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# The CB1R system within the nucleus accumbens of vervet monkeys

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# Résumé

La libération de dopamine (DA) dans le noyau accumbens (NAc) est centrale dans le circuit de récompense, et un déséquilibre de la concentration de la DA joue un rôle majeur dans la dépendance. Anatomiquement, le NAc peut être divisé en 2 parties, le cœur (« *core* ») et la capsule (« *shell* »), ces 2 régions reçoivent des projections dopaminergiques de l'aire tegmentale ventrale (ATV). La libération de DA dans la capsule est impliquée dans les sensations de récompenses associées à la dépendance, alors que le cœur fait partie du circuit moteur avec la substance noire et encode les patrons moteurs des mouvements des yeux et la locomotion. Chez les rongeurs, le système endocannabinoïde (eCB) est présent dans le NAc, est impliqué dans la neuromodulation, et semble jouer un rôle dans la libération de la DA. La plante de cannabis, contient entre autre le delta-9-tetrahydrocannabinol qui produit ses effets psychotropes en activant le récepteur cannabinoïde 1 (CB1R), en partie en altérant la libération de DA. En tant que tel, il est supposé que le système CB1R joue un rôle crucial dans la médiation des propriétés gratifiantes de la cannabis et d'autres drogues d'abus, et peut-être la valeur des récompenses naturelles. Cette étude vise à caractériser l'expression et la localisation de CB1R, et des enzymes métaboliques des eCBs, la NAPE-PLD et la FAAH dans le NAc du singe vervet (*Chlorocebus sabaeus*) à l'aide des méthodes d'immunobuvardage et d'immunohistochimie. Nous avons trouvé que CB1R, NAPE-PLD et FAAH sont exprimés dans le cœur et la capsule du NAc. Ces 3 protéines sont présentes dans les cellules *medium spiny neurons* et les *fast-spiking interneurons* GABAergiques. Ces protéines n'ont pas été toutefois retrouvées dans les projections dopaminergiques ou les astrocytes. Ces données démontrent que le système CB1R est présent dans le NAc du singe et est donc parfaitement positionné pour jouer un rôle dans le circuit de récompense en désinhibant la libération de DA. De façon beaucoup plus large, le système eCB du singe pourrait également jouer un rôle dans la perception, la motivation et la sélection d'action.

**Mots-clés**: CB1R, NAPE-PLD, FAAH, système endocannabinoïde, noyau accumbens, singe, immunohistochimie, immunofluorescence.

# Abstract

Dopamine (DA) release onto the nucleus accumbens (NAc) is central to the reward circuit, the dysregulation of which plays a role in addiction. The NAc can be anatomically divided into a core and shell. Both regions receive DA projections from the ventral tegmental area (VTA). VTA DA release onto the shell mediates feelings of reward associated with addiction, while the core is part of a motor circuit with the substantia nigra (SN) that encodes relevant motor patterns for eye movements and locomotion. In rodents, the endocannabinoid (eCB) system, which modulates neurotransmission, is present in the NAc, and plays a role in the modulation of DA release. Marijuana, which contains among others the active phytocannabinoid delta-9-tetrahydrocannabinol, produces its psychoactive effects by activating the cannabinoid receptor type 1 (CB1R), which may cause these effects by altering DA release. As such, it is hypothesized that the CB1R system plays a crucial role in mediating the rewarding properties of marijuana and other drugs of abuse, and possibly the value of natural rewards. Expression patterns of CB1R, the eCB synthesizing enzyme *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), and the eCB degradation enzyme fatty acid amide hydrolase (FAAH) in the NAc have not been described in monkeys and humans. It is therefore the goal of the present study to characterize the expression and localization of these components of the eCB system within the NAc of vervet monkeys (*Chlorocebus sabaeus*) using Western Blots and immunohistochemistry. We found that CB1R, NAPE-PLD, and FAAH are expressed across the NAc, both in the core and shell. CB1R, NAPE-PLD, and FAAH are localized in GABAergic medium spiny projection neurons (MSNs), and in fast-spiking GABAergic interneurons (FSIs). CB1R, NAPE-PLD, and FAAH did not co-localize with dopaminergic projections, or astrocytes. These data indicate that the CB1R system is also present in the monkey NAc and suggests that it may play an important role in the brain reward circuit through a disinhibitory action on DA release. Thus, the primate eCB system may play a considerable role in reward perception, motivation, and action selection.

**Keywords**: CB1R, NAPE-PLD, FAAH, endocannabinoid system, nucleus accumbens, primate, immunohistochemistry, immunofluorescence.

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# List of Abbreviations

2-AG: 2-arachidonoylglycerol

ACh: Acetylcholine

AEA: Anandamide

cAMP: Cyclic adenosine monophosphate

CB1R: Cannabinoid receptor type 1

CB2R: Cannabinoid receptor type 2

CeA: Central amygdala

D1DR: D1 dopamine receptor

D2DR: D2 dopamine receptor

DA: Dopamine

DAGL: Diacylglycerol lipase

eCB: Endocannabinoid

FAAH: Fatty acid amide hydrolase

FSI: Fast-spiking interneuron

GABA: γ-Aminobutyric acid

GFAP: Glial fibrillary acidic protein

GPR55: Orphan G-coupled protein receptor 55

M1 mAChR: M1 muscarinic acetylcholine receptor

M4 mAChR: M4 muscarinic acetylcholine receptor

MAGL: Monoacylglycerol lipase

MAPK: mitogen-activated protein kinase

mGluR: Metabotropic glutamate receptor

MSN: Medium spiny neuron

NAc: Nucleus accumbens

NAPE-PLD: *N*-acyl phosphatidylethanolamine phospholipase D

PFC: Prefrontal cortex

PPARgamma: Peroxisome proliferator-activated receptor gamma

PV: Parvalbumin

SNpr: Substantia nigra pars reticulata

SNc: Substantia nigra pars compacta

STN: Subthalamic nucleus

TH: Tyrosine hydroxylase

THC: $\Delta^9$-tetrahydrocannabinol

TRPV1: Transient receptor potential vanilloid 1

VTA: Ventral tegmental area

*"Truth has many dimensions, and the way you arrive at truth in complex situations is through many perspectives." – Eric Kandel*

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# Prelude

The nucleus accumbens (NAc) is a small area located in the striatum of the basal forebrain that is responsible for feelings of pleasure, reward, motivation, action selection, and various other cognitive functions. These functions are largely regulated by the release of dopamine (DA), which in large enough amount produces effects such as euphoria and movement. The processing performed by the accumbens is a key part of human survival, driving the urge to engage in basic actions related to survival such as drinking, eating, and reproduction. The dysregulation of these basic processes can be incredibly harmful, such as is the case when aberrant drug use hijacks the reward circuitry of the brain to associate drug use with survival over the actual necessities, producing a state of addiction. While the role of DA in healthy accumbal function and in addiction has been well studied, there is limited knowledge of the anatomy of the endocannabinoid (eCB) system in the primate NAc, a neuromodulatory system which may contribute to the control of DA release through complex mechanisms that remain not fully understood. Antagonism of the CB1R system in rodents attenuates drug self-administration across substances and also prevents relapse of drug-seeking behaviour. Through studying the anatomy of the eCB system in the primate NAc, we are presented with a better understanding of how this system may function in primate models of addiction, and lay the groundwork for future functional experiments. We are additionally provided with information in the context of visual neuroscience because neurons with responses to reinforcing or novel visual stimuli in the NAc may be affected by marijuana consumption, and may also be involved in the initiation of appetitive and aversive eye movements. We describe the structure of the NAc and its subregions relative to the striatum as a whole, the differential expression of eCB system proteins across NAc subregions, and the key cell type expression profiles of certain eCB proteins in the NAc. With this information, we are able to prepare further anatomical studies, as well as generate hypotheses for complementary electrophysiological and behaviour studies that could lead to new pharmacological therapies for the treatment of addiction and related neuropsychiatric conditions.

# Chapter 1: The Endocannabinoid System and the Reward Circuit

## 1.1 Introduction

The endogenous cannabinoid system, or endocannabinoid (eCB) system, is a signaling system activated by endogenous ligands which are similar in structure to the phytocannabinoids found in marijuana (Howlett et al., 2002). This system is found throughout the body and mediates a large number of physiological functions. It has been studied extensively and increasingly over the past few decades since the discovery and cloning of the cannabinoid receptor type 1 (CB1R), the receptor responsible for the psychoactive effects of marijuana's active constituents (Matsuda et al., 1990). Though famed for its relation to marijuana, the endogenous functions of the system are actually critical to many brain and immune functions. Of particular interest is its involvement in the reward circuit and related eye movements and locomotion originating from activation of the basal ganglia motor circuit. The well-conserved evolutionary nature of the subcortical structures such as the basal ganglia is a testament to their importance for survival (Di Chiara, 2002), and by extension so too is the eCB system essential due to its role in the homeostatic modulation of these circuits. Through appropriate circuit function, the saliency and rewarding value of a given stimuli is determined to influence approach behaviour towards the given stimuli. By increasing our knowledge of eCB signaling in the reward circuit, we may better understand the influence of eCB system modulation on motivation to respond to these stimuli.

### 1.1.1 The Endocannabinoid System

The eCB system is a key neuromodulatory system expressed throughout the central and peripheral nervous systems, and plays an important role in a diversity of neuronal systems (Piomelli, 2003). It is comprised of cannabinoid receptors type 1 (CB1R) and type 2 (CB2R), eCB synthesizing enzymes such as N-acyl phosphatidylethanolamine phospholipase D (NAPE-

PLD) and monoacylglycerol lipase (MAGL), eCB degradative enzymes such as fatty acid amide hydrolase (FAAH) and diacylglycerol lipase (DAGL), and the endogenous ligands of these receptors such as anandamide (AEA), 2-arachidonoyl glycerol (2-AG), and various others (for review see Howlett et al., 2002; Piomelli, 2003; Di Marzo & Piscitelli, 2015). There are also additional related receptors such as the transient receptor potential vanilloid 1 (TRPV1), the orphan G-protein coupled receptor 55 (GPR55), and the peroxisome proliferator-activated receptor gamma (PPARgamma) (Di Marzo & De Petrocellis, 2012). These molecules are best known for their role in retrograde neurotransmission, particularly at GABAergic terminals, though they may also act as glutamatergic terminals (Piomelli, 2003). Additionally, eCBs can also contribute to anterograde neuromodulation in some cases, particularly by AEA acting on CB1R and TRPV1 (Di Marzo & De Petrocellis, 2012).

The ligands of the eCB system differ from many other neurotransmitter systems in that they are lipid-based, synthesized rapidly on demand. They are also capable of travelling in a retrograde manner across the synapse (Piomelli, 2003). This is opposed to the classic neurotransmitters that are stored for anterograde release by most other systems. The CB1R is one of the most highly expressed GPCRs in the brain, while the CB2R, also found in the brain, is better known for its role in immune function and inflammation, particularly in the peripheral nervous system (Cabral et al., 2008; Turcotte et al., 2016). The eCB system is involved in a wide diversity of functions including appetite, energy balance and metabolism, reproduction, thermoregulation, the sensation of pain, mood, sleep, memory, reward, locomotion, and the mediation of the effects of a class of chemical ligands called cannabinoids. This includes endogenous cannabinoids, as well as exogenous cannabinoidss such as synthetic cannabinoids and phytocannabinoids found in cannabis.

### 1.1.2 Endocannabinoid Signaling

Retrograde transmission is calcium dependent, often being triggered by large amounts of postsynaptic activity that needs to be regulated presynaptically (Howlett et al., 2002), possibly serving to maintain a degree of homeostasis in synaptic firing. Upon depolarization of the postsynaptic membrane, the synthesis and release of eCBs from the cell membrane is rapidly initiated (Howlett et al., 2002). These signaling molecules then travel across the synaptic cleft,

possibly via diffusion or a transporter, and activate receptors on the presynaptic membrane (Figure 1). Since eCB receptors are G-protein coupled receptors (GPCRs), their activation initiates a signaling cascade. As a result, cAMP levels, and in turn PKA activty, are reduced (Elphick and Egertova, 2001).

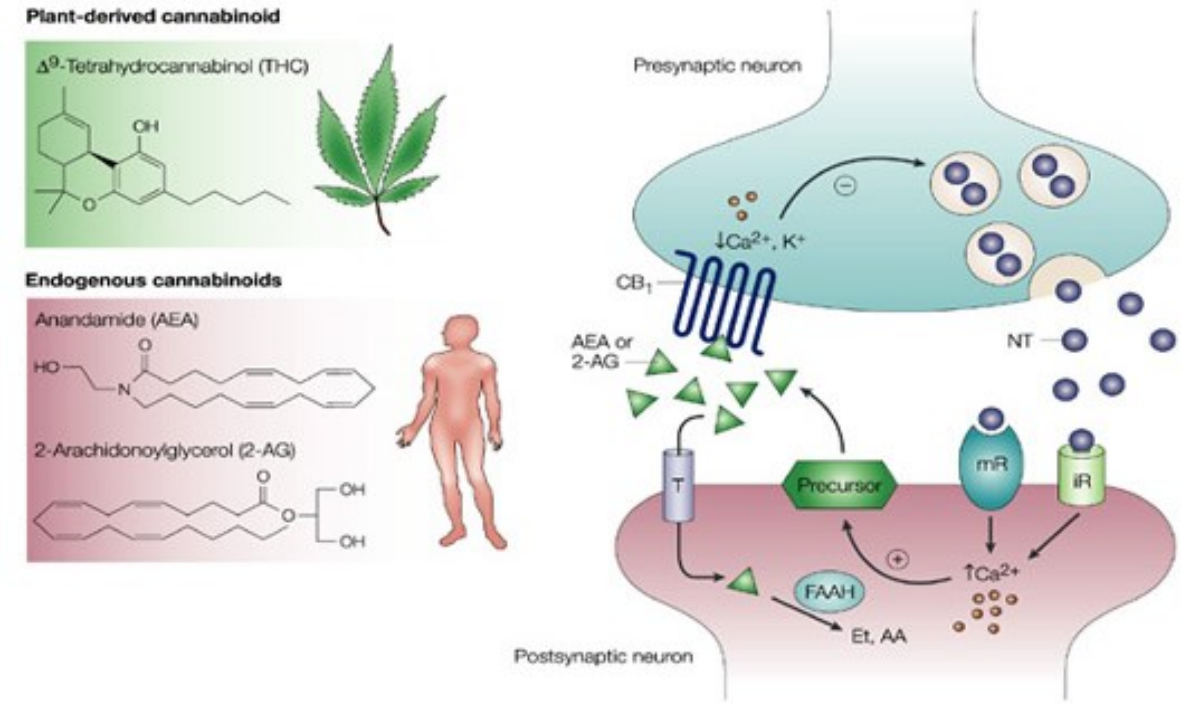


**Figure 1.** Endocannabinoid signaling (Guzman, 2003). Molecular structures of the endogenous cannabinoids AEA and 2-AG, as well as the plant-derived cannabinoid THC. Neurotransmitters (NT) are released presynaptically and activate postsynaptic ionotropic (iR) or metabotropic (mR) receptors, causing an increase in intracellular calcium. Membrane precursors are cleaved into AEA or 2-AG and activate presynaptic CB1Rs, which reduces intracellular calcium, resulting in less NT release. AEA reuptake into the postsynaptic membrane permits its hydrolysis by FAAH. THC acts similarly at CB1Rs to reduce NT release.

In addition to retrograde transmission, at least some eCBs may also act postsynaptically (Marinelli et al., 2008). While this has not yet been found with 2-AG, it has been demonstrated with AEA acting at postsynaptically expressed CB1R and TRPV1 (Di Marzo & De Petrocellis, 2012).

## 1.2 Endocannabinoid System Signal Transduction Pathway

A variety of receptors are part of or closely related to the eCB system, many of which are activated by more than one ligand, and many of which ligands act at more than one receptor simultaneously (Di Marzo & De Petrocellis, 2012). The endocannabinoid system is best known for its retrograde signaling, though it has also been found at times to engage in anterograde signaling, as well as autocrine or intrinsic signaling. The diversity of receptors and ligands allows different signaling types to be accomplished in each area of the brain in the way that is specifically needed. The eCB signaling pathway can cause different long lasting changes in different brain areas, such as homosynaptic long term depression (LTD) in some areas, but heterosynaptic LTD in others. Through this highly complex signal transduction pathway, the eCB system is capable of precise and diverse control of neuronal signaling across different brain areas.

### 1.2.1 Retrograde Signaling

Postsynaptic intracellular signaling results in the rapid postsynaptic synthesis and cleavage of AEA and 2-AG from the postsynaptic cell membrane (Piomelli, 2003). Calcium influx from postsynaptic activation causes the synthesis of NAPE as well as its hydrolytic cleavage by phospholipase-D (Okamoto et al., 2007). 2-AG production is mainly catalyzed by DAGL (Piomelli, 2003). eCBs then cross the synaptic cleft to bind to CB1Rs on the presynaptic terminal, possibly through either diffusion or with the help of extracellular lipid-binding proteins such as lipocalins (Piomelli, 2003). CB1Rs are usually coupled to $G_{i/o}$ proteins which activate signaling cascades causing an adenylyl cyclase mediated increase in type-A potassium influx and the direct inhibition of N-type voltage-dependent calcium channels, though $G_q$ coupling is also possible (Elphick & Egertova, 2001). Following CB1R activation, adenylyl cyclase is inhibited, reducing cyclic AMP levels. Reduced cyclic AMP levels cause a reduction in phosphorylation of type-A potassium channels by PKA, with a resulting inhibition of classical neurotransmitter release (Figure 2). Reduced PKA activity also reduces the phosphorylation of Raf, increasing its activity and the activation of the MAP kinase pathway (Elphick & Egertova, 2001). AEA reuptake from the synaptic cleft returns it to the postsynaptic neuron, where FAAH

catalyzes the hydrolysis of AEA (Deutsch and Chin, 1993). The degradation of 2-AG, however, mostly occurs directly within the presynaptic terminal via hydrolysis catalyzed by MAGL (Piomelli, 2003).

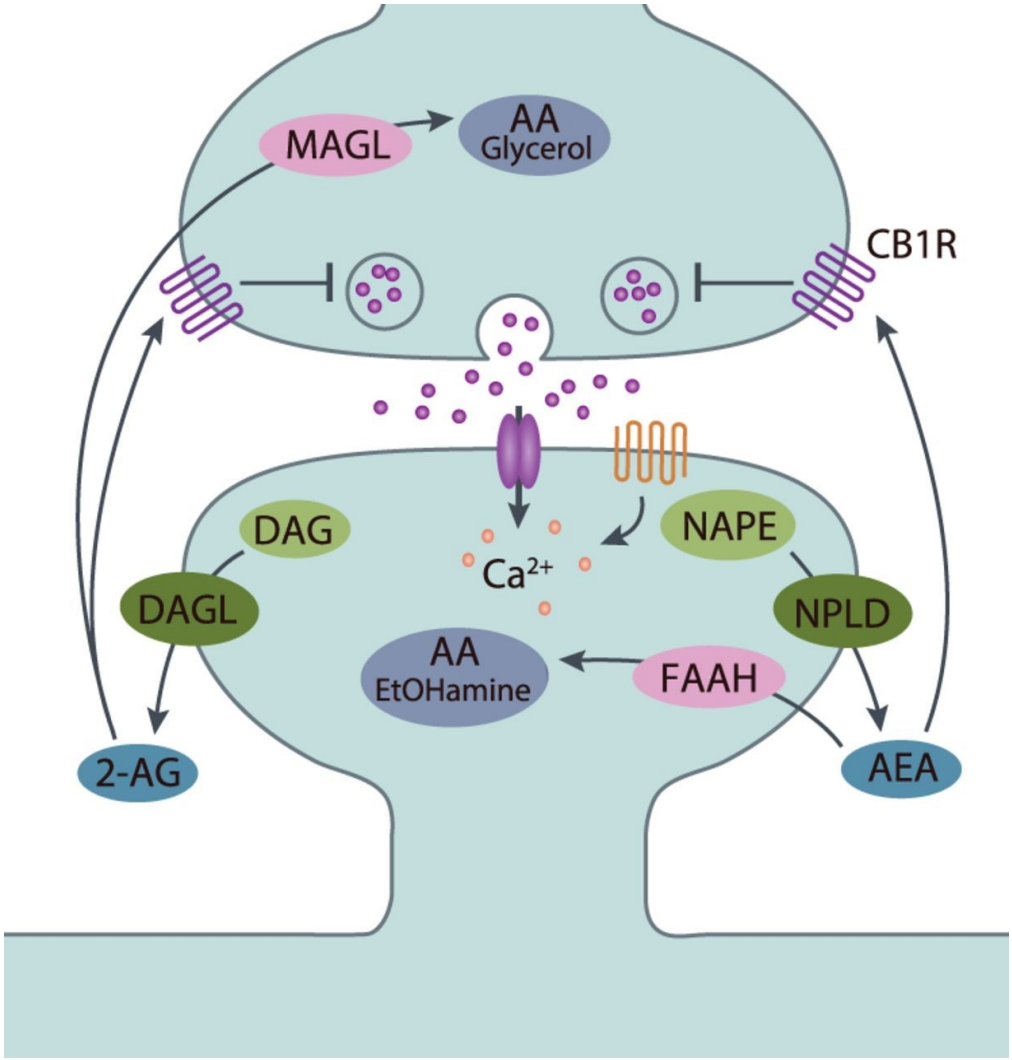


**Figure 2.** Retrograde endocannabinoid signaling (Zlebnik & Cheer, 2016). Retrograde signaling of endocannabinoids. Postsynaptic AEA synthesis from NAPE is catalyzed by NAPE-PLD (NPLD) and crosses the synaptic cleft to activate presynaptic CB1Rs. 2-AG is similarly produced from DAG by DAGL catalysis to also cross the synaptic cleft and activate CB1Rs. CB1R activation causes G-coupled proteins to reduce cyclic AMP, increasing potassium currents, and also causing a decrease in intracellular calcium. Release of classical neurotransmitters from synaptic vesicles is inhibited. AEA reuptake into the postsynaptic cell allows its breakdown by FAAH, while 2-AG is degraded presynaptically by MAGL.

### 1.2.2 Anterograde Signaling

Evidence for anterograde eCB signaling includes the finding of CB1Rs and NAPE-PLD at both the presynaptic and postsynaptic terminals, and FAAH and TRPV1 being predominantly found postsynaptically (Di Marzo and De Petrocellis, 2012). Taken together, this suggests that AEA may act in both retrograde and anterograde fashions at the CB1R, and also engage in anterograde signaling upon TRPV1. TRPV1 activation and CB1R activation at the same synapse may also have downstream signaling consequences due to cross-talk (Hermann et al., 2003).

### 1.2.3 Autocrine and Intrinsic Signaling

NAPE-PLD and FAAH have also been found concentrated postsynaptically in intracellular membranes, suggesting that anandamide may have a role in autocrine or intrinsic signaling (Di Marzo and De Petrocellis, 2012). Additionally, the depolarization of some cells has been found to reduce the firing rates of nearby interneurons, and that these effects are blocked by AM251, a CB1R inverse agonist (Kreitzer et al., 2002). This spread of eCB signaling to nearby interneurons may also result in a much wider indirect effect of eCB signaling on dendritic inputs to the depolarized cell since the nearby interneurons affected by local eCB spread can extend for hundreds of micrometers to contact other cells in the circuit. Spread of eCB signaling is also supported by that the depression of sIPSCs occurred not only in stimulated pyramidal neurons, but also in nearby pyramidal neurons that were not depolarized shortly after the stimulated pyramidal neuron depolarized in rat hippocampal sections (Wilson & Nicoll, 2001). The depression of signaling was distance-dependent and occurred most frequently in neighbouring pyramidal cells within 20um of the target cell. 2-AG is also believed to act postsynaptically at neocortical interneurons to produce a slow self-inhibition. AEA also has some evidence of potential for intrinsic signaling, since the enzymes NAPE-PLD and FAAH-1 have been found largely concentrated in intracellular membranes postsynaptically (Di Marzo and De Petrocellis, 2012).

### 1.2.4 Endocannabinoid-mediated Synaptic Plasticity

Synaptic plasticity can be affected by eCBs by both short term and long term mechanisms (Chevaleyre et al., 2006). One form of eCB-mediated short term plasticity is depolarization-induced suppression of inhibition (DSI), in which a retrograde eCB signal acts for less than one minute at GABAergic input upon postsynaptic depolarization of certain principal neurons in several brain regions, including the cerebellum and hippocampus (Llano et al., 1991; Pitler & Alger, 1992). DSI is calcium dependent and reduces only frequency, not amplitude of spontaneous IPSCs. Depolarization-induced suppression of excitation (DSE), occurs in a similar fashion as DSI, but acts at glutamatergic inputs. eCB mediated short term synaptic depression (eCB-STD) includes these forms of short term plasticity, as well as other transient retrograde synaptic suppression that involve eCB production caused by certain patterns of stimulation that do not require actual depolarization. Short term synaptic depression eCB production is initiated by two main mechanisms. Intracellular increase of calcium is necessary (Llano et al., 1991) and sufficient for eCB production (Wilson & Nicoll, 2001). Activation of metabotropic glutamate receptors or muscarinic acetylcholine receptors by exogenous agonists is also able to trigger eCB production (Fukudome et al., 2004), and may only require a small intracellular calcium increase (Galante & Diana, 2004), but may also be enhanced by increased intracellular calcium.

Both excitatory and inhibitory synapses may also have eCB-mediated long term depression (eCB-LTD) (Chevaleyre et al., 2006). eCB-LTD, like eCB-STD, requires the activation of presynaptic CB1Rs after postsynaptic eCB release caused by calcium influx or group I mGluR activation, but the maintenance of eCB-LTD does not require continued CB1R activation (Figure 3). Within the dorsal striatum, there has been found both high frequency stimulation (HFS) and medium frequency stimulation (MFS) eCB-LTD. HFS induced postsynaptic calcium influx and D2DR activation which were both needed to produce the release of AEA, and resulted in LTD (Giuffrida et al., 1999). MFS of afferents similarly requires the activation of both CB1Rs and D2DRs, but required neither postsynaptic calcium influx nor mGluR activation to produce LTD (Ronesi & Lovinger, 2005).

Within the NAc of the ventral striatum, MFS of cortical afferents produced LTD as well, but this LTD in the NAc was prevented when mGluR5s were selectively blocked (Robbe et al.,

2002). The application of a selective mGluR5 agonist was sufficient to also trigger this plasticity, and both MFS and the use of the mGluR5 agonist each did not produce LTD in the presence of CB1R antagonist nor in CB1R knockout mice, demonstrating the role of eCBs downstream of mGluR5 activation in this mechanism (Robbe et al., 2002). NMDAR, group II mGluR, D1DR, and D2DR blockades each did not affect the induction of cortical afferent LTD in the NAc, despite the importance of D2DR activation in the induction of LTD in the dorsal striatum. While D2DR activation is not required in the NAc for LTD as it is in the dorsal striatum, it is not precluded that additional D2DR activation might play a facilitatory role in increasing the amount of eCB production during the induction of LTD.

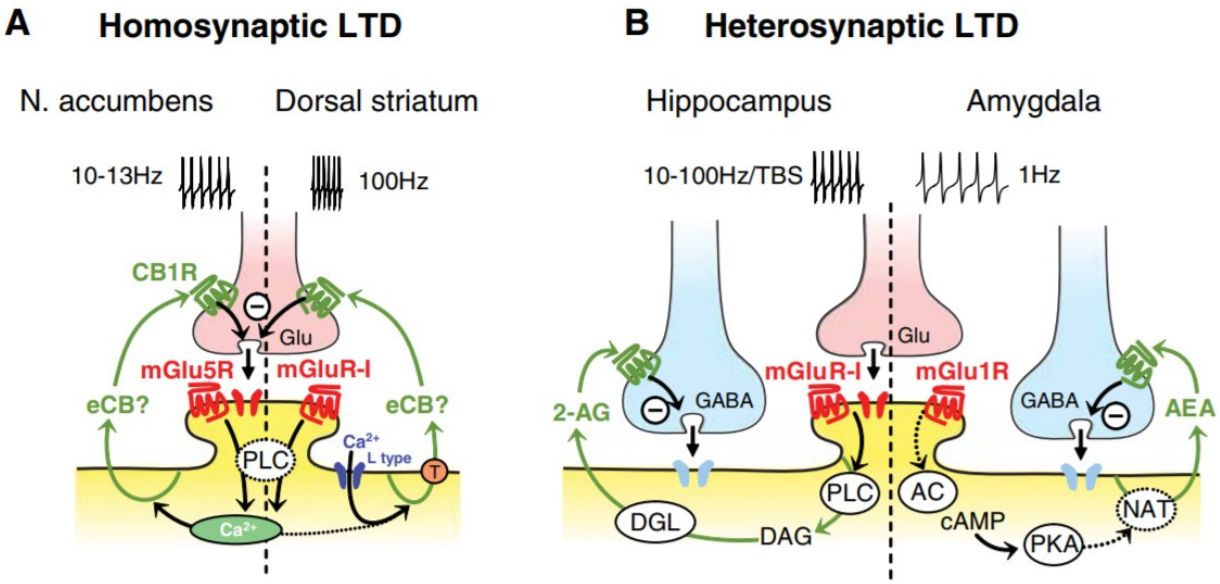


**Figure 3.** Homosynaptic and heterosynaptic eCB-LTD (Chevaleyre et al., 2006). A: Homosynaptic eCB-LTD in the NAc and dorsal striatum is induced by stimulation of excitatory inputs to medium spiny neurons with mGluR-I activation and increased postsynaptic calcium, including from intracellular stores in the NAc and L-type calcium channels in the dorsal striatum. D2 receptor activation is also needed in the dorsal striatum, but not the NAc. LTD in each results in decreased glutamate release. B: Heterosynaptic eCB-LTD in the hippocampus and basolateral amygdala (BLA) is initiated by glutamate release and mGluR-I activation and decreases GABA release. Increased postsynaptic calcium is not needed for LTD at these inhibitory synapses. In the hippocampus, 2-AG release results from PLC-DAGL activation, while in the amygdala AEA release requires the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathway.

While homosynaptic LTD at glutamatergic afferents has been well studied in the striatum, eCBs have also been found to mediate heterosynaptic LTD at inhibitory synapses in some brain regions. In the amygdala and hippocampus, repetitive excitatory input onto principal neurons in mice and rats has been found to result in eCB release and LTD at neighbouring inhibitory inputs (Marsicano et al. 2002; Chevaleyre & Castillo 2003). Similar to LTD in the NAc, its induction in these regions required the activation of mGluRs on principal neurons and the activation of CB1Rs on the terminals of the GABAergic neurons being depressed. Through the heterosynaptic depressive influence on GABAergic input, glutamatergic inputs may indirectly increase the sensitivity to excitability of their target principal neurons, and ultimately use eCB-LTD cooperatively at neighbouring inputs to contribute to a net excitatory effect. These changes may play an important role in the formation of new memories in the amygdala and hippocampus, including in relation to reward and aversion related memories having to do with their connectivity and activity with the NAc and dorsal striatum during goal-directed behaviour.

Inhibitory input long term depression (LTDi) in the hippocampus is likely mediated by the eCB 2-AG, opposed to AEA mediating eCB-LTD in the NAc, since LTDi here in rats is eliminated by blocking DAGL, the synthesis enzyme of 2-AG (Chevaleyre & Castillo 2003). While LTDi production of 2-AG may be best triggered by HFS in the hippocampus, in the amygdala it seems low frequency stimulation may be most effective at inducing LTDi. Lateral amygdala afferents receiving low frequency stimulation underwent LTDi at synapses with principal neurons in the BLA that was absent in the presence of CB1R antagonist nor in CB1R KO mice (Marsicano et al. 2002).

For amygdala eCB-LTD, as with the hippocampus but not the striatum, mGluR-I activation was both necessary and sufficient while calcium influx was not required (Azad et al. 2004). Amygdala eCB-LTD, however, differs from that of the hippocampus in that inhibition of PLD and DAGL did not interfere with LTDi, suggesting that here it may not be mediated by 2-AG (Azad et al. 2004). In fact, mice lacking FAAH, the degradative enzyme of AEA, LTDi was facilitated, suggesting that, like the striatum, AEA may be the eCB responsible for amygdala eCB-LTD (Azad et al. 2004). It remains to be investigated whether heterosynaptic LTDi may also occur in the NAc as it does in these regions.

### 1.2.5 Other Receptor Targets

Aside from the well-known cannabinoid receptors type 1 and type 2, the eCBs may also act at a larger diversity of receptors targets, including orphan G-protein coupled receptors (GPCRs), such as GPR18, GPR55, and GPR119, peroxisome proliferator-activated receptors (PPARs), such as PPARgamma, and transient receptor potential channels (TRPVs), such as TRPV1. Together, the wider action of eCBs and CBs across a greater diversity of receptors than the classical cannabinoid receptors has been referred to as the "endocannabinoidome" (Di Marzo & Piscitelli, 2015). There is also some belief that some effects of eCBs may be mediated by a not yet discovered cannabinoid receptor type 3.

Of the orphan GPCRs, it is GPR55 which is best known for its activation by both eCBs such as AEA and 2-AG, and by some exogenous cannabinoid agents as well (Di Marzo & Piscitelli, 2015). Lysophosphatidylinositol (LPI), and its 2-arachidonoyl derivative (2-ALPI), may be the natural endogenous ligands at this receptor in rodents (Oka et al., 2009), while lysophosphatidylglucoside (LPG) may be the endogenous ligand in monkeys (Bouskila et al., 2016).

### 1.2.6 Differential Mediation by 2-AG and AEA

While the majority of GPCRs each have only one endogenous ligand, the cannabinoid and related receptors have many, most notably 2-AG and AEA. This extra degree of flexibility allows for a more diverse range of signaling across the eCB system and may complement the already diverse nature of receptors being present both presynaptically and postsynaptically, and the unusual neuromodulatory properties of the eCB system having signaling mechanisms for both anterograde and retrograde transmission. This degree of flexibility may be particularly necessary to account for the high total levels of CB1R in the brain (Mackie, 2005), and the variation in the extent to which different brain areas require different signaling patterns, but of which other neurotransmitter systems may be incapable. It is important to consider the differences in 2-AG and AEA signaling when looking at a specific brain region because one may be more responsible for the mechanism of interest, such as findings that AEA and not 2-

AG may mediate eCB-LTD in the NAc (Giuffrida et al., 1999), or their precise balance may matter for maintaining the correct level of CB1R activation. This further highlights that the enzymes which control 2-AG and AEA levels may also differ in their importance across brain regions and necessitates region-specific differences in study.

AEA may serve as a high efficacy partial agonist at CB1Rs, while 2-AG may act as a lower efficacy full agonist (Sugiura et al., 1999). The efficacy of an agonist is the amount of receptor activation produced by a given amount of agonist binding with an affinity. Higher efficacy means that less ligand binding to the receptor is required for greater activation of the receptor, while partial agonism means that the ligand can only achieve partial effects at the receptor and not the maximal effect of a full agonist. Thus at low concentrations, a high efficacy partial agonist may have more effect than a lower efficacy full agonist, but at higher concentrations a lower efficacy full agonist can continue to cause more receptor effects.

AEA signaling may also act at postsynaptic TRPV1s to cause a reduction in 2-AG biosynthesis that may increase the ratio of retrograde AEA to 2-AG signaling (Maccarrone et al., 2008). Differences in the strength of each of the eCBs at a diversity of receptors may indicate an important homeostatic mechanism produced by finely balancing the ratio of one signaling molecule over the other, despite the overlap in targets. It is additionally interesting that TRPV1 channels may often be co-expressed with either or both of CB1R and CB2R (Di Marzo & De Petrocellis, 2012). While 2-AG may more commonly play the role of retrograde neuromodulator, in some locations such as the hippocampus AEA may play a role as a tonic retrograde mediator, opposed to the more classic mechanism of rapid production of eCBs for phasic and responsive retrograde signaling (Kim & Alger, 2010). When AEA does participate in phasic signaling, it seems most likely that it does so both presynaptically and postsynaptically via both CB1R and TRPV1 (Di Marzo & De Petrocellis, 2012), potentially influencing synaptic plasticity at both sides of the synapse.

Postsynaptically, AEA activation of TRPV1 may hyperpolarize neurons by reducing 2-AG synthesis by DAGLalpha, resulting in less retrograde inhibition of GABA release onto striatal MSNs (Maccarrone et al., 2008). It may also cause the endocytosis of AMPA receptors, resulting in LTD from attenuated glutamatergic signaling capacity (Grueter et al., 2010). Presynaptically, however, the activation of TRPV1 by AEA facilitates glutamatergic signaling

in striatal MSNs (Musella et al., 2009). Findings of both NAPE-PLD and 12-lipoxygenase, which converts arachidonic acid into 12-HPETE, together in some brain areas suggest that either or both of the TRPV1 ligands AEA and 12-HPETE may contribute to presynaptic LTD mediated by TRPV1 activation (Di Marzo & De Petrocellis, 2012).

In the bed nucleus of the stria terminalis (BNST), postsynaptic LTD may be mediated by TRPV1 activation by AEA, while 2-AG acts in a retrograde fashion to also produce short term depression and LTD at the same neurons (Puente et al., 2011). In this case, the separate presynaptic and postsynaptic action of AEA and 2-AG may synergize. AEA and 2-AG may also provide some other differing effects aside from their receptor activity. AEA may directly inhibit T-type calcium channels and TASK potassium channels to enhance or reduce CB1R signaling where needed (Di Marzo & De Petrocellis, 2012). The allosteric enhancement of glycine receptor excitability by AEA and GABA-A receptor excitability by 2-AG also allow for control of neuronal inhibition by the eCB system when eCB receptors are not even present (Di Marzo & De Petrocellis, 2012), and with the flexibility of being able to have separate effects on separate inhibitory channels.

Since different eCBs may each act on more than one target simultaneously, and since each target may have a different combination of ligands of varying capability acting on them simultaneously, there is then a large and complicated set of possible interactions of eCBs at varying receptors. Since eCBs may also synergize in effect, or alter their ratio of signaling against one another, it allows a diverse set of possibilities across brain areas that the eCB system may provide differences in homeostatic control in each area that are finely tuned to the needs of that area. This, in effect, creates a second level of plasticity to how eCBs may at a given point, in a given place, influence synaptic plasticity.

## 1.3 Endocannabinoids and the Reward Circuit

The eCB system is widely expressed throughout the central nervous system, including throughout the reward circuit (Figure 4) in mice, rats, monkeys, and humans (Herkenham et al., 1990; Gatley et al., 1996; Glass et al., 1997; Ong & Mackie, 1999). The reward circuit is a part

of the limbic system involved in emotional value attribution underlying motivation to approach stimuli based on whether they are rewarding. The central axis of the reward circuit is the release of DA from the ventral tegmental area (VTA) onto the NAc. The reward circuit also involves their interaction with the decision-making of the prefrontal cortex (PFC), hippocampal memory systems, other limbic structures involved in stress and anxiety, and basal ganglia motor circuits involved in initiating eye movements and locomotion related to approach. The eCB system is present in these areas and its modulation by eCBs plays a critical role in their regulation of signaling within the reward circuit (Zlebnik & Cheer, 2016).

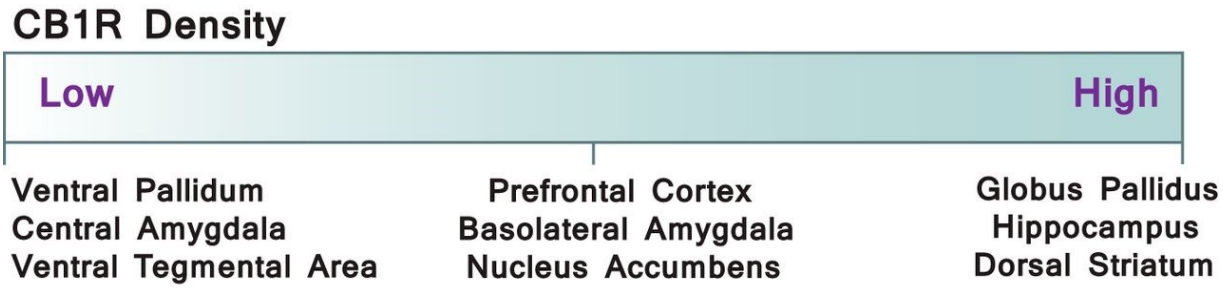


**Figure 4.** CB1R expression levels in several reward circuit brain regions (Zlebnik & Cheer, 2016).

### 1.3.1 Reward Circuit Anatomy

The reward circuit is a set of brain structures intimately linked to survival of the organism and of the species, and existing within the brain far down the evolutionary ladder. The primitive drives to feed, drink and reproduce are an important part of animal behaviour, even for a species as intelligent and evolved as humans. This system, however, has some susceptibility to substances of abuse to become "hijacked" into misattributing value from survival cues to drug acquisition and consumption. The key component of this system is the release of DA from the VTA mesoaccumbens neurons projecting onto the NAc (Figure 5), which activates the reward circuitry of the brain (Lupica and Riegel, 2005). The NAc is an ovoid structure in the ventral striatum of the basal forebrain that is subdivided into a shell, which is involved in reward perception, and a core, which forms a motor circuit related to approaching and avoiding

rewarding and aversive stimuli respectively (Groenewegen et al., 1999; Parkinson et al., 1999; Corbit et al., 2001).

Evolutionarily, the cannabinoids also affect non-mammalian vertebrates and some invertebrates, such as having affecting cell division and macromolecular synthesis in the protozoan Tetrahymena pyriformis, behavioural effects in ants, and effects on neurotransmitter release in lobsters (Elphick & Egertova, 2001). A CB1R orthologue gene, but none for CB2R, has been found in the puffer fish *Fugu rubripes*, and suggests that the CB1R is likely also found in various non-mammalian vertebrates such as amphibians, reptiles, and birds (Elphick & Egertova, 2001). For invertebrates, sea urchin sperm cells contain a receptor orthogolous to those in vertebrates, but locusts, *Drosphila melanogaster*, and *Caenorhabditis elegans* do not contain genes which are orthologues of mammalian cannabinoid receptors (Elphick & Egertova, 2001). Invertebrates, and possibly other member of the animal kingdom, may contain proteins capable of binding cannabinoinds, but most are not structurally related to the vertebrate CB1R and CB2R (Elphick & Egertova, 2001). Due to sea urchin CB1R, the CB1R may have began in early deutrerosomes and then branched several times, but the CB2R likely didn't diverge from CB1R until early mammals.

Inappropriate release of VTA DA in mammals can contribute to misattribution of survival cues to other sources, such as is the case when drugs of abuse increase the activation of the VTA-NAc circuitry through various methods (Lupica and Riegel, 2005). Opiates such as heroin and morphine inhibit the release of GABA onto VTA DA neurons, resulting in a disinhibition of DA release. Psychostimulants such as cocaine and amphetamines block reuptake of DA by the dopamine reuptake transporter (DAT) on axon terminals such that DA is not cleared from the synaptic cleft. Amphetamines additionally inhibit synaptic vesicle storage of monoamines, stimulating DA exocytosis. Nicotine causes a direct increase in the activity of DA axon terminals in the Nac projecting from the VTA, but more importantly causes a longer lasting increase in DA by increasing glutamate release onto VTA DA neurons that increases their release of DA. Ethanol also increases DA release in the NAc, but the precise mechanism remains not yet fully understood.

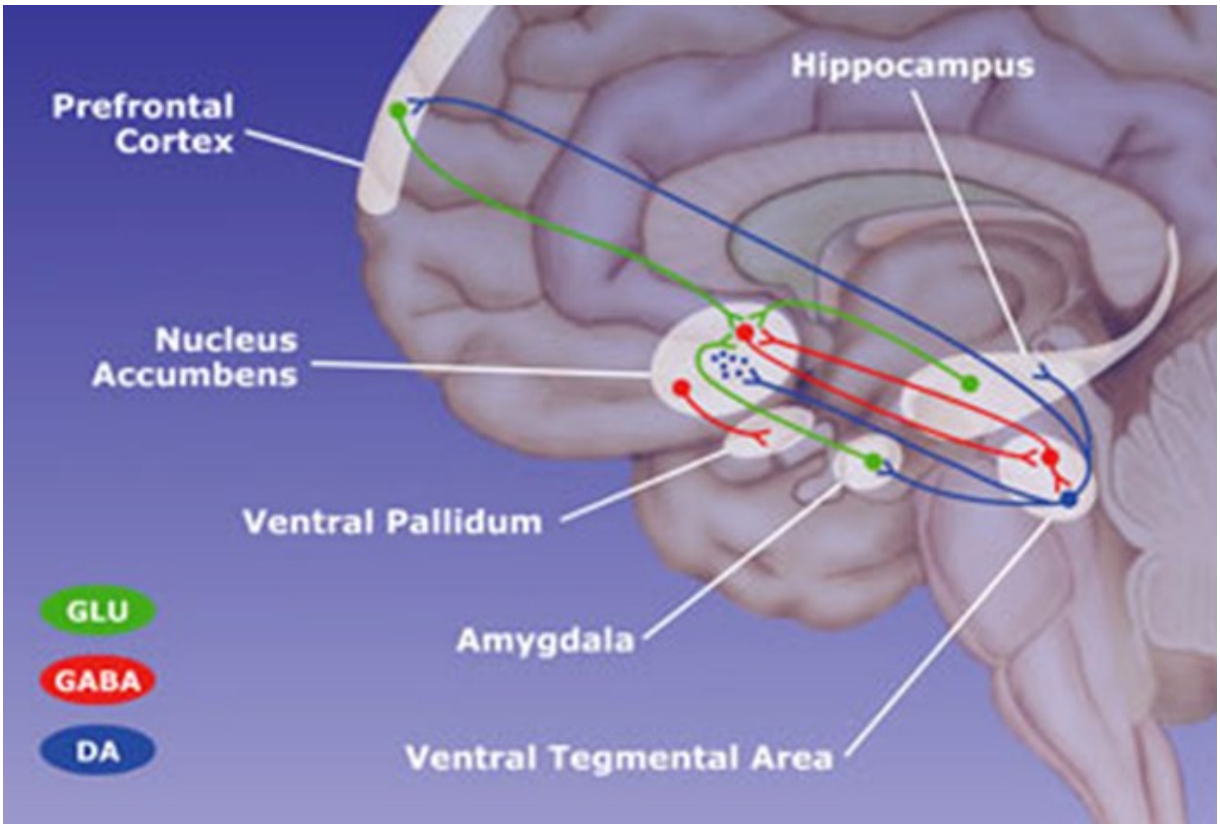


**Figure 5.** Reward circuit anatomy (Public figure). The central axis of the reward circuit is the release of DA from the VTA onto the NAc, though DA is also released onto several other areas. The NAc receives key excitatory input from the PFC, as well as excitatory inputs from the hippocampus and amygdala. The NAc sends inhibitory projections back to the VTA, as well as to other areas, including the ventral pallidum and substantia nigra.

In the case of the phytocannabinoids present in marijuana, such as the primary active constituent delta-9-tetrahydrocannabinol (THC), the mechanism of action may involve the attenuation of GABA release onto the VTA both directly and indirectly from the activation of CB1Rs on various cell types (Figure 6). Within the NAc, GABAergic medium spiny neurons (MSNs) are the main projection neurons, accounting for the majority of cells. These projections can be part of two different projection pathways. MSNs with DA D1-like receptors are part of the direct projection pathway, while MSNs with DA D2-like receptors are part of the indirect projection pathway. There are also various interneuron types, mainly various types of GABAergic interneurons such as those which are parvalbumin (PV)+. Additionally, there are

cholinergic interneurons which act at multiple receptors subtypes similarly to DA projections from the midbrain, but typically work against DA related behaviours.

The activation of CB1Rs on medium spiny neurons (MSNs) projecting to the VTA (Mackie, 2005) may directly reduce their release of GABA onto VTA DA neurons. CB1Rs on glutamatergic terminals projecting to the NAc from the PFC may also result in a reduction of glutamate onto NAc MSNs (Robbe et al., 2002), which may result in a reduction of their release of GABA onto the VTA. CB1Rs on fast-spiking parvalbumin positive interneurons (FSIs) (Winters et al., 2012) may interfere with their synchronization of populations of MSNs in the NAc (Younts & Castillo, 2014), having a net effect of lowering VTA DA neuron inhibition despite a reduction in direct MSN inhibition since MSNs are projected in clusters and require strong and coordinated activation to effectively inhibit VTA DA neurons (Pennartz et al., 1994). VTA DA neurons do not express CB1Rs (Herkenham et al., 1990) and thus are not affected directly, resulting only in their disinhibition and the dysregulated release of excess DA.

DA is released into the NAc tonically, but also at times in a phasic pattern that progressively decreases in amplitude and decreases in duration (Grace & Bunney, 1984). Behaviourally active cannabinoids have been shown in rats to increase both the tonic firing rate and phasic bursting activity of midbrain dopamine neurons onto the Nac, with the phasic bursting producing the most noticeable increase in transmitter release (French et al., 1997). There appear to be few cannabinoid binding sites in the VTA and SN, and direct injection of THC there does not cause much effect on DA release onto the Nac (French et al., 1997). This supports that the main effect of cannabinoids on midbrain DA release onto the Nac is likely not direct, though there may be some role on midbrain interneurons.

The main effect of cannabinoinds may then be on NAc efferents to the midbrain which have their transmitter release reduced, resulting in less inhibition of midbrain DA cell bodies and glutamatergic terminals onto them, resulting in an indirect increase of midbrain DA neuron activity onto the NAc, particularly of bursting patterns. The release of DA onto the NAc may also be implicated in more than just the perception of reward, but also the encoding and activation of motor patterns via a motor circuit through the substantia nigra (SN). The substantia nigra pars compacta (SNpc) has DAergic projections onto the NAc core which may be key to the activation of reward-related motor patterns (Groenewegen et al., 1999). The SN additionally receives input from NAc MSNs expressing CB1Rs on their terminals (Julian et al., 2003). When

the VTA is strongly activated torelease large amounts of DA on the NAc, it may be disinhibited by a resulting increase in CB1R activation. The SN may then be similarly disinhibited by heightened CB1R activation. The combination of dopaminergic effects on the NAc core and shell may underlie both the reward and motor aspects of motivated behaviour caused by drug consumption.

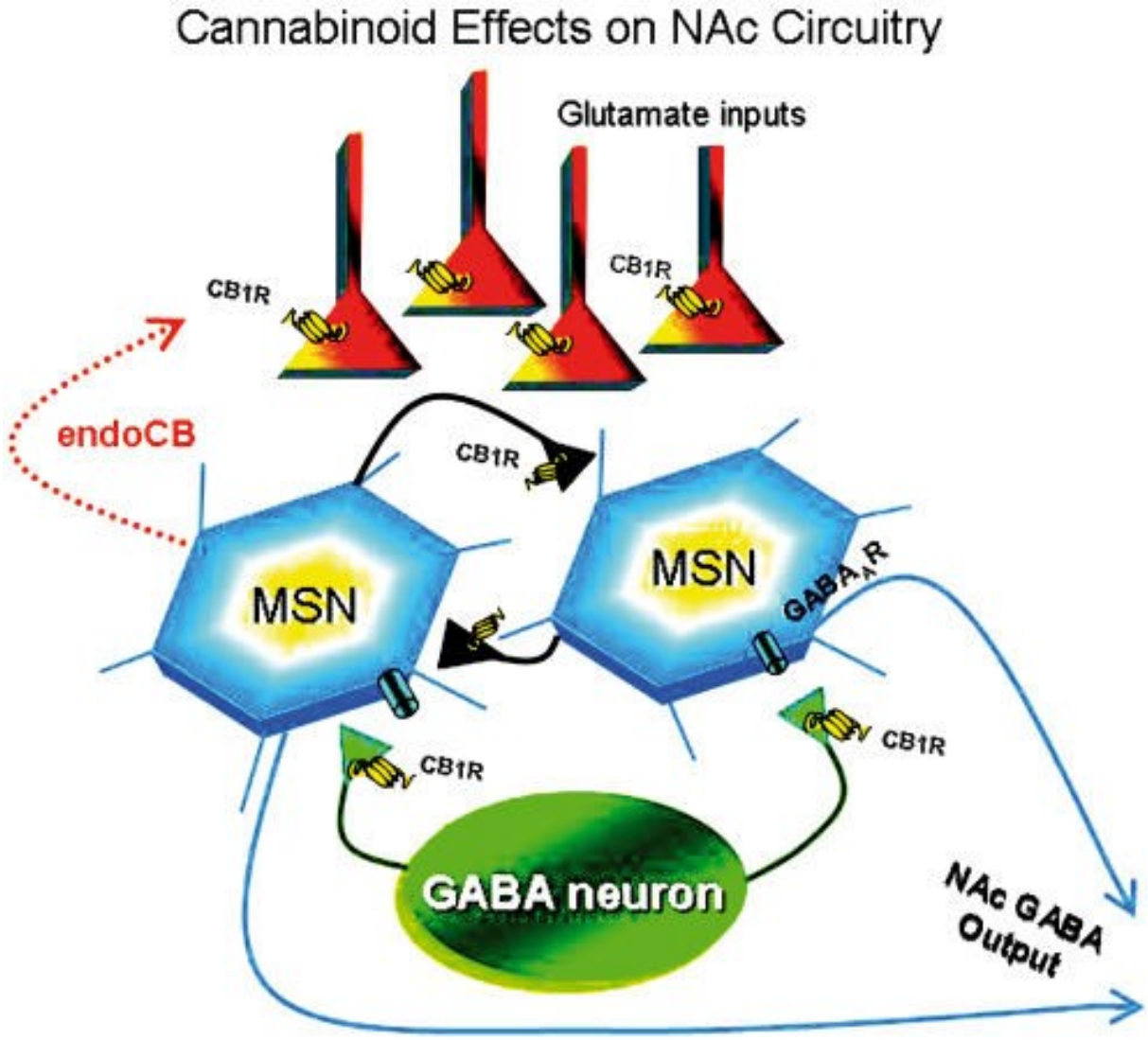


**Figure 6.** Cannabinoid effects on NAc circuitry (Lupica et al., 2004). Within the NAc, CB1Rs are present on GABAergic MSNs, their glutamatergic cortical afferents, and certain GABAergic interneurons. MSNs may have axon collaterals onto one another, which may be one site of their CB1Rs. CB1R activation may reduce MSN activity directly, reduce MSN activation from cortical inputs, and reduce MSN modulation by GABAergic interneurons. The net effect is reduced NAc MSN GABA output to efferent brain regions such as the VTA.

### 1.3.2 Prefrontal Cortex Influence

The PFC may also play an important role through its importance in decision-making and self-control. The PFC contains glutamatergic neurons expressing CB1R that form direct connections to the VTA and NAc (Parsons & Hurd, 2015). Glutamatergic activation of NAc medium spiny neurons (MSNs) plays a crucial role in activating their release of GABA onto the VTA to inhibit excess DA release that may otherwise cause unbalanced motivation for reward. The overactivation of DA release in the NAc may result in increased activation of D2DRs in MSNs that may contribute to eCB-LTD of these glutamatergic projections (Chevaleyre et al., 2006), and a resulting weakening of PFC augmentation of NAc MSN inhibition of dopaminergic VTA neurons.

### 1.3.3 Motor Circuit with Substantia Nigra

The NAc core contains MSN projections to the SN, which are affected by glutamatergic PFC projections, and the activation of which affects the release of DA by the SNpc back onto the striatum in rats (Robbe et al., 2002; Julian et al., 2003). DA plays a neuromodulatory role to activate the D1-like family of dopamine receptor, which includes dopamine receptor D1 and D5, and are present in the direct pathway of projections which inhibits neurons in the globus pallidus interior (GPi) (Silkis, 2001). DA also modulates the D2-like family of receptors, which includes D2, D3, and D4, oppositely, reducing the activation of the indirect pathway which inhibits the globus pallidus exterior (GPe) (Silkis, 2001). The GPe then provides greater inhibition of the subthalamic nucleus (STN), which is then able to produce less activation of its targets in the GPi (Silkis, 2001).

As a result of dopaminergic signaling on the direct and indirect pathways, GPi inhibition of the motor thalamus is blocked (Figure 7), resulting in increased activation of the motor cortex and the initiation of movement (Silkis, 2001). The activation of the D1 pathway is responsible for the disinhibition of approach behaviours, while the D2 pathway causes the inhibition of avoidance behaviours. Though there is a slight distinction between increasing approach and reducing avoidance, the effect of DA on these pathways is ultimately synergistic. In cases of

negative learning, the reduction of DA would result in the opposite change in direct and indirect pathway activation and inactivation, reducing approach behaviour and increasing avoidance behaviour. It can also be noted that acetylcholine (ACh) acts in opposition to DA in the direct and indirect pathways, also acting in a neuromodulatory fashion through multiple muscarinic receptors, most notably receptors M1 in the indirect pathway and M4 in the direct pathway (Silkis, 2001). There is a resulting net reduction of motor activity when ACh levels outbalance DA levels.

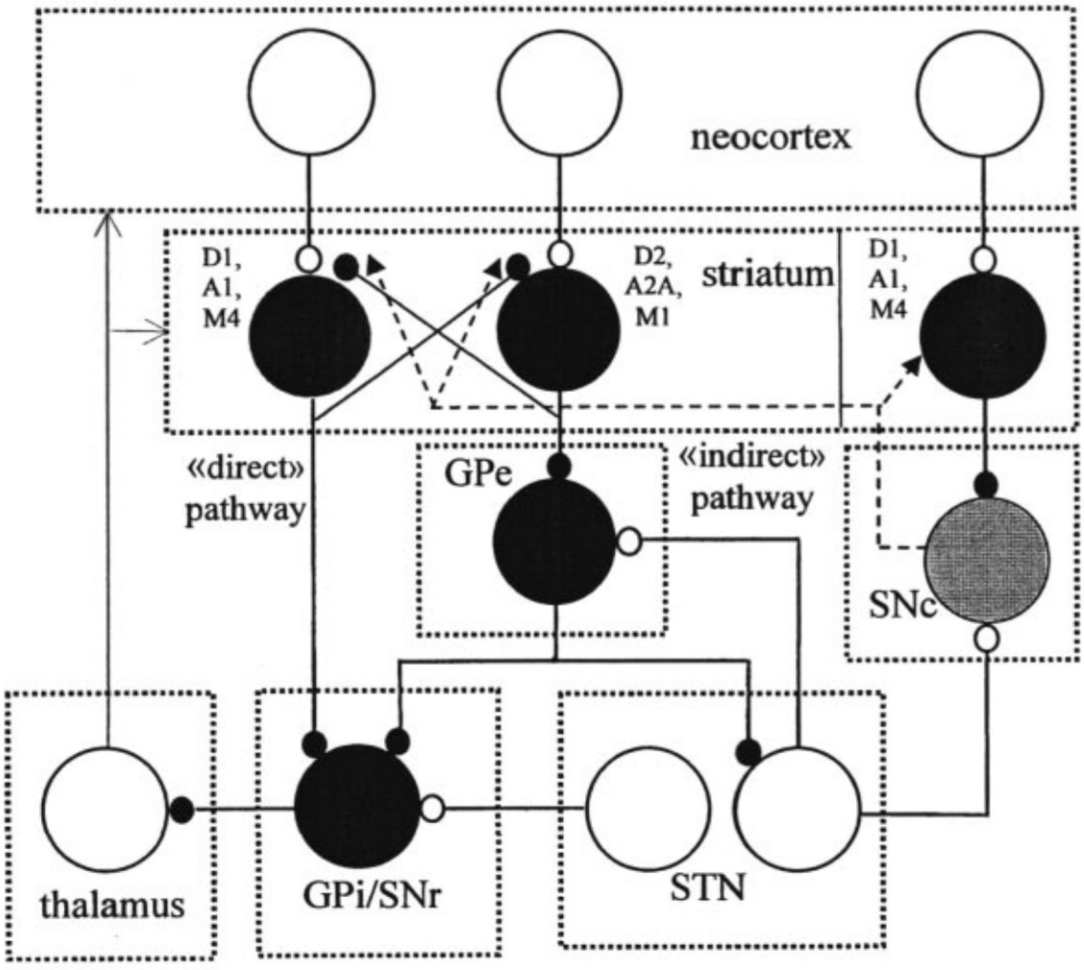


**Figure 7.** Basal ganglia motor pathway (Silkis, 2001). The striatum receives glutamatergic inputs from the cortex and dopaminergic inputs from the SNc which initiate the motor circuit. D1DRs and M4 AChRs are present in the direct pathway, while D2DRs and M1 AChRs are present in the indirect pathway. The direct pathway from the striatum inhibits the GPi/SNr to disinhibit the thalamus, resulting in increased motor activity of

approach behaviours. The indirect pathway from the striatum inhibits the GPe to disinhibit the STN. The STN increases activation of the GPi/SNr to inhibit the thalamus and reduce motor activity to cause avoidance behaviours. D1DRs enhance direct pathway activation and approach behaviours, while D2DRs reduce indirect pathway activation and avoidance behaviours. M1/M4 AChRs act in opposition to DA receptors in each pathway.

### 1.3.4 Stress Influence from the Extended Amygdala

The extended amygdala, which includes the central amygdala (CeA), the bed nucleus of the stria terminalis (BNST), and the sublenticular substantia innominata, has direct projections onto the NAc and the VTA. Glutamatergic projections onto these reward circuit structures from the basolateral amygdala contain CB1Rs on their terminals (Parsons & Hurd, 2015). The amygdala lies near the caudal end of the NAc, and is believed to be intimately related, particularly through its regulation of fear and stress. Stress is believed to play a role in models of relapse to drug-seeking behaviour, and the attenuation of stress to reduce drug self-administration and be preventative towards relapse (Mantsch et al., 2015). The eCB system is considerably expressed in the amygdala (Zlebnik & Cheer, 2016) and may be a critical mediator of stress in this structure.

Various studies have shown that certain stressors such as intermittent foot shock stress in rats can induce reinstatement of drug-seeking and self-administration of many rewarding substances, as well as palatable food rewards in some cases, and even operant responding previously reinforced by brain stimulation reward (Mantsch et al., 2016). This also applies to the reinstatement of drug-related conditioned place preference. The selective reinstatement on food in only some cases and not others may be due to the hypothalamic release of corticotropin-releasing factor (CRF) from stress which inhibits food intake through the hypothalamic-pituitary axis, but also has actions in other brain circuits such as the extended amygdala. Ventricular injections of CRF have been shown to induce reinstatement of various substances such as heroin, alcohol, and cocaine (Mantsch et al., 2016). Metyrapone, which inhibits corticosterone synthesis, also activated the CeA and caused heroin seeking reinstatement, supporting this role of CRF at the CeA in reinstatement.

Lateral tegmental noradrenergic nuclei are also activated by stress to release noradrenaline at the BNST and CeA, but when blocked do not prevent the effects of CRF injection. This suggests that noradrenaline is upstream of CRF in the stress circuit. The blocking of reinstatement after pharmacologically induced noradrenaline by CRF1 receptor antagonist injection in the ventral BNST suggests that the ventral BNST is the site of noradrenaline's interaction with CRF in producing stress (Mantsch et al., 2016). The median and dorsal raphe nuclei release of serotonin has also been implicated in reinstatement. Systemic injection of the serotonin reuptake inhibitor fluoxetine decreased stress induced reinstatement of alcohol seeking, while median raphe nucleus injection of an agonist reducing serotonergic firing and release had a similar effect to stress on reinstatement (Mantsch et al., 2016). In the dorsal raphe nucleus, increased GABA transmission from muscimol reinstated morphine conditioned place preference, while inhibition of GABA by bicuculline decreased stress induced reinstatement (Mantsch et al., 2016). Thus stress may cause lateral tegmental noradrenaline release onto the BNST and CeA, an interaction of noradrenaline at the ventral BNST affecting CRF release, an interaction between serotonin and CRF, and a resulting activity on extended amygdala projections onto the reward circuit, including directly onto the NAc, influencing reward-seeking behaviour.

### 1.3.5 Addiction and Prospective Pharmacological Approaches

According to the opponent-process model of motivation (Solomon and Corbit, 1974), there is an a-process which represents the positive hedonic or mood state and a b-process which represents the negative hedonic or mood state. The mood state or affective stimulus resulting from the use of a drug is the sum of these two processes. When a drug is first experienced with no history of use, the affective response to the drug is an initial positive hedonic increase in mood, that then declines into a decrease in mood below the original baseline of mood, but that should eventually return to the homeostatic baseline.

However, to appropriately match chronic demands, an allostatic set point may sometimes instead be reached which diverges significantly from normal homeostatic parameters (Koob & Le Moal, 2001). The b-process is a counteradaptive opponent process which balances the

activational a-process. When the b-process is fully effective and has a sufficient amount of time to take its course, homeostasis should be restored. However, if drug re-exposure occurs without an effective and complete b-process, then instead of returning to the original homeostatic state, there may be an incomplete recovery to a new, lower allostatic mood baseline (Figure 8). Repeated frequent drug use may compound incomplete recoveries and result in increasingly negative allostatic baseline mood states. Though individual incidences may create neglible changes, since the b-process is never actually quite perfect at recovering mood to baseline, over time increasing damage may accumulate to baseline mood and response to reward even with a recovery period (Koob & Le Moal, 2001). This damage can lead to progressively worse pathological states to the brain and body and resulting behaviour. Eventually, response to reward is altered and mood is lowered to a state where the substance is no longer taken to produce euphoria, but instead is taken to mitigate the dysphoria of being outside of the state to which the organism has become adapted (Solomon and Corbit 1974; Koob & Le Moal, 2001).

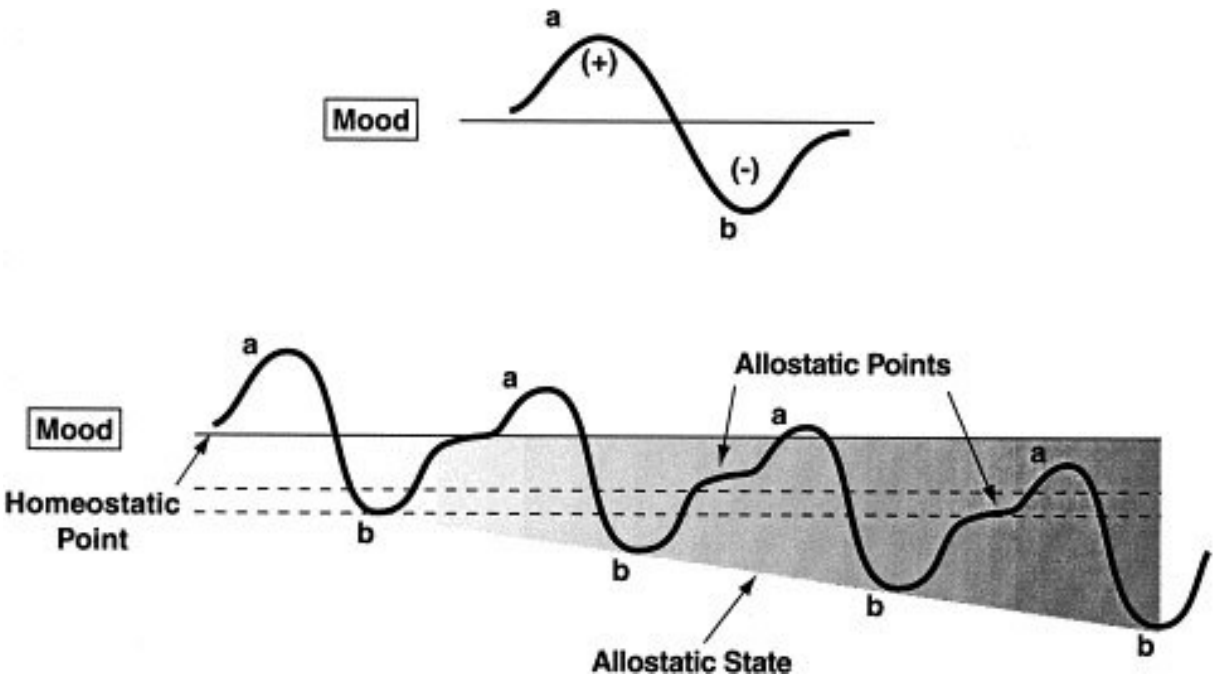


**Figure 8.** Model of decreasing allostatic mood set points during chronic substance use (Koob and Le Moal, 2001). Substance use causes an initial increase a in mood above the baseline, followed by a decrease b below the baseline. The recovery process b never fully returns to the original baseline, causing the natural homeostatic baseline point to degrade over time to progressively lower allostatic set points. Initially substance uses produces euphoria, but later is used to mitigate chronic dysphoria.

Extended abstinence from substances to detoxify may allow for partial recovery from the damage produced by the allostatic load resulting from substance abuse, but baseline mood is never able to fully return to its original homeostatic set point (Koob & Le Moal, 2001). This may be due to long-term changes in synaptic plasticity resulting from adaptation to the substance use or possibly from damage caused by the use of the drug.

If at least some of the unrecovered mood and altered reward response of an allostatic set point is from changes in synaptic plasticity, then it might be possible to pharmacologically correct these changes in plasticity. Even if some damage is irreversible, it still may be possible to functionally correct the effects of permanent damage resulting from drug use close to the original homeostatic baseline if it is possible to overcorrect remaining plasticity to also account for this damage. The eCB system is an important neurotransmitter system involved in reward and memory and has the capacity to influence synaptic plasticity. As a result, it is under investigation as a source of targets for pharmacological manipulation in varying ways that may influence the cessation of drug-seeking and self-administration, as well as relapse which may be based upon drug memory and long term synaptic plasticity changes compromising baseline mood and response to natural rewards.

CB1R antagonism has been effectively used to treat self-administration and prevent long-term relapse in rodent and primate animal models, across many different rewarding substances (Panlilio et al., 2010; Parsons & Hurd, 2015). The CB1R inverse agonist Rimonabant (SR141716) has also been used in humans to effectively treat obesity, though it was discontinued to negative side effects on mood, including depression, anxiety, and suicide (Christensen et al., 2007). The nature of these side effects on mood may just be a reflection of it being an inverse agonist which not only prevented the signaling of other molecules at CB1Rs, but also interfered with constitutive activity at these receptors, and may have actions at other receptor targets since it still had some inverse agonist properties in mice with the CB1R gene *cnr1* knocked out(Pertwee, 2005). This may then suggest that other classes of eCB system modulators, such as CB1R neutral antagonists or allosteric modifiers may be capable of producing the same beneficial effects in treating addiction that have been seen in animal models without the intense side effects that were seen with CB1R inverse agonism in humans. Considering the presence and localization of the CB1R system in the brain, and the well-

established rodent models of its signaling, it would not be surprising that over-antagonizing the constitutive activity of CB1R signaling in the reward circuit might fully explain intense adverse effects on mood. Too much CB1R antagonism could prevent the inhibition of various neurons involved in the circuitry responsible for the inhibition of dopaminergic VTA neurons, which could then greatly attenuate DA release onto the NAc and the associated feelings of reward and pleasure (Lupica & Riegel, 2005).

There is also, however, the distinct possibility that the intensity of the negative effects on mood associated with the inverse agonist, which are the exact opposite of the intense increase in mood produced by hedonic drugs, are precisely what are able to provide a long-term cure to addiction if it is able to reverse the synaptic plasticity changes from drug use. This negative effect on mood may be related to an opponent process on mood opposite to the one produced by hedonic drugs, that thus results in an increase in allostatic set points back closer to the original homeostatic one. If it is indeed the case that this is an essential part of the mechanism of the effectiveness of CB1R antagonism in treating addiction, then the return of CB1R antagonism use in humans in the future as a treatment on its own would be controversial. However, it may be possible to pair such a treatment with a second medicine which elevates or protects mood through a mechanism that doesn't require DA or impact synaptic plasticity, such that side effects could be mitigated during treatment while still allowing the restoration of damaged synaptic plasticity in the reward circuit.

There are, however, still numerous avenues of eCB system modulation remaining to be investigated which may be able to provide treatment without side effects that require secondary treatment. Aside from directly targeting the CB1R, it is also under investigation to indirectly affect signaling through controlling the levels of eCBs and their balance. FAAH inhibition to reduce the degradation of AEA, as well as AEA reuptake inhibition, have been under investigation. FAAH inhibition prevented relapse and reduced self-administration of nicotine in monkeys (Justinova et al., 2015). AEA reuptake inhibition via AM404 was able to reduce nicotine reinstatement in rats, though not reduce ongoing self-administration (Gamaleddin et al., 2013). This may produce a tonic, low amount of activation of CB1R, which may either hold the mood at a higher than average point through CB1R activation, or may produce a net reduction of CB1R activity below normal conditions by competitively interfering with the

endogenous binding of 2-AG, a more efficacious full agonist but that has lower affinity than AEA. It is also possible that any beneficial effects of altering AEA signaling may be in part mediated through the activation of TRPV1 (Maccarrone et al., 2008). One of the main constituents of marijuana, delta-9-tetrahydrocannabinol (THC) has also been considered as having therapeutic potential. The ability of exogenous cannabinoids to affect the stress circuit has caused THC to be tested in humans for the treatment of stress disorders, which is relevant to addiction since stress is a factor that has been associated with substance abuse (Roitman et al., 2014). Dronabinol, a partial CB1R agonist, has also been tested in humans as a withdrawal management therapy for opioid abuse (Lofwall et al., 2016). CB2Rs are now also being studied due to recent research finding their expression and electrophysiological and behavoiural significance in VTA dopaminergic neurons in rodents (Zhang et al., 2014), though their modulation seems to have different effects on different substances and potential species differences which remain to be addressed (Atwood & Mackie, 2010).

## 1.4 Objectives and Hypotheses

The objective of this study is to characterize the expression and the precise localization of the CB1R system within the NAc core and shell of vervet monkeys (*Chlorocebus sabaeus*) using Western blots and immunohistochemistry. Based on the rodent literature, we expect that CB1R, and the synthesizing and degradative enzymes of its endogenous ligand AEA, NAPE-PLD and FAAH respectively, are present in projection neurons, interneurons, and glia, but not in dopaminergic recipients, in the monkey NAc.

# Chapter 2: CB1R, NAPE-PLD, and FAAH in the Nucleus Accumbens of Vervet Monkeys

## Expression and localization of CB1R, NAPE-PLD, and FAAH in the vervet monkey nucleus accumbens

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Abstract

Extensive rodent literature suggests that the endocannabinoid (eCB) system present in the nucleus accumbens (NAc) modulates dopamine (DA) release in this area. However, expression patterns of the cannabinoid receptor type 1 (CB1R), the synthesizing enzyme *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), and the degradation enzyme fatty acid amide hydrolase (FAAH) in the NAc have not yet been described in non-human primates. The goal of this study is therefore to characterize the expression and localization of the eCB system within the NAc of vervet monkeys (*Chlorocebus sabaeus*) using Western blots and immunohistochemistry. Results show that CB1R, NAPE-PLD, and FAAH are expressed across the NAc rostrocaudal axis, both in the core and shell. CB1R, NAPE-PLD, and FAAH are localized in medium spiny neurons (MSNs) and fast-spiking GABAergic interneurons (FSIs). Dopaminergic projections and astrocytes did not express CB1R, NAPE-PLD, or FAAH. These

data show that the eCB system is present in the vervet monkey NAc and supports its role in the primate brain reward circuit.

## Introduction

The endocannabinoid (eCB) system is widely expressed in the central nervous system (CNS). It comprises the cannabinoid receptors type 1 (CB1R) and type 2 (CB2R), endogenous ligands (eCBs), and enzymes regulating the levels of eCBs[1,2,3]. The eCBs are lipophilic molecules that are synthesized "on demand" from the membrane of postsynaptic neurons after an increase in neural activity and calcium ion influx[1]. These endogenous ligands function as fast acting retrograde neuromodulators and are degraded rapidly[1]. The synthesis of anandamide, an eCB, is in part mediated by the release of N-acylethanolamines (NAEs) from N-acyl phosphatidylethanolamine (NAPE), by enzymes such as N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD)[4]. Its swift degradation is mostly mediated by the intracellular enzyme fatty acid amide hydrolase (FAAH)[5,6]. The expression of CB1R is found in many structures of the mouse, rat, monkey, and human brain, including the amygdala, cingulate cortex, prefrontal cortex (PFC), ventral pallidum, caudate, putamen, nucleus accumbens (NAc), ventral tegmental area (VTA) and lateral hypothalamus[7,8,9,10]. These regions are involved in reward, addiction and cognitive function. CB1R is also localized throughout the neocortex in rodents and primates[11,12,13].

Neurophysiological studies first demonstrated that cannabis exerts its addictive potential from activating the pleasure-reward circuitry of the brain, namely the VTA that synapses with the NAc[14]. NAc dopamine (DA) elevation is qualitatively indistinguishable whether it is produced by THC, opioids, amphetamine, cocaine, ethanol, nicotine, barbiturates, or addictive dissociative anesthetics such as phencyclidine[15,16]. The prevalence of treatment for cannabis dependence is greater than treatment for cocaine addiction in the USA[17], and its addiction potential has been further demonstrated by self-administration of THC in squirrel monkeys[17]. Within the NAc, there is a functional dissociation of the effect of VTA DA release onto the shell and core. The shell mediates feelings of reward while the core mediates locomotion toward rewards[18,19] through a motor circuit that includes the substantia nigra (SN)[20]. While it is known that the eCB system may influence these circuits, the detailed anatomy of eCB system components in these circuits has not yet been fully described.

Recent investigations have intensified efforts on the localization of an endogenous cannabinoid system in the NAc. CB1R is localized in the NAc of rodents[11,21,22], and is moderately expressed in the rat NAc[23]. It is also found in fast-spiking GABAergic interneurons (FSIs) in the NAc of mice[24] and is expressed by GABAergic interneurons in the rat NAc[25], but not in cholinergic or somatostatin-positive neurons in the rat NAc[25] or dopaminergic neurons in the basal ganglia of rats[7]. In the rat NAc, CB1R is present on GABAergic medium spiny neurons (MSNs)[26,27], and is also expressed in the mouse on the terminals of glutamatergic prefrontal cortical projecting neurons[28,29]. It has been proposed that cannabinoid receptors found on glutamatergic and GABAergic neurons modulate the activity of VTA DA neurons that project to the NAc[30]. Additionally, the SN has dopaminergic projections onto the NAc core[20] which may be similarly modulated. In the rat, the SN receives GABAergic projections from CB1R containing neurons in the NAc[27], suggesting an eCB role in the encoding of reward-related motor programs. The presence of CB1R has also been detected in the primate NAc[10], but not thoroughly investigated.

NAPE-PLD and FAAH distributions in the monkey NAc remain both unknown since all detailed immunohistochemical studies available to date have been carried out in rodents. NAPE-PLD plays a role in the rodent NAc signaling[22], and FAAH antagonists increase DA levels therein both with and without anandamide[31]. Furthermore, a large body of evidence shows that the eCB system modulates the neural activity within the NAc[14,30,32,33,34]. Since the NAc is a key player in addiction in rodent models and it contains components of the eCB system, it has been proposed that the latter may be involved in the mediation of addictive behavior. There is, however, no available data for the primate NAc and it is therefore the aim of this study to examine the expression and the precise localization of the eCB system components, namely CB1R, NAPE-PLD, and FAAH, in the vervet monkey NAc.

For its part, the CB2R is best known to be highly expressed in the immune system, including in brain microglia[35], but more recently has been found at low levels in some neurons[3]. This includes the finding of CB2R genes and receptors to be expressed in mice midbrain DA neurons, and therein to effect DA neuronal firing and related behaviour[36]. However, its function in the CNS is not yet as well understood[3] as CB1R on which the present investigation is focused. CB2R shares only 44% homology with CB1R[37], and as a result functions significantly differently. CB2R shows little modulation of calcium channels or inwardly rectifying potassium

channels in comparison to CB1R, which makes its signaling very different[38]. Its signaling is further complicated by species differences in CB2R response to identical drugs[3]. Despite common agonists, these receptors ultimately function differently. This is also reflected in differing affinity of their agonists. While 2-AG has high affinity at CB2R, anandamide serves as a weaker partial agonist of CB2R and has greater specificity to CB1R[3]. The significant difference in function of these two receptors makes them best studied separately. Here, the investigation of CB1R is complemented by the additional study of NAPE-PLD and FAAH, the synthesizing and degradative enzymes of anandamide.

Results

**Western Blot Analysis**

**CB1R, NAPE-PLD, and FAAH presence and specificity in the NAc.** We investigated the expression of three elements of the eCB system by evaluating the total amounts of CB1R and eCB-synthesizing (NAPE-PLD) and degradative (FAAH) enzymes in the monkey NAc. Immunoblots of three unfixed vervet NAc homogenates incubated with CB1R, NAPE-PLD, and FAAH antisera are shown in Fig. 1 and demonstrate their presence in the NAc. The specificity of the antibodies is shown by specific band recognition and blocking peptide signal abolishment. The CB1R blot recognized the expected major band at 60 kDa (Fig. 1a). The NAPE-PLD immunoblot shows as expected an intense band at 46 kDa (Fig. 1b), and the FAAH blot shows a dense expected band at approximately 63 kDa (Fig. 1c). Pre-incubation with the respective blocking peptides for NAPE-PLD and FAAH abolished the antibody signal for each (Fig. 1b, c), confirming the specificity of the antibody. However, for the CB1R antibody used here, a blocking peptide condition was not possible since there is not yet one commercially available. GAPDH loading controls for each immunoblot showed even levels of protein content across samples (n=3) for each condition, as well as even loading between conditions with and without their respective blocking peptides. We provide here for the first time a set of results in primates that further extends the data obtained in rodents[23].

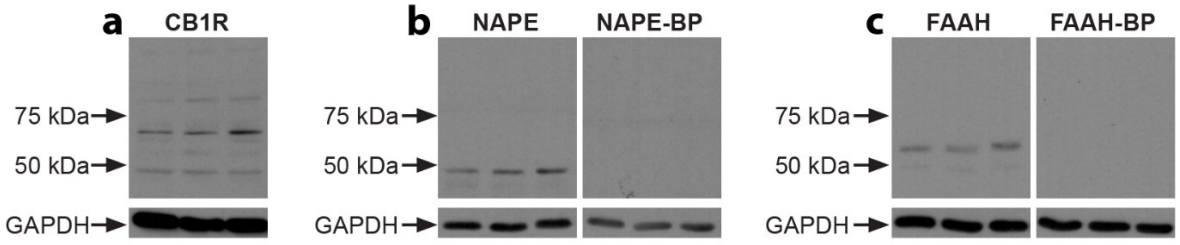


**Figure 9.** Article figure 1. Presence of CB1R, NAPE-PLD, or FAAH in the NAc of three vervet monkeys. WB analysis of total protein samples for the CB1R antibody (a) showing detection of the expected major protein band at 60 kDa. For the NAPE-PLD antibody (b), the expected band is seen at 46 kDa, and not detected when pre-incubated with its blocking peptide. For the FAAH antibody (c), the expected band is seen at 63 kDa, and not detected when pre-incubated with its blocking peptide. All lanes contained 10 µg of total protein. The lower blots show the expression of GAPDH and demonstrate loading in all lanes.

**DAB Single Labeling**

**Delineation of core and shell in the NAc.** To verify the precise location of the border between the core and shell of the NAc, DAB (3,3'-diaminobenzidine) immunostaining was carried out for calbindin-d28k (CB), a calcium binding and buffering protein that shows lower expression in the shell than the core[39]. Coronal sections of basal forebrain were taken, and 6 evenly spaced slices at a time were selected from across the rostrocaudal extent (Fig. 2). The border between the core and shell has been visualized with overlaid dashed lines. The demarcation of core and shell in the vervet monkey was found to be highly similar to previously published work in the macaque monkey[40]. Confirmation of the core and shell borders throughout the NAc allowed us to accurately determine the position of the nucleus and these subdivisions during confocal microscopy of our immunofluorescent experiments.

The vervet NAc has an irregular ovoid shape that varies across the rostrocaudal axis. The core expands as it progresses to the middle of the structure, and then becomes smaller once again as it reaches its caudal portion, and finally stretches into a thin oval as it subsides towards the most caudal extent. The shell encapsulates the ventral portion of the core throughout, always lying nearest to the apex of the heart-shaped striatum. The shell is largest in the middle of the rostrocaudal axis. The ventromedial beginning of the division of the core and shell can at times

be clearly seen by the nearby ventricle reaching between them, particularly in mid-rostrocaudal sections.

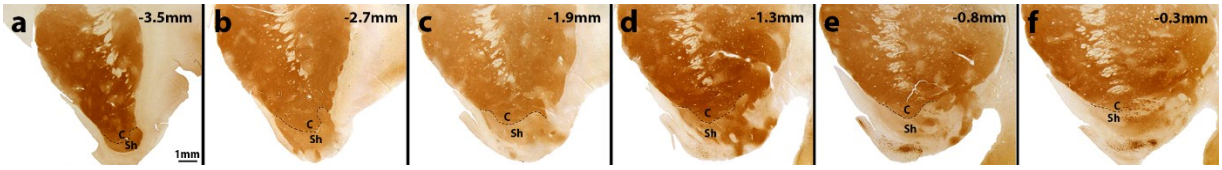


**Figure 10.** Article figure 2. Photomicrographs of calbindin-stained coronal sections of basal forebrain across the rostrocaudal axis. (a-f) The first rostral section of the NAc was taken at approximately 3.5mm anterior to the anterior commissure (AC) and the last caudal section was taken 0.3mm anterior to the AC. The total length of the NAc was approximately 4mm. Each slice distance relative to the AC is designated in the top right corner. The calbindin stain indicates the core and shell border of the NAc by overlaid dashed lines. The demarcation of core and shell is based on our own observation and previously published work[39,40]. Scale bar = 1 mm. C = core; Sh = shell.

**Spatial expression of the CB1R system in the NAc.** To visualize the localization of the CB1R system in the NAc, coronal serial brain sections containing the NAc were labeled with specific antibodies against CB, CB1R, NAPE-PLD, or FAAH. In the negative control condition, no primary antibody was used. Serial sections were taken from six representative levels across the rostrocaudal axis to compare the patterns of distribution. CB delimited anatomically the border between the core and the shell (Fig. 2a-f), as a reference for the rest of the series of slices, which were labeled for CB1R, NAPE-PLD, or FAAH. CB1R was detected throughout the NAc, but with higher expression in the dorsomedial and ventral shell in middle and caudal sections (Fig. 3c-f). In the caudal portion of the NAc, greater expression in the core was also present (Fig. 3d-f). NAPE-PLD and FAAH were homogeneously expressed across the rostrocaudal extent of the NAc (Fig. 3g-r). At low magnification, the entire NAc can be clearly seen and the distribution of eCB components visualized relative to the demarcation of the shell and core by CB. A consistent staining pattern across all monkeys was found.

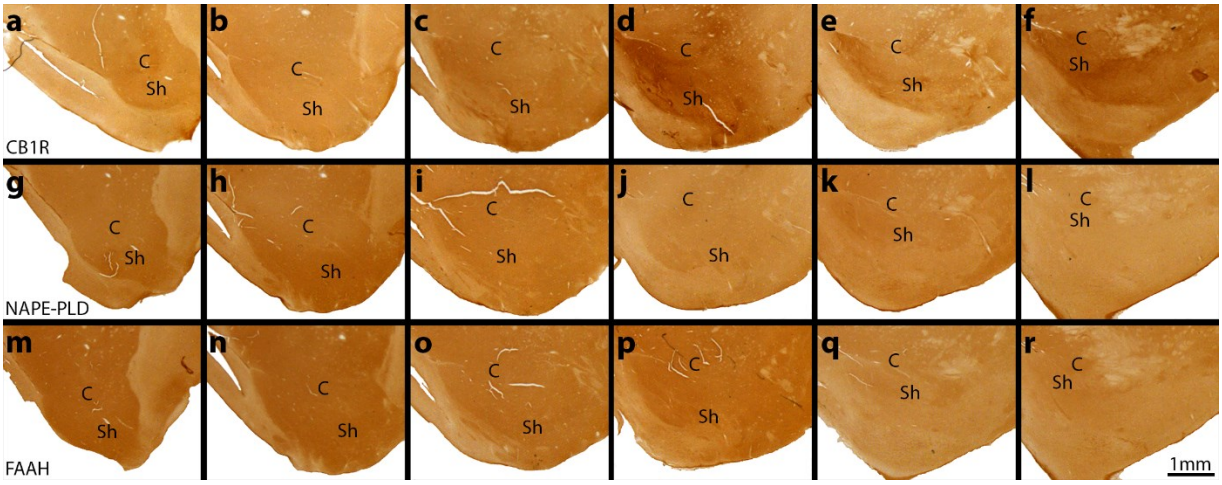


**Figure 11.** Article figure 3. Spatial distribution of CB1R, NAPE-PLD, and FAAH throughout the rostrocaudal extent of the NAc. Coronal serial sections were taken adjacent to the CB stained slices in Fig. 2. (a-f) Rostral sections show relatively homogenous staining of CB1R, but there is an increase of staining density in the medial portion of the shell beginning in mid-rostrocaudal sections (c). In (d) and further caudally, the CB1R expression is further increased in the medial shell. It is also noticeably augmented in the core and the ventral shell at these levels. (g-l) NAPE-PLD and (m-r) FAAH distributions remain relatively homogenous across the rostrocaudal extent. Scale bar = 1 mm. C = core; Sh = shell.

## Immunofluorescent Double Labeling

**CB1R, NAPE-PLD, and FAAH are expressed in medium spiny neurons (MSNs).** MSNs were marked with Ctip2, a transcription factor specific for their differentiation[41]. Double immunolabeling was performed against CB1R, NAPE-PLD, and FAAH (Fig. 4). The three CB1R system components were all clearly expressed in the soma of Ctip2-positive neurons.

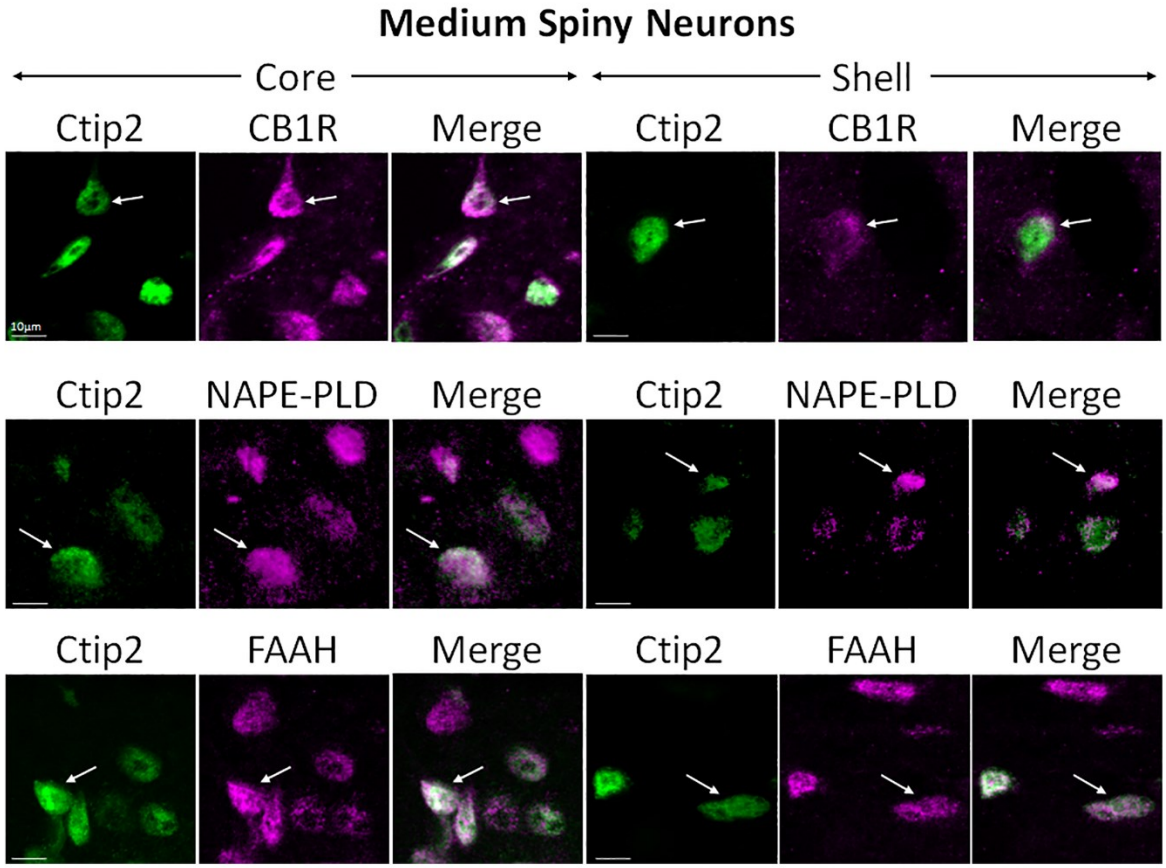


**Figure 12.** Article figure 4. Double-label immunofluorescence illustrating co-localization of CB1R-IR, NAPE-PLD-IR, and FAAH-IR with Ctip2. Confocal micrographs of NAc co-immunolabeled for CB1R, NAPE-PLD, or FAAH (magenta), and Ctip2 (green), a specific marker for MSNs, in core and shell. Arrows point at Ctip2-positive MSNs that express either CB1R, NAPE-PLD, or FAAH. Scale bar = 10 µm.

**CB1R, NAPE-PLD, and FAAH are expressed in fast-spiking GABAergic interneurons (FSIs).** The calcium binding protein PV was used to mark FSIs[42,43]. Double immunolabeling was performed against the CB1R system components (Fig. 5). PV can be seen throughout perikarya and fibers, extending down to axons. The eCB components can be seen most clearly in the cell bodies.

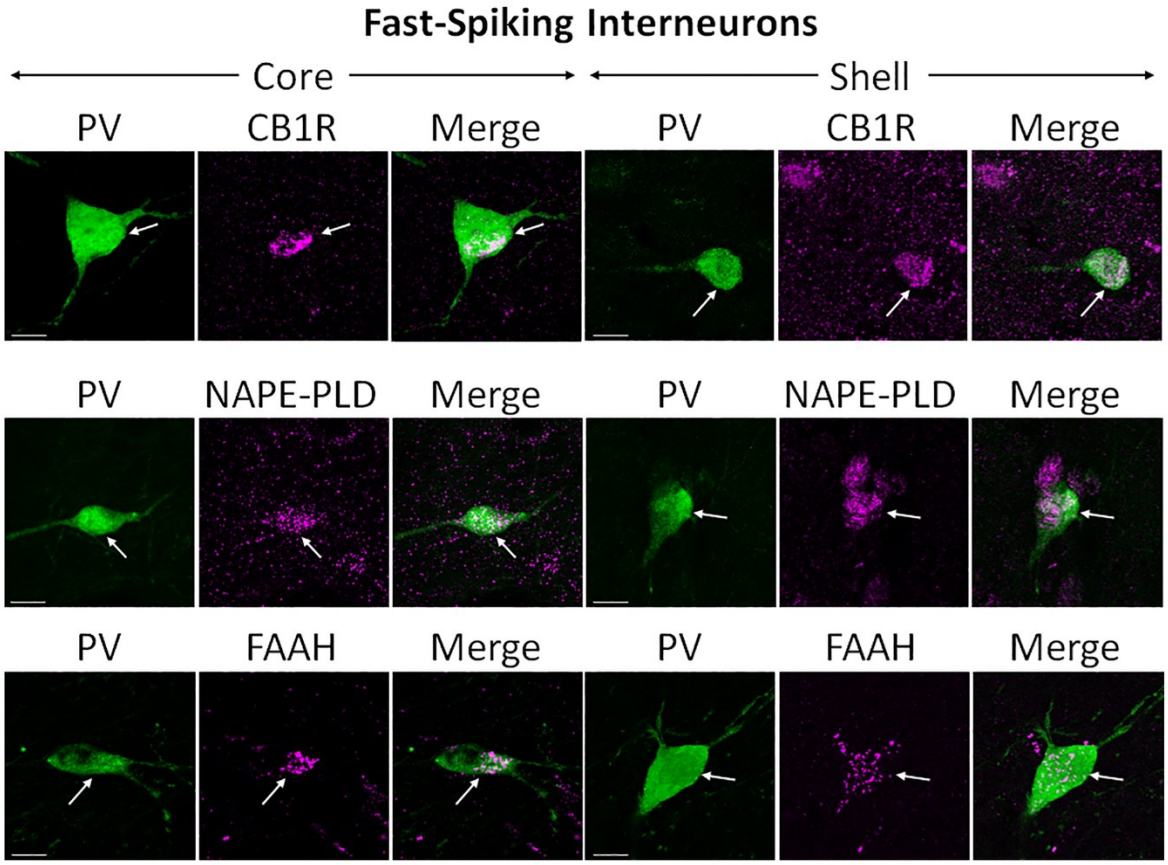


**Figure 13.** Article figure 5. Double-label immunofluorescence illustrating co-localization of CB1R-IR, NAPE-PLD-IR, and FAAH-IR with parvalbumin (PV). Confocal micrographs of NAc co-immunolabeled for CB1R, NAPE-PLD, or FAAH (magenta), and PV (green), a specific marker for FSIs, in core and shell. Arrows point at PV-positive interneurons that express either CB1R, NAPE-PLD, or FAAH. Scale bar = 10 μm.

**CB1R, NAPE-PLD, and FAAH are not expressed in DA-producing cells.** Tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis, was used as a specific marker of dopaminergic neurons. No co-localization was obtained when sections were stained with TH and CB1R (Fig. 6). Axon fibers and terminals stained with TH surround the multiple cell bodies labeled with CB1R, suggesting a complementary but not overlapping staining pattern. Sections immuno-stained with TH and NAPE-PLD or FAAH showed similar patterns of complementation without co-localization to that of TH with CB1R (Fig. 6). Lack of CB1R system expression in dopaminergic neurons is consistent with previous findings in rodents[7].

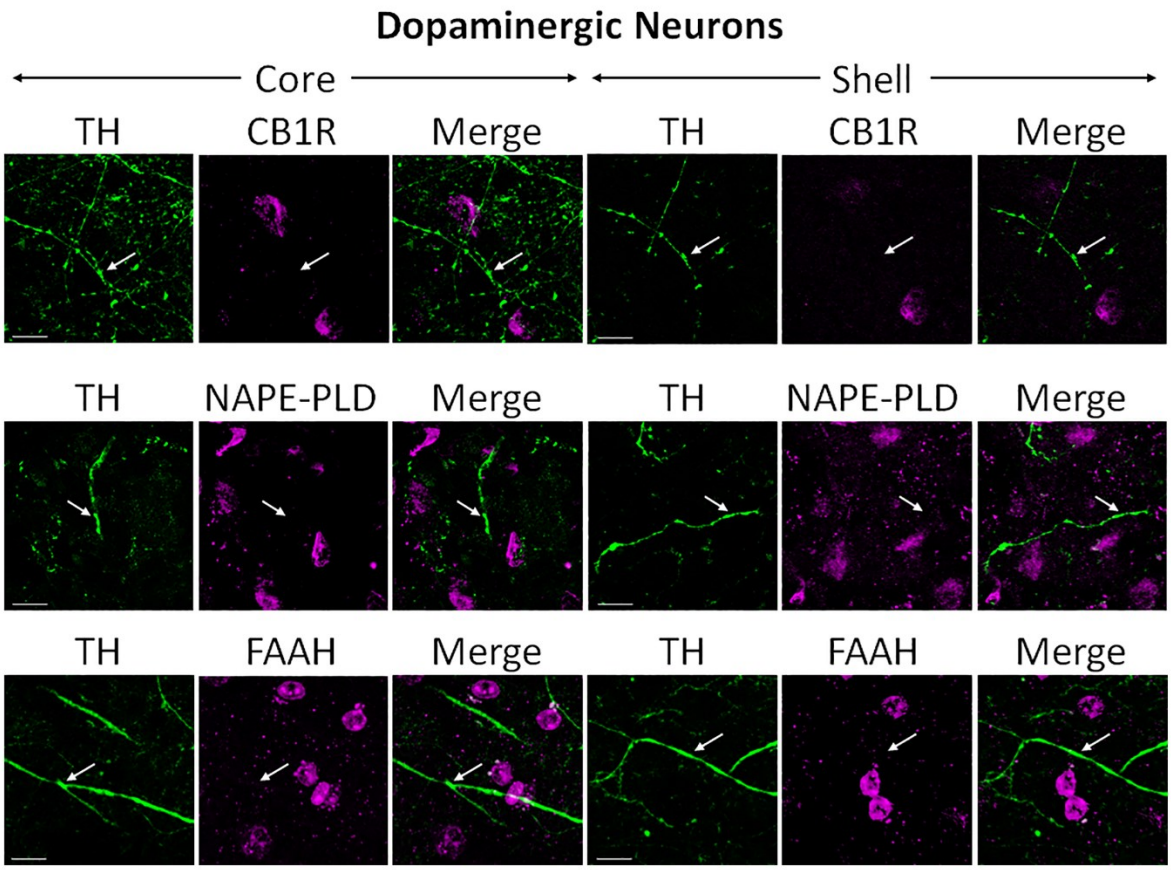


**Figure 14.** Article figure 6. Double-label immunofluorescence illustrating co-localization of CB1R-IR, NAPE-PLD-IR, and FAAH-IR with tyrosine hydroxylase (TH). Confocal micrographs of NAc co-immunolabeled for CB1R, NAPE-PLD, or FAAH (magenta), and TH (green), a specific marker for dopaminergic projections, in core and shell. Arrows point at TH-positive axons and terminals that do not express either CB1R, NAPE-PLD, or FAAH. Scale bar = 10 µm.

**CB1R, NAPE-PLD, and FAAH are not expressed in glial cells.** Glial fibrillary acidic protein (GFAP) was used to mark astrocytes. CB1R, NAPE-PLD, and FAAH were not expressed in GFAP-positive glial cells in the NAc (Fig. 7). GFAP immunoreactivity was clearly detected; individual glial cell bodies and processes can be seen. While CB1R, NAPE-PLD, and FAAH were expressed in neurons, they did not co-localize with GFAP-positive cells (Fig. 7).

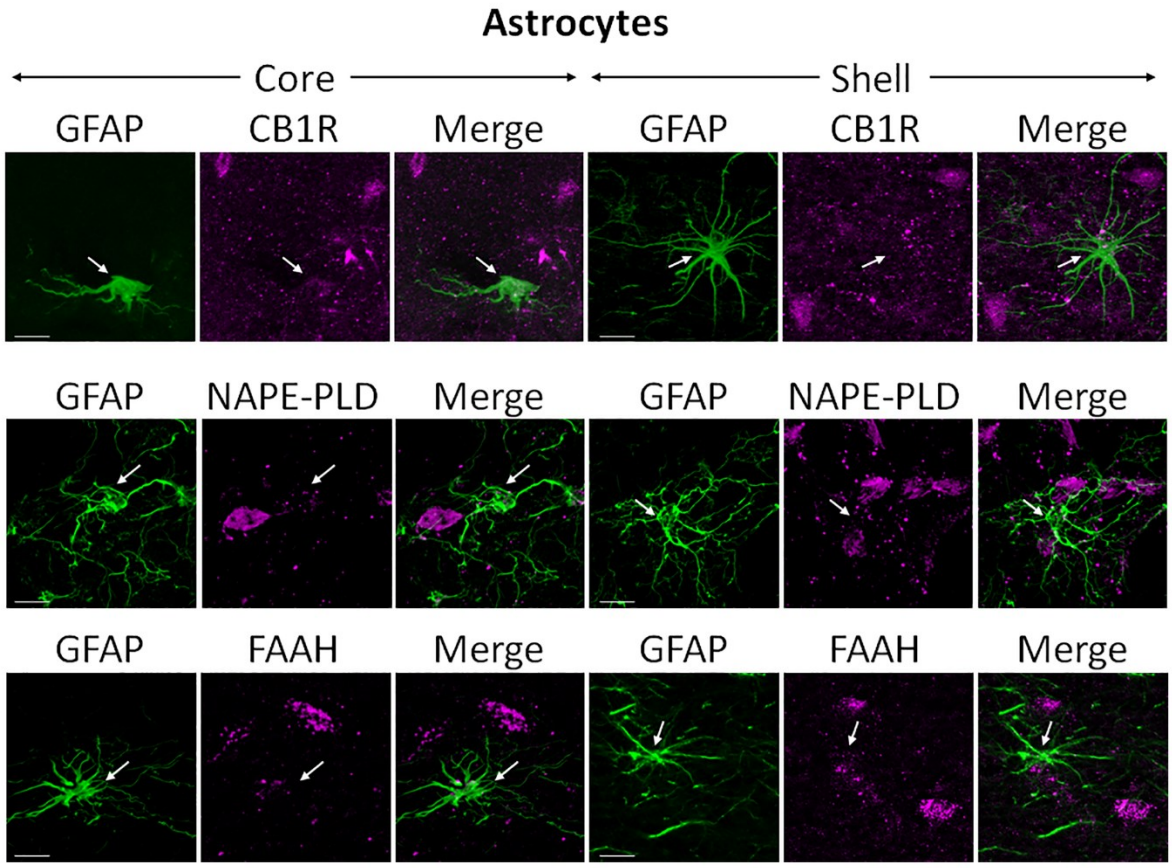


**Figure 15.** Article figure 7. Double-label immunofluorescence illustrating co-localization of CB1R-IR, NAPE-PLD-IR, and FAAH-IR with glial fibrillary acidic protein (GFAP). Confocal micrographs of NAc co-immunolabeled for CB1R, NAPE-PLD, or FAAH (magenta), and GFAP (green), a specific marker for astrocytes, in core and shell. Arrows point at GFAP-positive glial cells that do not express either CB1R, NAPE-PLD, or FAAH. Scale bar = 10 µm.

Discussion

This study reports for the first time the expression and localization of CB1R, NAPE-PLD, and FAAH in the NAc of vervet monkeys. Immunoblots of vervet monkey NAc tissue against CB1R, NAPE-PLD, and FAAH antisera were similar to those previously reported for rodents[44,45] and vervet monkey retinal and thalamic tissues[46,47]. The NAc can be anatomically separated into two distinct parts: the outer shell and interior core[48,49]. Each part plays a different role in behavior and addiction[50]. The core is responsible for major output onto the SN, and receives all inputs from the SN, as well as some inputs from the VTA onto its medial portion[20].

The shell largely projects to the VTA[51], though the SN also receives minor projections from the lateral shell. The shell also receives many projections back from the VTA[52], mostly onto its medial and ventral portions[20]. We found differences in CB1R expression in the core and shell at diverse points along the rostrocaudal axis, with a higher dorsomedial and ventral expression in the shell in middle and caudal sections, and increased core expression in mid-rostrocaudal sections of the NAc (Fig. 3). This may indicate a more pronounced role of eCBs in a circuit where the SN receives projections from the middle and caudal portion of the shell and the middle portion of the core. As for the VTA, the influence of eCBs stems from projections onto the middle portion of the NAc core, and its greater connections with the middle and caudal portions of the shell.

Recent research has shown that the NAc plays a key role in action selection; as such, abnormalities in accumbal signaling have been linked to the development of addictions and other neuropsychiatric conditions[53,54]. It has been hypothesized that DA transmission in the NAc is implicated in translating motivation into action[55], and reinforcement learning[56]. Mesolimbic DA neurons projecting onto the NAc have two modes of firing, either "tonic" or "phasic"[57,58], both of which are implicated in the development of drug addiction[59]. It has been reported that the eCB system plays a role in the modulation of both phasic and tonic DA firing in the NAc[60]. Although there seems to be moderate to low levels of CB1R in the NAc[9,11,61,62], a collection of work has shown that the CB1R antagonists and agonists modulate DA NAc signaling, in both rodents and primates[63,64]. Additionally, CB1Rs in the monkey brain have been imaged *in vivo* using various radioligands[65,66]. CB1R is known to be responsible for the psychoactive effects of marijuana, the effects of which have been blocked by a CB1R antagonist in marijuana smoking humans[67] and THC and anandamide self-administering monkeys[17]. This suggests the importance of CB1R in reward and addiction. CB2R might also play a role in the reward circuit. CB2R knockout mice have been shown to lack conditioned place preference for nicotine and to self-administer less nicotine[68]. A CB2R antagonist also blocked conditioned place preference from nicotine and reduced nicotine self-administration[68]. Interestingly, the CB2R agonist also reduced cocaine self-administration[69]. CB2R is also expressed in mouse VTA DA neurons that have reduced excitability in the presence of CB2R agonists and reduced cocaine self-administration[36]. However, the role of the CB2R in the reward circuit and in neurons has not been studied in as much detail as the CB1R, and the CB2R remains better known for its critical

role in immune function in the brain[35]. For these reasons, we have focused our attention on describing the anatomy of CB1R expression in the NAc.

We have found that CB1R, NAPE-PLD, and FAAH are expressed in both cell bodies and processes in MSNs and FSIs, but not in dopaminergic projections or astrocytes. While it is well known that eCBs act as retrograde neuromodulators[70], it has also been suggested that certain substrates, particularly anandamide, can act on CB1R postsynaptically or intrinsically[71], or in an autocrine fashion[72,73]. Our results show the presence of CB1R in cell bodies, including on the cell membrane, which suggests that eCBs may also act as postsynaptic or autocrine modulators in the monkey NAc. This is further supported by the presence of CB1Rs in neuronal cell bodies and dendrites in the rat striatum[27]. Since the dopaminergic neurons which innervate the MSNs do not express the eCB system, any anterograde eCB modulation would likely come from FSIs or glutamatergic terminals from the PFC, though eCB spillover from nearby MSN or FSI dendrites is also possible[74,75].

FSIs may act to synchronize the spike timing of larger populations of neurons[76], such as MSNs. It has also been reported that FSIs may inhibit themselves[77]. The presence of the eCB system in FSIs suggests that it plays a role in how the spike timing of MSNs is regulated by FSIs, the decreased synchrony of which could lead to weaker inhibition of dopaminergic neurons in the VTA that project onto the NAc. Specific outputs of the NAc come from ensembles of neurons that are clustered spatially close to one another and fire in a coherent and synchronous manner, and require a strong excitatory input[78], further supporting the importance of FSI synchronization of MSNs. The eCB system may also play a role in the gating of MSNs between their two possible resting potentials of a physiologically silent hyperpolarized “down” state and their slightly depolarized “up” state at which action potentials can be induced[79]. CB1R activation, whether on MSN cell bodies in the NAc or on MSN terminals in the VTA[30] and SN[27], may directly reduce inhibition of dopaminergic firing onto the NAc. Additionally, the presence of CB1Rs on NAc FSIs[24], which are important for the synchronization of ensembles of MSNs, may further contribute to the regulation of MSN output[80]. CB1Rs have been detected on glutamatergic neurons terminating in the NAc in mice which suggest that they may also reduce MSN output[29]. Since some MSNs are also glutamatergic in addition to being GABAergic[81], it may also be possible that CB1R affects glutamatergic signaling onto interneurons at the terminations of these MSN projections. Taken together, inhibition by CB1R activation on both

GABAergic and glutamatergic cells may reduce the release of GABA by MSNs projecting onto VTA and SN DA neurons, which in turn may increase DA in the NAc and other brain regions. This dysregulation of DA release could enhance reward perception and motor pattern activation, underlying addiction. These results suggest that the eCB system may play a crucial role in the modulation of the primate brain reward circuit that remains to be investigated.

Materials and Methods

**Animals.** Ten vervet monkeys were used in this study (3 females and 7 males aged 0.4 years (y), 0.5y, 0.75y, 2y, 2y, 2y, 2.5y, 3y, 5.5y, and 11y). The animals were born and raised in an enriched environment in the laboratories of the Behavioral Sciences Foundation (BSF; St-Kitts, West Indies), a facility that is recognized by the Canadian Council on Animal Care (CCAC). The brain tissue was donated by Prof. Roberta Palmour from McGill University, in collaboration with the BSF, from animals enrolled in an independent terminal project reviewed and approved by the local Institutional Review Board of the BSF. They were utilized in accordance with the CCAC requirement for reduction of animals sacrificed for experimental purposes.

**Tissue Preparation.** Brain sections that included the whole NAc were prepared following previously published methods[46,47,82]. Briefly, the animals were sedated with ketamine hydrochloride (10 mg/kg, i.m.), then euthanized with an overdose of sodium pentobarbital (25 mg/kg, i.v.) and perfused transcardially with 0.1M phosphate buffered saline (PBS, 0.1M) until complete exsanguination. The brain was then either rapidly frozen unfixed for Western blots (WB), or was bathed in a 4% paraformaldehyde solution in PBS for immunohistochemistry. The fixed brain was then stereotaxically blocked, removed from the skull, weighed, and the volume determined. The brain was finally cryoprotected in graded sucrose solutions and embedded in Shandon embedding media at -65°C. The blocks were sliced (40 µm) with a cryostat in a serial manner and stored, again according to previously published methods[82].

**Western Blotting.** To test the presence and specificity of the CB1R, NAPE-PLD, and FAAH antisera, WB were performed on unfixed vervet NAc tissue from 3 different monkeys. The entire NAc from one hemisphere was dissected from each monkey and homogenized by hand using a sterile pestle in RIPA buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 1% NP-40 [USB Corp.,

Cleveland, OH, USA], 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA), supplemented with a protease-inhibitor mixture (aprotinin 1:1,000, leupeptin 1:1,000, pepstatin 1:1,000, and phenylmethylsulfonyl fluoride 0.2 mg/ml); Roche Applied Science, Laval, QC, Canada). After the samples were centrifuged (4˚C, 10 minutes), the supernatant was extracted and content was equalized using Thermo Scientific Pierce BCA Protein Assay Kit (Fisher Scientific, Ottawa, ON, Canada). Ten µg of protein per well was loaded in a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel and electrophoresed. It was then transferred onto a nitrocellulose membrane filter (BioTrace NTll; Life Sciences, Pall, Pensacola, FL, USA) and washed 3 times 10 minutes in TBST (0.15 M NaCl, 25 mM Tris-HCl, 25 mM Tris, 0.5% Tween-20). It was blocked for an hour in 5% skim milk (Selection, Montreal, QC, Canada) in TBST, and left to incubate overnight in an IgG primary antibody raised in rabbit; anti-CB1R, anti-NAPE-PLD, and anti-FAAH at a concentration of 1:1,000 in blocking solution. For blocking peptide (BP) control conditions a ratio of 5:1 BP to antibody was pre-incubated for 1 hour before being diluted in blocking solution (final concentrations of NAPE-PLD 1:1,000, NAPE-PLD BP 1:200; FAAH 1:1,000, FAAH BP 1:200). On the following day, 6 washes in TBST of 5 minutes each preceded and followed incubation of the blot in secondary antibody conjugated to horseradish peroxidase (1:5,000; Jackson Immunoresearch, West Grove, PA, USA) in blocking solution for two hours. The blot was washed 6 times 5 minutes in TBST. Detection was done using a homemade ECL WB detection reagent (final concentrations of 2.50 mM luminol, 0.4 mM p-coumaric acid, 0.1 M TrisHCl, pH 8.5, 0.018% $H_2O_2$). After detection, the loading control was performed. The blot was washed 3 times 10 minutes in TBST, blocked for an hour in 5% skim milk in TBST, then incubated overnight in an anti-GAPDH IgM primary antibody raised in mouse at a concentration of 1:20,000. The next day, the blot underwent the same washes, incubation in secondary antibody, washes again, and detection, as above.

**DAB immunohistochemistry.** DAB immunostaining was performed in free-floating solution similarly to previously published methods[47]. Briefly, brain sections of 40 µm that included the NAc were cleaned 3 times for 10 minutes each in washing solution (0.1 M PBS buffer pH 7.4, 0.03% Triton X-100). The tissue was then protected from non-specific binding in a blocking solution (0.5% triton, 10% either normal donkey serum or normal goat serum, in 0.1M PBS) for 90 minutes. The tissue was then placed in primary antibody (Table 1) diluted in blocking

solution and left to incubate overnight at 4˚C. After washing the sections for 10 minutes once and 5 minutes twice in washing solution, the slides were incubated in secondary antibody (biotinylated goat anti-rabbit, donkey anti-rabbit, or donkey anti-mouse diluted 1:200 in blocking solution) for 2 hours. Tissue was then washed 3 times for 10 minutes and incubated for 1h in an avidin-biotin-conjugated horseradish peroxidase (Vectastain ABC kit, Burlingame, CA, USA) solution (1:500 in 0.1M PBS). Another 3 washes of 10 minutes were performed and the sections were treated with a DAB substrate, until the tissue was coloured (1 to 10 minutes). The tissue was then washed again for 3 times of 10 minutes and the sections were mounted on gelatinized slides and left to dry. They then underwent dehydration in graded ethanol, were cleared in xylene, and cover slipped with Permount mounting media (Fisher Scientific; Pittsburgh, PA, USA).

**Immunofluorescence.** Double-labeling were performed on the vervet monkey NAc, following previously published methods in the retina and dorsal lateral geniculate nucleus[47,83], but with minor changes. Tissue was treated the same as in the above DAB protocol for "day one", until primary antibody incubation. When the tissue was ready to be incubated in primary antibody, it was exposed to two primary antibodies at dilution rates mentioned in Table 1 and incubated overnight. On the second day, the tissue was washed in washing solution for 3 times 10 minutes. The tissue was then incubated in secondary antibody diluted in blocking solution (1:200). The slices were washed 3 times for 10 minutes in 0.1M PBS, then 1 time for 10 minutes in 0.1M PB. They were then mounted onto gelatinized slides and left to dry for approximately half an hour before coverslipping using Fluoromount G mounting medium (SouthernBiotech, Birmingham, AL, USA).

Equipment and Settings

**Brightfield Microscopy.** DAB slides were analyzed under a Leica microscope, using a 0.65X objective. The images were taken in Qcapture (Micro-Bright Field) software. All adjustments, such as size, colour, brightness and contrast, were performed using ImageJ and Adobe Photoshop (CS6; Adobe Systems; San Jose; CA, USA) and subsequently exported onto Adobe InDesign (CS6; Adobe Systems), where the final figure layout was completed.

**Confocal Microscopy.** Fluorescence was detected using a Leica TCS SP2 confocal laser scanning microscope with default Leica software (Leica Microsystems, Exton, PA, USA). Images were taken under a 63X objective, at resolutions of either 1080x1080 or 2160x2160 pixels. Green and far-red channels were used to detect images from the 40 µm slices. The green channel (488 nm) was used to detect cell markers and the far-red channels (647 nm) to detect CB1R, NAPE-PLD, and FAAH. To enhance some images, z-stacks were taken for optimization and averaged using ImageJ. Z-stacks allowed for visualization of cells along the X-Y, X-Z and Y-Z axes. All adjustments, such as size, colour, brightness and contrast, were performed using ImageJ and Adobe Photoshop CS6 and subsequently exported onto Adobe InDesign CS6, where the final figure layout was completed.

Antibody Characterization (for more info, please see Table 1)

**CB.** A monoclonal mouse anti-calbindin-d28k (CB, Cell Signaling Technology, Danvers, MA, USA, Cat# 13176, RRID: AB_2687400) was developed with a recombinant protein specific to the amino terminus of human CB. CB labels cell bodies, dendrites and their spines, and axons and their terminals, of MSNs in the basal ganglia of the monkey and rat, with the most intense labeling occurring in the matrix of the cytoplasm[84]. Primary antibody working dilutions and other detailed information are included in Table 1.

**CB1R.** A polyclonal rabbit anti-CB1R (CB1R, Calbiochem, Gibbstown, NJ, USA, Cat# 209550-100UL, RRID: AB_211563) was developed using the first 77 amino acid residues of rat CB1R. A major 60 kDa band in rat heart tissue[85], and minor 23, 72 and 180 kDa bands from various other tissues (manufacturer data sheet) are recognized by this antibody. It has been previously reported that this antibody is specific, using a CB1R knockout mouse retina[45]. It recognizes CB1R in other species, including the vervet monkey[46].

**CTIP2.** A monoclonal rat anti-Ctip2 antibody (Ctip2, ab18465, Abcam plc., Cambridge, UK, Cat# ab18465, RRID: AB_2064130) was developed using a synthetic peptide corresponding to amino acid 1-150 of the human Ctip2. It is a specific marker of GABAergic medium-sized spiny neuron (MSN) differentiation, which comprises over 90% of striatal neurons, and is not present in interneurons[41]. This antibody's use has been verified in primates[86].

**FAAH.** A polyclonal rabbit anti-fatty acid amide hydrolase (FAAH, Cayman Chemical, Ann Arbor, MI, USA, Cat# 101600, RRID: AB_10078701) was developed using a synthetic peptide corresponding to amino acid 561-579 of the rat FAAH. It recognizes a dense band at 63 kDa in FAAH recombinant protein (manufacturer data sheet). The antibody has been shown to have specificity in the vervet monkey[46].

**GFAP.** A monoclonal mouse anti-glial fibrillary acidic protein (GFAP clone GA5, Cell Signaling Technology, Danvers, MA, USA, Cat# 3670, RRID: AB_561049) was purified using pig spinal cord GFAP. It is a specific marker of astrocytes, in humans, mice, and rats (manufacturer data sheet). Its specificity has also been verified by immunofluorescence in the marmoset monkey brain[87].

**NAPE-PLD.** A polyclonal rabbit anti-N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD, Cayman Chemical, Ann Arbor, MI, USA, Cat# 10305, RRID: AB_10507996) was developed using part of a synthetic peptide from human NAPE-PLD. The amino acids (159-172), have been shown to be cross reactive in many species and recognizes an intense band at 46 kDa in human cerebellum tissue, as well as in mouse brain tissue (manufacturer data sheet).

**PV.** A monoclonal mouse anti-parvalbumin antibody (PV, Swant, Marly, Fribourg, Switzerland, Cat# 235, RRID: AB_10000343) was developed by hybridization of mouse myeloma cells with spleen cells from mice immunized with parvalbumin purified from carp muscles. PV labels fast-spiking GABAergic interneurons (FSIs)[42,43,88,89].

**TH.** A monoclonal mouse anti-tyrosine hydroxylase antibody (TH clone lnc1, EMD Millipore, Cat# MAB318, RRID: AB_2201528) was developed from tyrosine hydroxylase purified from PC12 cells and recognizes an epitope on the outside of the regulatory N-terminus. It detects TH in many mammalian species, including monkey and human (manufacturer data sheet). Its use has been verified in primates[90]. It was used to stain dopamine-producing cells, located in the shell whose axons originate in the VTA, but not from the SN[20,91], and in the core to axon projections from both the VTA and SN[20].

Table 1. Primary antibodies used in this study.

| Antibody | Immunogen | Source | Working Dilution | RRID |
|---|---|---|---|---|
| CB | Recombinant protein specific to amino terminus of human CB | Cell Signaling Technology, Danvers, MA, USA | DAB 1:500, IF 1:200 | AB_2687400 |
| CB1R | Fusion protein containing aa 1-77 of rat CB1R | Calbiochem, Gibbstown, NJ, USA | DAB 1:300, IF 1:200, WB 1:1,000 | AB_211563 |
| CTIP2 | Synthetic peptide corresponding to aa 1-150 of human Ctip2 | Abcam plc., Cambridge, UK | IF 1:200 | AB_2064130 |
| FAAH | Synthetic peptide corresponding to aa 561-579 of rat FAAH | Cayman Chemical, Ann Arbor, MI, USA | DAB 1:200, IF 1:200, WB 1:1,000 | AB_10078701 |
| GFAP | GFAP purified from pig spinal cord | Cell Signaling Technology, Danvers, MA, USA | IF 1:200 | AB_561049 |
| NAPE-PLD | Synthetic peptide from human NAPE-PLD aa 159-172 | Cayman Chemical, Ann Arbor, MI, USA | DAB 1:200, IF 1:200, WB 1:1,000 | AB_10507996 |
| PV | Parvalbumin purified from carp muscle | Swant, Marly, Fribourg, Switzerland | IF 1:200 | AB_10000343 |
| TH | TH purified from PC12 cells derived from rat pheochromocytoma; recognizes an epitope on the outside of the regulatory N-terminus of TH | EMD Millipore, Chemicon, Temecula, CA, USA | IF 1:200 | AB_2201528 |

CB: Calbindin-d28k; CB1R: cannabinoid receptor type 1; CTIP2: CTIP2 transcription factor; DAB: 3,3'-diaminobenzidine immunostaining; FAAH: fatty acid amide hydrolase; GFAP: glial fibrillary acidic protein; IF: immunofluorescence; NAPE-PLD: N-acyl phosphatidylethanolamine-specific phospholipase D; PV: parvalbumin; TH: tyrosine hydroxylase; WB: Western blot.

## Data Availability

The data generated and analyzed during the current study are available from the corresponding author on request.

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**Contributions**

MP and AFJ conceptualized the study. RK, JB, and LE performed the experiments and analyzed the data. RK wrote the main body of the text with JB and LE. RP prepared and provided whole vervet brains. MP and JFB supervised and provided equipment and funding. All authors reviewed the manuscript.

**Competing Interests**

The authors declare that they have no competing interests.

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# Chapter 3: Discussion

The reward circuit has been well studied in rodent models, including many aspects of eCB signaling, but until now, limited work had been completed in the non-human primate model. Our results have supported consistency between rodent and primate models, finding many similar results to those reported in rodent studies. We have validated our antibodies specifically in the primate NAc and have used them to support specific cell type expression profiles of the CB1R system in the vervet monkey which match findings in the rodent. The extent of divisibility of the larger and more complex monkey NAc have also allowed us to visualize in greater detail the differential nature of the rostrocaudal expression pattern, possibly providing new information on how CBs and eCBs may trigger different effects in the NAc core and shell. We have validated antibodies that work in rodent animal models as well as in the monkey model and have proposed a mechanism of action of marijuana in the reward circuit.

## 3.1 CB1R System Anatomy in the Nucleus Accumbens

While the NAc has been studied extensively in the rodent, limited work has been done in monkeys, and so we have first made sure to address the validity of our antibody signals in order to be sure they are accurate and specific. We have used Western blots to validate the specificity of our antibodies for CB1R, NAPE-PLD, and FAAH in homogenates of fresh NAc (Article Fig. 1). We have demonstrated that these antibodies detect proteins matching the correct molecular weight of our proteins of interest, and used blocking peptides when available. Indeed, for the NAPE-PLD and FAAH antibodies, we have also demonstrated the specific abolishment of the signal at our expected molecular weight and equal loading with the GAPDH or beta-actin loading controls. Also, by using more than one type of immunohistochemical technique, here both DAB immunostaining and immunofluorescence (Article Fig. 2-7), our results further support successful and specific signal of our antibodies across different protocols, and as such we are able to have necessary confidence in our results for their analysis.

### 3.1.1 Differential Expression Across the Rostrocaudal Axis

After initial validation of our antibodies by Western blotting, our next objective was to define the borders of the NAc in the vervet monkey similarly to what had previously been done in marmoset and rhesus monkeys (Meredith et al., 1996; Brauer et al., 2000). Using an antibody against calbindin-d28k, a calcium buffering protein expressed in higher densities in the core of the NAc, we were able to define the border of the core and shell across rostrocaudal depths and establish the overall shape of the structure within the striatum (Article Fig. 2). By taking directly adjacent brain slices containing the NAc from within the same animal, we were able to obtain the spatial expression patterns of CB1R, NAPE-PLD, and FAAH in the NAc with accuracy in reference to the calbindin-d28k expression patterns.

Interestingly, the CB1R showed particular variation in its expression (Article Fig. 3A-F). It was expressed at high levels in the dorsomedial and ventral shell in the middle and caudal portions, as well as high core expression in the caudal half, particularly in middle sections. The areas of the NAc that the SN receives projections from include minor projections from the middle and caudal portion of the shell and major projections from the middle portion of the core, and the core receives all dopaminergic nigral output onto the NAc (Heimer et al., 1991; Groenewegen et al., 1999). The higher expression of CB1Rs in these subregions may indicate a larger role in their modulation of these inputs, and may be the most important areas in the eCB modulation of translating motivation into action. The middle and caudal portions of the shell also have greater connectivity with the VTA, particularly onto its medial and ventral portions, which also provides projections back onto the medial portion of the core (Swanson et al., 1982; Heimer et al., 1991; Groenewegen et al., 1999). Thus the medial and ventral portions of the middle and caudal extent of the NAc may be the most important areas in relation to eCB regulation of reward perception and translation into motivation.

The importance of CB1R in the regulation of NAc-mediated cognition and behaviour may also serve as a method to further subdivide the core and shell of the NAc into a larger number of discrete regions. While the core and shell are known to mediate different aspects of NAc processing (Parkinson et al., 1999; Corbit et al., 2001), differences in expression within these regions may reveal further subdivision and specificity of processing in CB1R mediated effects on reward processing. Subregions of the core and shell with higher CB1R expression

levels may then play a greater role in the effects of cannabinoid modulation on NAc-mediated cognition and behaviour, and it would be interesting in the future to study if these further subdivisions can also be mutually dissociated in their importance regulating the functions with which the core and shell are currently respectively associated. Furthermore, that NAPE-PLD and FAAH expressions showed relative homogeneity across the NAc should not be immediately discounted (Article Fig. 3G-R). While it may seem unimportant on its own that there is little change across subregions, in the context of the great variability of CB1R expression across subregions, it provides important information about potential AEA synthesis and degradation rates. The functional significance of the difference in CB1Rs may be supported by the consistent enzyme rates, since it reduces the likelihood that differences in number of receptors are being needed to respond with the same sensitivity to greater or fewer eCBs. The abundance of CB1Rs does not always correlate with functionality and pharmacological relevance of cannabinoids across separate brain regions since mice with CB1R selectively knocked out in specific cell types of specific brain regions showed greater changes in effects of THC in glutamatergic neurons that had a lower expression of CB1Rs than another GABAergic set (Monory et al., 2007). However, since these different expression patterns are within the same brain region with the same neuron types and similar patterns of connectivity, it may be that for the same amount of eCB production that their effect may be more potent and important at these locations due to greater receptor availability. It could also be that the difference in the receptor levels reflect differences in the importance of their regulation of signaling in specific cell types in these areas with different CB1R sensitivity than neighbouring cells.

The difference in receptor expression levels across these subregions may also have interesting implications for functional selectivity and biased signaling. Functional selectivity is the ability of a receptor, particularly GPCRs, to be able to activate more than one different signal transduction pathway, and thus for different ligands to bias a given receptor's signaling further towards one pathway or the other (Kenakin, 2011). Differences in the expression level of the same receptor in different tissues of the same animal are also known to in some cases result in differences in the agonist properties of a given ligand at these different sites. The CB1R has been shown to signal via more than one pathway (Delgado-Peraza et al., 2016). By having different CB1R levels in different subregions of the NAc, it may impact the importance each of AEA and 2-AG play relative to one another in each of these regions, and may also affect which

downstream signaling pathways are more affected by a given ligand at different concentrations. This may also be true of how exogenous cannabinoids may have differing effects on CB1Rs in each subregion, and how these effects may also change at different concentrations. One example being how at differing doses cannabinoids may have seemingly opposite effects on anxiety (Viveros et al., 2005). Since hippocampal neurons showed activation of CB1Rs on glutamatergic neurons at much lower doses of the eCB agonist CP-55,940 than was required for GABAergic neurons (Rey et al., 2012), it is believed that there are brain-wide differences in dose-dependence of effect on CB1Rs at excitatory versus inhibitory synapses which are believed to have different sensitivities to CB1R activation, and may explain opposite dose-dependent effects of the same agonist. The different CB1R expression levels across the NAc may indicate that at different agonist concentrations, CB1R signaling pathways may be affected differently across subregions, possibly allowing for dose-dependent differences in the effect of CB1R on NAc processing.

### 3.1.2 Cell Type Expression Profiles

We have detailed cell type specific expression profiles of CB1R, NAPE-PLD, and FAAH in four key cell types: medium spiny neurons (MSNs), fast-spiking interneurons (FSIs), dopaminergic projections, and astrocytes (Article Fig. 4-7). MSNs are GABAergic and are the main projection neurons of the striatum, composing approximately 90-95% of all neurons in the striatum (Arlotta et al., 2008). MSNs are spatially clustered in ensembles that project together to the same areas and require strong input to fire effectively (Pennartz et al., 1994). FSIs are also GABAergic and play a critical role in the synchronization of large populations of MSNs (Younts & Castillo, 2014). Dopaminergic projections are the key input from the VTA and SN onto their respective subregions of the NAc that produce the perception of reward and the initiation of motor patterns (Parkinson et al., 1999; Corbit et al., 2001). Astrocytes also play an important role that is often underestimated by forming what is known as the tripartite synapse, whereby they play an indirect role in synaptic signaling by the extent to which they contribute to the reuptake and recycling of neurotransmitters from the synaptic cleft, as well as other aspects of

background maintenance important for healthy brain function (Cabral et al., 2008; Perea et al., 2009).

We found each of all 3 of our proteins of interest in MSNs and FSIs, but not in dopaminergic projections or astrocytes (Article Fig. 4-7). While study of NAPE-PLD and FAAH has been incomplete, even in rodent models, the CB1R has been previously found in MSNs and FSIs in the rodent NAc (Julian et al., 2003; Mackie, 2005; Winters et al., 2012), but not in dopaminergic projections (Herkenham et al., 1990). Here, our results are consistent with and supported by the rodent literature. It is of interest that in each case we found all three components present or absent, since it might not be expected that they would all be in the same cell type because CB1R would classically be most expected presynaptically, while NAPE-PLD and FAAH may be most expected postsynaptically, such that different cell types could have preferential expression of some components but not others. The cellular expression pattern found here in MSNs and FSIs may be due to both these cell types engaging in both sending and receiving classical retrograde eCB signaling. However, it may also support that AEA signaling is occurring not only in a retrograde manner, but possibly also in an anterograde, autocrine, or intrinsic fashion (Di Marzo & De Petrocellis, 2012). That all three components are absent in dopaminergic projections strongly supports a lack of direct eCB effect on them.

In terms of astrocytes, the expression and the role of the CB1R are unclear and debated in the literature (Atwood & Mackie, 2010). While the CB1R may or may not be present in astrocytes in some brain regions in some species, it is not present in the NAc of the vervet monkey. Our findings are consistent with rodent findings in the other three cell types we have studied here, both for positive and negative results. It remains possible that CB1Rs are still present in these astrocytes, but that their expression levels are simply below the sensitivity of detection of our methods. However, if this is the case, it would be questionable how functionally significant to our model they would be at such low levels. If the eCB system does play a role in astrocytes, then perhaps future study of CB2Rs would be of interest in this cell type.

### 3.1.3 Proposed Mechanism of the CB1R System in Reward

Based on our own findings and limited other work in primates, in conjunction with the extensive rodent literature, we have proposed a mechanism of how the CB1R system may contribute to reward in the primate NAc, and how its dysregulation may be a critical factor in addiction (Figure 16). While there may be slight differences in rodent NAc subregions and pathways, thus far cell-type expressions have been consistent. A reward stimulus results in the activation of the VTA, which then releases DA onto the NAc shell to produce the perception of reward (Lupica & Riegel, 2005). MSNs in the NAc are either excited or inhibited by the increase of DA from the VTA based on whether they are expressing more D1-like or D2-like DA receptors, which relates to their projection pathway (Silkis, 2001). Glutamatergic input from limbic areas such as the hippocampus and amygdala may affect whether MSNs are in either a physiologically silent hyperpolarized state, or a slightly depolarized state at which action potentials can be induced (O'Donnell & Grace, 1995). MSNs also receive activation by glutamate from PFC projections (Robbe et al., 2002), which may be critical for the strong excitatory input required to fire (Pennartz et al., 1994), and may represent the influence of self-control. Finally, for the coherent and synchronized manner in which MSNs fire, FSIs inhibit themselves as well as populations of spatially assembled MSNs (Younts & Castillo, 2014). Ensembles of MSNs then project together to their target brain areas (Pennartz et al., 1994).

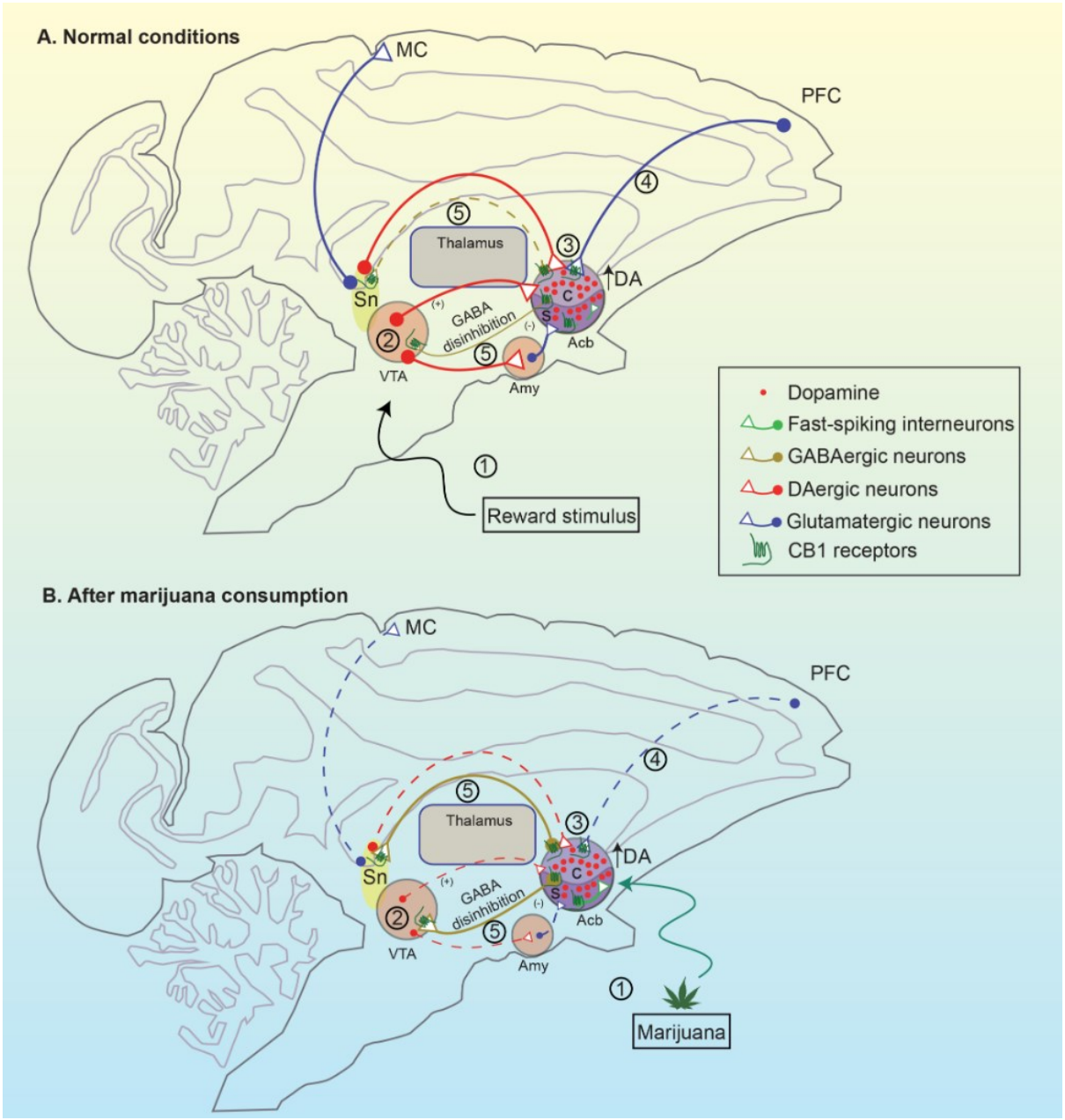


**Figure 16.** CB1R influence on the reward circuit. Influence of CB1Rs on key components of the reward circuit during normal conditions with a natural reward stimulus (A), and after marijuana consumption (B). Solid and dashed lines in B represent increased and decreased activity respectively. A rewarding stimulus is perceived (1) and triggers the release of DA from the VTA onto NAc MSNs that inhibit the VTA (2). MSN depolarization, and possibly also the activation of D2DRs, enhances eCB production. Within the NAc, MSNs are inhibited by local FSIs (3) and also receive strong excitatory input from the PFC (4). Background glutamatergic input from other limbic areas such as the amygdala and hippocampus may not depolarize MSNs, but may maintain their excitability opposed to a physiologically silent state. CB1Rs present on MSNs, FSIs, and

PFC projections may be activated. NAc MSN GABA output onto the VTA and SN is reduced (5), permitting greater DA release, and thus reward perception and motor activation through a circuit eventually connecting the SN to the motor cortex (details not pictured). In the presence of THC and lesser active cannabinoids from marijuana, greatly increased CB1R activation further reduces activity of many connections, but not of dopaminergic neurons. This results in greater disinhibition and increased release of DA, underlying the strongly rewarding psychoactive properties of marijuana.

Clusters of MSNs project onto the VTA such that DA signaling may alter their direct inhibition of projections from the VTA that release DA back onto the NAc core and shell, and may also alter their inhibition of VTA interneurons, indirectly affecting VTA DA neurons (Lupica & Riegel, 2005). NAc core and shell MSNs that receive input from the VTA then project to the SN where they may alter the inhibition of dopaminergic projections back onto the NAc core (Groenewegen et al., 1999). DA release onto the NAc core from the VTA and SN initiates a motor circuit between the NAc core and SN that encodes motor patterns related to obtaining the reward perceptions experienced by the NAc shell from the VTA DA release induced by the reward stimulus (Groenewegen et al., 1999; Corbit et al., 2001).

Through the basal ganglia motor circuit, the NAc core and SN cooperate to affect activation of the motor cortex (details not pictured in Figure 16). NAc core projections to the SN, which are affected by glutamatergic PFC projections, control the release of DA by the SNpc back onto the striatum. MSNs with D1-like DA receptors are activated and project through the direct pathway which inhibits cells in the globus pallidus interior (GPi) (Silkis, 2001). MSNs with D2-like DA receptors have their output reduced and project through the indirect pathway which inhibits the globus pallidus exterior (GPe) (Silkis, 2001). The STN then receives greater inhibition from the GPe, causing less glutamatergic activation of STN recipients in the GPi (Silkis, 2001). Increased dopaminergic signaling ultimately results in reduced GPi inhibition of the motor thalamus, increasing activation of the motor cortex and the initiation of movement. Through this mechanism, increased DA from the reward stimulus results in D1 pathway activation that disinhibits approach behaviours, and the D2 pathway inhibition of avoidance behaviours.

CB1Rs are present on the cell bodies of MSNs and FSIs in the NAc (Article Fig. 4-5; Mackie, 2005; Winters et al., 2012), as well as the terminals of Glut projections onto the NAc from the PFC (Robbe et al., 2002), and GABA projections onto the VTA and SN from the NAc (Julian et al., 2003; Lupica & Riegel, 2005), but not on DA neurons from the VTA and SN onto the NAc (Article Fig. 6; Herkenham et al., 1990). When DA levels are elevated in the NAc, the depolarization of MSNs causes an increase in intracellular calcium concentration that may increase the production of eCBs. Since dopaminergic projections do not express CB1Rs, they may not be directly affected by the presence of eCBs and continue to release DA. Glutamatergic inputs onto MSNs, however, are affected by these eCBs and their activation of MSNs to inhibit midbrain dopaminergic neurons is reduced. FSIs synchronizing MSN populations may also have their neurotransmitter release reduced by their CB1R activation, and MSNs may even have an autocrine or intrinsic effect on their own CB1Rs. Furthermore, since some MSNs are glutamatergic in addition to being GABAergic (Perreault et al., 2012), it may also be possible that CB1R activation affects MSN glutamatergic signaling onto interneurons in the VTA and SN that also contribute to DA neuron regulation. Reduced excitatory input from glutamatergic projections and direct reduction of their own neurotransmitter release may both contribute to reduced MSN regulation of dopaminergic targets in the VTA and SN. While FSIs are inhibitory towards MSNs, their reduced output may weaken the crucial synchrony of MSN clusters and have a net effect of further reducing the strength of MSN inhibition of dopaminergic targets. The resulting reduction of both glutamatergic and GABAergic signaling of each other part of the circuit by CB1R activation, but not directly on dopaminergic neurons themselves, may result in their disinhibition and the increased release of DA in the NAc and other brain regions.

### 3.1.4 Potential Implications of the Role of the CB1R System in Reward

CB1R activation may play a fundamental role in the natural increase of DA release in response to rewarding stimuli in the reward circuit. CB1R activation may then not only be responsible for the psychoactive effects of marijuana, but also play a disinhibitory role in the increase of DA produced by the mechanisms of many different drugs of abuse. The mechanism of reward of THC might then be described as producing reward by hijacking the natural

mechanism of DA disinhibition through the overactivation of CB1Rs, and might simply be the same as the above mechanism but to a greater extent of activation. Dysregulation of DA release may enhance reward perception and motor pattern activation underlying addiction and dysregulation of CB1R activation may underlie marijuana's dysregulation of DA release. However, greater regulation of CB1R activation may then also be able to attenuate dysregulated DA release produced by other mechanisms. This is supported by the success of CB1R antagonism in rodent and primate models of drug self-administration in causing the attenuation of this behaviour (Panlilio et al., 2010; Parsons & Hurd, 2015). That the CB1R inverse agonist Rimonabant caused depressive symptoms in humans (Christensen et al., 2007) also supports this model in two ways. That a high level of CB1R antagonism produced very negative effects on mood supports its importance in reward. Secondly, since it is an inverse agonist that also interferes not only with activation by ligands, but also constitutive activity (Pertwee, 2005), its intense effect on mood supports that constitutive CB1R activity may be necessary for natural levels of reward and mood, while it is abnormal levels produced by drugs that cause the euphoria of marijuana. This leaves interesting consideration for how future investigation may lead to the correct modulation of the eCB system in the reward circuit that mood may be balanced while treating addiction.

It is further worth considering whether the CB1R may play a role in long term synaptic plasticity changes related to addiction, and how it may then be through the alteration of such plasticity that relapse to drug-seeking behaviour and long-term damage to reward sensitivity and mood might be treatable. This necessitates electrophysiological study, with MSNs being a key starting point due to their fundamental level of DA neuron regulation. Due to the role of the PFC in decision-making, long-term synaptic plasticity changes to its glutamatergic projections to NAc MSNs may also play a critical role in the maintenance of self-control in relation to rewarding stimuli and their cues. eCB-LTD of these projections may be pivotal in shifting the balance of control over rewarding behaviours away from cortical decision-making areas towards subcortical structures. FSIs will also need to be evaluated despite their population being much lower due to the potential far-reaching implications of their role in network synchrony. Further elucidation of eCB-mediated effects on synaptic plasticity in the NAc and connected brain regions and the functional influence on behaviour of these changes will be important for

understanding how the eCB system may be used to treat not only acute symptoms of addiction, but also to fix persisting changes to the brain (Koob & Le Moal, 2001) causing craving and relapse years after cessation of drug consumption.

## 3.2 Future Directions

While the anatomical work began here provides a significant improvement to understanding the anatomy of the eCB system in the primate NAc by addressing the most important proteins and cells, there still remains several eCB system proteins, and several interneuron cell types remaining to be catalogued. There are also varying receptor expression profiles within cell types such as the difference between the projection pathways of D1DR and D2DR expressing neurons. Furthermore, there are many other brain areas such as the VTA and amygdala which play interesting roles in addiction and relapse. Finally, not only are there these anatomical questions remaining, but there is also the matter of demonstrating with electrophysiology that the expression levels of these proteins in each cell type represents a significant level of functional effect on excitability in the presence of specific modulators. From this point, there will then be the need for animal behavioural studies to better understand how predicted modulations of a fully mapped eCB system in the reward circuit will actually affect animal behaviour in relation to addictive behaviours such as drug self-administration and relapse to drug-seeking behaviour.

### 3.2.1 Remaining Anatomy

In addition to the NAc anatomy completed here, there remains additional eCB system proteins, NAc cell types, and the receptor expression profiles of those cell types which indicate important information about their connectivity. CB1R, the receptor responsible for the psychoactive effects of THC, and NAPE-PLD and FAAH which synthesize and degrade AEA, an eCB with high affinity for the CB1R and perhaps the key eCB responsible for eCB-LTD in the NAc, were analyzed. However, the CB2R and TRPV1, which are also believed to potentially play roles in the reward circuit, as well as other related receptors such as GPR55 and

PPARgamma of which less is known, remain to be investigated. In addition, there are the synthesizing and degrading enzymes of 2-AG, DAGL and MAGL, as well as other enzymes relating to additional ligands of the various endocannabinoidome receptors.

The cell types analyzed here include MSNs, the projection neurons of the NAc, FSIs, a class of interneurons important for population synchronization, dopaminergic projections, a key type of input from the VTA and SN in reward perception and action initiation, and astrocytes, a critical part of the tripartite synapse. This set of cells cover many crucial aspects of signaling within the NAc, but is not exhaustive of all possible information. Better understanding of glutamatergic projections onto MSNs in the primate NAc is the most important future step, though cholinergic and nitric oxide interneurons may also offer further insight into the role of the eCB system in the primate NAc. It may additionally be of interest to investigate microglia, which like astrocytes are not neurons, but still may have indirect effects on signaling. By studying these cell types, the structure of the eCB system in the NAc circuitry of the primate reward circuit may be better understood, as well as by the future study of the VTA, SN, extended amygdala, and the various other interacting areas that contribute to the reward processing completed by the NAc.

Receptor expression profiles within given cell types will also provide another layer of information, especially in the context of MSNs. Studying the triple colocalization of eCB system proteins with cell type markers and either DA receptor or mAChR subtypes could provide more information on the importance of eCB signaling in the separate projection pathways indicated by different DA receptors, as well as the role of eCBs in cells which receive either excitatory or inhibitory modulation by different receptor subtypes for DA and ACh. Due to the highly consistent expression pattern of CB1R, NAPE-PLD, and FAAH across the cell types examined here, it is most likely that receptor expression profiles relating to different projection pathways would not indicate a qualitative difference in expression, but could be interesting to study for the possibility of finding a quantitative difference in expression between excitatory and inhibitory responding subtypes of cells.

### 3.2.2 Future Physiology and Behavioural Study

The completed anatomical results provide an important framework for future electrophysiology by delineating the structure and size of the NAc and its core and shell, as well as which subregions have differences in their expression of CB1R system components. Which cell types express CB1R, NAPE-PLD, and FAAH further allows for the hypothesis of whether a cell with a given spiking pattern will be affected by the application of an exogenous eCB system modulator during recording. This essential groundwork opens the door to allowing the electrophysiological study of these cell types in these regions with specific CB1R system modulators to test the functional significance on neuronal firing of these primate structural findings. It will be particularly interesting to see the changes in firing of populations of MSNs in differing NAc subregions during CB1R modulation, and potential plasticity changes from modulation. There will also be the need for complementary behavioural studies of the effects of CB1R system modulators in association with electrophysiological ones to demonstrate the functional behavioural significance of changes in neural activity such that a physiological mechanism and behavioural implications may support one another. Self-administration tests and reinstatement tests with addictive substances both in the acute presence of and after prolonged treatment with specific modulators will help in understanding the behavioural implications of the effect of CB1R modulators on the reward circuit. Through the combination of future anatomical, physiological, and behavioural studies, a complete model of the structure, function, and impact of the eCB system on the primate reward circuit may eventually be fully understood and manipulated to provide new therapeutic benefits to patients suffering from addiction, and possibly other related neuropyschiatric conditions.

# Conclusion

The eCB system is clearly present in the primate reward circuit, possibly playing a key role in the indirect regulation of DA release, and should continue to be considered as containing potential targets for the pharmacological treatment of addiction. While CB1R antagonism in humans has been met with unfortunate and intolerable side effects in the past, the inverse agonist used in these cases is only one of several ways which the CB1R might be modulated, and the eCB system as a whole may still be targeted in many other ways. We have demonstrated that the anatomical structure of the primate reward circuit, at least in the NAc, shares a high degree of similarity to that of the rodent, perhaps due to the ancient and critical evolutionary role of this structure in survival. The primate NAc can be easily divided into a core and shell with different spatial expression of the CB1R, which may allow the exploitation of functional selectivity to allow the targeting of specific aspects of NAc processing preferentially depending on the ligand used and its concentration. The key cell types in rodent models of the eCB system in the reward circuit, furthermore, show the same expression, or lack thereof, of the CB1R. While the remaining pieces of the eCB system must still continue to be verified in primates, our findings do support the translatability of much of the extensive rodent literature that has been performed in this area. Electrophysiology and behavioural studies now have an anatomical framework within the monkey NAc and will be required in conjunction with eCB system modulation in order to continue to progress the understanding of the many complex ways the eCB system can influence the many complex connections of the primate reward circuit. Through the continuation of this work, we may ultimately uncover new pharmacological therapies for the treatment of neuropsychiatric conditions such as addiction.

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