects on the pursuit of reward and on anxiety. Importantly, interference with endocannabinoid signaling at the CB1R is sufficient to produce therapeutically relevant effects on body weight and food intake. This profile of effects indicates that CB1R neutral antagonists have the potential to be effective and safe adjunctive pharmaceuticals to treat obesity with.

Chapter 3: CB1R signaling plays an important role in regulating activity of neurons in the ventral tegmental area of freely moving animals. We identified two neuronal populations that were differentially affected by a CB1R agonist, depending on their response to the dopamine receptor agonist apomorphine. Whereas putative dopamine neurons were excited by the CB1R agonist, putative non-dopamine neurons were inhibited by it. Within these populations, a

CB1R inverse agonist did indeed produce opposite effects, which were not seen with a neutral antagonist. This suggests that CB1R constitutive activity is indeed a relevant modulator of VTA neuronal activity.

Chapter 4: Constitutively active MORs regulate GABAergic inputs onto dopamine neurons in the ventral tegmental area. The extent of this constitutive activity is dynamic as it increases when an animal is withdrawn from an opiate-dependent state. We show that a likely cause for this is an increase in cAMP-dependent signaling, which is known to occur during morphine withdrawal. Given the association between hypofunctionality of the mesolimbic dopamine system and aversive withdrawal effects, this highlights the potential of MOR neutral antagonists in the treatment of opiate overdose and addiction.

Chapter 5: Orexin, a neuromodulator that has much in common with the hedonic effects of cannabinoids and opioids, also regulates GABAergic signaling in the ventral tegmental area. We show that orexin has different effects in identifiable subsets of dopamine neurons in the ventral tegmental area. This finding reinforces the notion of physiological and functional heterogeneity of neurons that have often been viewed as a homogeneous set.

Chapter 6: In this general discussion we evaluate our findings against the backdrop of the current societal problems involving obesity and drug addiction. We also outline future directions of research that may further contribute to the development of successful therapeutic strategies for these detrimental diseases.

Chapter 2

CONSTITUTIVE CANNABINOID 1 RECEPTOR ACTIVITY IS HIGHLY RELEVANT FOR SAFER OBESITY TREATMENT

Frank J. Meye
Viviana Trezza
Louk J. M. J. Vanderschuren
Geert M. J. Ramakers
Roger A. H. Adan

(Submitted)

ABSTRACT

The cannabinoid 1 receptor (CB1R) was put forward as a promising drug target for anti-obesity medication. However, the first marketed CB1R antagonist/inverse agonist rimonabant was discontinued, as its use was occasionally associated with negative affect and suicidality. In artificial cell systems, CB1Rs can become constitutively active in the absence of ligands. Here, we show that such constitutive CB1R activity also regulates GABAergic and glutamatergic neurotransmission in the ventral tegmental area and basolateral amygdala, regions which regulate motivation and emotions. We show that CB1R inverse agonists like rimonabant suppress the constitutive CB1R activity in such regions, and cause anxiety and reduced motivation for reward. The neutral CB1R antagonist NESS0327 does not suppress constitutive activity and lacks these negative effects. Importantly however, both rimonabant and NESS0327 equally reduce weight gain and food intake. Together, these findings suggest that neutral CB1R antagonists can treat obesity efficiently and more safely than inverse agonists.

INTRODUCTION

The combination of a sedentary lifestyle and overconsumption of energy-dense foods has culminated in an obesity epidemic. This is particularly alarming as obesity is a strong risk factor for cardiovascular disease, cancer, and type II diabetes[195]. Adjunctive pharmacological strategies that facilitate a healthy life style can be used to treat obesity[196-198]. The cannabinoid 1 receptor (CB1R) was a prominent candidate target, due to its key role in energy balance[104,117,199,200]. The CB1R antagonists/inverse agonist rimonabant was shown to clinically reduce body weight in humans, but was associated with psychiatric side effects, like anxiety, depression, and even suicidality[24,199,201,202]. Due to these side effects, CB1R antagonists/inverse agonists were abandoned as viable medicine.

Notably however, the CB1R is a constitutively active receptor in artificial cell systems, as it can adopt active conformations in the absence of any ligand[15,19,203]. Rimonabant suppresses CB1R constitutive signaling, while such inverse agonism is not observed with novel neutral CB1R antagonists like NESS0327[15,19,125,130,203-206]. CB1R constitutive activity occurs in artificial cell systems, but its physiological occurrence and role in behavior remain unclear. We now show that CB1R constitutive activity regulates neurotransmission in the mesocorticolimbic system, which plays an important role in anxiety and depression[127,128,207-209]. We also show that the suppression of CB1R constitutive activity has potentially harmful effects on anxiety and motivation for reward. The neutral CB1R antagonist NESS0327 lacks these effects, and is just as capable of reducing weight gain and food intake as SR141716A.

MATERIAL AND METHODS

Animals

Electrophysiological experiments were performed in C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) born in our own animal facility. Mice were group housed together with littermates in Macrolon cages (34 x 20 x 13.5 cm). The age of mice ranged from postnatal day 14-21 (VTA) or 22-42 (BLA).

Behavioral experiments were performed in male Wistar rats (Charles River, Sulzfeld, Germany) housed in pairs in Macrolon cages (37.5 x 22.5 x 15.0 cm). At

arrival in our animal facility, rats typically weighed between 250-300 grams. Rats were handled daily, but otherwise left to recover for at least one week prior to experimentation.

Both mice and rats were kept under controlled conditions (ambient temperature of 20-21˚C, 60-65% relative humidity). Unless otherwise specified, animals had ad libitum access to chow (CRM(E), Special Diet Services, Witham, UK) and water, and were housed on a 12/12h light cycle with lights on at 7:00 am. Animals were experimentally naïve when tested. All animal experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in agreement with Dutch laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Drugs

The AMPA/Kainate receptor antagonist DNQX, the $GABA_A$ receptor antagonist bicuculline, the voltage-gated Na^+-channel blocker tetrodotoxin (TTX), the direct CB1/2R agonist WIN55,212-2 , the FAAH inhibitor URB597 (which inhibits hydrolysis of the endocannabinoid anandamide), the MGL inhibitor URB602 (which inhibits hydrolysis of the endocannabinoid 2-AG), the neutral CB1R antagonist O-2050 and the CB1R inverse agonist AM251 were all purchased from Tocris (Bristol, UK). The neutral CB1R antagonist NESS0327 was purchased from Cayman Chemical (Ann Arbor, MI, USA) and the CB1R inverse agonist SR141716A was a generous gift from the NIMH Chemical Synthesis and Drug Supply Program. For electrophysiological experiments, these compounds were dissolved in DMSO and bath-applied, with the final bath concentration of DMSO never exceeding 0.1%. For behavioral experiments we dissolved SR141716A and NESS0327 in a vehicle of 1% DMSO, 4% polyethylene glycol and 5% TWEEN-80. Drugs were injected i.p. (ml/kg bodyweight).

Electrophysiology

Experiments were conducted essentially as described earlier[66,210]. Mouse brains were rapidly removed, then stored and sliced in pre-carbogenated (95% O_2 / 5% CO_2) enriched-artificial cerebrospinal fluid (ACSF), consisting of in mM: NaCl 124, KCl 3.3, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 2.6, $NaHCO_3$ 20, glucose 10, and ascorbic acid 1. For the VTA, slices were cut in a horizontal plane, whereas for recordings in the BLA slices were cut coronally. Slices were left to recover for 1 hour at 32˚C in carbogenated normal ACSF ($MgSO_4$ concentration of 1.3 mM,

no ascorbic acid). During recordings, a slice was continuously superfused with carbogenated normal ACSF at a rate of 2-3 ml/min. Whole-cell voltage clamp recordings were made using an EPC-10 patch clamp amplifier (HEKA). Recordings were obtained using borosilicate glass pipettes with a resistance of 3-5 MΩ. Pyramidal neurons in the BLA were identified based on their morphology. Dopamine neurons in the VTA were identified on the basis of a prominent I_h (≥100 pA). In a subset of recorded cells, post hoc immunohistochemical double staining was performed for the dopamine-marker tyrosine hydroxylase (TH) and neurobiotin, a marker infused into recorded cells. These findings supported the correct identification of cells as dopaminergic, as in our hands I_h-positive cells were also positive for TH, as also found before[66].

The pipette solutions for mIPSC recordings in the VTA had the following composition, in mM: K-gluconate 78, KCl 77, HEPES 10, EGTA 1, Mg^{2+}-ATP 2, Na^{+}-GTP 0.4, pH=7.4. Pipette solution for mEPSC recordings in the BLA consisted of, in mM: Cs-methanesulphonate 120, CsCl 17.5, HEPES 10, BAPTA 5, Mg^{2+}-ATP 2, Na^{+}-GTP 0.4, pH=7.4. Recordings were performed at -60 mV for mIPSCs and -70 mV for mEPSCs. To isolate mIPSCs, recordings were made in the presence of 1 µM TTX and 20 µM DNQX, whereas mEPSCs were isolated using 1 µM TTX and 20 µM bicuculline. Data were sampled at 20 KHz. Only recordings in which the product of the uncompensated series resistance and cell capacitance was stable over time (<20% change) were included in the final analysis.

Behavioral procedures

Motivated behavior

Sixteen male Wistar rats weighing 350-400 grams at the start of the experiment were kept on a reversed light schedule (lights on at 7.00 pm). Rats were fed with 20 grams chow per day to maintain body weight. Throughout the experiment, water was available ad libitum, except during operant conditions. Lever pressing for sucrose pellets (45 mg, formula F, Research Diets, New Brunswick, NJ, USA) was performed as reported previously[59]. Rats were first trained to respond for sucrose pellets under a fixed ratio 1 (FR1) schedule of reinforcement in operant chambers (30.5 x 24.2 x 21.0 cm; Med Associates, Georgia, VT, USA). These chambers were placed in light- and sound-attenuating cubicles, and were equipped with a ventilation fan, two levers, and a pellet dispenser with white cue lights above them. Under the FR1 schedule, a single lever press resulted in

delivery of one sucrose pellet. Presses on the inactive lever were recorded, but did not produce any effect. Sessions lasted 30 minutes or until 60 rewards had been obtained. Once animals responded stably on the FR1 schedule, they were shifted to the progressive-ratio (PR) schedule of reward delivery. Under PR schedules lever presses still yielded single sucrose pellet rewards, but the response requirement for each subsequent reward increased progressively, according to the equation: Response ratio = $[5*e^{0.2*\text{reward number}}]-5$. A session continued until the animal failed to meet the response requirement within 1 hour. Baseline responding was considered stable when the variation in rewards obtained over at least three consecutive sessions was no more than two rewards, and there was no increasing or decreasing trend in the baseline. Rats were tested once per day for five days per week. On a test day when drugs were administered, animals received two injections with cannabinoid antagonists and/or vehicle (60 and 30 min prior to the test). Drug test sessions were separated by at least two sessions without treatment, for purposes of washout. The different drug conditions were administered in a random order for every animal, and typically every animal received every treatment.

Anxiety-like behavior

Sixty-two male Wistar rats, weighing between 330-400 grams, were tested once in the elevated plus maze test, as reported previously[211]. The elevated platform was made out of gray Plexiglas and consisted of two open arms (50 x 10 x 0 cm) and two closed arms (50 x 10 x 40 cm), which extended from a common centre square (10 x 10 cm). The platform was elevated 60 cm above the floor. Animals were observed by means of a video camera above the platform, which was connected to both a video recorder and a television monitor. The protocol of the test was as follows: an animal was injected with vehicle and/or cannabinoid antagonists 60 (injection 1) and 30 (injection 2) minutes prior to the test. Subsequently, the animal was placed in the common centre square of the plus maze, facing a closed arm. The animal was then observed for a period of 5 minutes. Three different parameters were analyzed, using Observer 3.0 software (Noldus Information Technology BV):

1. % Time spent in the open arms (%TO): (total seconds spent in the open arms of the maze / 300) x 100%
2. The number of stretched-attend-postures (SAPs) made from the exit of a closed arm towards an open arm. A SAP was characterized as a forward

elongation of the body, with static hind quarters, which was followed by a retraction to the original position.

3. The total number of exploratory head dips made over the edge of the open arms.

We have previously shown that the anxiolytic drug diazepam showed a strong anxiolytic profile on all parameters (i.e. more time spent in the open arms, more exploratory head dips, and less SAPs)[211].

Locomotor Activity

To test for potentially sedative effects of the cannabinoid ligands, the locomotor activity of seventy-two Wistar rats, weighing between 300-350 grams, was assessed as previously described[212]. Before placement in a locomotor box (50 x 35 x 40 cm), rats were injected with either vehicle or a CB1R antagonist (60 minutes prior), and vehicle or CB1R agonist WIN55,212-2 (30 minutes prior). Subsequently, rats were left to explore the environment for 1 hour. The horizontal distance travelled was tracked automatically using a video tracking system (Ethovision, Noldus Information Technology BV, Wageningen, Netherlands), which determined the position of the animal at a rate of 5 Hz.

Bodyweight and food intake

Seventy-two male Wistar rats, weighing between 250-350 grams at the start of the experiment, were tested. To monitor their food intake, rats were individually housed. Prior to testing, rats had been habituated to these cages and food intake was established during a 5 day baseline. Then, for 8 consecutive days, animals received a cannabinoid drug or vehicle injection 30 minutes prior to onset of the dark phase. Food intake during the dark phase was recorded by placing the food trays on scales which were continuously digitally monitored using Scales software (Department Biomedical Engineering, UMC Utrecht, The Netherlands).

Analysis

The occurrence and kinetics of synaptic events were scored in MiniAnalysis (Synaptosoft, Decatur, CA, USA). Event distributions of frequency and amplitude were fitted in Igor Pro (Wavemetrics, Lake Oswego, OR, USA) using a lognormal curve: $f(x) = A*\exp[-0.5*(\ln X - \mu) / \sigma)^2] / (x*\sigma*\sqrt{2\pi})$, as described before[210,213]. In this equation X represents the measured instantaneous frequency or amplitude of an event, A is the relative area under the curve, μ is the mean and σ the

standard deviation of the underlying normal distribution. To assess drug effects in electrophysiological experiments, ANOVAs and Bonferroni post hoc tests were performed on the means of the transformed normally distributed data. To determine percentage changes, the mean of the lognormal distribution (m) was calculated using the equation: $m = \exp(\mu + \sigma^2)$. Behavioral data were statistically analyzed using Repeated Measures ANOVA, One-Way ANOVAs or Two-Way ANOVAs, followed by Bonferroni post hoc tests. All statistical testing was performed in SPSS 17.0 (Chicago, IL, USA). Data are reported as mean ± SEM.

RESULTS

CB1Rs on GABAergic afferents to ventral tegmental area dopamine neurons are constitutively active

Dopamine neurons in the ventral tegmental area (VTA) play a crucial role in appetitive behavior[25,214,215], but also in depression and anxiety[127,128,136,207]. Cannabinoids disinhibit VTA dopamine neurons by acting on CB1Rs on

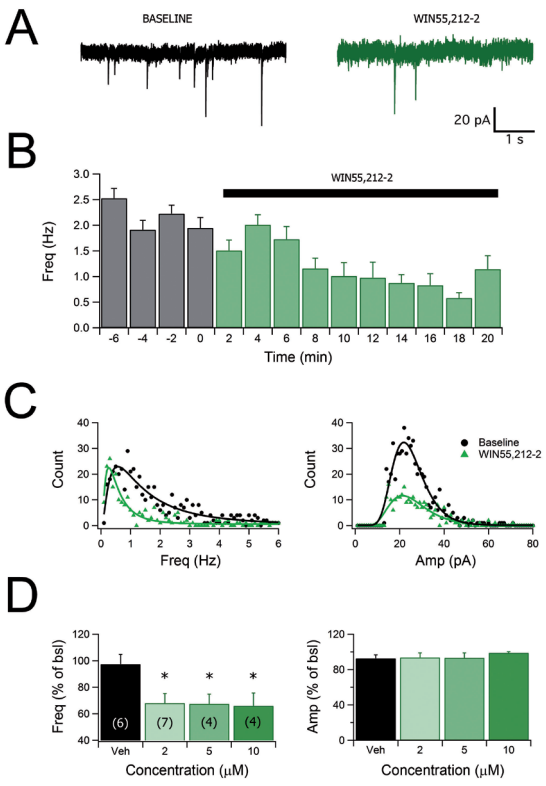

Figure 1

CB1R agonist WIN55,212-2 reduces the GABAergic mIPSC frequency in VTA dopamine neurons. (**A**) Current traces from a representative recording during baseline conditions and in the presence of WIN55,212-2 (2 μM). The vertical scale bar indicates 20 pA, and the horizontal scale bar indicates 1 second. (**B**) Timeline of the effect of WIN55,212-2 (2 μM) from a recording. (**C**) Representative lognormal distributions for baseline (black circles) and WIN55,212-2 (green triangles) with fitted curves for frequency (left) and amplitude (right). (**D**) WIN55,212-2 reduced mIPSC frequency, but not the amplitude compared to baseline. The number of cells is in parentheses. *$P < 0.05$

GABAergic nerve terminals[83,86]. We determined whether these CB1Rs also exhibit constitutive activity in the absence of (endo)cannabinoids. We recorded from VTA dopamine neurons and isolated synaptic GABAergic miniature inhibitory postsynaptic currents (mIPSCs). In accordance with a presynaptic localization of CB1Rs[83,86], the CB1/2R agonist WIN55,212-2 reduced mIPSC frequency ($F(3,17)=3.98$, $P < 0.05$; Repeated Measures ANOVA), but not amplitude ($P > 0.05$) compared to vehicle (Fig. 1).

If some CB1Rs on these GABAergic nerve terminals are constitutively active, suppression of this constitutive activity with an inverse agonist would be expected to produce an effect opposite to that of the agonist. Acute administration of CB1R inverse agonist SR141716A (rimonabant)[15,203] indeed increased mIPSC frequency ($F(5,24)=9.60$, $P < 0.001$; Repeated Measures ANOVA; Fig. 2). This effect was dose-dependent, with an EC_{50} of 9.25 ± 6.46 nM and a maximal increase in mIPSCs of 55.98 ± 10.22% at 100 nM (Figure 2*D*). The effect of SR141716A was mimicked by AM251 (1 μM; $F(1,9)=11.11$, $P < 0.01$; Repeated Measures ANOVA), another highly potent inverse CB1R agonist with high specificity[216,217] (Fig. 2D).”

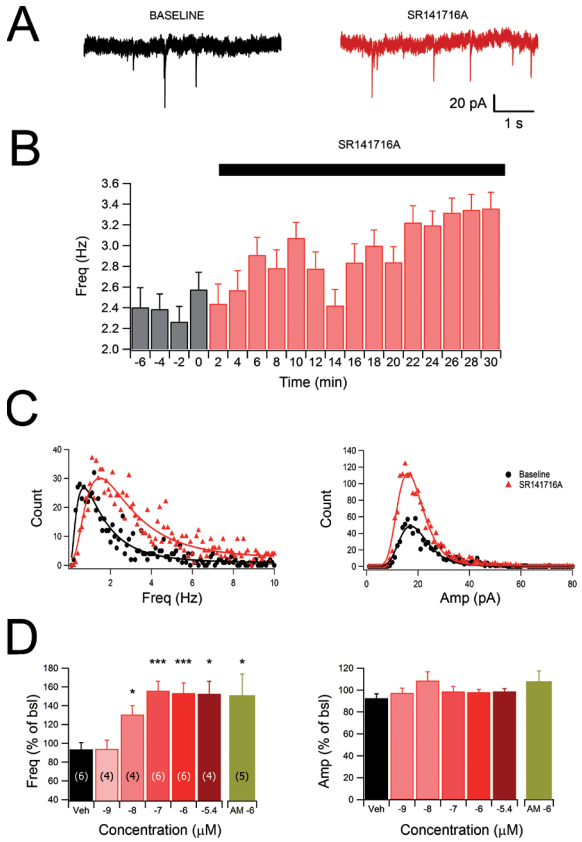

Figure 2
CB1R inverse agonists increase GABAergic mIPSC frequency in VTA dopamine neurons. (**A**) Current traces from a representative recording during baseline conditions and in the presence of CB1R inverse agonist SR141716A (1 μM). The vertical scale bar indicates 20 pA, and the horizontal scale bar indicates 1 second. (**B**) Timeline of the effect of SR141716A (1 μM) in a recording. (**C**) Representative lognormal distributions with fitted curves for baseline (black circles) and SR141716A (red triangles) for mIPSC frequency (left) and amplitude (right). (**D**) Dose-response curve for inverse agonists SR141716A and AM251 (AM) on mIPSC frequency (left) and amplitude (right). The number of cells is in parentheses. Both SR141716A and AM251 increased the mIPSC frequency compared to baseline. Neither compound affected mIPSC amplitude. *$P < 0.05$; ***$P < 0.001$.

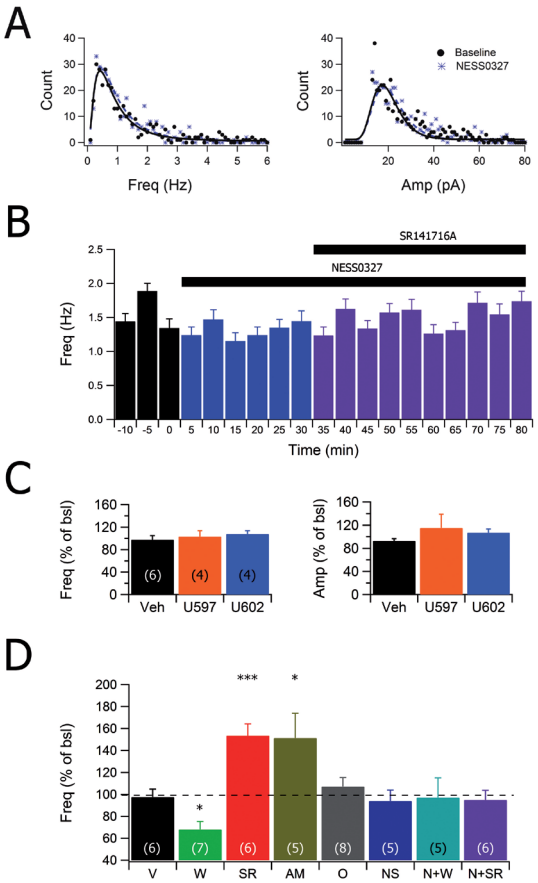

Figure 3
Inverse agonists suppress CB1R constitutive activity on GABAergic projections to VTA dopamine neurons. (**A**) Representative log-normal distributions with fitted curves for baseline (black circles) and CB1R neutral antagonist NESS0327 (0.5 μM; blue stars) for mIPSC frequency (left) and amplitude (right). (**B**) Timeline of an experiment, showing that NESS0327 does not affect mIPSC frequency itself, but does block the effect of SR141716A. (**C**) The indirect CB1R agonists URB597 (1 μM) and URB602 (50 μM), which would enhance an anandamide or 2-AG tone, resp., if it were present, do not affect mIPSC frequency or amplitude. (D) Overall summary of effects of cannabinoid ligands on mIPSC frequency, with the number of cells in parentheses. Whereas CB1R agonist WIN55,21-2 (W; 2 μM) decreases the frequency, inverse agonists AM251 (AM; 1 μM) and SR141716A (SR; 1 μM) increase it. Importantly, neutral antagonists O-2050 (O; 1 μM) and NESS0327 (NS; 0.5 μM) do not affect mIPSCs, but NESS0327 blocks the effects of both WIN55,212-2 (N+W) and SR141716A (N+SR). $*P < 0.05$; $***P < 0.001$.

The apparent inverse agonistic effect of SR141716A could also be caused by interference with ongoing endocannabinoid-CB1R signaling. To rule out this possibility, we tested neutral CB1R antagonists, which would also interfere with endocannabinoids acting on CB1Rs, while leaving any constitutive signaling intact. At concentrations sufficient for maximal occupancy of the CB1R[15,205], the specific neutral antagonists NESS0327 (0.5 μM) and O-2050 (1 μM) did not affect mIPSC frequency (Fig. 3*A, D;* $P > 0.05$; Repeated Measures ANOVA). Pre-administration of NESS0327 fully blocked the effects of subsequently administered WIN55,212-2 or SR141716A (Fig. 3*B, D;* $P > 0.05$; Repeated Measures ANOVA), illustrating the CB1R-dependence of their effects. To corroborate the lack of an endogenous tone in our slice preparations, we also tested the effects of two indirect cannabinoid agonists, URB597 and URB602. These ligands interfere with the degradation of anandamide and 2-AG respectively, and only exert an ef-

fect in the case of ongoing endocannabinoid-CB1R signaling[134,218]. Even at high concentrations and up to 30 minutes of incubation, neither the FAAH inhibitor URB597 (1 μM) nor the MGL inhibitor URB602 (50 μM) affected mIPSC frequency (Fig. 3C; $P > 0.05$; Repeated Measures ANOVA).

CB1Rs on glutamatergic afferents to the basolateral amygdala are constitutively active

We determined whether CB1R constitutive activity also occurs at glutamatergic synapses in the basolateral amygdala (BLA). These synapses play an important role in anxiety[219] and are subject to modulation by cannabinoids in a CB1R-dependent manner[220]. We patch clamped BLA pyramidal neurons and isolated glutamatergic miniature excitatory postsynaptic currents (mEPSCs). The results indicate the occurrence of constitutively active CB1Rs on glutamatergic terminals in the BLA ($F(4,21)=14.96$, $P < 0.001$; Repeated Measures ANOVA, Ligand Class x Treatment interaction, followed by Bonferroni post hoc testing). WIN55,212-2

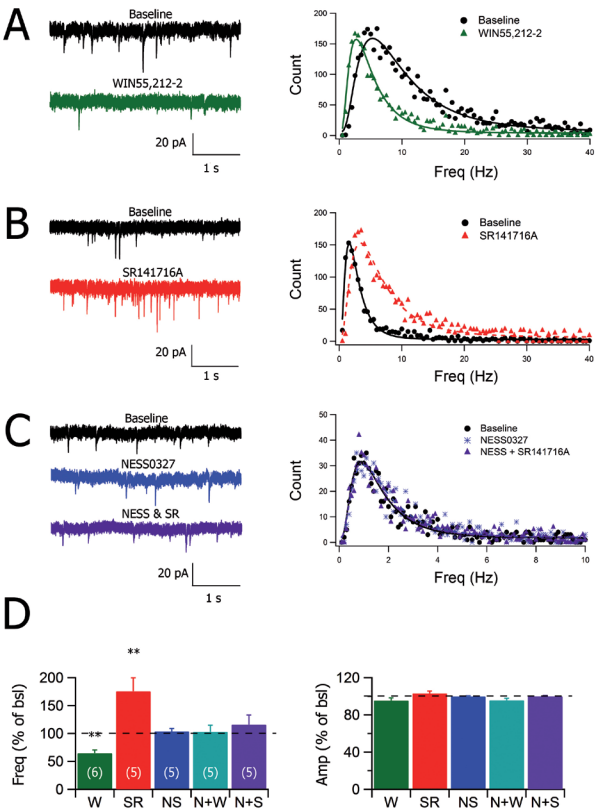

Figure 4
CB1R constitutive activity regulates glutamatergic transmission in BLA pyramidal neurons. Representative traces (left) and lognormal distributions (right) before and after administration of: (**A**) CB1R agonist WIN55,212-2 (2 μM; green triangles), (**B**) CB1R inverse agonist SR141716A (1 μM; red triangles), and (**C**) CB1R neutral antagonist NESS0327 (0.5 μM) with (purple triangles) and without (blue stars) co-administered SR141716A (1 μM). In A-C, vertical scale bars indicate 20 pA, and horizontal scale bars 1 second. (**D**) Overall effects of cannabinoid ligands on mEPSC frequency and amplitude in the BLA. The number of cells is in parentheses. W: WIN55,212-2; S(R): SR141716A; N(S): NESS0327. Cannabinoid ligands differentially affected mEPSC frequency compared to baseline. No effects were observed on mEPSC amplitude. $^{**}P < 0.01$.

(2 μM) reduced mEPSC frequency (Fig. 4*A, D*), while SR141716A (1 μM) enhanced it (Fig. 4*B, D*). NESS0327 (0.5 μM) did not affect mEPSC frequency itself, but did prevent the action of WIN55,212-2 and SR141716A (Fig. 4*C, D*). None of the ligands affected mEPSC amplitude (Fig. 4*D*).

A CB1R neutral antagonist and inverse agonist reduce weight gain equally

We observed that while neither type of CB1R antagonist directly affected locomotor activity (Fig. 5). Both antagonists were able to block the reduction

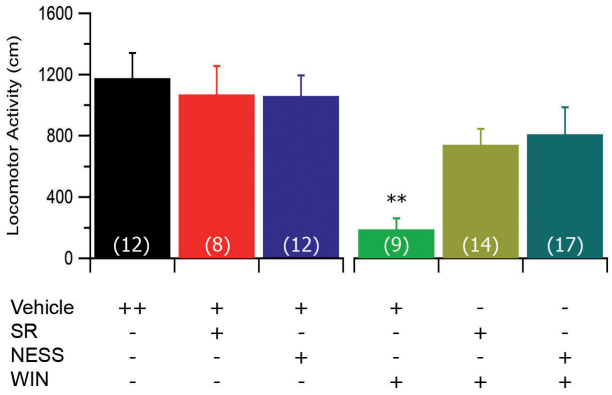

Figure 5

Both types of CB1R antagonist prevent the effect of WIN55,212-2 on locomotor activity. The CB1R agonist WIN-55,212-2 (3 mg/kg) reduced locomotor activity. Pre-treatment with either the neutral CB1R antagonist NESS0327 (0.1 mg/kg) or the inverse CB1R agonist SR141716A (1.0 mg/kg) similarly suppressed the sedative effect of WIN55,212-2 (3 mg/kg). $^{**}P < 0.01$. The number of animals is shown in parentheses.

in locomotor activity induced by WIN55,212-2 (3 mg/kg; $F(3,48)=5.54$, $P < 0.01$; Two-Way ANOVA). At 1 mg/kg SR141716A and 0.1 mg/kg NESS0327, both antagonists did so to an equal extent (Bonferroni post hoc analysis; $P > 0.05$).

We then investigated whether the CB1R neutral antagonist NESS0327 and the inverse agonist SR141716A differed in their ability to reduce body weight and food intake in a separate study. Rats pretreated with either NESS0327 or SR141716A, dose-dependently gained less weight than vehicle controls (Fig. 6*A*; $F(48,512)=5.10$, $P < 0.001$; Repeated Measures ANOVA, Treatment x Time interaction). After 8 days of treatment the dose-dependent reduction in body weight gain was very evident (Fig. 6*B*; $F(6,64)=8.17$, $P < 0.001$; One-Way ANOVA, Bonferroni post hoc testing). The maximal effect of SR141716A (at 1 mg/kg) and NESS0327 (at 0.1 mg/kg) was associated with a similar reduction in average food intake during the treatment period (Fig. 6*C*; $F(2,21)=6.41$, $P < 0.01$; One-Way ANOVA, Bonferroni post hoc testing). The CB1R neutral antagonist NESS0327

and the inverse agonist SR141716A reduced body weight and food intake equally well (Fig. 6*C*; *P*>0.05; One-Way ANOVA Bonferroni post hoc testing).

Constitutive CB1R activity regulates anxiety and reward processing

As we established a role of CB1R constitutive activity in neuronal circuitry involved in processing emotions and rewards, we investigated whether this was indeed associated with behavioral effects on such parameters. We observed that SR141716A (1 mg/kg) produced strong anxiogenic effects in the elevated plus maze on the amount of time spent in the open arms (%TO; F(1,58)=2.21, *P* < 0.05; Two-Way ANOVA, Treatment 1 x Treatment 2 interaction, Bonferroni post hoc testing; Fig. 7*A*), the number of stretched attend postures (SAPs, F(1,58)=15.46, *P* < 0.001; Two-Way ANOVA, Treatment 1 x Treatment 2 interaction, Bonferroni post hoc testing; Fig. 7*B*) and the

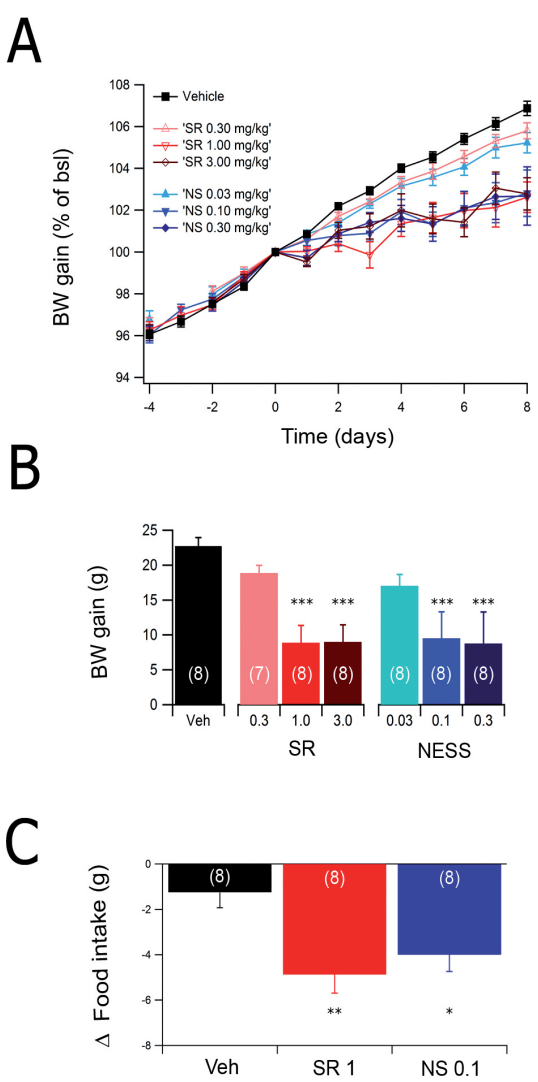

Figure 6

Both types of CB1R antagonist reduce body weight gain and food intake.

(**A**) The inverse CB1R agonist SR141716A and the neutral CB1R antagonist NESS0327 both dose-dependently reduce weight gain over time. (**B**) Dose-dependent changes in weight gain after 8 days of cannabinoid antagonist administration. NESS0327 (0.1 mg/kg) and SR141716A (1.0 mg/kg) produced a comparable maximal reduction in body weight gain. (**C**) Associated changes in food intake after cannabinoid antagonist administration. NESS0327 (0.1 mg/kg) and SR141716A (1.0 mg/kg) produced the same reduction in food intake. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. The number of animals is shown in parentheses.

number of head dips (F(1,58)=16.45, $P < 0.001$; Two-Way ANOVA, Treatment 1 x Treatment 2 interaction, Bonferroni post hoc testing; Fig. 7*C*). The neutral CB1R antagonist NESS0327 (0.1 mg/kg) was not anxiogenic itself and blocked the effect of SR141716A on all parameters, showing their CB1R-dependence (Bonferroni post hoc testing; $P > 0.05$).

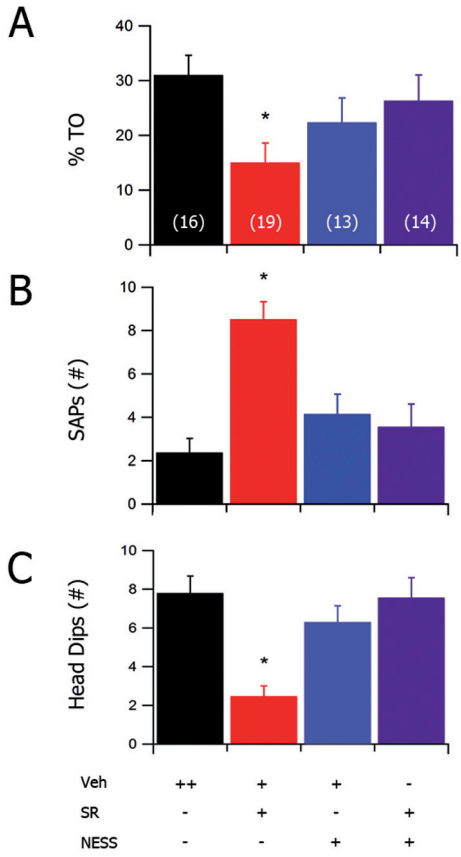

Figure 7
Suppression of CB1R constitutive activity is anxiogenic in the elevated plus maze. (**A**) The CB1R inverse agonist SR141716A, but not the neutral antagonist NESS0327, increases anxiogenic responses in the elevated plus maze on the percentage of time spent in the open arms (%TO), (**B**) the number of stretched attend postures (SAPs), and (**C**) the number of head dips. $^{**}P < 0.01$; $^{***}P < 0.001$ The number of animals is in parentheses.

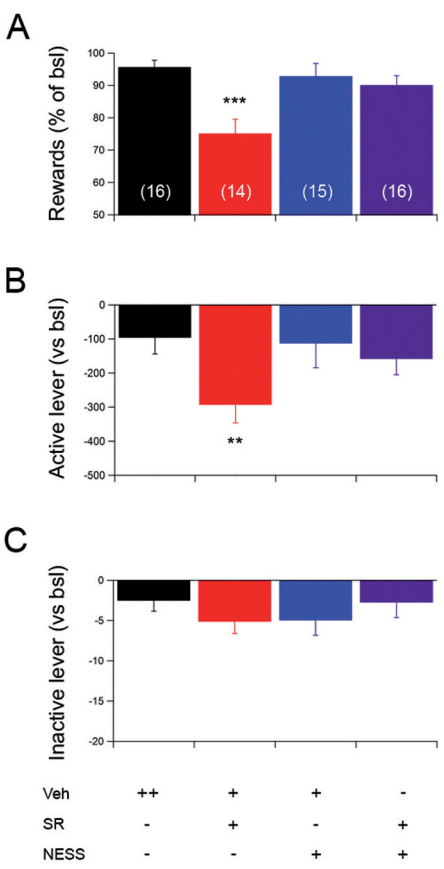

Figure 8
Suppression of CB1R constitutive activity reduces motivation for reward. (**A**) The CB1R inverse agonist SR141716A, but not the neutral antagonist NESS0327, lowers the amount of sucrose rewards obtained and (**B**) the amount of presses on the active lever. (**C**) Neither compound had an effect on the amount of presses on the inactive lever, which was not coupled to any reward. $^{**}P < 0.01$; $^{***}P < 0.001$. The number of animals per condition is in parentheses.

In a progressive ratio set-up, SR141716A (1 mg/kg) clearly reduced the motivation for sucrose reward. NESS0327 (0.1 mg/kg) did not affect motivation and blocked the effect of SR141716A ($F(3,36)=5.37$, $P < 0.05$; Repeated Measures ANOVA, Drug x Session interaction, Bonferroni post hoc testing; Fig. 8*A*). A similar profile of effects was observed for the number of presses on the active lever ($F(3,36)=2.88$, $P < 0.05$; Repeated Measures ANOVA, Drug x Session interaction, Bonferroni post hoc testing; Fig. 8*B*). There were no effects of the drugs on presses on the inactive lever ($P > 0.05$; Repeated Measures ANOVA, Drug x Session interaction; Fig. 8*C*), indicating that the cannabinoids had no general disruptive effect on behavior.

DISCUSSION

CB1R inverse agonists were welcomed as drugs to treat obesity until evidence indicated that they can produce mood disorders and suicidality[24,199,201,202,221]. We now present evidence that detrimental side effects of CB1R inverse agonists are due to interference with the constitutive activity that CB1Rs exhibit. We show that this constitutive CB1R activity plays an important role in regulating GABAergic and glutamatergic neurotransmission in brain regions implicated in anxiety and depression[127,128,136,207-209,222]. Most importantly, we demonstrate that a neutral CB1R antagonist, which leaves the constitutive CB1R activity intact, is just as efficient in reducing weight gain as a CB1R inverse agonist, but lacks potentially harmful effects on anxiety and motivation.

Our findings indicate that constitutive CB1R activity does not just occur in artificial systems, but also regulates neurotransmission in native brain tissue. The effect of SR141716A cannot readily be attributed to a mechanism of action other than suppression of constitutive CB1R activity[203]. We determined that the inverse agonistic effect of SR141716A was CB1R-mediated, as it was fully blocked by the specific neutral antagonist NESS0327[205]. The dose-response curve of SR141716A on mIPSC frequency in the VTA suggested a low nanomolar affinity, comparable to a previously reported established CB1R-mediated effect of SR141716A[19]. Moreover, the effect of SR141716A on mIPSC frequency was mimicked by AM251, another inverse agonist for the CB1R[216,217]. We also determined, using both neutral antagonists and indirect agonists, that the inverse agonistic effect of SR141716A in our slice preparation was not due to interference with ongoing

endocannabinoid signaling. These findings highlight a role for CB1R constitutive activity in regulation of neurotransmission in mesocorticolimbic regions under close to physiological conditions.

The behavioral differences between SR141716A and NESS0327 suggest that CB1R constitutive activity has important functions *in vivo* as well. Our findings indicate that while CB1R constitutive activity does not play a large role in food intake and body weight regulation, it is significantly involved in anxiety and motivation for reward. This is particularly relevant in light of the adverse effects of the discontinued weight loss drug rimonabant, which include anxiety and depression[24,201]. The CB1R neutral antagonist NESS0327 was not anxiogenic in our study, a finding supported by a previous report that found no anxiogenic effect of another CB1R neutral antagonist AM4113[130]. We now provide evidence that such findings can be attributed specifically to the lack of interference with CB1R constitutive activity. Interestingly, endocannabinoid-CB1R signaling, likely in the BLA, is known to be involved in both anxiety and fear[133,134]. This suggests that, despite endocannabinoid involvement in emotional processing, prominent anxiety mainly occurs when CB1R constitutive activity is suppressed on top of interference with endocannabinoid signaling. Similarly, endocannabinoids may well contribute to motivated behaviors[223], but our findings suggest that reduced motivation for sucrose reward may not be observed until CB1R constitutive activity is suppressed. It is clear that decreased motivation for (food-related) rewards could be a useful quality of a weight-loss drug, but it is conceivable that it also encompasses a great risk. Lack of motivation and drive (avolition) is an important component of major depressive disorder[135,136] and there is evidence to suggest that an impaired mesocorticolimbic dopamine system is at the root of this[128,136]. In this light, our findings offer a potential mechanism by which rimonabant could produce such symptoms, and suggest that neutral antagonists like NESS0327 may be less likely to produce them.

Whereas NESS0327 and SR141716A differed on potentially harmful effects, they showed great similarity in their therapeutically relevant effects on body weight and food intake. This corresponds well with previous reports that interference with endocannabinoid signaling at the CB1R is sufficient for effects on energy balance[104,107,125,204,224,225]. Therefore, CB1R neutral antagonists, which only interfere with endocannabinoid-CB1R signaling, may prove to be drugs that are efficient in treating obesity, while lacking many of the deleterious side effects associated with CB1R inverse agonists.

Acknowledgements

We thank Top Institute Pharma (D1-105) for funding this project and NIMH Chemical Synthesis and Drug Supply Program for supplying rimonabant for the purposes of this study. Finally, we thank Prof. Ad IJzerman, Prof. Bert Leufkens, Dr. Mario van der Stelt and Dr. Lex van der Ploeg for helpful suggestions on the manuscript.

Chapter 3

EVIDENCE FOR A ROLE OF CB1R CONSTITUTIVE ACTIVITY IN THE MODULATION OF VTA NEURONAL FIRING FREQUENCY IN THE FREELY MOVING RAT

Frank J. Meye[a]
Geoffrey van der Plasse[a]
Mieneke C. M. Luijendijk
Geert M. J. Ramakers[b]
Roger A. H. Adan[b]
[a,b] *Authors contributed equally to this work*

(In preparation)

ABSTRACT

Cannabinoids impinge on the dopamine reward circuitry at the level of the ventral tegmental area (VTA), where they engage the endogenous cannabinoid system. However, the role that this endogenous system itself plays in the regulation of VTA neuronal activity remains largely unclear. In the current study we address this issue by investigating the effect of various cannabinoid ligands on VTA neuronal firing, by means of *in vivo* electrophysiological recordings in freely moving rats. We show that the cannabinoid 1/2 receptor agonist WIN55,212-2 evokes a dual response in the VTA. Whereas WIN55,212-2 generally excites putative dopamine neurons, it inhibits putative non-dopamine neurons. We then investigated whether tonic activation of the cannabinoid 1 receptor (CB1R) contributed to the firing frequency in these VTA neuronal subsets. We assessed the effect of a CB1R neutral antagonist and of an inverse agonist, in order to characterize two separate mechanisms of tonic CB1R activation: endocannabinoid-dependent CB1R signaling and agonist-independent constitutive CB1R activity. First we observed that the neutral CB1R antagonist NESS0327 did not elicit homogenous responses in either one of the two VTA subsets, indicating that these neurons are not tonically regulated by endocannabinoids. Contrarily, the inverse agonist SR141716A evoked responses in the two VTA neuronal subsets which were directly opposite to those evoked by the agonist WIN55,212-2; such that putative dopamine neurons were inhibited and non-dopamine neurons excited by it. These findings suggest that the endogenous cannabinoid system regulates VTA dopamine neuron activity during basal conditions in an agonist-independent manner, rather than through endocannabinoid-CB1R signaling. We discuss how these findings may have important repercussions for drug design.

INTRODUCTION

Cannabinoids are commonly-used recreational drugs that hijack an endogenous system consisting of endocannabinoids and G protein-coupled cannabinoid receptors[226]. The cannabinoid 1 receptor (CB1R) is ubiquitously represented in the brain, but especially its occurrence in the mesolimbic dopamine system is deemed relevant in the context of reward processing[227]. This neural network is rooted in the midbrain where dopaminergic neurons from the ventral tegmental area (VTA) send projections through the medial forebrain bundle to regions like the nucleus accumbens (NAc)[43].

Cannabinoids acting on CB1Rs localized to GABAergic nerve terminals in the VTA[82,83] and NAc[84] disinhibit dopamine neurons[85], and accordingly increase NAc dopamine levels[81,228]. Indeed, it has been shown in anaesthetized animals that cannabinoid 1/2R agonists such as delta-9 tetrahydrocannabinol (delta-9-THC) and WIN55,212-2, enhance the firing frequency and burst firing of presumed dopamine neurons in the VTA[77,79,80,229]. However, it is currently unclear whether the endocannabinoid system itself is tonically involved in regulating neuronal activity in the VTA. One study reported that the CB1R antagonist/inverse agonist SR141716A caused a small but significant reduction in firing frequency and burst firing of putative VTA dopamine neurons[80]: an effect that was not readily observed at lower doses[77,79]. Importantly, any effect of the CB1R antagonist/inverse agonist SR141716A can arise from interference with two separate activation mechanisms that CB1Rs have at their disposal: (1) Interference with agonist-dependent activation via endocannabinoids (the antagonistic property), and (2) Interference with agonist-independent constitutive activity (the inverse agonistic property)[15,16,203,230]. Consequently, the mechanism(s) by which the endocannabinoid system controls VTA neurons is currently unknown.

The current study aims to unravel the contributions of tonic constitutive CB1R activity and endocannabinoid-dependent CB1R signaling to the regulation of neuronal activity in the VTA under baseline conditions. We approach this issue by performing *in vivo* electrophysiological recordings in the VTA of awake animals. Moreover, we employ a pharmacological strategy that involves both a CB1R inverse agonist and a neutral antagonist, which differentially interfere with agonist-dependent and constitutive signalling[203].

MATERIAL AND METHODS

Ethical approval

All experiments were approved by the Animal Experimentation Committee of Utrecht University and were carried out in agreement with Dutch Laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Subjects

Data were collected from 31 recording sessions, obtained from 6 male Wistar rats (Charles River), weighing on average 359 ± 11.9 g at the time of surgery. Upon arrival, the animals were housed in standard Macrolon type-IV cages, handled and weighed daily, and kept under a normal day/night cycle (white light on from 7 am until 7 pm). Food and water was available ad libitum throughout the experiment. Following surgery (see below), the animals were transferred to individual cages (41x41x40 cm) where they remained for the duration of the experiment.

Surgery and electrophysiology

Animals were anaesthetized with 5% isoflurane in a 30%:70% N_2O:O_2 mixture. Following induction of anesthesia, the isoflurane level was progressively reduced and maintained at 1.75%. The animals were mounted in a Kopf stereotaxic frame. After incision of the skin, additional local anesthetic (Xylocaine spray; 10%, Astra) was applied to the skull. Body temperature was monitored and maintained at 37.5 ºC using a heating pad. Eyedrops (Cera, Naaldwijk, Netherlands) were applied to prevent dehydration and s.c. saline was given during surgery to maintain fluid balance. After exposure of the cranium, 9 small holes were drilled into the cranium to accommodate surgical screws, one of which served as ground. Another larger hole was drilled over the ventral tegmental area in either the left or right hemisphere (centre of the hole 5.6 mm posterior, 1.2 mm lateral to bregma). The dura was opened and the bundle of tetrodes, extending ~3 mm from the drive, was lowered under a 5° angle, and implanted in the brain. The headstage holding the tetrodes (Harlan 4 drive, Neuralynx, Montana, US) was then anchored to the screws with dental cement (GC Fuji PLUS capsules). To protect the brain from the dental cement, and prevent blood from entering the tubing of the headstage, the drill hole was first filled with mineral oil (Sigma-Aldrich, Steinheim, Germany).

Immediately after surgery, all tetrodes were further lowered into the target area (DV coordinate, ~8.0 mm). Following surgery, the animals were given at least one week of recovery before recordings started. Electrophysiological recordings were performed using a Multineuron Acquisition Processor (MAP) recording system (Plexon, Dallas, Texas, USA). Signals from the individual leads of the tetrodes (Pt/Ir wire, Neuralynx, Tucson, AZ) were passed through a unity-gain amplifier (20x) and amplified a second time with a Plexon 16-channel preamplifier (PBX3, 16 s-pr). Subsequently, the signal was fed into the MAP unit for filtering and further amplification of individual channels. Digitized output (40 kHz) was then stored on a 64-bit Windows XP workstation. A 1 ms data sample was stored whenever the signal crossed a preset voltage threshold so that the width of recorded spikes was 40 data points. Recordings were made in a chamber that was identical in size and shape to the home cage.

Drugs

To assess responses of VTA neurons to cannabinoid ligands, the CB1/2R agonist (*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55,212-2; Tocris [BristolAnn Arbor, MI, USA]) at 1 mg/kg, the CB1R inverse agonist N-piperidino-5-(4-clorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716A) at 1 or 3 mg/kg, and the CB1R neutral antagonist 8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydro-N-1-piperidinyl-benzo[6,7]cyclohepta[1,2-c] pyrazole-3-carboxamide (NESS0327; Cayman Chemical [Ann Arbor, MI, USA]) at 0.1 mg/kg were injected i.p. following baseline recordings. Prior to such injections, the drug vehicle (1% DMSO, 4% polyethylene glycol, 5% TWEEN-80 and 90% distilled water) was administered i.p. to control for neuronal responses to the injection itself. Apomorphine (0.1 mg/kg) was administered at the end of each recording session. This non-selective dopamine receptor agonist helps identify putative VTA dopamine neurons, as those typically possess dopamine 2 (D2) autoreceptors through which D2R agonists suppress firing frequency[231-233]. Drugs were dissolved in the following concentrations: WIN: 0.5 mg/ml; SR: 0.5 or 1.5 mg/ml; NESS: 0.05 mg/ml; Apo: 0.05 mg/ml). The injection volume was therefore 2 ml/kg body weight. A washout period of at least two days was kept between all recording sessions.

Data analysis

Single units were isolated by offline cluster cutting procedures (Offline Sorter version 3, Plexon, Dallas, USA). Before a cluster of spikes was accepted as belonging to a single unit, several parameters were checked visually: the averaged waveforms across the four leads of each tetrode, the cluster plots showing spike parameter distributions such as peak amplitudes and principal components, the autocorrelogram and the inter-spike interval histogram. Since the absence of spike activity during the refractory period is indicative of good isolation, units of which the autocorrelogram and the spike interval histogram revealed activity during this period (<1500 μs) were removed from the analysis. After initial sorting, data were analyzed in Matlab (R2007b, The Mathworks, Inc., MA) where an algorithm was devised to calculate firing frequency (spikes/min) across drug periods, waveform kinetics, as well as the percentage of spikes occurring in bursts. A burst was identified using well-established criteria[234]. Its onset was characterized by a duo of action potentials with an interspike interval less than 80 ms, while its termination was defined by an interspike interval of more than 160 ms. In our final analysis we excluded cells with a baseline firing frequency less than 0.1 Hz, to prevent obscuring of drug-induced suppressions of neuronal activity[235].

Firing activity was evaluated in bins of 30 seconds each. To evaluate drug effects we normalized the firing frequency during baseline to 100% (10 minutes preceding drug injection). We then expressed the neuronal activity per bin, in a 15 minute time window following drug/vehicle administration as a percentage of baseline. Subsequently we averaged this normalized response over these 15 minutes. To exclude non-specific effects of the injection on neuronal activity we considered the time period of 5 to 20 minutes after injection to assess the effects of our compounds. Comparisons between the average firing frequency for these two timeframes (-10 to 0 minutes and 5 to 20 minutes with respect to injection) were made by means of Repeated Measures ANOVA. In all such analyses we made use of a grouping variable consisting of the dichotomized response to the non-specific dopamine agonist apomorphine. We characterized putative VTA dopamine neurons as being inhibited by at least 20% by apomorphine[233]. We characterized neurons that were not inhibited by apomorphine in this manner as putatively non-dopaminergic.

Histology

The final position of the tetrodes was marked by passing a current (25 μA, 10 s) through one of the leads of each tetrode in order to induce a lesion and initiate gliosis. During this procedure the animals were anaesthetized (see above). After 24 hours, the animal was decapitated and the brain was removed and stored overnight in 4% paraformaldehyde (PFA). Subsequently, brains were kept in a solution of 30% sucrose and 0.05% sodium azide until histological analyses. Coronal brain sections of 40 μm were cut using a vibratome (Leica, Rijkswijk, NL) and Nissl-stained to identify the position of the tetrodes.

RESULTS

Neuronal responses in the VTA

Recordings in the VTA of rats (n=6) were performed, for which the tetrode placement was validated (Figure 1). Within this recorded region, multi-channel recordings allowed for manual clustering of distinguishable individual neurons (Figure 2). During recordings the drug vehicle was administered to test for effects of injection itself. Within the first 5 minutes after injection, a vehicle-dependent increase in neuronal activity was often observed which faded to base-

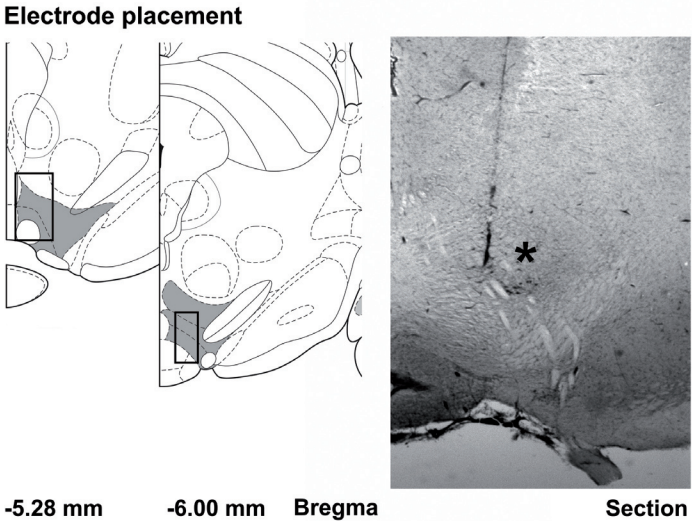

Figure 1
Localization of tetrode recording sites. Left: The shaded grey areas in the two brain atlas figures delineate the VTA (figure adapted from[244]). Numbers below the figures indicate the anterior-posterior coordinate relative to bregma. Rectangular boxes show the sites from where the recordings were made. On the left a histological section is shown. The black asterisk marks a tetrode end-point.

line after those 5 minutes. Therefore, the time period of 5 to 20 minutes after injection was considered to assess the effects of the cannabinoid ligands, as well as the effect of vehicle in a comparable timeframe.

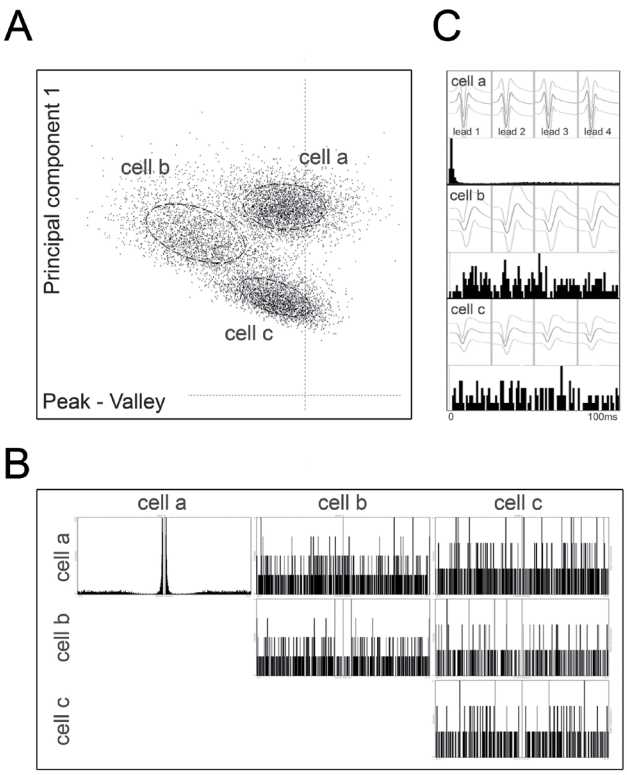

Figure 2
Cluster-plot, auto- and cross-correlograms and waveforms of 3 example neurons measured on a single tet-rode. (**A**) A plot of the principal component analysis (y-axis) and peak-valley (x-axis) shows 3 separate clusters, indicating 3 distinct neurons (the dashed circles in this plot). (**B**) Auto – and cross-correlations between the three neurons depicted in (A). Auto-correlations (diagonal) are calculated to assess if individual spike-clusters (neurons) do not show spiking activity during their refractory period (indicative of contamination with spikes from a separate cluster). Cross-correlations were performed to analyze if separate neurons exhibit correlated firing activity. These data indicate unrelated firing activity between these three neurons. (**C**) This graph shows the waveforms of each recorded neuron as well as the interspike-interval. The top graphs show the waveforms of neurons a-c as recorded on each of four tetrode leads (i.e. individual electrodes that are combined to make up a tetrode). The bottom half of each graph shows the interval between successive action potentials.

The CB1/2R agonist WIN55,212-2 affects VTA neuronal populations in a manner dependent on the effect of apomorphine

Administration of the CB1/2R agonist WIN55,212-2 elicited responses in a population of neurons in the VTA (Figure 3*A*, left). WIN55,212-2 (1 mg/kg, i.p.) exerted a heterogeneous effect on VTA neurons, ranging from increases to decreases (Figure 3*A*, middle). In order to isolate these subpopulations by means other than the effect of WIN55,212-2 itself, the non-specific dopamine receptor

agonist apomorphine was applied (0.1 mg/kg, i.p.). Dopamine neurons typically possess the D2 autoreceptor, making them susceptible to apomorphine-induced inhibition, whereas non-dopamine neurons are often not affected by D2R agonism, or have their activity enhanced by it[231-233]. To attribute VTA neurons to one of these two clusters (putatively dopaminergic, or non-dopaminergic), a cut-off criterion of 20% change from baseline was used[233] for the effect of apomorphine. VTA neurons inhibited by apomorphine by more than 20% were assigned to the putative dopaminergic cluster (n=29). The remaining VTA neurons, which were not inhibited in this way, were assigned to the non-dopamine cluster (n=41). Putative VTA dopamine neurons were often excited by WIN55,212-2. Conversely, putative VTA non-dopamine neurons were often inhibited by WIN55,212-2 (Figure 3*A*). Statistical analysis showed an interaction between the effect of WIN55,212-2 and the response to apomorphine ($F(1,68)=6.84$, $p<0.05$, APO-group x WIN interaction, Repeated Measures ANOVA). This revealed the overall excitatory WIN55,212-2 effect (188.88 ± 30.55% of baseline) in the APO-decrease group, and the overall inhibitory effect (84.45 ± 25.70% of baseline)

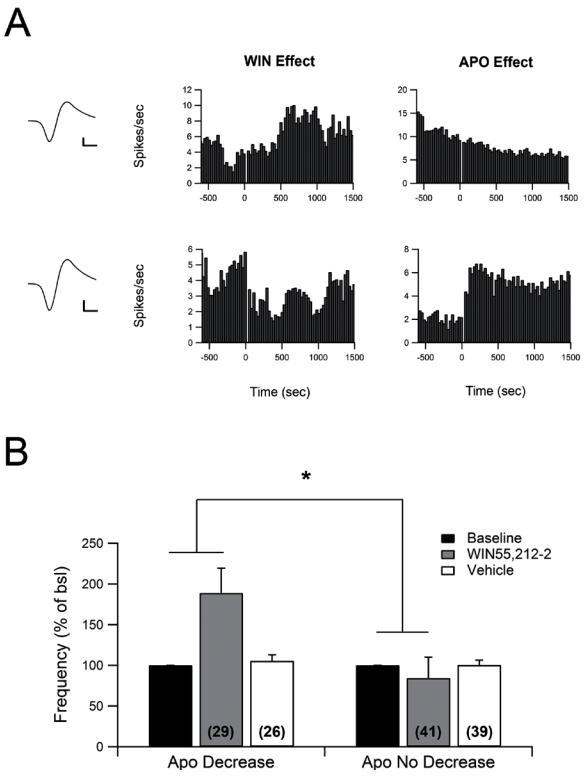

Figure 3
The CB1/2R agonist WIN,55-212 excites putative VTA dopamine neurons and inhibits putative VTA non-dopamine neurons. (**A**) Examples of VTA neurons affected by 1 mg/kg WIN55,212-2. *Left*: The waveform of these two individual neurons, showing comparable kinetics. Scale bars indicate 0.2 ms (horizontal) and 10 μV (vertical). *Middle*: the response of the corresponding neurons to administration of 1 mg/kg i.p. WIN55,212-2. Injection occurred at time point zero. *Right*: The corresponding effect of 0.1 mg/kg apomorphine on these neurons. (**B**) The overall effect of WIN55,212-2 and vehicle across the two subsets of VTA neurons. Bars depict averages ± SEMs. Number of neurons in parentheses. **p<0.05 for the WIN x Apo-cluster interaction*.

in the APO-no-decrease group. The vehicle injection itself had no effect on firing frequency, neither within nor across apomorphine subgroups (F<1 for main effect of WIN and for interaction, Repeated Measures ANOVA) (Figure 3*B*). In the apomorphine-decrease group, apomorphine itself reduced AP frequency to 42.96 ± 5.64% of baseline ($F(1,28)=164.19$, $p<0.001$), whereas in the group of neurons that did not show a decrease, apomorphine enhanced AP frequency to 112.36 ± 5.34% of baseline ($F(1,40)=5.36$, $p<0.05$). No effect of WIN55,212 on the percentage of spikes in bursts was observed, neither in general ($F(1,13)=1.49$, $p=0.25$, Main effect of WIN), nor in an APO-group dependent manner (F<1, APO-group x WIN interaction, Repeated Measures ANOVAs).

With extracellular recordings, subdivisions in the VTA are also often made based on baseline firing frequency, and/or waveform characteristics such as the width of the action potential. However, we found no correlation between the effect of WIN55,212 and either action potential basal frequency ($r=-0.08$, $p=0.52$, $n=70$) or AP half-width ($r=-0.06$, $p=0.63$, $n=70$), while there was indeed a significant correlation between the effects of WIN55,212-2 and apomorphine ($r=-0.39$, $p=0.001$, $n=70$, Pearson Correlations). Therefore, we considered apomorphine response as the most suitable determinant of the expected cannabinoid effect. Across the two apomorphine subpopulations there was no difference between AP half width (APO-Decrease: 194.83 ± 8.37 µs; APO-No Decrease: 200 ± 7.04 µs; $F(1,68)=0.22$, $p=0.64$). Interestingly, there was a strong trend towards a difference in the basal firing frequency however, as neurons inhibited by apomorphine (putatively dopaminergic) tended to have a lower firing frequency (1.90 ± 1.22 Hz) than neurons that were not inhibited by apomorphine (4.99 ± 1.02 Hz; $F(1,68)=3.78$, $p=0.06$). VTA dopamine neurons have indeed been reported to have lower firing frequency than VTA non-dopamine neurons (e.g. GABA neurons)[233].

No evidence for tonic endocannabinoid-modulation of VTA neurons during basal conditions

In order to determine whether endocannabinoids regulate the VTA during basal conditions, the response of VTA neurons to the highly potent CB1/R neutral antagonist NESS0327 at 0.1 mg/kg was assessed. This dose provides sufficient occupancy of CB1Rs in the nervous system to block the central effects of cannabinoids, as we (Chapter 2) and others[205] have shown. If endogenous cannabinoids modulate VTA activity, systemic administration of this neutral CB1R antagonist would block this endogenous signaling and consequently affect

neuronal activity. A set of 34 neurons was recorded (18 of which were inhibited by apomorphine). NESS0327 itself did not produce an apparent overall effect on neuronal firing, neither across subgroups, nor within them (F<1 for main effect of NESS0327 and for its interaction with apomorphine-response) (Figure 4*A, B*). Similarly, burst firing was unaffected by NESS0327 in general (F<1 for main effect of NESS0327 and for its interaction with apomorphine-response).

For several neurons (n=15) WIN55,212-2 (1 mg/kg) was administered 30 minutes after NESS0327. These neurons were again divided based on response to apomorphine, with 5 neurons being suppressed by apomorphine versus 10 that were not. In the presence of NESS0327, there was no longer an indication of a WIN55,212-2 effect (F<1 for main effect of WIN and for its interaction with apomorphine-response) (Figure 4*B*). This indicates that NESS0327 could effectively prevent agonist-induced CB1R activation, while not producing a concerted effect on the VTA population on its own accord.

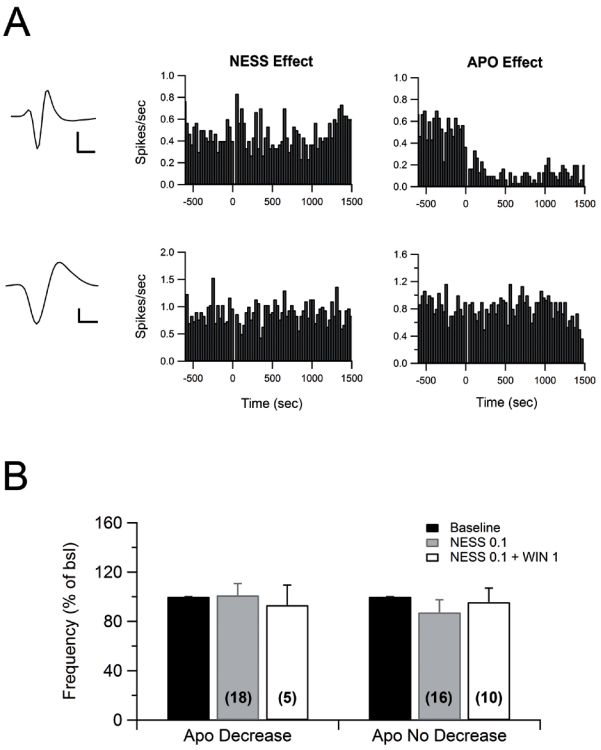

Figure 4

The neutral CB1R antagonist NESS0327 did not affect VTA neurons. (**A**) *Left*: Representative waveforms of neurons in the VTA subjected to i.p. 0.1 mg/kg. Scale bars indicate 0.2 ms (horizontal) and 10 μV (vertical). *Middle*: peri-event time histogram showing the response of these neurons to 0.1 mg/kg NESS0327 administration at time point zero. *Right*: The effect of these neurons to 0.1 mg/kg apomorphine. (**B**) The overall effect of NESS0327 across the two subsets of VTA neurons, as well as the effect of WIN55,212-2 (1 mg/kg) in the presence of NESS0327 (0.1 mg/kg). Bars depict averages ± SEMs. Number of neurons in parentheses.

The inverse agonist SR141716A differentially affects VTA neuronal subpopulations

The lack of an effect of the CB1R neutral antagonist NESS0327 points towards the absence of tonic activation of CB1Rs by endocannabinoids during basal conditions. However, this does not exclude the possibility that agonist-independent constitutive signaling contributes to VTA neuronal activity. To investigate this possibility the CB1R inverse agonist SR141716A was administered which, like NESS0327, prevents endocannabinoid-CB1R signaling, but importantly also suppresses CB1R constitutive activity[15,16,203,205]. SR141716A was administered at two doses: a dose of 1 mg/kg which was previously reported to be without an effect on VTA neurons[77,79], and a higher dose of 3 mg/kg.

At 1 mg/kg SR141716A (n=39; 27/39 exhibited a decrease by apomorphine), there was no significant overall effect, ($F(1,37)=1.39$, $p=0.25$, SR141716A main effect), nor was there a significant interaction of SR141716A at 1 mg/kg with apomorphine-response ($F(1,37)=1.97$, $p=0.17$, APO-group x SR1 interaction, Repeated Measures ANOVAs; data not shown). Similarly, there was no effect of SR141716A at 1 mg/kg on the percentage of burst firing ($F<1$ for both the main effect of SR141716A, and for the interaction with apomorphine-response).

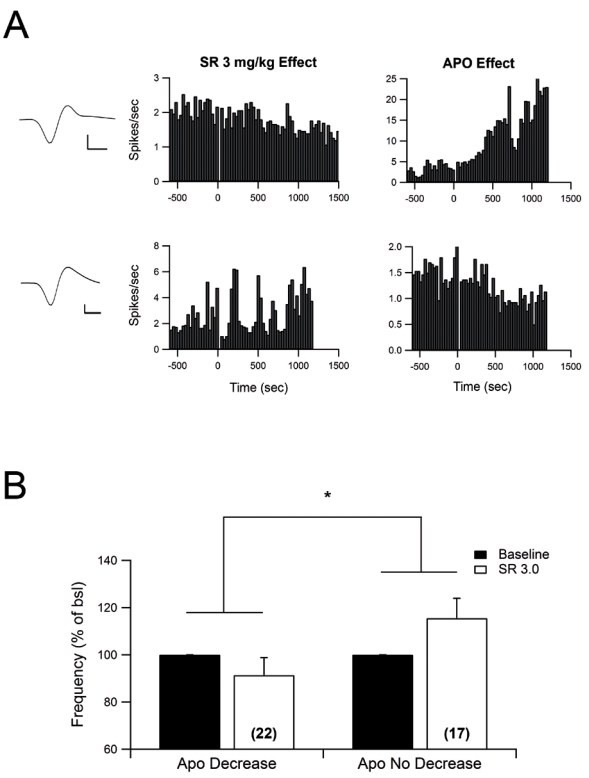

Figure 5

The CB1R inverse agonist SR141716A has inverse agonistic effects on VTA neurons. (**A**) Examples of VTA neurons responding to SR141716A (3 mg/kg). *Left*: Representative waveform of two individual neurons recorded during an experiment in which SR141716A was administered at 3 mg/kg. Scale bars indicate 0.2 ms (horizontal) and 10 µV (vertical). *Middle*: The response of these neurons to 3 mg/kg i.p. SR141716A. Injection occurred at time point zero. *Right*: The response in these neurons evoked by 0.1 mg/kg apomorphine. (**B**) The overall effect of SR141716A at 3 mg/kg across the two subsets of VTA neurons. Bars depict averages ± SEMs. Number of neurons in parentheses. **p<0.05 of the SR x Apo-cluster interaction.*

At the dose of 3 mg/kg, SR141716A (n=39; 22/39 decreased by apomorphine) differentially affected the two VTA subpopulations ($F(1,37)=4.57$, $p<0.05$, APO-group x SR3 interaction, Repeated Measures ANOVA) (Figure 5*A, B*). This interaction reflected that in putative VTA dopamine neurons, SR141716A 3 mg/kg was associated with a small decrease in firing frequency to 91.40 ± 7.45% of baseline. Conversely, in putative VTA non-dopamine neurons SR141716A caused a small increase in firing frequency to 115.49 ± 8.47% of baseline. This effect did not extent to burst firing, which was unaffected by SR141716A 3 mg/kg both in general as well as in different apomorphine-responsive subsets ($F<1$ for both the main effect of SR141716A and its interaction with the apomorphine-response).

Overall these findings reveal an inverse agonistic effect of SR141716A at 3 mg/kg (but not the 1 mg/kg) dose on neuronal firing in the VTA. Overall this suggests that CB1Rs, rather than tonic endocannabinoids, regulate activity of VTA neurons under baseline conditions.

DISCUSSION

Exogenous cannabinoids engage the mesolimbic dopamine system, and do so in part by directly altering activity of neurons in the VTA[77,79,80,229,236]. We now provide evidence that the endogenous cannabinoid system itself also contributes to the regulation of VTA neuronal activation, and may specifically do so by tonic constitutive agonist-independent CB1R activation.

In particular our findings indicate that overall the CB1/2R agonist WIN55,212-2 increased activity of a population of putative VTA dopamine neurons, while it suppressed activity of putative non-dopaminergic (e.g. GABAergic) neurons. Subsequently we determined the role of the endocannabinoid system in these VTA neuronal populations. We observed that the highly potent neutral CB1R antagonist NESS0327[205] did not consistently affect their firing frequency, while this concentration was sufficient to block the effect of WIN55,212-2. This suggests that during basal conditions (i.e. conditions not related to any task, or reward processing), there is minimal or no tonic endocannabinoid-CB1R signaling relevant for VTA activity. However, this still allows for a contribution of tonically active CB1Rs due to agonist-independent processes[15,16,203]. In this case, the inverse CB1R agonist SR141716A would suppress such constitutive activity

and affect neuronal firing. Accordingly, SR141716A at 3 mg/kg decreased activity of putative VTA dopamine neurons, whereas putative non-dopamine neurons showed increased activity. These responses were directly opposite to those produced by the CB1R agonist WIN55,212-2. A previous study in anaesthetized animals also reported that SR141716A at 2 mg/kg reduced firing frequency of putative VTA dopamine neurons to a similar extent, and suggested this was due to interference with tonic endocannabinoid signaling[80]. We now identify that the actual underlying mechanism for this is more likely suppression of CB1R constitutive activity, rather than interference with endocannabinoid-CB1R signaling. Also, since the effect of SR141716A on putative VTA dopamine neurons has now been observed in both anaesthetized and awake animals, it is unlikely to be caused by any anesthesia-related disruption of the endogenous cannabinoid system[237,238], or by indirect consequence of a particular behavioral phenotype. The lack of a significant effect of 0.5 mg/kg[80] or 1 mg/kg[77,79] may be attributable to the observation that 1 mg/kg SR141716A is likely to lead to considerable, but far from maximal CB1 receptor occupancy[239,240]. Given that the effect is subtle, interference with overall CB1R activity may only become noticeable at the spike output level at higher doses of SR141716A, at least during basal conditions (i.e. conditions not related to reward processing). A likely mechanism underlying the effect we observed on dopamine neurons is that constitutively active CB1Rs on their GABAergic afferents, actively suppress GABAergic transmission (Chapter 2). Since, CB1Rs on the glutamatergic afferents to these neurons may not utilize this activation mechanism during basal conditions[70], this would allow an inverse agonist like SR141716A to induce a shift towards reduced disinhibition (i.e. less activation) of VTA dopamine neurons. Alternatively, the effect may be the summation of a more elaborate network regulated by tonically (constitutively) active CB1Rs that includes projections onto non-dopamine neurons[86].

A direct comparison between such an *in vitro* and the current *in vivo* approach is somewhat hindered by the arduous task of correct identification of VTA dopamine and non-dopamine neurons by means of extracellular recordings[233]. In these experiments we exclusively relied on D2R agonism induced by apomorphine[241], which did prove to be an adequate predictor of the overall effect of the cannabinoid ligands. However, given the increasingly acknowledged diversity of VTA dopamine neurons[232,233,242], we cannot exclude the possibility that our two VTA subgroups do not completely match with the dopamine and non-dopamine (e.g. GABA) neuronal clusters in the VTA. Nevertheless, our sub-

population identification, in which CB1/2R agonist WIN55,212-2 generally activated putative VTA dopamine neurons, matches well with other reports of the known cannabinoid-induced increase in dopamine in VTA projection targets[81,228]. We therefore feel it is justified to provisionally label our subgroups as predominately dopaminergic and non-dopaminergic.

Future studies also need to address how the contribution of CB1R activation – be it constitutive or agonist-induced – to VTA neuronal activity is altered when an animal switches from undirected towards goal-directed behavior (e.g. conditions where reward is actively pursued and/or experienced). There is already some evidence that rewarding stimulation of the medial forebrain bundle evokes a role for the endogenous cannabinoid system in shaping VTA neuronal responses[236]. The potentially separate roles that endocannabinoids and constitutive CB1R signaling may play in such behaviors remain largely to be unraveled.

The current findings may also have clinical relevance requiring further exploration. In so far as that SR141716A (rimonabant) once showed promise as a weight loss drug, but was discontinued due to serious side effects, including depressed feelings[24]. A hypofunctional dopamine system has been proposed to play an important role in the loss of the sensation of pleasure and drive that is so characteristic for clinical depression[127,136]. In our study, SR141716A acutely induced suppression of putative dopamine neurons *in vivo*. It is therefore a distinct possibility that the side effect of SR141716A on depression occurs in part by drug-induced hypofunctionality of the dopamine system. In this light it is especially noteworthy that the highly potent neutral CB1R antagonist NESS0327 did not affect VTA neuronal populations. There are now indications that neutral CB1R antagonists are capable of reducing food intake just like CB1R inverse agonists[125,204,243], (also see Chapter 2), which makes these current findings all the more relevant for potential development of safer treatment for obesity medication by use of neutral CB1R antagonists rather than inverse agonists. However, prior to that it would be of critical importance to investigate the more long-term effects of chronic administration of CB1R inverse agonists and neutral antagonists on VTA functionality.

Acknowledgements

The authors would like to thank Top Institute Pharma (D1-105) and the European Union Seventh Framework Programme for funding this project. We also thank the NIMH Chemical Synthesis and Drug Supply Program for supplying SR141716A for the purpose of this study.

Chapter 4

MORPHINE WITHDRAWAL ENHANCES CONSTITUTIVE MU OPIOID RECEPTOR ACTIVITY IN THE VTA

Frank J. Meye
Ruud van Zessen
Marten P. Smidt
Roger A. H. Adan
Geert M. J. Ramakers

(Submitted)

ABSTRACT

Mu opioid receptors (MORs) in the ventral tegmental area (VTA) are pivotally involved in addictive behavior. While MORs are typically activated by opioids, they can also become constitutively active in the absence of any agonist. In the current study we show that MOR constitutive activity is highly relevant in the VTA because it regulates GABAergic input to dopamine neurons. Specifically, suppression of MOR constitutive activity with the inverse agonist KC-2-009 enhanced GABAergic neurotransmission onto VTA dopamine neurons. This inverse agonistic effect was fully blocked by the specific MOR neutral antagonist CTOP, which had no effect on GABAergic transmission itself. We next show that withdrawal from chronic morphine further increases the magnitude of MOR constitutive activity. We demonstrate that this increase can be an adaptive response to the detrimental elevation in cAMP levels known to occur during morphine withdrawal. These findings offer important insights in the physiological occurrence and function of MOR constitutive activity, and have important implications for therapeutic strategies aimed at normalizing MOR signaling during addiction and opioid overdose.

INTRODUCTION

Endogenous opioids acting at the mu opioid receptor (MOR) serve a pivotal function in reward processing and addictive behaviors[8,66,156,160-162,165,166,245,246]. This is especially the case for MORs regulating the ventral tegmental area (VTA)[247-249], which typically suppress GABAergic inputs to VTA dopamine neurons[149,150,152,250,251].

Aside from activation by (endogenous) opioids, MORs can also exhibit constitutive signaling in the absence of any agonist[180-182]. Constitutive MOR activity has been demonstrated in artificial cell systems[180-182,252-254], and in some native tissues[183,184,255,256]. Intriguingly, there is evidence that reduction of constitutive MOR activity exacerbates the expression of physical and psychological opioid withdrawal symptoms[21,184,257-259]. Part of the neuroanatomical substrate for such adverse effects is likely a hypofunctional mesolimbic dopamine system[154,168,189-193]. Reduced dopamine signaling during morphine withdrawal can arise due to increased GABAergic signaling onto VTA dopamine neurons[260]. This enhancement of inhibitory signals has been reported to occur as a consequence of morphine withdrawal-induced elevations in cAMP (cAMP superactivation) in GABAergic terminals[169,194]. Together, these findings point to an important role of GABAergic signaling onto VTA dopamine neurons in the occurrence of opiate withdrawal symptoms.

Currently, separate lines of evidence implicate the VTA on the one hand, and constitutively active MORs in general on the other hand, in the process of opiate withdrawal. However, no converging findings have shown a role for MOR constitutive activity in the regulation of the VTA during opiate withdrawal. Furthermore, the actual physiological relevance of MOR constitutive activity, be it in an opiate-naïve or an opiate-experienced state, remains poorly understood. In the current study we provide evidence that MOR constitutive activity already controls the GABAergic inputs of VTA dopamine neurons during a drug-naïve state, and exerts even more prominent control during morphine withdrawal.

MATERIALS AND METHODS

Animals

All experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in agreement with Dutch law (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC). Electrophysiological experiments were performed in heterozygous Pitx3-GFP mice (n=90, P14-30), obtained by crossing C57Bl6-Jico wild-type with Pitx3$^{gfp/gfp}$ mice. These animals have selective GFP expression in dopaminergic neurons in the midbrain, allowing for their accurate identification[38,261-264]. We established that agonist-dependent MOR regulation was similar to that in wild type C57Bl6-Jola mice.

For morphine withdrawal experiments, mice were treated twice daily with morphine (10 mg/kg) (or saline for controls) for 5 consecutive days. This protocol has been demonstrated to induce morphine dependence[194,265]. This regimen was followed by 3-4 days of morphine abstinence, after which animals were sacrificed for electrophysiological experiments.

Electrophysiology

Electrophysiological experiments were conducted as described earlier[66,210]. Animals were anaesthetized with isoflurane and then decapitated. Subsequently, the brain was rapidly removed and stored in ice cold carbogenated (95% O_2 / 5% CO_2) enriched-artificial cerebrospinal fluid (ACSF), consisting of (in mM): NaCl (124), KCl (3.3), KH_2PO_4 (1.2), $CaCl_2$ (2.5), $MgSO_4$ (2.6), $NaHCO_3$ (20), glucose (10), and ascorbic acid (1). Horizontal brain slices of 200 µm were cut in the same chilled medium on a Vibratome (Leica, Rijswijk, The Netherlands). After cutting, slices were incubated in carbogenated normal ACSF (final $MgSO_4$ concentration of 1.3 mM, no ascorbic acid). Slices were left to recover for 1 hour at 32˚C. During recordings, a slice was mechanically stabilized and continuously superfused with carbogenated normal ACSF at a rate of 2-3 ml/min at room temperature.

Dopaminergic VTA neurons were identified as green fluorescent neurons within an anatomically circumscribed region, consisting of the area rostral to the medial lemniscus (ML), medial to the medial terminal nucleus of the accessory optical tract (MT) and lateral of the interpeduncular fossa (IPF). In these neurons whole-cell voltage clamp recordings were obtained with an EPC-10 patch clamp amplifier, using Patchmaster v2x32 software (HEKA Electronics, Germany). Recordings were made using borosilicate glass pipettes filled with

(in mM): K-gluconate (78), KCl (77), HEPES (10), EGTA (1), Mg^{2+}-ATP (2), Na^{+}-GTP (0.4), pH=7.4, which yielded a pipette resistance of 3-5 MΩ. Data were sampled at 20 kHz. Immediately after patching, cells were voltage-clamped at -60 mV and allowed to recover for at least 10 minutes before baseline recordings. Miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of 1 μM TTX and 10 μM DNQX. To ensure stable recordings, cells were discarded when the product of uncompensated series resistance and cell capacitance varied too strongly over time (>20% change).

For all recorded cells the membrane capacitance was calculated in Patchmaster v2x32 software (HEKA Electronics, Germany), using a rectangular test pulse (5 mV, 5 ms) to evoke transient capacitance currents. H-currents for cells were determined by a protocol where neurons were clamped for 2 seconds to incrementally (-10 mV increment) more hyperpolarizing values spanning from -70 to -120 mV. The maximal evoked current at -120 mV was taken as the value of the H-current[66,210].

For analysis, events were scored using MiniAnalysis software (Synaptosoft, Decatur, CA, USA) and distributions of frequency and amplitude were fitted in Igor Pro (Wavemetrics, Lake Oswego, OR, USA) with a lognormal curve: $f(x) = A*\exp[-0.5*(\ln X - \mu) / \sigma)^2] / (x*\sigma*\sqrt{2\pi})$, as described before[210,213]. In this equation X represents the measured instantaneous frequency or amplitude of an event, A is the relative area under the curve of the distribution, μ is the mean and σ the standard deviation of the underlying normal distribution. The mean of the lognormal distribution (m) was then calculated using the equation: $m = \exp(\mu + \sigma^2)$ to allow for the determination of percentage-wise changes. For the calculation of mIPSC kinetics, the decay time to 50% of the peak value for every mIPSC was calculated in MiniAnalysis (Synaptosoft, Decatur, CA, USA). To assess drug effects, two-tailed Repeated Measures or One-Way ANOVAs were performed on the means of the normally distributed data. The Mann-Whitney U test was performed in the case of non-parametric tests. All statistical analyses were done in SPSS 17.0 (Chicago, IL, USA).

Drugs

The AMPA/Kainate receptor antagonist DNQX, the voltage-gated Na^{+}-channel blocker tetrodotoxin (TTX), the MOR agonist DAMGO and the MOR neutral antagonist CTOP were from Tocris Bioscience (Bristol, UK). Forskolin was from Sigma-Aldrich (UK). Morphine-HCl was from O.P.G. (Utrecht, The Netherlands). The MOR inverse agonist KC-2-009 was a kind gift from Dr. Kenneth Rice at the NIH Synthesis program.

RESULTS

MORs on GABAergic projections to VTA dopaminergic neurons are constitutively active

In order to determine the contribution of MOR constitutive activity in the VTA, we first determined the effect of MOR activation itself. MOR agonists have been reported to hyperpolarize VTA GABA neurons[150], and to reduce their release probability[149]. We patch clamped Pitx3-GFP-positive VTA dopamine neurons and isolated GABAergic mIPSCs in the presence of TTX (1 µM) and DNQX (10 µM). As expected, administration of the MOR agonist DAMGO (1 µM) reduced the GABAergic mIPSC frequency to 55.15 ± 5.40% of baseline (n=14, F(1,13)=28.34, p<0.001, Repeated Measures ANOVA, Fig. 1*A,B*). DAMGO did not affect the amplitude of mIP-

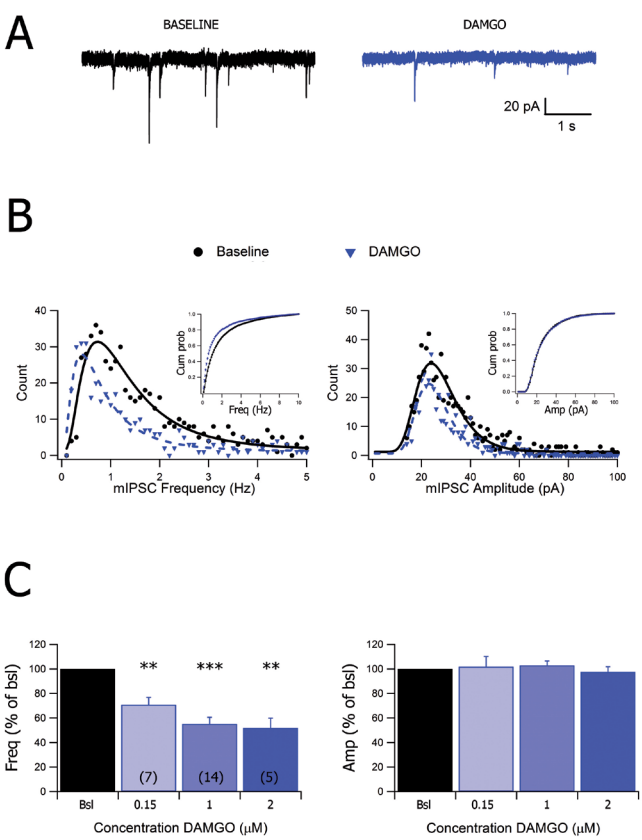

Figure 1

The MOR agonist DAMGO suppresses mIPSC frequency onto VTA dopamine neurons. (**A**) Example traces of mIPSCs in the absence (black) and presence (blue) of 1 µM DAMGO. mIPSCs frequency is diminished after administration of the MOR agonist. (**B**) Distributions of mIPSC acute frequency (left) and amplitude (right) during baseline (black circles) and after administration of 1 µM DAMGO (blue triangles), in a representative experiment. The lines represent the fitted lognormal curves to extract means and standard deviations for the baseline (black solid) and DAMGO (blue dashed) conditions. DAMGO induces a clear leftward shift in the mIPSC frequency distribution. The inset represents the cumulative probability plot of mIPSC frequency (left) and amplitude (right) of all experiments before (black circles) and after 1 µM DAMGO (blue triangles). (**C**) Average acute frequency (left) and amplitude (right) after DAMGO treatment as percentage of baseline, with the number of experiments in parentheses. DAMGO reduces mIPSCs across various doses. ***p<0.01; ***p < 0.001*.

SCs (F(1,13)=0.12, p=0.73, Repeated Measures ANOVA, Fig. 1*B,C*), in accordance with reports that MORs are located presynaptically on GABAergic nerve terminals in the VTA[266]. DAMGO exerted its effect on frequency at various doses (F(1,23)=47.00, p<0.001, Repeated Measures ANOVA, Fig. 1*C*). In wild type mice, DAMGO (1 μM) reduced mIPSC frequency to a comparable extent (56.94 ± 9.53% of baseline, n=5, (F1,4)=20.41, p<0.05, Repeated Measures ANOVA, data not shown), indicating a normal MOR-system in the Pitx3-GFP heterozygous animals.

Activation of MORs decreases inhibitory signals onto VTA dopamine neurons. If some of these MORs are already constitutively active, the suppression of their constitutive activity would increase inhibitory signals onto VTA dopamine neurons. We administered the MOR inverse agonist KC-2-009 (1 μM)[181]

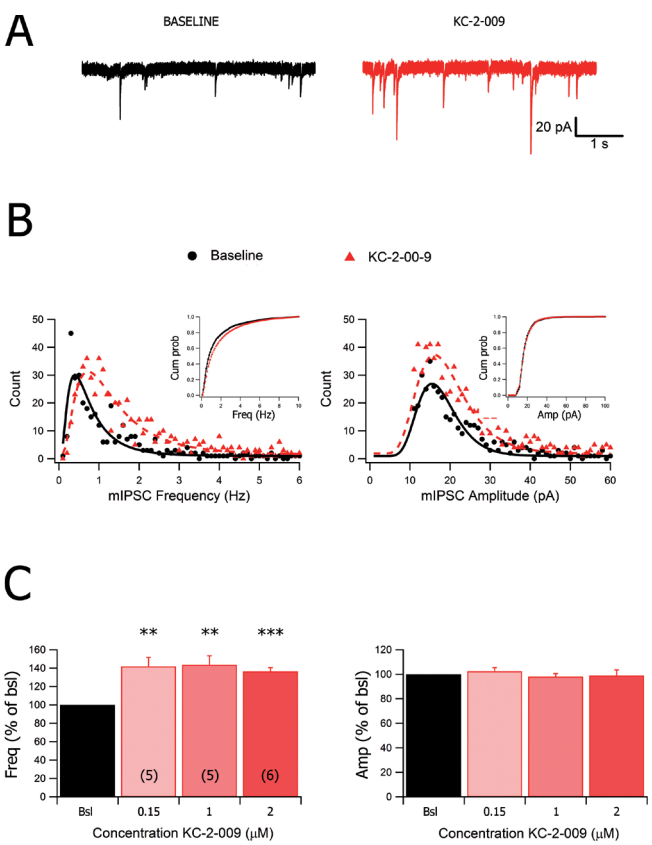

Figure 2
The MOR inverse agonist KC-2-009 enhances mIPSC frequency onto VTA dopamine neurons. (**A**) Representative traces of mIPSCs in the absence (black) and presence (red) of 1 μM KC-2-009. Administration of the MOR inverse agonist increases mIPSC frequency. (**B**) Exemplar lognormal distributions of mIPSC acute frequency (left) and amplitude (right) during baseline (black circles) and after administration of 1 μM KC-2-009 (red triangles). The MOR inverse agonist induces a rightward shift in the mIPSC frequency distribution, indicating a higher frequency of GABA transmission. In the inset are the cumulative probability plot of mIPSC frequency (left) and amplitude (right) of all experiments before (black circles) and after 1 μM KC-2-009 (red triangles). (**C**) Average acute frequency (left) and amplitude (right) as a percentage of baseline after various doses of KC-2-009. KC-2-009 clearly increased mIPSC frequency at all these doses. The number of experiments per dose is indicated in parentheses. ***p<0.01; ***p < 0.001.*

to suppress constitutive MOR activity and indeed observed a clear increase in mIPSC frequency to 143.70 ± 9.77% of baseline (n=5, F(1,4)=41.91, p<0.01, Repeated Measures ANOVA, Fig. 2*A,B*). The effect of KC-2-009 on mIPSC frequency was without a concomitant effect on amplitude (F(1,4)=0.04, p=0.85, Repeated Measures ANOVA, Fig. 2*B,C*), similarly in line with a presynaptic locus of action. The effect of KC-2-009 on mIPSC frequency was clear at various concentrations (F(1,13)=144.59, p<0.001, Repeated Measures ANOVA, Fig. 2*C*). The absence of dose-dependence over this range indicated that the maximal effect of KC-2-009 was already obtained at 100 nM (F(2,13)=0.17, p=0.85, Repeated Measures ANOVA Treatment x Dose, Fig. 2*C*).

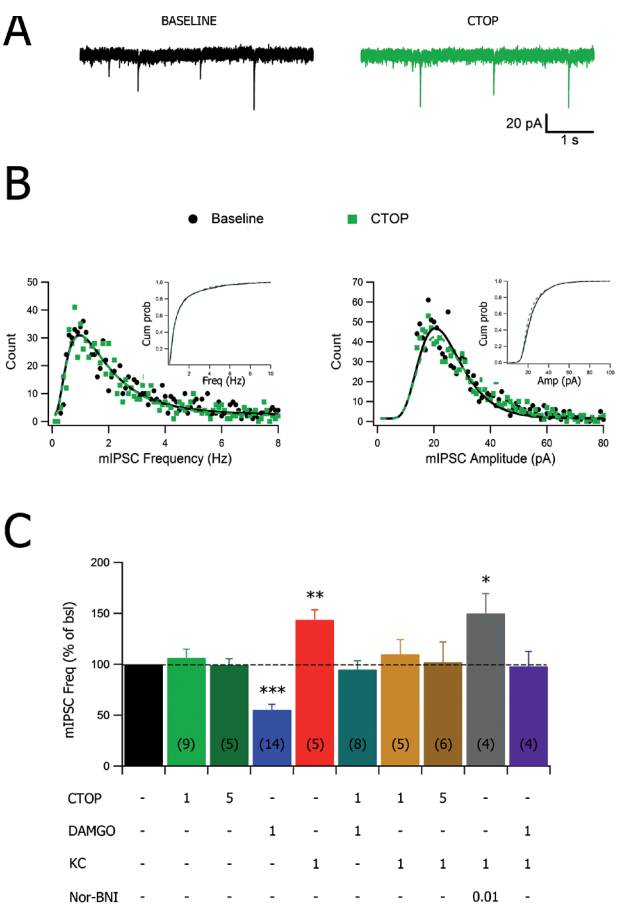

Figure 3

The inverse agonistic effect of KC-2-009 is due to suppression of MOR constitutive activity. (**A**) Representative traces of mIPSCs in the absence (black) and presence (red) of 1 µM of the MOR neutral antagonist CTOP, which did not affect mIPSC frequency or amplitude. (**B**) Associated exemplar log-normal distributions of mIPSC acute frequency (left) and amplitude (right) during baseline (black circles) and after administration of 1 µM CTOP (green squares). CTOP did not change the mIPSC frequency or amplitude distributions. The insets are the cumulative probability plots for mIPSC frequency (left) and amplitude (right) of all experiments before (black circles) and after 1 µM CTOP (green squares). (**C**) Overview of the effect of various MOR ligands on mIPSC frequency in the VTA, with the number of experiments indicated in parentheses. Whereas the neutral antagonist CTOP did not affect mIPSCs itself, it did block the effects of both DAMGO and KC-2-009. Contrarily, the KOR antagonist Nor-BNI could not block the effect of KC-2-009. Pretreatment with KC-2-009 also prevented the effect of DAMGO. **p<0.05; **p<0.01; ***p < 0.001*.

Alternative explanations for the inverse agonistic effect of KC-2-009 other than suppression of MOR constitutive activity are (1) interference with endogenous opioids acting at the MOR (i.e. interference with agonist-dependent, rather than constitutive MOR activation), (2) a MOR-independent effect, or (3) a specific effect on a subset of MORs with different downstream coupling. To address these alternative possibilities we tested the MOR-specific neutral antagonist CTOP, which is also fully capable of blocking MOR activation by endogenous opioids[258]. CTOP (1 μM) by itself affected neither mIPSC frequency (n=9, $F(1,8)=0.08$, $p=0.79$, Fig. 3*A,B*) nor amplitude ($F(1,8)=0.85$, $p=0.38$, Repeated Measures ANOVAs; Fig. 3*B,C*) compared to baseline. The lack of a direct effect of CTOP on GABAergic transmission was not due to insufficient MOR occupation. At a higher concentration, CTOP (5 μM) did not affect mIPSC frequency either (n=5, $F(1,4)=0.05$, $p=0.83$, Repeated Measures ANOVA, Fig. 3*C*). Moreover, preadministration of either 1 or 5 μM CTOP blocked the effect of subsequently administered KC-2-009 (1 μM) ($F(1,9)=0.04$, $p=0.85$, Repeated Measures ANOVA, Fig. 3*C*). Likewise, the effect of the MOR agonist DAMGO (1 μM) was blocked by preadministration of the neutral MOR antagonist CTOP (1 μM) (n=8, $F(1,7)=1.55$, $p=0.25$, Repeated Measures ANOVA, Fig. 3*C*). It has been reported that KC-2-009 also has low affinity for the kappa opioid receptor (KOR)[181]. While the efficacy of KC-2-009 in our assay suggested a MOR-dependent effect, we still tested whether the effect of KC-2-009 was indeed not mediated by the KOR. In the presence of the KOR antagonist Nor-Binaltorphimine (Nor-BNI; 10 nM), at a dose which should block KOR-, but not MOR-dependent effects[267], KC-2-009 (1 μM) still clearly increased mIPSC frequency to 149.87±19.45% of baseline (n=4; $F(1,3)=17.91$, $p<0.05$, Repeated Measures ANOVA, Fig. 3*C*).

These findings indicate that the effect of KC-2-009 is both MOR-dependent and not due to interference with signaling of endogenously present opioids acting at the MOR in our slice preparation. One unexplored hypothetical situation would be that KC-2-009 had specific effects on a subset of MORs different from those affected by DAMGO (e.g. different MOR isoforms), with these MORs potentially having alternate downstream signaling properties [268].In this particular scenario, DAMGO and KC-2-009 would have different loci of effect (i.e. different MOR-subsets). In order to test this possibility, we preadministered KC-2-009 (1 μM), let its enhancing effect on mIPSC frequency stabilize, and then administered DAMGO (1 μM). If DAMGO would affect a different subset of MORs than KC-2-009, it would not compete with DAMGO for binding, permitting its inhibiting effect to occur in the presence of a stabilized KC-2-009 effect. Instead,

we observed that the effect of DAMGO was blocked by pre-administered KC-2-009 (n=4; $F(1,3)=0.36$, $p=0.59$, Repeated Measures ANOVA, Fig. 3*C*). This finding is indicative of a similar locus of effect (i.e. the MOR) of both DAMGO and KC-2-009.

We also explored whether there were indications that the neurons from DAMGO and KC-2-009 experiments had inadvertently been sampled from distinct VTA dopamine neuron subpopulations. Such subpopulations can have distinct anatomical connections[25], potentially innervated by separate GABAergic afferents subject to differential MOR control. Important markers for VTA subpopulations are H-current and cell size[242]. We found no difference between neurons from DAMGO and KC-2-009 experiments (DAMGO: 18.11 ± 3.05 pF; KC: 19.57 ± 2.53 pF, $F<1$, One-Way ANOVA), which is an index for cell size, or on H-current (DAMGO-cells: 175.00 ± 78.77 pA; KC-cells: 101.82 ± 40.38 pA, $F<1$, One-Way ANOVA). Neither did we observe differences in mIPSC basal input frequency (DAMGO-cells: 1.93 ± 0.23 Hz; KC-cells: 1.67 ± 0.33 Hz; $F<1$, One-Way ANOVA), or mIPSC decay time kinetics (Pre-DAMGO: 2.13 ± 0.16 ms; Post-DAMGO: 2.20 ± 0.18 ms; Pre-KC: 2.00 ± 0.09 ms; Post-KC: 2.01 ± 0.06 ms; $F<1$ for main effects and Drug x Treatment interaction, Repeated Measures ANOVA). These findings suggest that the *a priori* selection of cells in these two experimental groups had not been biased. Overall, the inverse agonistic effect of KC-2-009 is best explained by suppression of the constitutive activity that MORs on GABAergic afferents to VTA dopamine neurons exhibit.

Withdrawal from chronic morphine enhances MOR constitutive signaling

During morphine withdrawal, MOR inverse agonists evoke stronger withdrawal symptoms (e.g. withdrawal jumping, paw tremors and conditioned place aversion) than MOR neutral antagonists[21,184,257,258]. This suggests that MOR constitutive activity becomes more important during morphine withdrawal, especially in regions involved in the expression of withdrawal symptoms, like the VTA[189,191-193,269]. Therefore, we investigated whether the constitutive MOR activity in the VTA would be enhanced during morphine withdrawal.

We pretreated mice with morphine (10 mg/kg twice daily) for 5 days to induce opiate dependence[194,265], followed by 3-4 days of abstinence to examine withdrawal. A characteristic feature of morphine withdrawal is an increase in GABAergic activity in regions like the VTA[260], leading to enhanced GABAergic neurotransmission onto VTA dopamine neurons[169,194], associated with detrimental opiate withdrawal symptoms[194]. Accordingly we observed that withdrawal

from chronic morphine pretreatment (n=15) increased the frequency of mIPSCs onto VTA dopamine neurons with 66.08% ($F(1,48)=4.09$, $p<0.05$, One-Way ANOVA, Fig. 4*A*), compared to control [saline pretreated (n=6) pooled with untreated controls (n=29), which were statistically similar, $F(1,33)=0.21$, $p=0.65$, One-Way ANOVA]. We did not observe an effect of pretreatment on mIPSC amplitude ($F(1,48)=1.73$, $p=0.20$, One-Way ANOVA, data not shown).

If MOR constitutive activity is indeed enhanced in the VTA during morphine withdrawal, the inverse agonistic effect of KC-2-009 should be enhanced. We compared the effect of MOR inverse agonist KC-2-009 (compared to the cell its own baseline, to control for any effects of morphine pretreatment on input frequency itself) after three conditions: (1) Three or four days after chronic saline

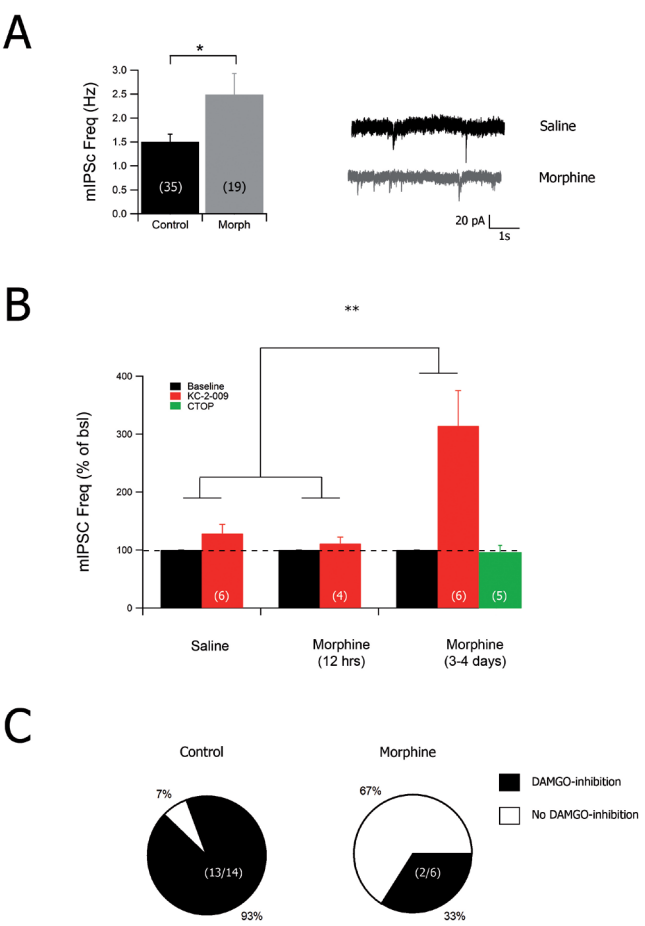

Figure 4

Morphine withdrawal enhances MOR constitutive activity in the VTA. (**A**) Morphine withdrawal increased the baseline frequency of mIPSCs onto VTA dopamine neurons compared to control. (**B**) The MOR inverse agonist KC-2-009 (1 μM) led to a greater increase in mIPSC frequency after 3-4 days of withdrawal from chronic morphine, compared to either saline treatment or only 12 hours of morphine abstinence. The MOR neutral antagonist CTOP was still without effect after 3-4 days of withdrawal, suggesting there was no increased release of endogenous opioids in the slice preparation. Note that effects are with respect to the baseline of the cell, correcting for any effects of morphine withdrawal on baseline frequency itself. (**C**) Percentage of VTA dopamine neurons suppressed by the MOR agonist DAMGO during control conditions (left) or after morphine withdrawal (right). After morphine withdrawal, DAMGO only reduced mIPSC frequency in a minority of cells. The number of experiments is indicated in parentheses. *$p<0.05$; **$p < 0.01$.

treatment, (2) A short 12 hour abstinence after chronic morphine treatment, and (3) Three or four days of abstinence after chronic morphine treatment. We observed a main effect of KC-2-009 (1 μM) on mIPSC frequency ($F_{1,13}=32.13$, $p<0.001$, Repeated Measures ANOVA, Fig. 4*B*). Importantly, the effect of KC-2-009 was stronger after 3-4 days of morphine withdrawal (baseline: 1.79 ± 0.45 Hz; KC: 5.15 ± 1.30 Hz) than in the other conditions ([saline baseline: 1.58 Hz ± 2.09 Hz; KC: 2.09 ± 0.57 Hz]; morphine 12 hrs baseline: 1.64 ± 0.85 Hz; KC: 1.85 ± 1.00 Hz), as revealed by a significant Pretreatment x Acute Drug Effect interaction $F(2,13)=10.46$, $p<0.01$, Repeated Measures ANOVA, Fig. 4*B*).

The increased effect of the MOR inverse agonist KC-2-009 is in accordance with enhanced MOR constitutive signaling. However, the increased effect could also have been due to interference with endogenous opioids potentially released during morphine withdrawal. To examine this possibility, we tested the effect of the MOR neutral antagonist CTOP after 3-4 days of morphine withdrawal. Under these conditions CTOP still did not affect mIPSC frequency (n=5, $F(1,4)=0.02$, $p=0.90$, Repeated Measures ANOVA , Fig. 4*B*), indicating that the enhanced effect of KC-2-009 could not be explained by a withdrawal-induced increase in endogenous opioids in our slice preparation.

The enhanced inverse agonistic effect of KC-2-009 during withdrawal could be due to a general increase in MOR density, raising the amount of both constitutively active and inactive MORs in absolute terms in a similar ratio. In this scenario, a MOR agonist would also be predicted to have an increased effect. Alternatively, morphine withdrawal could lead to a shift from inactive to constitutively active MORs, in which case a MOR agonist would be predicted to have a diminished effect. In agreement with this second scenario, DAMGO (1 μM; n=6) only suppressed GABAergic input (≥20%) in a minority of dopaminergic neurons (2/6 cells) after morphine pretreatment, which differed significantly from the vast majority of dopaminergic neurons that were disinhibited by an equimolar concentration of DAMGO in a drug-naïve state (13/14 cells; Mann-Whitney U test, $p<0.05$, Fig. 4*C*).

Enhancement of cAMP levels increases MOR constitutive signaling

The withdrawal symptoms after chronic morphine treatment are caused by adaptive changes such as the upregulation of the cAMP-dependent cascade[270]. In the VTA, such cAMP superactivation has been reported to increase GABAergic input to VTA dopamine neurons[169], and is responsible for negative withdrawal

symptoms[194]. Enhanced MOR constitutive activity could be an adaptive countermeasure to partly suppress the withdrawal-induced cAMP overshoot and concomitant enhanced GABAergic transmission in the VTA. Therefore, we tested whether activation of the cAMP cascade was sufficient to enhance MOR constitutive activity. We treated slices of drug-naïve animals with forskolin (FSK) to mimic the cAMP superactivation component of the morphine withdrawal state. Compared to control (n=29), FSK treatment (100 nM; n=18) evoked an increase in the frequency of mIPSCs to 231.78% ($F(1,45)=17.97$, $p<0.001$, One-Way ANOVA, Fig. 5*A*), just like actual morphine withdrawal. We did not observe a significant effect of FSK treatment on mIPSC amplitude ($F(1,45)=1.15$, $p=0.29$, One-Way ANOVA, Fig 5*A*). In these experiments slices were incubated with FSK for at least 40 minutes to obtain a stable effect, which then served as a new baseline for subsequent drug administration. KC-2-009 (1 µM) enhanced mIPSC frequency overall ($F(1,10)=31.71$, $p<0.001$, Repeated Measures ANOVA, Main effect). However, compared to control (n=6, 133.98±12.58% of baseline),

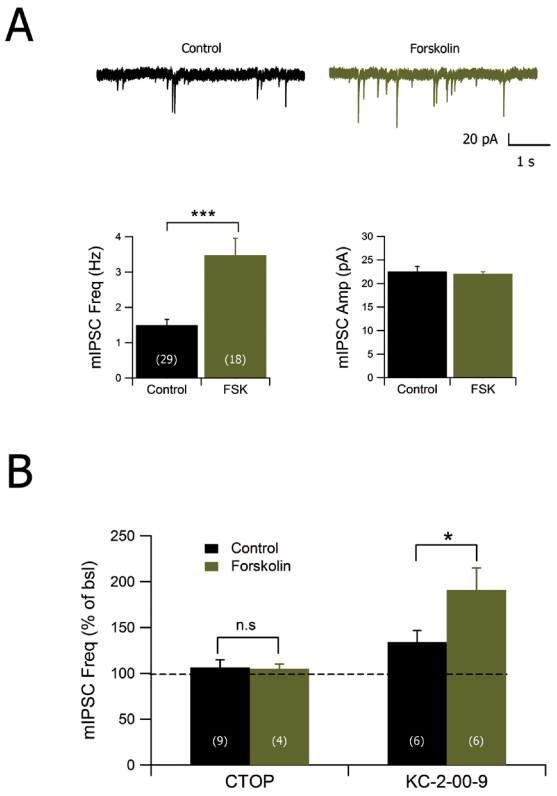

Figure 5

Pretreatment with adenylate cyclase activator forskolin is sufficient to enhance MOR constitutive activity in the VTA. (**A**) Top: Representative mIPSC traces in the absence and presence of 0.1 µM forskolin (FSK). Bottom: FSK increased the baseline frequency of mIPSCs onto VTA dopamine neurons compared to control. (**B**) Differential effects of the MOR neutral antagonist CTOP and the inverse agonist KC-2-009, in the absence and presence of FSK. Percentage change is with respect to the stabilized FSK-containing baseline. FSK pretreatment enhanced the effect of KC-2-009, whereas it did not change the effect of CTOP. Number of experiments indicated in parentheses. **p<0.05; ***p < 0.001.*

pretreatment with FSK (n=6, 190.78±24.17% of the stabilized FSK-containing baseline) led to an increased effect of KC-2-009 on mIPSC frequency, as indicated by a significant Pretreatment x Acute Drug Effect interaction ($F(1,10)=6.32$, $p<0.05$, Repeated Measures ANOVA, Fig. 5*B*). Again, no effects on amplitude were observed ($F<1$ for main effects or interaction, data not shown). Acute administration of the neutral antagonist CTOP after FSK treatment (n=4) or control (n=9) did not increase mIPSC frequency ($F<1$ for main effect of CTOP or interaction with FSK pretreatment, Repeated Measures ANOVA, Fig. 5*B*). Therefore, the enhanced effect of KC-2-009 was not due to either FSK-induced release of endogenous opioids or an ongoing effect of FSK itself. Instead, these data indicate that when the cAMP cascade is activated, GABAergic inputs onto VTA dopamine neurons are enhanced, as also is the case during morphine withdrawal. Moreover, this aspect of morphine withdrawal is sufficient to increase MOR constitutive activity in the VTA.

DISCUSSION

MOR signaling in the VTA plays an important role in reward processing and aspects of addiction[247-249], including opiate withdrawal[169,194]. We now show that constitutive agonist-independent MOR activation regulates GABAergic control of VTA dopamine neurons. Furthermore, such MOR constitutive activity is especially prominent during morphine withdrawal.

We demonstrate that MOR-dependent inverse agonism by KC-2-009 on mIPSC frequency in the VTA is best explained by suppression of constitutive MOR activity. Indeed, the effect of KC-2-009 was reliably blocked by the selective MOR neutral antagonist CTOP. While KC-2-009 also has low affinity for the KOR[181], its inverse agonistic effect was not blocked by the selective KOR antagonist Nor-BNI. Moreover, the effect of KC-2-009 was not due to interference with endogenous opioid signaling, since CTOP had no effect on mIPSC frequency itself. Finally, pretreatment with KC-2-009 blocked the effect of DAMGO, suggesting they act on a similar target (i.e. the MOR). Together these data show that a population of MORs in the VTA is constitutively active and involved in regulating inhibitory GABAergic transmission onto dopamine neurons in the VTA. This finding is in accordance with the report that MOR inverse agonism at the level of G

protein binding and adenylate cyclase activity occurs in morphine-experienced mouse brain homogenates, including punches of the midbrain, but not in MOR knockout animals[183]. Such findings suggest that constitutively active MORs and agonist-activated MORs may share much of the same downstream signaling cascade. However, future experiments need to validate whether the pathway used by constitutively active MORs in regions like the VTA is similar to that of, for instance, DAMGO-activated MORs.

The physiological occurrence of MOR constitutive activity is highly relevant for certain aspects of drug addiction. It has been proposed that MORs become increasingly constitutively active during morphine withdrawal as an adaptation to the sudden lack of morphine that the system has grown accustomed to[258]. Such enhanced MOR constitutive activity can last for more days when the exposure to opiates has been greater[183]. We indeed observed that after 3-4 days of withdrawal from chronic morphine treatment, the effect of the MOR inverse agonist KC-2-009 on GABAergic inputs to VTA dopamine neurons was strongly enhanced. Such enhancement of MOR inverse agonism after withdrawal from a MOR agonist also occurs in artificial cell systems and in brain homogenates[183,184]. Interestingly, we did not observe a pronounced increase in the efficacy of KC-2-009 after only 12 hour abstinence from morphine. This suggests that the changes in MOR constitutive activity are not a direct response to the chronic administration of morphine itself, but become pronounced later in the withdrawal period. We also observed that the effect of the MOR agonist DAMGO was diminished during morphine withdrawal, as has been reported earlier[265]. This suggests a withdrawal-induced shift towards more constitutively active MORs, allowing for less additional agonist-induced activation. Therefore, enhanced MOR constitutive activity may also contribute to the opiate tolerance that develops after chronic use[271]. Since our finding indicates that enhanced MOR activity by an agonist can increase MOR constitutive activity, we cannot entirely rule out that the magnitude of constitutive MOR activity in our slice preparation was relatively heightened by the release of opiates during euthanasia. However, it is important to note that there is strong evidence to suggest that MOR constitutive activity also occurs in freely moving animals[21,184,257,258], supporting its physiological relevance.

A critical component of morphine withdrawal is the occurrence of cAMP superactivation, which is likely responsible for the negative withdrawal state[169,194,272]. This cAMP increase may in itself trigger adaptive counteractive pro-

cesses, like enhanced MOR constitutive signaling, to suppress cAMP[180,183,254,258]. Indeed, cAMP-dependent kinases have been reported to increase MOR phosphorylation, thereby elevating constitutive activity[257,258]. In accordance with such findings in artificial cell systems, we now show that acute treatment with forskolin to activate the cAMP-pathway is sufficient to enhance MOR inverse agonism in the VTA. The exact molecular cascade of events through which enhanced adenylate cyclase activity elevates MOR constitutive activity requires further elucidation. It also remains to be determined whether continued elevation of cAMP levels is required for the enhanced MOR constitutive activity, or if the changes in MOR constitutive activity represent a sequential step that can even outlast the changes in cAMP levels.

Compounds like naloxone and naltrexone are currently commonly prescribed to treat opiate overdose and addiction. However, these ligands act like MOR inverse agonists in systems previously exposed to opioids, and produce serious adverse effects[13]. There are strong indications that a hypofunctional dopamine system stands at the root of many of these negative effects[189,191-194,269]. Our findings confirm previous reports that during morphine withdrawal, GABAergic signaling onto VTA dopamine neurons is enhanced[169,194]. This is likely an important contributor to the hypofunctionality of VTA dopamine neurons during morphine withdrawal. Notably, morphine withdrawal is also associated with alterations in the nucleus accumbens (NAc) shell, a prominent target region of the VTA. Extended (10 days) but not acute (12 hours) withdrawal from a similar morphine treatment schedule was found to enhance both the excitability of medium spiny neurons (MSNs), as well as their glutamatergic input[273]. Interestingly, in another study morphine withdrawal was linked to enhanced c-Fos expression specifically in MSNs in the NAc that express the dopamine 2 (D2), rather than the dopamine 1 (D1) receptor[274]. Since dopamine signaling oppositely affects excitability of D2R-MSNs and D1R-MSNs[49,275], morphine withdrawal-induced VTA hypofunctionality[154,168,189-193], would likely shift the balance in the NAc towards more activation of D2R-MSNs. Whereas striatal activation of D1R-MSNs seems to represent rewarding processes, activation of striatal D2R-MSNs appears to be more linked to anti-reward or aversive processes[39,45,274,276]. Interference with constitutively active MORs on GABAergic terminals to VTA dopamine neurons would be expected to even further diminish dopamine signaling in regions like the NAc shell, leading to a further shift towards activity in D2R-MSNs: a potential correlate for the expression of adverse symptoms. Indeed, enhancing

dopamine signaling in the NAc shell ameliorates anxiety-like symptoms caused by morphine withdrawal[277]. Despite the importance of this particular VTA projection to the NAc shell, it is clear that morphine withdrawal is a multi-facetted state to which many other brain regions also contribute, including for instance the amygdala, and periaquaductal gray[278-280]. Interestingly, MORs serve important regulatory functions in such regions as well[146,279]. This opens up the possibility that constitutively active MORs in these regions also serve an important role in morphine withdrawal that may be affected by MOR inverse agonists.

Although MOR neutral antagonists and inverse agonists can both produce opiate withdrawal effects (e.g. jumping, tremors and place aversion), there is now strong evidence that neutral MOR antagonists do so to a lesser extent than inverse agonists[21,184,257,258]. MOR neutral antagonists therefore have the potential to be safer therapeutic agents to treat both opiate addiction and overdose. These reports connect well with our current findings that MOR constitutive activity regulates GABAergic signaling in a key brain region involved in opioid withdrawal[189,191-193,269].

Chapter 5

IDENTIFIABLE VTA DOPAMINE SUBPOPULATIONS ARE DIFFERENTIALLY AFFECTED BY THE OREXIN/HYPOCRETIN SYSTEM

Frank J. Meye
Eljo Y. van Battum
Roger A. H. Adan
Geert M. J. Ramakers

(Submitted)

ABSTRACT

Orexin A is a neuropeptide involved in behaviors particularly relevant for drug abuse and hedonic feeding. To an important extent the orexin system affects these behaviors by impinging on the mesolimbic dopamine system. Orexins excite dopamine neurons in the VTA and enhance their glutamatergic input. In the current study we show that GABAergic projections to VTA dopamine neurons are also subject to modulation by orexin A. However, this regulation is heterogeneous, leading to increases in GABAergic inhibition in some neurons, and decreases in others. The type of effect of orexin A could be determined *a priori* using the variables cell capacitance (reflecting cell size), the magnitude of the hyperpolarization-activated current (I_h), and the anatomical position of the cell within the VTA. We show that orexin A increases GABAergic inhibition in a population of large VTA dopamine neurons, with a large I_h, predominately in the lateral VTA. Conversely, orexin A either reduces GABAergic inhibition, or does not affect it, in a population of small dopamine neurons in the medial VTA with a small I_h. Differential effects of orexin A were also observed on action potential frequency. Whereas a population of small VTA dopamine neurons was excited by orexin A, another population of large VTA dopamine neurons was not. These findings offer important insights in the way the orexin system regulates the dopamine reward system.

INTRODUCTION

The hypothalamic orexins/hypocretins are neuropeptides with an established role in wakefulness and feeding[281,282]. Interestingly, it has recently become apparent that these neuropeptides exhibit close crosstalk with the dopamine system[283-285]: a network involved in reward processing[25]. This is likely in part mediated by the direct projection to the VTA from the hypothalamic neurons that express these neuropeptides[286-288]

The orexin system in the mesolimbic dopamine system is involved in the rewarding aspects of drugs of abuse as well as those of palatable food[7,285,289,290]. Accordingly, orexins typically excite VTA dopamine neurons[283,291]. This is in part due to direct effects on orexin 1 and 2 receptors located on VTA dopamine neurons themselves[284,291]. However, orexins also modulate the synaptic inputs to these neurons. In particular NMDA receptor mediated glutamatergic transmission is acutely enhanced by orexin A[61,290], and such modification is further enhanced by exposure to either cocaine or high fat food[290]. These findings suggest that the effects of orexin on glutamatergic synapses in the VTA play an important role in reward processing. It remains unclear however, how orexins regulate GABAergic control of VTA dopamine neurons, which also plays a vital role in processing salient stimuli[292-294]. We approached this issue in the current study, with a combination of slice physiology and pharmacology.

METHODS

Animals

In all experiments C57Bl/6-Jola mice (The Jackson Laboratory, USA) ranging from postnatal day 13 to 21 were used. Mother and pups were held together in standard transparent, iron grid-roofed, Plexiglas cages and were provided with a paper tissue as nest material. The animals were housed under a 12-hour light/dark cycle with lights on at 7.00h and off at 19.00h. Housing conditions were 21˚C and 65% humidity. Mothers were fed ad libitum with normal chow and had unlimited access to drinking water. All procedures were carried out in agreement with Dutch laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Preparation of brain slices

Mice were anaesthetized with isoflurane and quickly sacrificed through decapitation. Brains were rapidly removed and placed in ice-cold carbogenated (95% O_2; 5% CO_2) enriched artificial cerebrospinal fluid (ACSF) containing in mM: 124 NaCl, 3.3 KCl, 1.2 KH_2PO_4, 2.6 $MgSO_4$, 2.5 $CaCl_2$, 20 $NaHCO_3$, 10 D-glucose and 1 ascorbic acid, pH=7.4. Horizontal slices of 200 µm thickness were cut using a vibratome (Leica VT100S, Leica Microsystems, Germany). After dissection, the slices were transferred to a holding chamber and incubated for one hour at 32˚C in carbogenated (95% O_2; 5% CO_2) standard ACSF (1.3 mM $MgSO_4$, no ascorbic acid).

Electrophysiology

Whole-cell patch-clamp recordings were performed at room temperature with an EPC-10 patch-clamp amplifier (HEKA Electronics, Germany) and Patchmaster v2x32 software (HEKA Electronics, Germany). Slices were placed in the recording chamber and were constantly submerged in carbogenated (95% O_2; 5% CO_2) standard ACSF at the rate of 3 ml/min through a mini peristaltic pump system (Ismatec, Switzerland). Cells were visualized using infrared differential interference contrast video microscopy (Olympus BX61WI, Olympus Optical Co., Germany). Patch-pipettes were made of borosilicate glass (Harvard apparatus, USA) using a horizontal pipette puller (P-87, Sutter instruments, USA). Pipette resistance varied between 3.5 and 5.0 MΩ. For recording miniature inhibitory postsynaptic currents (mIPSCs) we used an internal medium containing in mM: 78 K-Gluconate, 77 KCl, 10 HEPES, 1 EGTA, 2 ATP and 0.4 GTP, pH=7.4. For recording action potentials (APs) the pipette medium contained in mM: 155 K-Gluconate, 10 HEPES, 1 EGTA, 2 ATP, 0.4 GTP.

The location of the VTA was identified as laterally from the interpeduncular fossa and medially from the medial terminal nucleus of the accessory optic tract (MT). VTA dopaminergic neurons were identified by the presence of a hyperpolarization-activated current (I_h) of at least 100 pA evoked by a voltage step from -60 to -120 mV. During mIPSC recordings, neurons were voltage-clamped at -60 mV. To isolate mIPSCs, TTX (1 µM) was added to the ACSF to block voltage-dependent Na^+-channels, and DNQX (10 µM) was added to block AMPA/kainate receptors. During AP recordings, neurons were initially injected with enough current to keep the membrane potential at approximately -60 mV. Neurons were clamped on that value for the remainder of the experiment when drug effects were as-

sessed. Afterwards, the position of the recorded cells was drawn into a schematic template from a mouse brain atlas[295], while blind to the effect of orexin.

Drugs

Orexin A, TTX and DNQX were all purchased from Tocris (Bristol, UK). Drugs were dissolved in distilled water and stored in aliquots at –20˚C before use. All drugs were bath-applied.

Data analysis

Membrane capacitance was calculated in Patchmaster v2x32 software (HEKA Electronics, Germany), using a rectangular test pulse (5 mV, 5 ms) to evoke transient capacitive currents. In a separate experiment we validated that such calculated capacitance values give a good indication of cell size, by correlating them with direct estimation of neuronal somatic surface area. To this end we photographed neurons with Qcapture v3.1.1 (Qimaging, 2007) at 800X magnification with differential interference contrast microscopy (Olympus BX61WI, Olympus Optical Co., Germany). We then calculated the surface circumscribed by the traced contours of the somata of these neurons in ImageJ v1.34s (NIH, USA), while being blind to the associated calculated membrane capacitance value. This calculation yielded a high and significant correlation with the cell capacitance estimates ($r=0.75$, $p<0.001$, Pearson Correlation; $n=93$; data not shown).

H-currents were evoked by a protocol of six hyperpolarization steps from a -60 mV baseline to values ranging from -70 to -120 mV (2 seconds per step). In order to calculate the maximal I_h, a single exponential fit was made on the current response to the -120 mV voltage step (Igor Pro, Wavemetrics, Lake Oswego, OR, USA), using the equation: $f(x) = y0 + A*\exp(-(x-x0) / \tau)$. Here y0 represents the asymptotic current response to the voltage step, which was subtracted from the initial current (50 ms after the voltage step) to obtain the maximal I_h.

APs were detected using MiniAnalysis software (Synaptosoft, Decatur, CA, USA). Characterization of AP kinetics was performed in Igor Pro (Wavemetrics, Lake Oswego, OR, USA). For kinetics calculations it was necessary to determine the start of an AP. To find this value we differentiated the recorded voltage trace and determined when the differentiated AP exceeded a threshold value of 20 mV/ms[296]. The 'half-width' of an action potential was taken to be the time between the half-maximal value of the AP (calculated from AP start to apex) on the upstroke and the downstroke. Membrane resistance was calculated by de-

termining the IV-relationship in current clamp over a range of injected currents spanning from -100 pA to -50 pA.

Synaptic events were detected and analyzed using MiniAnalysis software (Synaptosoft, Decatur, CA, USA). Subsequently, histograms were made of the lognormal distributions of the frequency and amplitude of mIPSCs. To assess the mean and standard deviation of the underlying normal distributions, we fitted lognormal curves over the histograms (Igor Pro, Wavemetrics, Lake Oswego, OR, USA)[210,213]. In the applied equation: $f(x) = A*\exp[-0.5*(\ln X - \mu) / \sigma)^2] / (x*\sigma*\sqrt{2\pi})$, X is the measured instantaneous frequency or amplitude of an event, A is the area under the fitted curve, and μ and σ are the mean and standard deviation of the underlying normal distribution, respectively. To assess drug effects in electrophysiological experiments, Repeated Measures ANOVAs were performed on the means (μ) of such transformed normally distributed data. All statistical testing was performed in SPSS 17.0 (Chicago, IL, USA). To determine percentage changes, the mean of the lognormal distribution (m) was calculated using the equation: $m = \exp(\mu + \sigma^2)$[213].

RESULTS

Orexins directly affect glutamatergic synapses onto VTA dopamine neurons, likely with a presynaptic locus of effect[290]. We investigated whether a similar orexin-mediated regulation existed for GABAergic inputs. We isolated mIPSCs onto presumed VTA dopamine neurons ($I_h \geq 100$ pA). After a baseline period of at least 30 minutes, we administered orexin A at a concentration of either 100 nM or 200 nM. Within this population of VTA putative dopamine neurons, orexin A exerted a heterogeneous response on mIPSC frequency (Fig. 1*A*). Both type of responses (increases and decreases) were observed irrespective of the dose (100 or 200 nM), with comparable magnitudes. Therefore, these neurons were pooled together for subsequent analyses (n=15 for 100 nM, n=6 for 200 nM). Whereas in some neurons orexin A increased mIPSC frequency with more than 20%, a reduction in mIPSC frequency was observed in other cells. Amplitude was typically not affected (Fig. 1*B*).

We aimed to determine whether certain neuronal characteristics could predict the effect of orexin A. Important determinants of VTA dopamine neuron

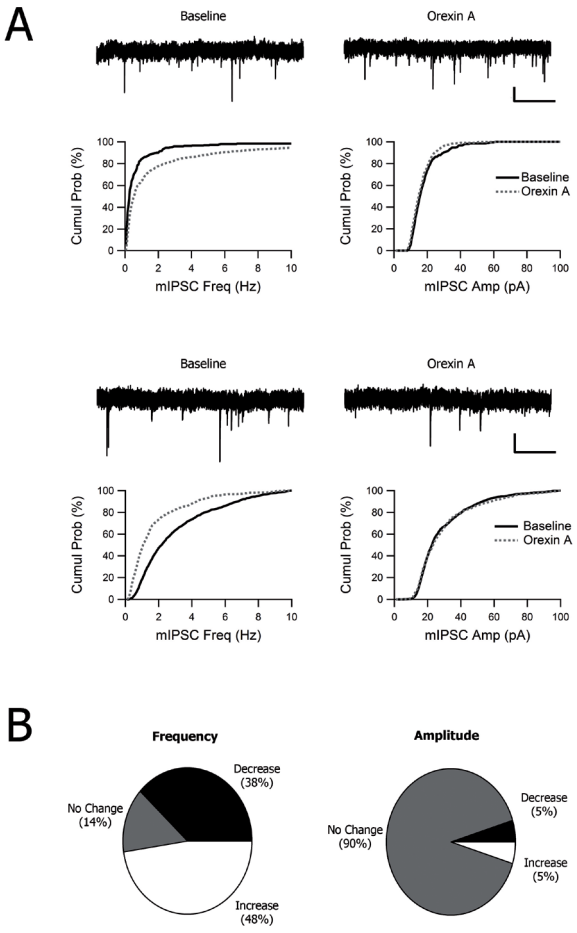

Figure 1

Orexin A exerts a heterogeneous effect on mIPSC input to VTA dopamine neurons. (**A**) An example of a VTA dopamine neuron in which orexin A increased the mIPSC frequency (top), and one in which it decreased this frequency (bottom). For each example representative mIPSC traces are shown during baseline and in the presence of 100 nM orexin (above). Scale bars indicate 20 pA (vertical) and 2 seconds (horizontal). Below the traces, exemplar cumulative probability plots are shown for both the mIPSC frequency (left) and amplitude (right). (**B**) An overview of the distribution of effects of orexin A on mIPSC frequency (left) and amplitude (right). Threshold levels for determining increases and decreases were a ≥ 20% change from baseline.

subsets include the size of the neuron, the magnitude of its I_h and its anatomical position[242]. We investigated whether the observed variance in the effect of orexin A on mIPSC frequency could partly be explained by incorporating such parameters. In order to assess cell size we estimated the capacitance of the membrane: a proportionally related variable. We later validated this approach in a separate set of neurons for which we also had direct estimates of cell surface (see methods). Cell capacitance was positively correlated with the effect of orexin A on mIPSC frequency ($r=0.44$, $p<0.05$, Pearson's R correlation; n=21; Fig. 2*A*). This reflected that orexin A typically increased mIPSC frequency onto larger VTA dopamine neurons in our sample, whereas such inputs were decreased or not affected in smaller cells. We also observed that the effect of orexin A on mIPSCs seemed to vary according to the anatomical position of the neuron alongside the medial-lateral axis. There was a strong trend of a significant positive correlation between the effect of orexin A on mIPSCs and how lateral the neuron was

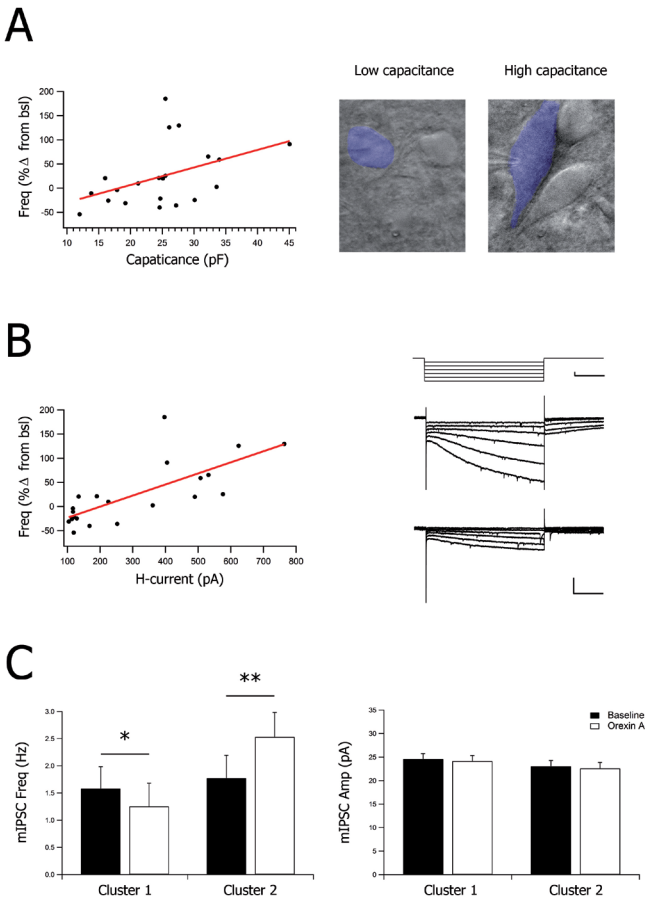

Figure 2

Cellular markers predict the type of effect orexin A exerts on mIPSC frequency in the VTA. (**A**) Left: The effect of orexin A on mIPSC frequency correlated with the membrane capacitance. Right: Examples of patched neurons (in pseudo-blue) with a small and a large recorded membrane capacitance. (**B**) Left: The correlation between the effect of orexin A and the size of the I_h. Right: Examples of I_h for the different response types to orexin A. In response to the I_h protocol (right, top, vertical scale bar indicates 10 mV, horizontal scale bar indicates 0.5 second) neurons in which orexin A increased mIPSC frequency typically expressed a large I_h (mid), whereas neurons in which orexin A decreased or did not affect mIPSCs showed a relatively smaller I_h that was still at least 100 pA (low). Scale bars indicate 20 pA and 0.5 second. (**C**) An unbiased clustering approach on membrane capacitance, I_h and medial-lateral position yielded two separate clusters of VTA dopamine neurons. In Cluster 1 orexin A reduced mIPSC frequency, whereas in Cluster 2 it increased their frequency (left). In neither cluster did orexin A affect the amplitude of the mIPSCs (right).

positioned (r=0.42, p=0.06, Pearson's R correlation; n=21). No such correlations were observed for the anatomical position along the anterior-posterior (r=0.10, p=0.66; Pearson's R correlation; n=21) or the dorsal-ventral axes (r=0.09, p=0.72; Pearson's R correlation; n=21). Finally, the size of the I_h was highly related to the effect of orexin A on mIPSC frequency (r=0.74, p<0.001, Pearson's R correlation; n=21; Fig. 2*B*). In neurons with a large I_h, orexin A typically increased mIPSC frequency, whereas in cells with a smaller I_h (but still ≥ 100 pA) orexin A typically either reduced mIPSC frequency or was ineffective. The I_h was not simply greater due to the cells being larger with a proportional increase in channel

numbers due to larger surface area. When we assessed I_h density, by dividing the I_h magnitude by the membrane capacitance, we observed that this corrected variable was also strongly correlated with the effect of orexin A (r=0.68, p=0.001, Pearson's R correlation; n=21).

We determined whether the three variables of I_h, membrane capacitance and medial-lateral position could be used *a priori* to predict the effect orexin A would exert on GABAergic input to a particular VTA dopamine neuron. We performed an unbiased cluster analysis (SPSS 17.0, Chicago, IL, USA; Schwarz's Bayesian Criterion), which yielded two separate clusters of cells. Cluster 1 consisted of 11 neurons with relatively low membrane capacitance (20.04 ± 1.66 pF), a small I_h (139.67 ± 11.51 pA), and a relatively medial position in the VTA (0.54 ± 0.01 mm from the midline). Cluster 2 consisted of 10 neurons with significantly higher membrane capacitance (30.18 ± 1.98 pF, $F(1,19)=15.59$, $p=0.001$, One-Way ANOVA), a large I_h (490.85 ± 46.11 pA, $F(1,19)=59.60$, $p<0.001$, One-Way ANOVA), and a more lateral position in the VTA (0.72 ± 0.04 mm from the midline, $F(1,19)=18.21$, $p< 0.001$, One-Way ANOVA). We assessed the effect of orexin A on mIPSC frequency in these two clusters and observed a cluster-specific effect ($F(1,19)=14.16$, $p=0.001$, Repeated Measures ANOVA, Drug x Cluster interaction, Fig. 2D). Specific contrasts showed that in Cluster 1, orexin A reduced the frequency of mIPSCs ($F(1,10)=5.02$, $p<0.05$), whereas in Cluster 2 it increased their frequency ($F(1,9)=17.33$, $p<0.01$). A linear regression analysis suggested that the predictors Membrane capacitance, I_h, and medial-lateral position, together explained 56% of the variance of the effect of orexin A on mIPSC frequency.

Given the subset-specific effects of orexin A on GABAergic input to VTA dopamine neurons, we wondered whether similar heterogeneity could be observed at the spike output level of identifiable VTA dopamine neuronal subsets. Indeed, while orexins are known to excite VTA dopamine neurons, they do not uniformly do so[291]. We patch clamped a set of VTA neurons with an I_h of at least 100 pA and recorded the AP frequency of these neurons during baseline and in the presence of orexin A (100 nM). We observed that orexin A also produced a heterogeneous effect on action potential frequency. In some neurons, orexin A increased the frequency, whereas in others we did not observe a change in firing (Fig. 3*A*). We again attempted to use predictive parameters to explain this variance. It turned out that this set of neurons was overall sampled from more lateral parts of the VTA (0.82 ± 0.03 mm; n=21) than the previous set (0.62 ± 0.03 mm; n=13; $F(1,33)=20,22$, $p<0.001$, One-Way ANOVA). In this set of neurons

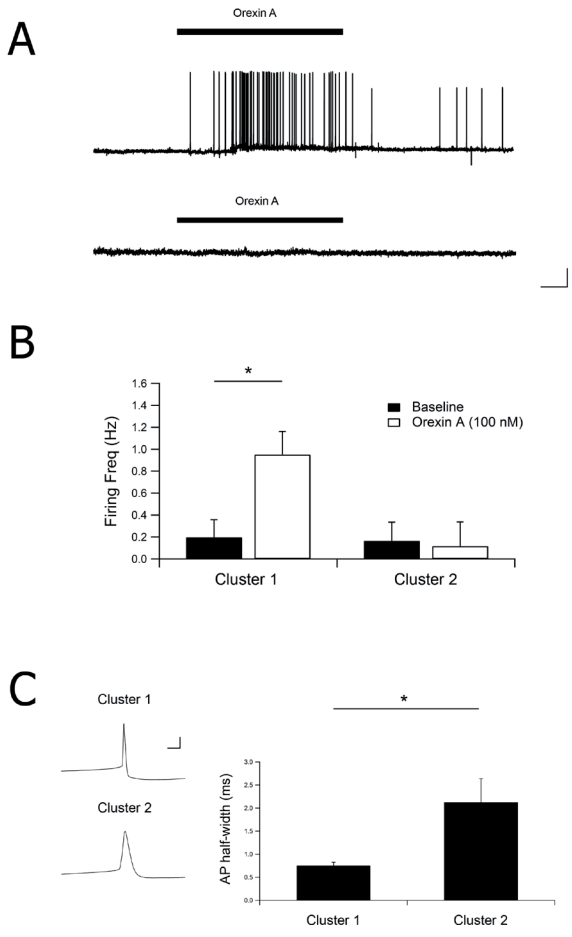

Figure 3

Orexin A exerts a heterogeneous effect on VTA dopamine neuron action potential frequency. (**A**) In response to orexin A, some neurons increased their firing frequency (top), whereas others did not (bottom). Scale bars indicate 20 mV and 1 minute. (**B**) An unbiased clustering on the membrane capacitance again yielded two separate clusters. In Cluster 1 (with relatively low membrane capacitance) orexin A increased firing frequency, whereas it did not in Cluster 2. (**C**) These clusters also differed notably in the width of the action potential. Representative examples of action potentials for both clusters are shown left. Scale bars indicate 20 mV and 4 ms. The overall difference in width is shown right.

neither anatomical positioning (r=0.20, p=0.56, Spearman's rho) nor I_h (r=0.24, p=0.44, Spearman's rho) was correlated to the effect of orexin A. However, membrane capacitance exhibited a clear negative correlation (r=-0.61, p<0.05, Spearman's rho). We performed the unbiased cluster analysis (SPSS 17.0, Schwarz's Bayesian Criterion) using membrane capacitance as the sole determinant, which again yielded two clusters of neurons. Cluster 1 consisted of 7 neurons with low membrane capacitance (17.90 ± 0.87 pF), while Cluster 2 consisted of 6 neurons with a significantly higher membrane capacitance (30.20 ± 2.17 pF, F(1,11)=27.27, p<0.001, One-Way ANOVA). There was a subset-specific effect of orexin A on AP frequency across clusters (F(1,11)=4.90, p<0.05, Repeated Measures ANOVA, Drug x Cluster interaction; Fig. 3B). This interaction reflected that in the population of relatively small cells (i.e. low membrane capacitance) orexin A significantly increased the AP frequency. Contrarily, there was no such effect in the population of relatively bigger cells (i.e. high membrane capacitance).

Of additional interest was that the APs were considerably more narrow in Cluster 1 (0.74 ± 0.07 ms) than in Cluster 2 (2.38 ± 0.59 ms; $F(1,11)=7.93$, $p<0.05$; Fig. 3C). This may at least in part be due to the difference in membrane capacitance, although the overall correlation between AP width and membrane capacitance fell short of significance ($r=0.51$, $p=0.08$, Pearson's R correlation; $n=13$). We also observed a strong trend towards a difference in membrane resistance in the two clusters, as cells from Cluster 1 generally had lower membrane resistance (221.00 ± 30.37 MΩ) than neurons from Cluster 2 (407.50 ± 84.91 MΩ; $F(1,11)=4.85$, $p=0.05$, data not shown). Overall these findings highlight the existence of two separate clusters of putative dopamine neurons in the VTA with differential responses to orexin A.

DISCUSSION

Orexins impinge on the mesolimbic dopamine system and serve an important role in the goal-directed pursuit of reward[283-285]. Orexins affect VTA dopamine neurons by direct excitation[291,297], and by increasing their excitatory glutamatergic inputs[61,290]. In this study we present evidence that orexin A also regulates GABAergic control of VTA dopamine neurons, but in a heterogeneous manner. We observed that orexin A increased GABAergic inputs to bigger VTA dopamine neurons, which were typically positioned in more lateral parts of the VTA and generally also possessed a large I_h. In smaller VTA dopamine neurons with a relatively smaller I_h, which were also generally more medially positioned, orexin A either did not affect GABAergic inputs or decreased them. We further observed that orexin A generally excited smaller, but not larger VTA dopamine neurons. Together, these findings suggest that orexin A excites a population of small VTA dopamine neurons and reduces their GABAergic inhibition. Contrarily, orexin A does not excite another subpopulation of larger VTA dopamine neurons, and increases their GABAergic input.

Our findings indicate that orexin A differentially affects VTA subpopulations. The characteristics of these subgroups are highly reminiscent of ones identified by a previous report, which identified a medial VTA subpopulation of small calbindin-expressing dopamine neurons with a small I_h, and another lateral VTA subpopulation of large calbindin-negative dopamine neurons with a large I_h[242].

Notably, across the two experiments described in our study, only membrane capacitance adequately accounted for the variance of the orexin A effect in both the mIPSC and AP experiments. This suggests that neuronal size is an adequate predictor for the overall effect of orexin A on a dopamine neuron. In line with this, a previous study reported that intraventricular orexin infusion predominately raised the amount of Fos-positive small, rather than large, VTA dopamine neurons. These small neurons were especially present in the caudomedial part of the VTA, but not exclusively so, as both large and small neurons were found throughout the VTA[298]. This finding could potentially account for why the effect of orexin A on GABAergic inputs to VTA dopamine neurons correlated well with the anatomical position and I_h, as small cells are simply more abundant in medial parts of the VTA, where H-currents are also generally smaller[242].

VTA subpopulations differ in their projection targets[232,298,299]. In this light it is interesting that orexin A was shown to increase dopamine signaling in the prefrontal cortex (PFC) and in the nucleus accumbens (NAc) shell, while no changes were observed in the NAc core[284,300]. Our subgroup of large VTA dopamine neurons in which orexin A increased GABAergic signals, while not increasing the firing frequency, may have therefore predominately projected to the NAc core. The other subpopulation of smaller VTA dopamine neurons, in which orexin A did not increase GABAergic inputs, and increased the firing frequency, may have projected more to regions such as the PFC and the NAc shell. In support of this notion, an immunohistochemical study observed that neurons projecting to the NAc shell and PFC are generally smaller than those projecting to the NAc core[298]. The distinction in the effect of orexin on dopamine signaling in the NAc shell and core is noteworthy. The functional differences between NAc core and shell are still uncertain, but the NAc shell has been put forward as a region that is especially involved in motivational and rewarding aspects of both food and drugs of abuse[43,301]. There is evidence that orexin projections directly target the NAc, but mainly connect with the shell rather than the core[285]. Together, this suggests a circuit in which enhanced activity of orexin neurons in regions like the lateral hypothalamus elevates orexin levels in the NAc shell directly, and also raises dopamine levels in the NAc shell via activation of VTA neuronal subsets. Such convergence of orexin and dopamine would then not occur in the NAc core. In the NAc shell, the simultaneous administration of orexin B and dopamine altered the firing frequency at least additively, and possibly synergistically[302]. However, it remains to be determined how convergent dopamine signaling in the NAc shell shapes the effect of orexin on explicit reward behaviors.

Our findings provide further insights in the mechanism by which the heterogeneous effects of orexin in the VTA can come about. However, the exact signaling mechanisms utilized by orexin A in the different VTA subpopulations remain elusive. The effects of orexin A on mIPSC frequency rather than amplitude suggest a presynaptic expression of the effect. It is unclear however, where the induction of the effect takes place. It is possible that the induction and expression are both presynaptic. For instance, the increase in mIPSC frequency in larger VTA dopamine neurons could occur due to orexin A targeting orexin receptors located specifically on GABAergic nerve terminals synapsing on such large neurons. The heightened release of GABA could then occur due to heightened calcium signaling in the nerve terminal[303]. The decrease in mIPSC frequency in relatively smaller VTA dopamine neurons could also have a presynaptic locus of induction. For instance, orexin 2 receptors have been shown to be able to non-canonically couple to the inhibitory $G_{i/o}$ instead of G_q proteins[304]. Alternatively, the observed effects of orexin A on mIPSC frequency could have a postsynaptic site of induction. In this case, a secreted retrograde messenger could translate the postsynaptic induction into presynaptic expression. Among the candidate retrograde messengers are nitric oxide, arachidonic acid and endocannabinoids[305]. The orexin system is known to be associated with all these systems[306-308]. There is evidence that endocannabinoids and nitric oxide occur in the VTA[86,309]. Of note is that nitric oxide may especially be prevalent in dopaminergic neurons in the medial VTA[309], which could indicate a role in the effects of orexin A on smaller dopamine neurons, which predominate in this part of the VTA[298]. Further experiments need to address the exact molecular cascades involved in the differential effects of orexin A in the VTA.

Overall, we have shown that different subpopulations of VTA dopamine neurons respond differently to orexin A. In relatively smaller VTA dopamine neurons, orexin A decreases the GABAergic inputs or left it unaltered, and these neurons increase their firing frequency. Contrarily, in bigger VTA dopamine neurons, orexin A increased the GABAergic input, and these neurons did not fire more. These findings offer important insight in the working mechanisms of orexin A in the neural circuitry of the reward system.

Acknowledgements

The authors would like to thank Top Institute Pharma (D1-105) for funding this project.

Chapter 6

GENERAL DISCUSSION

1. Context

This thesis emerges against the backdrop of two separate and alarming developments: (1) The high global prevalence of both obesity and drug addiction: problems that greatly impact afflicted individuals and society as a whole[182,310]. (2) The general inefficiency in the therapeutical drug design process, where many potentially useful pharmaceuticals fail to become clinically applied[311]. The findings in this thesis bear on both of these issues. We identify new activation mechanisms of known drug targets, and show how this knowledge may be implemented for therapeutic gain.

1.1 Obesity and drug addiction

Obesity and drug addiction - while disparate at the surface - share a lot of main features. Conceptually, both revolve around the overcommitted pursuit of transiently pleasurable stimuli that become detrimental when overused. Mechanistically, both problems are rooted in the neural circuitry normally dedicated to optimize chances of survival and procreation (i.e. the mesolimbic reward system)[3,312]. Despite knowing that overconsumption of food or drugs of abuse is bad, the afflicted individual (i.e. the obese patient or drug addict) is still driven towards them, exactly because those stimuli so potently hijack the neural reward system[2], with serious health consequences as a result[195,310].

Remedying this situation is no easy fix. Repressive governmental policies aimed to reduce the availability of drugs of abuse have often turned out to be extremely limited in their efficacy at best[313]. The onus should therefore perhaps instead be on the individual (and its brain). Those that have succumbed to uncontrollable substance abuse and food intake may benefit most from proper counseling, supplemented with adjunctive pharmaceutical intervention to help facilitate the choice for a healthier life style[314,315]. However, it is exactly in the realm of such adjunctive drug therapy that many advances still need to be made.

1.2 The inefficiency of the drug design process

In recent decades the money spent on drug design has increased, while the output of effective and safe -and therefore useful- therapeutic agents has at best remained equal. A cause for this may be the inclination of pharmaceutical companies to preferentially switch to new and speculative drug targets, as opposed to refining compounds for known targets[311]. There are clear problems with this approach, as therapeutics that did not make the cut but which could

have been truly optimized after elaborate refinement, may now be discarded –together with the target- as a whole.

The cannabinoid 1 receptor (CB1R) 'antagonist' rimonabant serves as a striking example in this respect. Given the known involvement of the endocannabinoid system in appetite, it was reasonable to assume that interference with this system would be useful in the treatment of obesity[117]. When it became evident that rimonabant not only reduced food intake and weight, but was also associated with serious psychiatric problems in a subset of users[24], this spelled the end of not only rimonabant but likely of CB1R antagonists in general. However, at the time there were already strong indications that rimonabant was not a very typical antagonist, but rather an inverse agonist that also suppressed agonist-independent constitutive activity of CB1Rs, at least in artificial systems[14,16,18,19,316]. Given that rimonabant effectively has a dual mode of action: (1) interference with endocannabinoids signaling at the CB1R, and (2) interference with constitutive agonist-independent CB1R activity, it was unknown how much either mechanism contributed to the beneficial and the adverse effects. All the more so since it remained largely unclear whether constitutive CB1R activity was anything more than an artifact encountered in essays remote from physiological conditions. This kind of information was lacking, but is for obvious reasons vital to proper drug design, especially if we consider that there are now neutral CB1R antagonists available that do selectively interfere with just endocannabinoid-CB1R signaling, leaving CB1R constitutive activity intact[203].

Using rimonabant as an example, we arrive at the conclusion that discontinuing a drug because of its potential safety hazard is evidently necessary, but abandoning an entire target without an exact understanding of its actual working mechanisms, can be wasteful. In this thesis we have sought to understand how some important neuromodulatory targets, namely the cannabinoid, opioid and the orexin system, regulate the functionality of the mesolimbic dopamine system. This neural circuit is not only involved in both drug addiction and obesity, but also in potential realms for psychiatric side effects, such as depression and anxiety[3,127,128,203,207-209,312,317]. This makes it crucially important that pharmaceuticals impinging on this system do so in a manner balanced enough to prevent adverse effects. This in turn requires a precise understanding of how the targeted neuromodulators regulate this system.

2. Summary of thesis results

The current thesis is dedicated to describing the effects of the neuromodulatory cannabinoid, opioid and orexin systems on the reward network, in particular in the ventral tegmental area (VTA). These neuromodulators are involved in both feeding and processing drugs of abuse, and are interesting therapeutic candidates to combat these afflictions, but many important aspects of the control they exert in the reward system remain unknown. In this thesis we have expanded on this knowledge base.

Chapter 2:

We show in an *ex vivo* slice preparation that a population of CB1Rs in the VTA and the basolateral amygdala (BLA) are constitutively active in the absence of endocannabinoids. Aside from determining the physiological occurrence of this activation mechanism, which was previously unidentified in the reward system, we also establish that this has therapeutic implications. CB1R inverse agonists were marketed as weight loss agents, but were discontinued due to side effects like anxiety and depression. In behavioral experiments we demonstrate that the beneficial effects of CB1R inverse agonists (reduced food intake and body weight) stem from interference with endocannabinoids acting at CB1Rs. Conversely, we provide evidence that the adverse effects of CB1R inverse agonists (anxiety and anhedonia) stem from interference with CB1R constitutive activity. We make a case that CB1R neutral antagonists will be efficient weight loss agents devoid of many of the deleterious side effects associated with CB1R inverse agonists.

Chapter 3:

Having established that constitutive CB1R activity influences synaptic activity in the reward system, as well as reward behavior (Chapter 2), we now provide evidence that it also contributes to the activity of VTA dopamine (and non-dopamine) neurons. To this end we recorded action potentials in the VTA of freely moving rats, using stereotactically implanted tetrodes. This *in vivo* approach is powerful as it offers the high spatial and temporal resolution of electrophysiological measurement, in a fully intact neuronal circuit. We show that CB1R agonism has differential effects in two identifiable subpopulations of VTA neurons, preferentially exciting putative dopamine neurons and inhibiting putative non-dopamine neurons. We found that CB1R inverse agonism occurs within

these subpopulations, while the subpopulations are largely not subjected to tonic endocannabinoid signaling during such basal conditions. This points to a role of constitutive CB1R agonist-independent signaling in regulating the firing frequency of VTA neurons *in vivo.*

Chapter 4:

Mu opioid receptors (MORs) are functionally closely related to CB1Rs and often play an important role in similar behavior and physiology. Accordingly, we demonstrate in this chapter that MORs regulating GABAergic control over VTA dopamine neurons also exhibit constitutive activity. Strikingly, this activation mechanism becomes increasingly dominant during a state of morphine withdrawal. As such, it can partly counteract the withdrawal-induced increase in GABAergic signaling which is known to be associated with adverse withdrawal effects. We propose that MOR constitutive activity in the VTA during opioid withdrawal is a protective adaptive response that should not be abruptly interfered with, to minimize the detrimental withdrawal symptoms that facilitate relapse and addiction. This has important implications for the utility of the MOR inverse agonists that are currently in use for treatment of overdose and addiction.

Chapter 5:

In this final experimental chapter we investigate the role of the orexin system in the VTA. Orexins are neuropeptides that have been shown to be involved in both food intake and drug reward. However, evidence suggested that the effects of orexins may be dependent on specific subsets of VTA neurons. We reveal an identifiable cluster of VTA dopamine neurons in which orexin A reduces the inhibitory input and increases the firing frequency. Another identifiable subset of neurons is not activated by orexin A, and instead is subjected to increased inhibitory input by this neuropeptide. Based on available literature we argue that these different subsets have different projection targets in the brain.

3. Points of interest and limitations

3.1 Constitutive activity: how and why?

A major part of this thesis has been dedicated to showing the physiological occurrence and behavioral relevance of constitutively active CB1Rs and MORs. It is therefore relevant to speculate on why there even is constitutive activity of G protein-coupled receptors (GPCRs). After all, with the mere 'tools' of an endogenous agonist and endogenous receptors, it is already possible to exert an array of effects. For instance, if there is a basal tone of activation of the GPCR due to a basal tone of the endogenous ligand, it is already possible to bidirectionally modulate this system by either lowering or increasing the levels of the endogenous ligand, or of the receptor. What use then, does this additional layer of complexity of constitutive receptor activity provide?

There are a couple of positions that can be taken in regard to this question. One possibility is that constitutive GPCR activity simply automatically arises as a by-product of stochastic processes. Specifically, it could be the consequence of GPCRs continuously altering their conformations; a portion of those conformations being associated with downstream signaling. With such dynamism of the receptor population it would be expected that, at any given time, a proportion of receptors adopt conformations suited for signaling. This fraction of receptors then makes up the constitutive activity of the population[318].

Another possibility is that, given the multiple active conformations that GPCRs possess, it is possible that 'ligand-biased agonism' occurs. This entails that different types of agonists for the same receptor have different effects, as they induce different conformational states of the receptor, and consequently engage a different downstream molecular cascade[319]. Extending this idea, it would be possible that an endogenous GPCR system also implements this kind of flexibility by having its endogenous ligand produce an effect at the GPCR through downstream pathway X, while its constitutively active form signals through pathway Y. There is both some evidence against and for this idea. For instance, in artificial systems a CB1R agonist and inverse agonist were shown to make use of much of the same molecular cascade[16,18,19]. Contrarily, for the G protein-coupled melanocortin 4 receptor it was shown that the endogenous inverse agonist agouti-related peptide could also engage a different G protein than the agonist[320].

These latter findings highlight two things: that all inverse agonism/constitutive signaling may not be alike, but also that there are endogenous ligands that may be said to be inverse agonists. Interestingly one has been proposed for the CB1R: the haemoglobin-derived hemopressin, which is quite similar in many respects to rimonabant[321-323]. However, it remains to be determined if and how hemopressin actually plays a role in the regulation of CB1Rs in the brain. If it does, it would provide a mechanism by which constitutive activity can be endogenously modulated (although it will also modulate agonist-dependent signaling). There is also evidence emerging that constitutive activity can be endogenously modulated through other routes. For instance, studies suggest that cAMP-mediated phosphorylation of MORs can enhance their constitutive activity[257,258]. We have also provided indirect evidence in favor of this, as we observed a strong increase in MOR inverse agonism in the VTA after forskolin-induced enhancement of the cAMP cascade (Chapter 4). In general, such findings suggest that the extent and role of constitutive activity for GPCRs can be subject to modulation and are therefore likely tissue and region-specific.

3.1.1 Constitutive activity: limitations and future perspectives

One problem with constitutive activity is that it is difficult to establish through other means than by inverse agonists (versus neutral antagonists), which makes it somewhat difficult to corroborate by non-pharmacological means. Also, while you can pharmacologically suppress constitutive activity, it is less straightforward to selectively enhance it. Nevertheless, there are methods to address this issue. The extent of constitutive activity that a GPCR exhibits is related to its structure, which is why mutations can lead to receptors that are either more, or less, constitutively active[324]. It is therefore feasible to over-express a highly or lowly constitutively active form of, for instance, the MOR compared with an over-expressed standard MOR as a control. Subsequently, the physiological and behavioral effects of MOR constitutive activity may be investigated via a specific non-pharmacological approach. Such experiments would lend additional credence to the findings obtained with inverse agonists and neutral antagonists. When constitutive activity is assessed by pharmacological means however, we feel it is an important control to attempt to block an inverse agonistic effect with the neutral antagonist. We have done so for both the CB1R inverse agonistic effect of SR141716A (which was blocked by the CB1R neutral antagonist NESS0327), and for the MOR inverse agonistic effect of KC-2-009 (which was blocked by the MOR neutral an-

tagonist CTOP). By doing this important control, potential alternative explanations for the inverse agonism, such as off-target effects, are controlled for.

3.2 Extrapolation of these findings

Our experiments have mainly been performed in mice and to a lesser extent in rats. When extrapolating our findings to humans we do so based on the high degree of similarity between the rodent and human brain in terms of basic neuroanatomy and physiology. Moreover, manipulations of neuromodulatory systems like the cannabinoids have clear effects on behaviors such as food intake across species [325,326]. Finally, rodents can exhibit addictive behaviors[3,52]. It therefore stands to reason that rodent models of reward behavior and its underlying neural circuitry can be extrapolated to the human situation with reasonable confidence.

3.2.1 Extrapolation of these findings: Limitations and future perspectives

Despite the many similarities at the neuroanatomical and physiological level of the reward systems across species, rodent models are not a perfect substitute. This has important implications that should always be borne in mind. For instance, in Chapter 2 we refer to the potential of the inverse CB1R agonist rimonabant to induce loss of bodyweight, but also depression in humans[24]. Investigating these issues in animals is not free of complications, for different reasons, and to a varying extent. Bodyweight can obviously be recorded straightforwardly in humans and rodents alike, but it must be considered that the metabolism speed is quite different in these species. Therefore, the effects in rodents could easily manifest itself on a different time scale than in humans. Indeed, we observed that antagonism at the CB1R rapidly reduced food intake and body weight gain in rats. A meta-analysis of the effect of rimonabant in humans suggested that body weight was reduced, but did require committed use over an extended period of time[24]. This illustrates that while the animal model can be valid in qualitative terms of a behavior, there is no logical necessity to assume predictive validity for some of the quantitative aspects.

Measuring and extrapolating depression in an animal model is a problem of an entirely different caliber. Animal models for depression-like behaviors often revolve around the tendency of reduced pleasure (anhedonia) and drive (avolition). These phenomena are presumed to be at the core of a neuropsychiatric disorder like depression[135,136], and are much more readily accessible in an animal model than the actual disorder itself. Accordingly, in Chapter 2 we chose to as-

sess whether the CB1R inverse agonist rimonabant and the neutral antagonist NESS0327 affected the motivation for a reward. This was done to gain insight in whether or not these ligands could induce a state of avolition: reduced drive for something pleasurable. We did indeed find that rimonabant, but not NESS0327, reduced this drive. Such findings highlight a role for CB1R constitutive activity in hedonic processing and motivation. From that point of view there is a clearly delineated route that flows from: Use of rimonabant → Suppression of CB1R constitutive activity in reward processing regions → Disrupted activity in such regions → Reduced motivation and drive → Susceptibility for depression. However, it of course remains hard to definitively prove in animal models, in which only certain features of depression can be modeled rather than the whole complex constellation, that this route and not some other, is why rimonabant use is indeed associated with depression. If it is, then based on the animal experiments there is good cause to assume that compounds like NESS0327 are an improvement. However, this definitely requires more validation in different types of depression models and over longer periods of treatment.

4. Conclusions

Overall this thesis has provided new insight in the mechanisms by which the cannabinoid, opioid and orexin systems regulate neuronal communication in the reward system. In particular we have shown that the hitherto putative agonist-independent activation mechanism of CB1Rs and MORs does actually occur in the reward system. We have also shown that interference with this activation mechanism is associated with detrimental side effects, while it contributes little to the desired therapeutic effects. Consequently, we make the case for neutral CB1R and MOR antagonists which leave constitutive signaling intact. Finally, we have shown that both the neuromodulatory orexin and cannabinoid system can have very distinct effects on VTA subsets of neurons. We also provide tools to characterize these subsets *a priori*. In conclusion, this thesis has expanded the understanding of how important neuromodulators regulate the brain reward system, and has outlined strategies to therapeutically utilize these insights.

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Addendum

CURRICULUM VITAE

Frank Julius Meye was born on April 19th 1984 in Utrecht, The Netherlands. In 2002 he graduated from secondary school (Gymnasium, Dr. F. H. De Bruijne Lyceum, Utrecht), and studied Psychology (Cognitive Neuroscience track) at the University of Utrecht. After having obtained his Bachelor of Science degree in 2005 *(cum laude)*, he continued with a Master study in Neuroscience and Cognition at the same university. During this Master program he spent 6 months of internship under supervision of Dr. Geert Ramakers at the Rudolf Magnus Institute of Neuroscience in Utrecht; as well as 9 months of internship under supervision of Dr. Gregory DiGirolamo at the Department of Experimental Psychology in Cambridge, United Kingdom. In 2007 he obtained his Master of Science degree *(cum laude)*, and started a PhD project under supervision of Prof. dr. Roger Adan and Dr. Geert Ramakers at the Rudolf Magnus Institute of Neuroscience in Utrecht; a project financially supported by Top Institute Pharma (G protein-Coupled Receptor Forum). In May 2012 he joined the research group of Dr. Manuel Mameli at the Institut du Fer à Moulin in Paris, France. He was recently awarded a Fyssen Fellowship financially supporting two years of post-doctoral research at this institute.

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SAMENVATTING IN HET NEDERLANDS

OBESITAS EN VERSLAVING

Obesitas en drugsverslaving zijn zeer vaak voorkomende aandoeningen die een grote impact hebben op het individu en de samenleving als geheel. Oppervlakkig gezien zijn deze stoornissen het product van vrijwillige keuzes. Er is echter steeds meer evidentie dat deze keuzes vaak niet zo vrijwillig zijn en dat de problematiek in veel gevallen in het brein stoelt. Bepaalde hersensystemen zijn gespecialiseerd in het herkennen en verkrijgen van 'beloning' (bijv. voedsel). Een dergelijk beloningssysteem is heel praktisch onder de - historisch gezien frequente - omstandigheid van voedselschaarste. De schaduwzijde ervan komt echter aan het licht in een welvarende samenleving, waar calorierijk voedsel en recreatieve drugs overal verkrijgbaar zijn. Zo ontstaat er een ongelukkige combinatie van een op beloning gefocust brein en een overvloed aan beschikbare beloningen die schadelijk zijn bij overgebruik. Niet iedereen is in staat om in dergelijke omstandigheden goed maat te houden. Waarom de één dit wel kan en de ander niet, ligt waarschijnlijk voor een groot deel aan verschillen tussen mensen qua gevoeligheid van dat beloningssysteem en de controlerende mechanismes ervan.

In het traject om mensen die met obesitas- en verslavingsproblematiek kampen te helpen, moet vaak voorkomen worden dat mensen terugvallen in hun oude gedrag van overconsumptie. Tijdelijke successen zijn goed haalbaar, maar vooral het volhouden van zo een regime is lastig. Vanuit therapeutisch perspectief is het daarom ook vooral zaak om aan dat aspect iets te doen. Er zijn sterke indicaties dat patiënten het meest gebaat zijn bij een levensstijlverandering, met farmacologische ondersteuning om de nieuwe levensstijl te vergemakkelijken en in stand te houden. Juist op dit terrein van ondersteunende medicatie is er echter nog een wereld te winnen, omdat de beschikbare middelen vaak zelf ook problemen opleveren die de bruikbaarheid ervan beperken. Hierbij valt te denken aan bijwerkingen variërend van fysieke ontwenningsverschijnselen tot zelfs neuropsychiatrische problemen als angst en depressie.

Dit proefschrift heeft als aanname dat veel van de problematiek van dergelijke farmaca te wijten is aan het gebrek aan inzicht in hoe ze precies werken in het brein, bijvoorbeeld in het beloningssysteem. In dat geval is het ontwikkelen van betere farmaca zeer gebaat bij een vergroot inzicht in hoe het beloningssysteem gereguleerd wordt, en op welke wijze dergelijke medicatie hierop aangrijpt. Dit proefschrift richt zich met name op de regulatie van het beloningssysteem door drie belangrijke modulatoren: cannabinoïden, opiaten en orexines.

EEN NADERE KIJK OP HET BELONINGSSYSTEEM

Een belangrijk neuraal circuit betrokken bij beloningsverwerking is het mesolimbisch dopamine systeem. Een cruciale nucleus in dit netwerk is het ventrale tegmentum (VTA), waar dopamine neuronen de motivatie voor (en verwerking van) beloning coderen. Deze neuronen projecteren naar verschillende gebieden, waaronder de nucleus accumbens en de prefrontale cortex. Dergelijke gebieden en connecties vormen het hart van het mesolimbisch dopamine beloningssysteem.

Neuromodulatoren reguleren bovenstaand circuit en hebben daarom een grote invloed op beloningsverwerking. Van deze endogene systemen, waaronder cannabinoïden, opiaten en orexines vallen, is bekend dat ze onder andere een grote invloed uitoefenen op de inhiberende GABAerge en/of op de excitatoire glutamaterge inputs die de dopamine neuronen ontvangen. Op deze wijze beïnvloeden ze de activiteit van de dopamine neuronen zelf. Van de cannabinoïde 1 (CB1R) en mu opiaat receptor (MOR) is bekend dat ze zich onder andere op de presynaptische uiteinden van axonen bevinden die synaptische contacten maken met de dopamine neuronen. Vooral de lokalisering van deze receptoren op inhiberende GABAerge projecties wordt geacht een grote rol te spelen in de directe excitatie van dopamine neuronen, omdat de (endogene) cannabinoïden en opiaten die op deze receptoren aangrijpen, deze inhiberende inputs onderdrukken (disinhibitie). Ondanks dergelijke inzichten, is er nog veel onbekend over hoe CB1Rs en MORs effect sorteren.

BEVINDINGEN VAN HET PROEFSCHRIFT:

Wanneer CB1Rs en MORs tot expressie worden gebracht in artificiële celsystemen, zijn ze ten dele spontaan actief. Deze *constitutieve activiteit* kan men onderscheiden van de meer standaard wijze van receptor-activatie die gemedieerd wordt door een agonist (een cannabinoïd respectievelijk een opiaat) die aan de receptor bindt. Er is echter weinig bekend over de rol van dergelijke constitutieve receptor-activiteit in het brein, zeker niet specifiek in het mesolimbisch dopamine systeem. In *hoofdstuk 2* laten we zien dat CB1Rs die gelokaliseerd zijn op GABAerge projecties naar VTA dopamine neuronen, inderdaad constitutief actief zijn. Om dit aan te tonen zijn elektrofysiologische metingen

verricht in de VTA van hersenplakken van de muis. Vervolgens zijn de effecten bepaald van verschillende farmacologische klassen van CB1R liganden op de GABAerge inputs naar VTA dopamine neuronen. Onder andere is gekeken naar de effecten van CB1R inverse agonisten en neutrale antagonisten. Dit zijn beide antagonisten die voorkomen dat CB1Rs geactiveerd worden door (endogene) agonisten. Daarentegen is bekend dat deze antagonisten verschillende effecten hebben op CB1R constitutieve activiteit; inverse agonisten onderdrukken die, terwijl neutrale antagonisten hem intact laten. Constitutieve CB1R activiteit kan dus onthuld worden door de effecten van CB1R neutrale antagonisten en inverse agonisten te contrasteren. Via een dergelijke benadering tonen wij aan, dat constitutief actieve CB1Rs de GABAerge signalen naar dopamine neuronen reguleren. Dat dit ook daadwerkelijk leidt tot veranderingen in de excitatie van dergelijke neuronen tonen wij aan in *hoofdstuk 3*. Hier beschrijven we elektrofysiologische metingen die verricht zijn in vrij-bewegende ratten, om aan te tonen dat er ook in een intact brein evidentie is voor CB1R invers agonisme in de VTA.

Tevens laten we zien dat constitutieve CB1R activiteit een belangrijke rol speelt in angstgedrag en de motivatie voor beloning in gedragsmatige tests *(hoofdstuk 2)*. Dit blijkt uit de observatie dat een CB1R inverse agonist ook daar een kwalitatief ander effect heeft (hij bevordert angst en verlaagt motivatie voor beloning) dan een neutrale antagonist (geen effecten op die factoren). Opmerkelijk is dat CB1R constitutieve activiteit geen belangrijke rol lijkt te spelen in voedselinname en gewichtsregulatie. Een CB1R neutrale antagonist en een inverse agonist zijn namelijk even goed in staat om voedselinname te verminderen, met corresponderende effecten op gewicht.

De CB1Rs en MORs zijn functioneel gezien nauw verwante receptoren. Te verwachten valt daarom, dat MOR constitutieve activiteit ook fysiologische relevantie kan hebben. In *hoofdstuk 4* tonen we inderdaad aan dat een vergelijkbaar principe van constitutieve activiteit opgaat voor MORs. Ook deze receptoren reguleren GABAerge inputs op VTA dopamine neuronen. Niet alleen laten we zien dat dergelijke MORs tot op zekere hoogte constitutief actief zijn, maar ook tonen we aan dat deze constitutieve activiteit is toegenomen in een periode na morfinegebruik. Dergelijke verhoogde basale MOR activiteit, die ook wordt waargenomen na verhoging van de cAMP-cascade, fungeert waarschijnlijk als een compensatoir mechanisme. Tijdens morfine abstinentie (na gebruik) treedt namelijk een verhoging op van de GABAerge inhibitie van VTA dopamine neuronen, die geassocieerd is met afkickverschijnselen. De ver-

hoogde MOR constitutieve activiteit bevordert onderdrukking van dergelijke inputs, wat als (onvolledig) tegenwicht kan dienen voor deze aversieve staat.

Een derde relevante neuromodulator is het orexin-systeem. Deze neuropeptiden grijpen ook aan op het mesolimbisch dopamine netwerk, maar in *hoofdstuk 5* demonstreren we dat ze dit op een zeer selectieve wijze doen. Slechts een subset van VTA dopamine neuronen - herkenbaar aan onder andere hun relatief klein oppervlak - wordt namelijk geëxciteerd door orexin A. Dit in tegenstelling tot een andere subgroep van grotere VTA dopamine neuronen, waarbij orexin A zelfs de GABAerge inhibitie vergroot. Deze bevindingen impliceren dat neuromodulatoren heel specifieke effecten kunnen sorteren binnen subpopulaties van het mesolimbisch dopamine beloningssysteem, en dat dit netwerk niet als een homogeen blok beschouwd moet worden.

THERAPEUTISCHE IMPLICATIES VAN DIT PROEFSCHRIFT

De bevindingen van dit proefschrift zijn niet slechts fysiologisch van belang, maar hebben ook therapeutische relevantie. Gezien de rol van het endogene cannabinoïde systeem in voedselinname, werd het anti-obesitas middel rimonabant – een CB1R 'antagonist' – ontwikkeld. Ondanks dat gebruikers van rimonabant gewicht verloren, werd het middel toch gediscontinueerd vanwege de ernstige bijwerkingen die het teweegbracht in een subset gebruikers. Vooral de gevoelens van angst en depressie waar rimonabant mee werd geassocieerd, waren aanleiding om niet alleen dit middel stop te zetten, maar om ook de therapeutische bruikbaarheid van CB1R antagonisme sterk in twijfel te trekken. Dit proefschrift is relevant in het licht van deze ontwikkelingen, omdat het laat zien dat rimonabant niet als een doorsnee CB1R antagonist fungeert in het brein, maar als een inverse agonist die aanwezige constitutieve activiteit onderdrukt *(hoofdstuk 2 & 3)*. CB1R inverse agonisten hebben derhalve daadwerkelijk een dubbele werking (in het brein):

(1) Ze blokkeren activatie van de CB1R die gemedieerd wordt door de endogene agonisten (de endocannabinoïden);
(2) Ze interfereren met de constitutieve CB1R activiteit die optreedt in absentie van agonisten.

Nu in dit proefschrift wordt aangetoond dat beide activatiemechanismes rele-

vant zijn in het brein, wordt het zaak om te achterhalen in welke mate de goede en slechte eigenschappen van rimobanant moeten worden toegeschreven aan interferentie met de twee afzonderlijke mechanismes. In dit proefschrift worden al stappen gezet in die richting. We demonstreren in diermodellen dat de neutrale CB1R antagonist NESS0327, die wel interfereert met endocannabinoïd- maar niet met constitutief-gemedieerde activiteit, voedselinname en gewichtstoename net zo vermindert als rimonabant dat doet. Daarentegen produceert de neutrale antagonist niet de effecten van angst en anhedonie (verlies van plezier/motivatie: een cruciaal kenmerk van depressie) die de inverse agonist rimonabant wel produceert *(hoofdstuk 2)*. Gezamenlijk suggereren deze bevindingen dat CB1R constitutieve activiteit dan wel niet een grote rol speelt in processen die betrekking hebben op voedselinname en gewichtsregulatie, maar des te meer in de regulatie van emoties en motivatie. De implicatie hiervan is dat CB1R neutrale antagonisten, in potentie, veiligere stoffen zijn voor de bestrijding van obesitas dan het gediscontinueerde middel rimonabant en CB1R inverse agonisten in het algemeen.

Ook de constitutieve activiteit van de MORs die we aantonen in het mesolimbisch dopamine systeem, is van therapeutisch belang *(hoofdstuk 4)*. Zeker gezien het feit dat MOR antagonisten gebruikt worden als medicatie voor verslaving, en ook worden ingezet in de bestrijding van opiaten-overdosering. We tonen aan dat de constitutieve activiteit van MORs in het mesolimbisch dopamine systeem alleen maar toeneemt nadat het brein eenmaal is blootgesteld aan opiaten. Tevens identificeren we die toename als een mechanisme dat waarschijnlijk afkickverschijnselen deels voorkomt. Momenteel zijn de MOR 'antagonisten' naloxone en naltrexone in gebruik voor de behandeling van opiaten-overdosering en drugsverslaving. Dit zijn beide stoffen waarvoor indicaties bestaan dat ze invers agonistische effecten hebben, vooral in situaties waar MORs eerder aan opiaten zijn blootgesteld. Gezien onze bevindingen kan dus worden verondersteld dat naltrexone en naloxone ook de beschermende compensatoire verhoging van MOR constitutieve activiteit tenietdoen, met mogelijk onnodige bijwerkingen tot gevolg. Derhalve is het aan te raden om bij voorkeur neutrale MOR antagonisten in te zetten in de bestrijding van opiaten-overdosering en drugsverslaving. Deze stoffen zijn net zo goed in staat te interfereren met opiaten die via de MOR werkzaam zijn: het therapeutisch beoogde werkingsmechanisme. Daarentegen zijn er nu goede indicaties, dat de neutrale antagonisten minder bijwerkingen zullen hebben dan de huidige middelen met MOR invers agonistisch potentieel.

ACKNOWLEDGEMENTS

Er zijn heel veel mensen die belangrijk zijn geweest in de afgelopen periode. Om die waardering goed tot uiting te brengen zijn 'pen en papier' waarschijnlijk niet voldoende, maar ik zal een poging wagen:

Geert. Ik weet nog dat je een interessant college over synaptische plasticiteit gaf tijdens de master. Alsof er op fast-forward gedrukt is zijn we nu zo'n 7 jaar en, als mijn *back-of-the-envelope* berekening een beetje klopt, ~10.000 glaspipetten verder. Ik heb erg veel van je geleerd in die periode en het is niet overdreven om te zeggen dat je een heel grote impact op me hebt gehad op wetenschappelijk gebied. Daarmee doel ik zowel op het inhoudelijke aspect, als op je nuchtere benadering ervan.

Je groep is door de jaren heen natuurlijk vaak van samenstelling veranderd. De grote constanten waren (academische) vrijheid, een uitstekende werksfeer en een uitdagende omgeving, en je rol daarin was voor mij cruciaal. Je bent iemand die me altijd ontzettend heeft weten te motiveren voor de wetenschap, en ik kan me niet herinneren dat ik ooit, zelfs na een mindere dag, ooit met iets anders dan frisse moed weer naar het lab ben gekomen. Kortom, onze lange samenwerking is me altijd bijzonder goed bevallen, en die zal ik zeker missen.

Roger. Ook van jou heb ik heel veel meegekregen. Je bent iemand die heel sterk is in stappen-vooruit-denken, en je hebt veel gedaan om me wereldwijs te maken wat betreft de veelvoudige aspecten van een carrière in de wetenschap. Daar ben ik je erg dankbaar voor.

Los daarvan heb ik veel bewondering voor de wijze waarop je parate kennis voor zeer uiteenlopende projecten binnen je groep hebt, en hoe je altijd met een scherpe kritische blik naar de voortgang van de projecten hebt gekeken. Je adviezen gaven vaak blijk van een hele sterke wetenschappelijke intuïtie, en zijn vaak van groot belang geweest bij het doen van de juiste experimenten. Je optimistische inslag en doelgerichtheid zijn een belangrijke drijfveer geweest, en ik ben heel blij dat ik die gedurende de afgelopen periode van zo nabij heb meegemaakt.

Esther. We hebben elkaar goed leren kennen in de lange tijd dat we een kamer deelden op het werk, en natuurlijk op de borrels, filmavonden etc. Ik kan met zekerheid zeggen dat je een zeer veelzijdig persoon bent. Als onderzoeker ben je heel secuur, en bovendien iemand die aanstekelijk veel plezier kan halen uit excelmacro's en kleurcoderingen. Als interieurdesigner ben je, laten we het 'experimenteel' noemen, gezien alle waanzinnige objecten die je met grote regelmaat op mijn muur plakte, en je herschepping van ventilatoren en beeldschermen in aluminiumfolie. Ik ben blij dat de profeet in je, je minst-ontwikkelde aspect is. Je hebt me ooit eens verteld dat je bij onze eerste ontmoeting dacht dat je waarschijnlijk 'weinig last' van me zou krijgen. Als het goed is treed je kort na het printen van dit boek op als een van mijn paranimfen, met alle bijbehorende lasten ;).

"Aha, jij bent dus **Bart**". Dat was ergens op een afdelingsborrel toen jij als student ongeveer aan de master begon en ik net met mijn PhD startte, toen ik ook nog geen idee had dat er zoveel adembenemende hacky-sack, Jianzi en tafelvoetbalsessies in het verschiet lagen. Ik heb er in ieder geval erg van genoten, en los daarvan ben je in die lange tijd dat we samen hebben gewerkt iemand waar ik ook veel bewondering voor heb gehad. Er lijkt bijna geen labtechniek te zijn die je niet al in de vingers hebt, verder bouw je met plezier je eigen versterkers voor metingen (uhmm, werkt ie al?), en het allerbelangrijkst: je rendang en saté zijn werkelijk voortreffelijk.

Ray en Lars. Ik denk dat het voor onze afdeling een aderlating was toen jullie er niet meer werkten, en ik vond het persoonlijk erg jammer. Jullie waren natuurlijk niet alleen qua inhoudelijke kennis en technische beheersing heel belangrijk voor velen, maar ook op sociaal vlak eigenlijk compleet onmisbaar. Ik heb zelf het geluk gehad dat ik jullie tot goede vrienden kon rekenen, en dat dat ook in stand bleef nadat we niet langer dezelfde werkvloer deelden. Cruciaal hierin waren natuurlijk ook al die gamesavonden (hier wil ik natuurlijk ook even **Alwin en Edwin** noemen als belangrijke schakels), borrels, bowlingevenementen, Wii-tennis-sessies enz, die gelukkig gewoon doorgingen. Het waren altijd zeer welkome afleidingen, en ze hebben in hoge mate bijgedragen aan het plezier dat ik tijdens het promotieonderzoek heb gehad.

Edwin. Samen hebben we ontelbare uren doorgebracht in het lab, en hebben we het ganse aanbod van De Brink in vele cycli voorbij zien komen. Ergens tussen de vis met broccoli en de kip met nasi heb ik je uiteindelijk goed leren kennen. Zelfs naar Groningse maatstaven ben je denk ik onverstoorbaar te noemen. Ik heb je in ieder geval nooit ergens door uit het veld zien laten slaan. Zelfs de opeenvolgende Ajax-kampioenschappen lijken je geen permanente schade te hebben toegebracht. Op een gegeven moment hebben we je op het lab omgedoopt tot 'Biggie-pedia': een treffende karakterisatie van je formaat en je encyclopedische kennis van vrij willekeurige onderwerpen. Die lijn doortrekkend, kan ik zeggen dat ik ook heb genoten van de 'Biggie-media', zoals wanneer je ons bijvoorbeeld uitnodigde voor een sessie van je muziekvereniging, en je verhalen over Friese misstanden. Veel succes en ik geloof vast dat je je droompositie in Groningen uiteindelijk zult weten te vinden.

Anup. I've enjoyed your stay at the RMI a lot. One reason being that you're a skilled electrophysiologist who doesn't take dogmas for granted and is never happy until he knows exactly what's going on in the experiment. Your urge to theorize has also led to a lot of nice discussions. It's a pity that your 'main goal' to automatize patch clamping to the extent that it can be done at home with a webcam and a mouse hasn't been fulfilled yet, but if anyone can do it... However, the main reason I liked your stay at the RMI is that we quickly became good friends and had a lot of fun together, both inside and outside of the lab (Norwegian fjords come to mind for instance). I don't think I've ever met anyone who simultaneously had as many hobbies as you did. At one point it seemed you were roller-skating, skateboarding, surfing, diving, swimming and playing guitar. I think you then casually informed me and Edwin during dinner that you were seriously considering underwater hockey…

Anyway, it's been a great ride and I'm sure I'll be seeing much more of both you and Edwin (even beyond your pending visit to Paris).

Myrte. We hebben een flinke tijd ongeveer parallelle paden bewandeld. Gezien je duracell-hardloop-skills is het ongelooflijk dat ik, zelfs metaforisch, ongeveer gelijke tred hield. Ik was er in ieder geval erg blij mee dat je in dezelfde groep je

PhD ging doen als ik. Dat betekende namelijk dat ik vaak alleen maar even opzij hoefde te kijken om te zien hoe iemand een bepaald probleem kundig aanpakte of een obstakel gracieus omzeilde. Los daarvan ben je ook een erg spontaan en aangenaam persoon waar volgens mij iedereen op het RMI, en zeker ook ik, het altijd erg goed mee heeft kunnen vinden. Heel veel plezier in Oxford. Na de 'formal dinner' ervaring die we ooit in Cambridge hebben gehad zul je er volkomen klaar voor zijn. Het wordt een gegarandeerd succes.

Ook **Antoin** en ik gaan natuurlijk ver terug. Een bepaalde favoriete wetenschapper van me karakteriseerde zichzelf ooit een keer bescheiden als: *'a man whose tastes far exceed his skills'*. Bij jou heb ik echter altijd het gevoel gehad dat je niet alleen brede (academische) *'tastes'* hebt, maar ook nog eens de nodige *'skills'* om er wat aan te doen. Dat is een gouden combinatie natuurlijk, en ik ben ook heel benieuwd naar de ervaringen die ook jij in Oxford gaat opdoen.

Koushik. Your presence at the RMI was most akin to a whirlwind. Activities and stories seemed to shadow you closely, and it was, and still is, always enormous fun to hang out together. Aside from that, you're a great scientist (yes I'm using the word on purpose to irk you a bit), whose approach to science I find inspirational. You seem to be even happier mixing herbs than reagents though, and we've certainly had some of our most glorious moments preparing tender oven-cooked fish. Since you're so much of a world-citizen, I can't be sure where we'll meet again, I'm just sure that we will.

Geoffrey. Waarschijnlijk vind je elk woord dat ik hier over je schrijf er één teveel. Dus bij voorbaat excuus, maar je zult er toch even aan moeten geloven. Ik kende je natuurlijk al langer, als een 'zeer gewaardeerde collega die op dezelfde golflengte zat wat een heleboel zaken betrof'. Maar vooral in die relatief recente maanden waarin we tot elkaar 'veroordeeld' waren voor de *in vivo* metingen heb ik je nog wat van dichterbij meegemaakt. Dit was volledig tot mijn plezier. Ik heb gemerkt dat je een vraag stellen kan leiden tot uiterst informatieve en/of de meest droogkomische antwoorden. Ik hoop dat je boekenkast nog een groenig boek kon gebruiken...

Susan & Fransesca. I've greatly enjoyed sharing the office with all of you. We always had a very nice atmosphere and I'll definitely miss that. **Margriet**, verbaal sparringsspartner *par excellence*, ook jou zal ik erg missen. We hielden elkaar aardig scherp, dus mijn medeleven alvast aan je nieuwe slijpsteen.

Ralph. De 3de GPCR schakel. Het was altijd fijn om met je over de experimenten e.d. te praten. Het is jammer dat onze projecten niet nog wat meer naar elkaar zijn toegegroeid. Het had me leuk geleken om nog wat nauwer samen te werken.

Viviana. It was a great joy to collaborate with you. Aside from your high efficiency, you're also a very pleasant person to work with and talk to.

Natasha / Angela / Henk / Marloes. When Geert told me our lab's 'critical mass' was going to more than double, I couldn't have wished for nicer people to join. **Ewoud / Arjen / Jesse / Jules / Eneda / Marek / Petra / Anne / Ela**. I've greatly enjoyed all the discussions and drinks we've had throughout the years. **Leo, Henk S. & Hugo**: zonder jullie was er natuurlijk weinig van de RMI-sfeer over.

I've also had the great fortune to work together with some very talented students. **Loek / Eljo / Ruud & Granville**. Without exception, it was a great experience to work with all of you. I've certainly learned a lot from it, and you've all played pivotal roles in the progress of the thesis in many ways. I also had a lot of fun during our collaborations, and I'll follow your own developments with great interest. Thanks for everything!

Ik wil ook **Jan, Wout, Mark, Marjolein, Roger K., Ria, Vicki, Sandra & Krista** bedanken. Jullie zijn altijd enorm hulpvaardig geweest bij van alles en nog wat. Verder mogen ook zeker **Keith, Maike, Rea, Rahul & Han** niet onvermeld blijven. Ik noem ook vooral **Mieneke** die me geholpen heeft bij de gedragsexperimenten, en natuurlijk cruciaal was voor de *in vivo* electrofysiologie.

Louk. Ook jij erg bedankt voor je waardevolle input en adviezen. Verder ook zeker dank aan **Marten, Martien, Pierre, Jeroen, Peter & Marian**.

Ook wil ik Top Instituut Pharma bedanken, met in het bijzonder het GPCR Forum natuurlijk. Los van het feit dat dit samenwerkingsverbond de financiering van het project mogelijk maakte, is het ook een collaboratie gebleken waar ik veel van heb geleerd. De bijeenkomsten waren altijd nuttig, informatief en strak geregeld, daar dank ik ook in het bijzonder **Margot** en **Ad** voor. **Danny**, jij ook erg bedankt voor je hulp tijdens onze korte samenwerking.

Ook dank ik de leescommissie, bestaande uit Prof. dr. **Mansvelder**, Prof. dr. **Ramsey**, Prof. dr. **Kenemans** & Prof. dr. **Hoogenraad**.

Heel veel dank aan **Amy** voor al haar snelle en professionele werk bij de lay-out van het boek en het maken van de cover. Ik ben net zo blij met het resultaat als met het feit dat ik het niet zelf met mijn eigen beperkte grafische vaardigheden hoefde te doen. Ik heb de samenwerking als zeer prettig ervaren en kan en zal je aan iedereen aanraden..

Werken op een lab heeft vele leuke kanten, maar een minder aspect is dat het een komen-en-gaan van mensen is, waar groet en afscheid elkaar vaak te rap opvolgen. Zeker in dat licht, zijn bepaalde vriendschappen/relaties voor mij des te specialer. In dit kader noem ik dan ook **Mario, Daan, Harald en Wilco**. We kennen elkaar al erg lang, en het was voor mij in ieder geval heel waardevol dat we elkaar met regelmaat zagen en spraken. Ik vind het ook daarom erg leuk dat een van jullie, Wilco, als mijn andere paranimf zal optreden. Ook noem ik verder zeker **Lucien** en **Petra**, het was erg leuk om jullie met regelmaat weer te zien. Lucien, het wordt leuk puzzelen nu. Ik kijk ernaar uit.

Tot slot dank ik mijn **ouders**. Jullie onvoorwaardelijke steun en interesse is me heel veel waard geweest. Ik heb me altijd heel vrij gevoeld, en verder heb ik

heel veel gehad aan jullie eigen positieve en nuchtere instelling. Ook is het niet onbelangrijk dat jullie goed konden aanvoelen of het even wél of niet een goed moment was om naar de vorderingen te informeren. Jullie ongedwongen liefdevolle houding op de achtergrond heeft bij mij veel inspiratie en kwaliteiten naar de voorgrond gebracht. Het heeft daarom, in hoge mate, belangrijke bijdragen aan het proefschrift geleverd. Bedankt!