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The impact of acute and chronic obesity-related inflammatory states on neuronal activity in the nucleus accumbens core and shell

Anita Kabahizi

Département de Neurosciences

Faculté de Médecine

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Department of Neuroscience, Faculty of Medicine

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The impact of acute and chronic obesity-related inflammatory states on neuronal activity in the nucleus accumbens core and shell

Presented by

Anita Kabahizi

Has been evaluated by a jury composed of the following persons

Dr. Graciela Pinyero

President-Rapporteur

Dr. Stephanie Fulton

Research Director

Dr. Rosemary Bagot

Jury Member

Résumé

L'inflammation systémique induite par l'obésité augmente la neuroinflammation et la réactivité gliale dans le noyau accumbens (NAc), associées à des comportements de type dépressif et anxieux. Les neurones à épines moyennes (MSN) du NAc font partie intégrante des circuits neuronaux qui contrôlent la motivation et l'humeur. Les différences fonctionnelles dans les entrées et les sorties des sous-régions du NAc – le cœur du NAc (NAcC) et la coquille du NAc (NAcSh) – fournissent une base pour étudier la divergence fonctionnelle dans les sous-territoires. Nos résultats biochimiques et chimiogénétiques préliminaires suggèrent que l'inflammation causée par une alimentation chronique riche en graisses entraîne une diminution de l'excitabilité des récepteurs D1 de la dopamine (D1R) des MSN. Notre objectif était d'étudier l'impact des états inflammatoires aigus et chroniques en étudiant l'impact du LPS et de l'alimentation riche en graisses saturées (HFD) sur l'activité des MSN du NAc et la plasticité synaptique. Pour ce faire, nous avons utilisé deux méthodes électrophysiologiques, les enregistrements de champ extracellulaire et le patch clamp intracellulaire à cellules entières, pour étudier la potentialisation à long terme (LTP) et l'activité excitatrice cellulaire dans le NAc en réponse à l'inflammation aiguë et chronique. Nos résultats suggèrent que le LPS peut induire des changements dans la LTP dans les champs de neurones du NAcC. Cela suggère que la neuroinflammation aiguë peut induire des changements dans la transmission du signal entre les synapses du NAcC. Dans les cellules patchées, nous avons constaté que les entrées excitatrices sur les MSN D1R du NAcC et du NAcSh présentaient une fréquence réduite, en réponse au LPS. Nous avons également constaté que le LPS peut induire une réduction de l'amplitude maximale des entrées inhibitrices sur les MSN D1R du NAcC et du NAcSh. Après 12 semaines d'un régime à base d'huile de palme (graisse saturée) amenant à l'obésité, les entrées excitatrices sur les MSN NAc D1R n'ont pas montré de

changements significatifs. Collectivement, nos données suggèrent qu'un défi aigu au LPS, mais pas un défi chronique au Palm, peut provoquer des changements aigus dans l'activité neuronale du NAc qui pourraient être médiés par des changements dans la signalisation de la dopamine.

Mots-clés : obésité, neuroinflammation, humeur, dopamine, système mésolimbique

Abstract

Obesity induced systemic inflammation upregulates neuroinflammation and glial reactivity in the nucleus accumbens (NAc) which is associated with depressive- and anxiety-like behaviors. Medium spiny neurons (MSNs) of the NAc are integral populations of the neural circuitry controlling motivation and mood. Functional differences in the inputs and outputs of NAc subregions- NAc core (NAcC) and NAc shell (NAcSh)- provide a basis to study functional divergence in the subterritories. Our preliminary biochemical and chemogenetic findings suggest that inflammation caused by chronic high-fat feeding results in decreased excitability of dopamine D1 receptor (D1R) MSNs. Our aims were to investigate the impact of acute and chronic inflammatory states by studying the impact of LPS and Palm saturated high-fat diet (HFD) on NAc MSN activity and synaptic plasticity. Given this, we used two electrophysiology methods, extracellular field recordings & intracellular whole-cell patch clamp, to study NAc long-term potentiation (LTP) and cellular excitatory activity in response to acute and chronic inflammation. Our results suggest that LPS may induce changes in LTP in neuron fields in the NAcC. This suggests that acute neuroinflammation may induce changes in signal transmission between synapses of the NAcC. In patched cells, excitatory inputs onto D1R MSNs of the NAcC and NAcSh displayed reduced frequency in response to LPS. We also demonstrate that LPS induces a reduction in the peak amplitude of inhibitory inputs onto both NAcC and NAcSh D1R MSNs. After 12 weeks on a saturated Palm diet, NAc D1R MSN excitatory inputs displayed no significant changes. Collectively, our data suggests that an acute LPS, but not chronic Palm challenge may illicit acute changes in NAc neuronal activity, perhaps mediated by changes in DA signaling.

Keywords: obesity, neuroinflammation, mood, dopamine, mesolimbic system

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List of Abbreviations and acronyms

DIO: diet-induced obesity
T2DM: type 2 diabetes mellitus
BMI: body mass index
IL-6: interleukin-6
TNFα: tumor necrosis factor
IL-1β: Interleukin-1β
NFkB: Nuclear factor kappa-light-chain-enhancer of activated B cells
MAPK: mitogen-activated protein kinases
CRP: C reactive protein
LPS: lipopolysaccharide
HFD: high-fat diet
BDNF: brain-derived neurotrophic factor
VTA: ventral tegmental area
PFC: pre-frontal cortex
NAc: Nucleus accumbens
DA: dopamine
AMPAr: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
NMDAr: N-methyl-D-aspartate
LTP: long-term potentiation
NAcC: Nucleus accumbens core
NAcSh: Nucleus accumbens shell
MSN: medium spiny neuron
DA D1 receptor: D1R
DA D2 receptor: D2R
Na^+: sodium
K^+: potassium
RMP: resting membrane potential
GABA**:** Gamma-aminobutyric acid
Ca^{2+}: calcium
LTD: long-term depression
$Mg2^+$: magnesium
fEPSP: field excitatory postsynaptic potential
IHC: immunohistochemistry
BBB: blood-brain barrier
EPSC: spontaneous excitatory post-synaptic currents
IPSC: spontaneous inhibitory post-synaptic currents
HFS: high frequency stimulation

To mom, Tia and Ru

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1. Introduction

1.1. Obesity

1.1.1. Prevalence, pathophysiology, and consequences of obesity

Obesity can be defined as an abnormal and excessive accumulation of fat mass and adipose tissue that may result in health impairments (1). Energy balance, or the matching of energy intake and energy expenditure, is necessary to maintain a consistent bodyweight (2). Thus, an imbalance between caloric intake and energy expenditure is a result of and is enhanced by caloric deficit leading to weight loss or caloric excess leading to weight gain, metabolic dysregulation, and obesity. In a post-industrial world, where high-fat and calorically dense foods are more accessible than ever, it is no surprise that diet-induced obesity (DIO) is on the rise. The nutritional profile of the human population toward increased consumption of processed and animal-derived foods, saturated fats and refined sugars has subsequently reduced the intake of vegetables, fruits, fibers, and fish, which are necessary components of a human diet (3). In Canada, there has been a gradual and steady incline in obesity rates amongst the population, rising from 10% to 26% over the last four decades (4). Since 2016, it is stated to affect at least 1.9 million Canadian adults (5). Globally, the prevalence of obesity has increased to pandemic proportions (6). Its pervasiveness and marked increase may also reflect healthcare systems' inability to recognize it as a progressive, chronic disease and thus not treat it in its early stages (7). Moreover, obesity may contribute to the prevalence of other morbidities such as type 2 diabetes mellitus (T2DM), hypertension, cardiovascular diseases, cancers and even mood disorders such as depression (8).

Body Mass Index (BMI) is the quick and surrogate indicator for identifying and categorizing overweight and obese humans. BMI is calculated by dividing a person's weight in kilograms by their height in meters squared (kg/m^2). However, BMI has proven to be an imperfect

system on an individual scale, failing to consider fat *vs* lean mass and the complex effects of age, sex and even ethnicity, all factors that may deter the mathematical precision of the value (9) (10). However, BMI remains a standard for determining obesity. For adults, the categories of the BMI range include normal BMI (ranges 18.5 to 24.9 kg/m^2), overweight BMI (25 kg/m^2), obese BMI (30 kg/m^2), and severe obese BMI (40 kg/m^2), for both sexes. Between 1975 and 2016, the average BMI for adults ranged from 22.1 to 24.8 kg/m^2 for women and 21.7 to 24.5 kg/m^2 for men (11). However, it has also been established that a better indicator of metabolic dysfunction is abdominal obesity, which is reflected by waist circumference (12,13). Evidence suggests that waist circumference is associated with visceral obesity and greater metabolic, psychiatric and neurological impairments and when coupled with BMI, predicts health risks more acutely than BMI does alone (14–16).

The study of the etiology of obesity and metabolic dysfunction is necessary to fully understand its mechanisms and causal links to other diseases to produce effective interventions, preventions, and therapies. In individuals with obesity and T2DM, low-grade inflammation has been strongly associated to changes in glucometabolic pathways (17). These changes may result in metabolic syndrome, otherwise known as the aggregation of many disorders, which raise the risks of developing cardiovascular diseases, insulin resistance, T2DM and neurological deficits (18). Individuals with metabolic syndrome have higher levels of proinflammatory cytokines in their blood and these markers may negatively affect the metabolism of peripheral tissues (19,20). Thus, one consequence of DIO is systemic and chronic low-grade inflammation.

1.2 Systemic inflammation in diet-induced obesity

Broadly, inflammation can be defined as a sequence of events orchestrated to conserve tissue, organ integrity and homeostasis. This process relies on the release of certain mediators and expression of specific receptors to restore balance to the body. Understanding the processes that give rise to inflammation of the periphery may lead to a fuller comprehension of disorders like obesity and how it impacts the brain.

1.2.1 Chronic, systemic inflammation

Inflammation can be defined as "acute", and involves the delivery of plasma and its components alongside leucocytes to a site of injury (21). This process leads to a production of various inflammatory mediators and is induced by tissue-resident macrophages and mast cells. Rather than being injury triggered, "low-grade" chronic and systemic inflammation is metabolically triggered in obesity. This inflammatory state does not occur alongside infection or tissue injury but is a prominent feature of metabolic syndrome and is maintained for a long period of time (22). Chronic inflammation is characterized by chronic activation of macrophages and lymphocytes, as well as the proliferation of connective tissue and blood vessels (23). The secretion of proinflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor (TNFα), interleukin-1β (IL-1β**)** and inflammatory adipokines like leptin further characterize metabolic syndrome (Figure 1) (24). Obesity, a core feature of metabolic syndrome, is associated with chronic inflammation (25). Changes in insulin resistance, hypertension and some malignancies are all linked to obesity's strong inflammatory component (26,27). Serum levels of inflammatory cytokines are elevated in overweight and obese people (28) and compared to BMI, measurements of body fat have a stronger correlation with such inflammatory markers (29–31).

Additionally, inflammatory responses induced by DIO may vary by sex. Given that obesity is a risk factor for some autoinflammatory and autoimmune diseases, there are likely mechanistic

links between obesity and autoimmune dysfunction that include sex-dependent changes in the production of proinflammatory cytokines (32).

1.2.2 Proinflammatory cytokines

A proinflammatory cytokine is a molecule secreted from immune cells and peripheral macrophages that results in increased inflammation. Typically, homeostasis exists between proinflammatory and anti-inflammatory cytokines to maintain health. However, during metabolic duress, excessive and chronic production of proinflammatory cytokines may occur. In obesity, one possible mechanism as to how adipose tissue accumulation can cause metabolic syndrome is via the development of systemic inflammation, as seen by elevated infiltration of immune cells into adipose tissue and increased circulation of proinflammatory factors (33).

TNFα is a pleotrophic polypeptide released by most macrophages, although fibroblasts, neutrophils, lymphocytes, and adipocytes also release this cytokine (33). In the context of obesity, the major source of TNFα release are immune cells stimulated by the accumulation of and by factors released from adipose tissue. TNFα influences the release of other proinflammatory cytokines including IL-6 and IL-1 by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and mitogen-activated protein kinases (MAPK) signaling pathways. Furthermore, adiponectin mRNA is downregulated by TNFα. TNFα expression, alongside one of its receptors, rises in the plasma and adipocytes of obese people and positively correlates with rising BMI (34). It has been shown that adipocyte-derived TNFα directly affects obesity-induced insulin resistance and that obese mice's adipose tissue secretes TNFα (35).

IL-1β is a prominent cytokine and candidate for therapeutic targeting for auto-inflammatory diseases (36). It is produced by macrophages and growing evidence suggests that it is involved in obesity-associated insulin resistance, primarily modeled in rodents (37). It has been

demonstrated that the increased macrophage-adipocyte interaction associated with obesity, including macrophage-derived factors, impairs insulin action in mouse 3T3-L1 adipocytes (38). One study showed that the inhibitory effect of macrophages on insulin signaling in human adipocytes depends on IL-1β (39). They also showed that proinflammatory cytokines that have been produced excessively can be greatly reduced by inhibiting IL-1b activity, receptors or synthesis.

Il-6 can be produced by different cell types in adipose tissue along with immune cells that mediate inflammation (40). This cytokine can exert action through binding to the membrane-bound alpha receptor (Il-6R) (41). Reports show positive associations between plasma Il-6 levels and various indices of obesity (42). As adipose tissue is thought to be the source of one-third of the total circulating levels of IL-6, the presence of inflammation is indicated by the strong stimulation of hepatocytes by IL-6 to produce and secrete C-reactive protein (CRP) (43). In human subcutaneous adipose tissue, obesity increases the gene and protein expression of IL-6R and IL-6, which positively correlates with the local expression of a number of inflammatory markers (44). In the CNS, the Il-6 receptor is also expressed, primarily in the hypothalamus, and the levels of Il-6 in the brain are negatively correlated with fat mass in obese subjects (45).

CRP, produced and synthesized in the liver, is a sensitive indicator of systemic inflammation (41). The liver is thought to play a key role in inflammation because it excretes free fatty acids and metabolizes triacylglycerol, which promotes adipose tissue to generate the cytokine IL-6, causing hepatocyte expression and CRP release (Figure 1) (46). Human hepatocytes are the principal site of CRP synthesis and secretion, and IL-6 and IL-1 play a major role in its regulation (43). Numerous cross-sectional studies have also connected CRP and obesity.

Overall, peripheral inflammation induced by DIO has been shown to be associated with a host of molecular changes, including the secretion of proinflammatory cytokines. It is, however, important to note that DIO is correlated with other disorders, such as changes in adiposity and metabolic dysregulation.

1.2.3 Adipose tissue accumulation and metabolic dysregulation in obesity

A highly dynamic organ, adipose tissue is composed of specialized cellular constitutes and depots in specific anatomical placements. In a state of energy balance, whole-body adipose tissue is considered a reservoir tissue to store excess fat. It is comprised primarily of white adipose tissue that is found around major organs and blood vessels in the abdominal cavity, whereas brown adipose tissue makes up only 4.3% of all adipose tissue in the adult human (47). White adipocytes contain a single, enormous lipid droplet that pushes all other organelles, including the nucleus, to the cell's edge and they are typically spherical in shape (48). Normally, white adipose tissue plays a primary role in the control of energy balance and secreting paracrine factors to regulate metabolic tissues (49). However, during obesity, the storage of excess energy becomes severely dysregulated, and white adipose tissue may not expand correctly. In fact, the expansion and remodeling of the tissue may result in vascular dysfunction that affects distinct depots (47). Metabolic complications of obesity are then first associated with alterations in differentiating adipose precursor cells and subcutaneous adipose tissue adipogenesis - the storage capacity of subcutaneous adipose tissue is overloaded and the addition of more caloric excess results in fat accumulation in ectopic tissues (50).

The irregular expansion and accumulation of white adipose tissue can then be associated with systemic inflammation, fibrosis, hypoxia, altered adipokine secretion and mitochondrial

dysfunction (51). Furthermore, immune cells and vasoconstricting mediators infiltrate visceral adipose tissue, contributing to vascular dysfunction and disease (52). Chronic, low-grade inflammation of proliferating adipose tissue results in a phenotypic change in white adipose tissue, where inflamed adipocytes secrete proinflammatory cytokines, further denoting obesity as an inflammatory immune disease (Figure 1) (53,54). Lipopolysaccharide (LPS), triglycerides and free fatty acids have been proposed as mediators of adipose tissue inflammation (55). Accumulating evidence also supports that inflammatory cytokines like TNFα and IL-6 are elevated in tissue macrophages of obese individuals (56). These changes induce the activation of other inflammatory factors, such as NFkB, which can negatively impact insulin activity in adipocytes (20). Since adipokines are involved in and regulate insulin signaling, glucose uptake and other metabolic processes, obesity-mediated inflammation may underlie the development of insulin resistance and T2DM along with dyslipidemia (57,58).

In terms of sex differences, male individuals are known to accumulate greater amounts of visceral adipose tissue and females greater subcutaneous depots (59). Some murine model studies have shown that males may exhibit greater diet-induced fat mass expansion than their female counterparts (in both visceral and subcutaneous depots) and that this may be due to the effects of sex hormones (60,61).

1.2.4 Gut inflammation in DIO

There are more than 100 trillion microbiota in the human gut that play key roles in regulating energy metabolism by utilizing symbiotic relationships with the body (62). Changes in the gut microbiome are associated with metabolic and immune disorders. The ingestion of a high-fat diet (HFD) may change this ecosystem and can play an important role in the development of

obesity (55). Due to the complicated etiology of this disorder, the precise mechanisms by which certain alterations in the makeup of the gut microbiota are related to the emergence of obesity and metabolic diseases in humans are still unknown. Additionally, LPS, an endotoxin component of the cell walls of Gram-negative bacteria, has been recognized as a trigger for systemic inflammation due to its impact on the release of pro-inflammatory cytokines (Figure 1) (63). A recent study found that after consuming a high-fat diet for four hours, individuals with obesity, insulin resistance, and/or T2DM had elevated plasma levels of LPS (64).

Given to the association between chronic, low-grade systemic inflammation and the development of obesity, it is important to address other disorders correlated with obesity, like mood deficits.

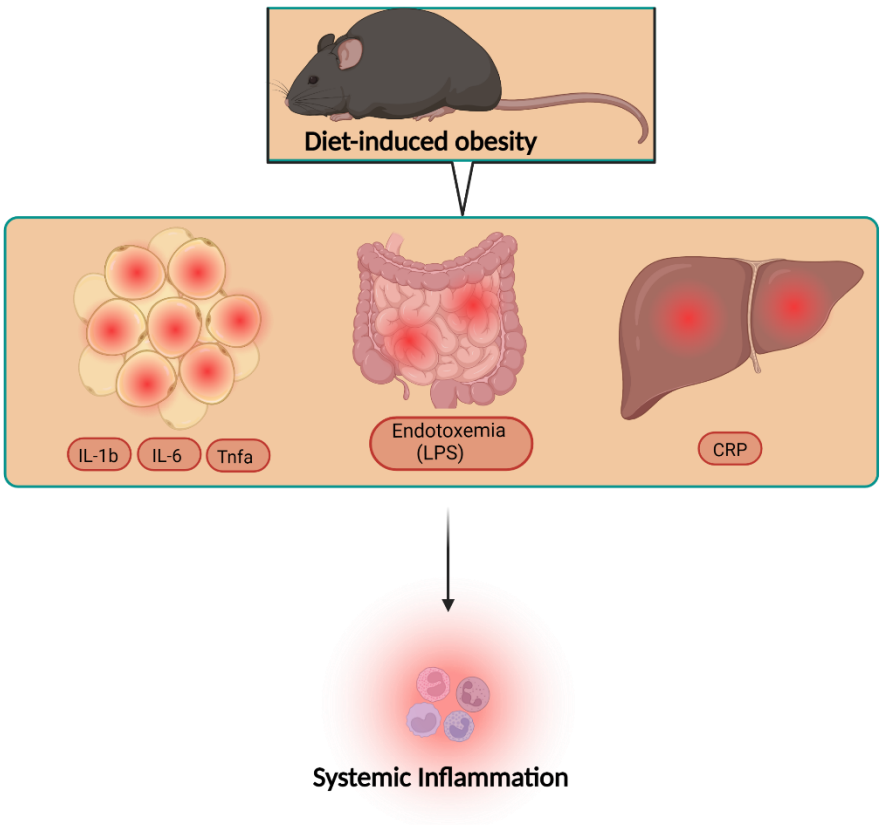

Figure 1. The linking mechanisms between diet-induced obesity and systemic inflammation

DIO recruits different organs to induce systemic inflammation via the release of proinflammatory cytokines.

1.3 Risk of mood deficits in obesity

1.3.1 Depression, anxiety comorbidity with obesity

Given the prevalence of obesity, it is important to understand its impact on other disorders. Major depressive disorder (depression) is a mood disorder that causes persistent feelings of sadness and loss of interest or motivation (65). Obesity and depression are among the most common health conditions worldwide and there is a strong association between the two. The correlation between obesity and depressive-like symptoms is 30% higher than the general population (66). It has also been documented that obesity may closely correlate with other mood disorders and emotional

states like bipolar disorder and anxiety, as an elevated BMI may be predictive of depressive and anxiety-like behaviors (67,68) Furthermore, it is crucial to emphasize that obese individuals considered metabolically healthy, without associated cardiometabolic risk factors, do not appear to have a higher risk for depression (69). One study observed that atypical depression was associated with higher rates of BMI, obesity and weight circumference in both sexes (70).

Given the close relationship of obesity and depression, it is hypothesized that they may also share biological mechanisms, including previously mentioned inflammatory and metabolic dysfunctions. In the brain, brain-derived neurotrophic factor (BDNF) works to develop neurons, promote their survival and is critical for synaptic plasticity. In both depression and obesity, animal and human models have shown reduced BDNF expression, implicating a similar impairment in the brain (71–74). Moreover, peripheral immune activation at the onset of obesity may correlate with the increase in circulation of proinflammatory cytokines during depression (75). In fact, one study found increased low-grade systemic inflammation in atypical (immuno-metabolic depression) but not in non-atypical depressive subtypes which are more often associated with obesity and metabolic syndrome (76). The study goes on to suggest that obesity may even be the driving factor of metabolic issues and inflammation, specifically in atypical depression. Similarly, increased levels of inflammatory markers may also be present in anxiety disorders, although further study is necessary (77). Interestingly, the effects of obesity and even acute peripheral inflammation (such as LPS induced inflammation) have been proven to disrupt neurogenesis and induce cognitive and emotional impairments (78).

In the context of obesity and mood disorders, and the neurological alterations that can mediate their comorbidity, one important candidate for study is the midbrain and limbic cortical circuit.

1.4 Midbrain and limbic cortical circuitry involved in mood regulation

Midbrain and limbic structures include the ventral tegmental area (VTA) that innervates the prefrontal cortex (PFC), amygdala and nucleus accumbens (NAc)- regions in which executive, affective and motivational functions arise (Figure 2) (79). These regions are involved in a variety of psychological processes and, when dysregulated, are implicated in disorders like schizophrenia, addiction, attention-deficit hyperactivity disorder and even depression (80). Dopamine (DA) has been studied in the context of both rapid synaptic transmission and long-term synaptic plasticity, and is an integral neuromodulator in these circuitries (45-47).

Located in the midbrain, the VTA is a major source for dopaminergic neurons that are crucial for normal mesolimbic function (81). These functions include emotional processes like reward-mediated drive, aversion, stress modulation, drug addiction, learning and memory. The VTA`s neuronal heterogeneity partially reflects its functional diversity - with about 60% of its neuronal composition made up of DA neurons, 35% GABAergic and approximately 5% glutamatergic (82).

Outputs of VTA DA neurons include the (medial) PFC and amygdala. In 1979, Brozoski and colleagues were the first to reveal that DA in the PFC was essential for working memory (83). They also found that depleting DA in the dorsolateral PFC was as bad for cognition as eliminating the cortex altogether. Circuitry from the PFC to the NAc centers around the dynamics of synaptic and extra synaptic glutamate levels, which gives rise to the glutamate homeostasis theory (84). The amygdala, in addition to processing emotional cues, may also work with dopaminergic systems to determine the prominence of specific stimuli (85). During Parkinson's disease, the amygdala's responsiveness during emotion perception tasks was increased following DA repletion, providing additional evidence for the influence of DA systems on its function (86).

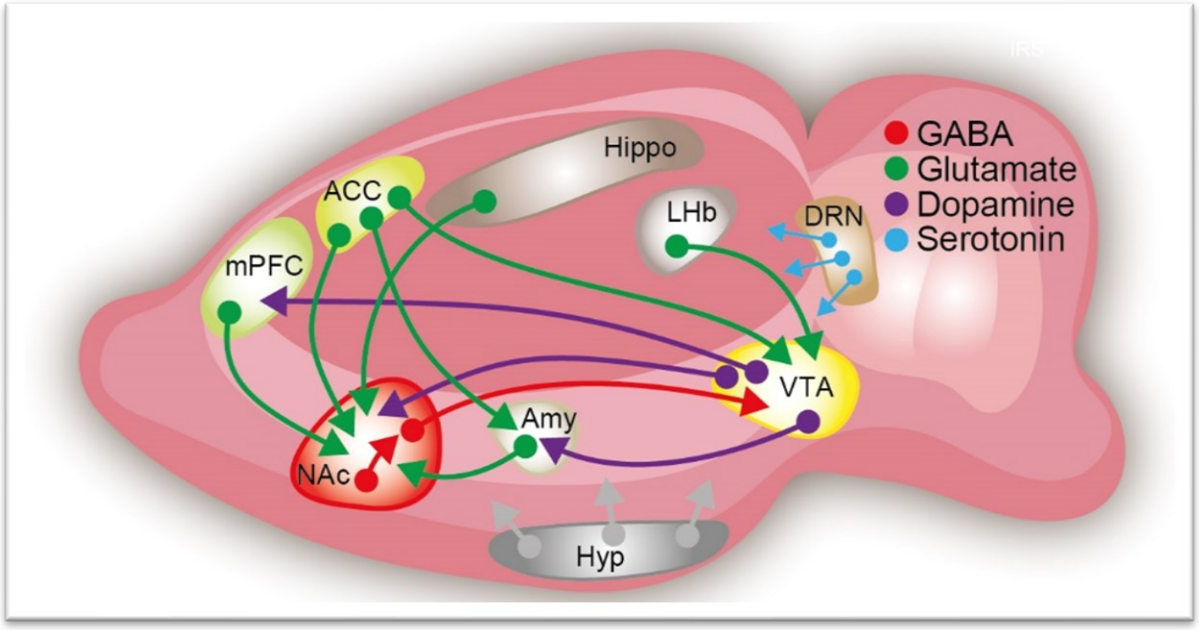

Figure 2. Central regions implicated in depression and anxiety (67).

1.4.1 Nucleus accumbens (NAc)

The associative learning process, where the brain connects sensory stimuli with certain motor behaviors and anticipated rewards, is fundamental to adaptation and survival. These processes are at least partially regulated by DA action in the NAc- the most well-studied and characterized dopaminergic circuit in the brain. It`s comprised of VTA DA neurons that project to the NAc, a part of the ventral striatum (87). The NAc is of key importance for translating motivational inputs into behavioral outputs, as it is a convergence point for pathways involved in motivated responses to salient rewarding and aversive stimuli (88). The NAc has also been implicated in appetitive motivation in both rodent and human studies (89). Chronic exposure to addictive drugs of abuse can also disrupt plasticity in the NAc. Several changes occur in glutamatergic synapses that target the region, most prominently after withdrawal from chronic drug exposure (90). In the NAc, glutamatergic inputs have been described to modulate

cholinergic interneuron activity (91). These inputs originate directly from the thalamus (primarily the paraventricular nucleus) to the NAc and the striatum. Glutamatergic transmission into the NAc seems to be mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAr), however this also evidence suggesting N-methyl-D-apartate (NMDAr) are also responsible for EPSPs in the NAc (92). Additionally, mesolimbic DA systems that interact with glutamate signals to the NAc arise from many regions, like the PFC, hippocampus, basolateral amygdala, and thalamus (93). From the PFC, there is evidence to suggest that a glutamatergic pathway may induce a facilitatory effect on DA release in the NAc by first acting on DA cells in the VTA (93). Some studies have also shown that glutamate spillover from terminals may directly stimulate or inhibit the release of DA in the NAc acting on DA terminals (93,94).

In terms of synaptic plasticity, it was found that NMDAr-dependent long-term potentiation (LTP) in the NAc is evoked more frequently in adolescent (3-week-old) mice than adult (6–20-week-old) mice, suggesting that LTP is developmentally regulated in this region (95). However, it was shown that AMPAr transmission was not regulated by age. Here, a detailed report on the neuroanatomy and activity occurring in the NAc will be described to affirm its relevance in morbidities like obesity and depression.

1.4.1.1 NAc core and shell

The NAc comprises “core” (NAcC) and “shell” (NAcSh) subregions. They give way to subnuclear organization which may correspond to differences in neural processing like reward motivation and adaptive motor responses (96). In fact, the subregions have been shown to exert opposing influence on drug taking, impulsive actions and reward delay discounting (97–100). However, the distinction between the two subregions should be prefaced with the fact that the true

anatomical organization of the NAc is not only exclusive to core-shell, but to a vast mixture of sub territories.

The NAcC has less neuroactive substances represented in its subregion than the NAcSh (101), and contains a greater amount of GabaA receptor (102). Using this receptor, GABA can mediate tonic inhibitory effects in NAcC that may reduce neuroactive metabolism. Similarly, the amount of DA transporter is also greater in NAcC than NAcSh (103). In fact, following the stimulation of the medial forebrain bundle, both the release and uptake of DA is three times greater in the NAcC than NAcSh (103), while, counterintuitively, the basal level of extracellular DA is also significantly higher in NAcC than NAcSh (104,105). Thus, DA may be responsible for reducing the neurochemical diversity in NAcC. Behaviorally, the NAcC is recognized as a main target to incentivized actions due to reward-predictive stimuli (106–108). It can also function as an intermediate between the dorsal striatum and NAcSh because it initializes motor actions and acquisition of reward-cue associations (109). Lesions and inactivation of perturbations in the NAcC of DA transmission have been shown to depress behavioral responses to incentive stimuli- including Pavlovian and discriminative stimulus tasks (110,111). In contrast, other pharmacological and lesion studies of the NAcC or its DA inputs showed that this subregion may not always be necessary for animals to discern between cues predicting differently valued outcomes or directing different actions to perform (112–114). Meanwhile, Sicre and colleagues observed that the NAcC is required for responses to incentive stimuli- which orients the attention towards reward seeking- but not instructive stimuli- which informs about the action to perform (115).

The NAcSh is composed of a more diverse amount of neuroactive substances than NAcC- including 5-HTreceptors, DA D3 receptors, calretinin, and cocaine-and amphetamine-regulated

transcripts and peptides that play a role in reward, feeding and stress and may function as an endogenous psychostimulant (96,101). It is interesting to note that the caudomedial shell contains some of the same neurochemicals as the extended amygdala (116). It has also been shown that a number of drugs can induce the expression of the immediate early gene, cFos, preferentially in the NAcSh (117,118). The NAcSh is implicated in tasks like reward prediction and affective processing (109). Sackett and colleagues observed that in the NAcSh, DA release dynamics were consistently higher overall, lasted longer, and tracked the preferred reward magnitude over the course of a cue period (119). Additionally, the lever push for reward showed distinctive DA signaling characteristics in NAcSh but not in the NAcC, demonstrating that information about reward value is tracked differently and uniquely by the NAcSh, where DA is released (119).

In terms of connectivity, NAcSh and NAcC outputs project to separate structures and sub territories (Figure 3). The NAcC innervates the dorsolateral part of the ventral pallidum (120). The ventromedial section of the subcommissural ventral pallidum, which projects to the medial segment of the thalamic mediodorsal nucleus, is selectively innervated by NAcSh (121). Along with the ventromedial section, NAcSh also substantially innervates the lateral preoptic-lateral hypothalamic continuity, which powerfully projects to the mesencephalic VTA, and serves as the NAcSh third primary location of output termination (116,122). However, the NAcC also primarily projects to the entopeduncular nucleus including a part that invades the lateral hypothalamus (120).

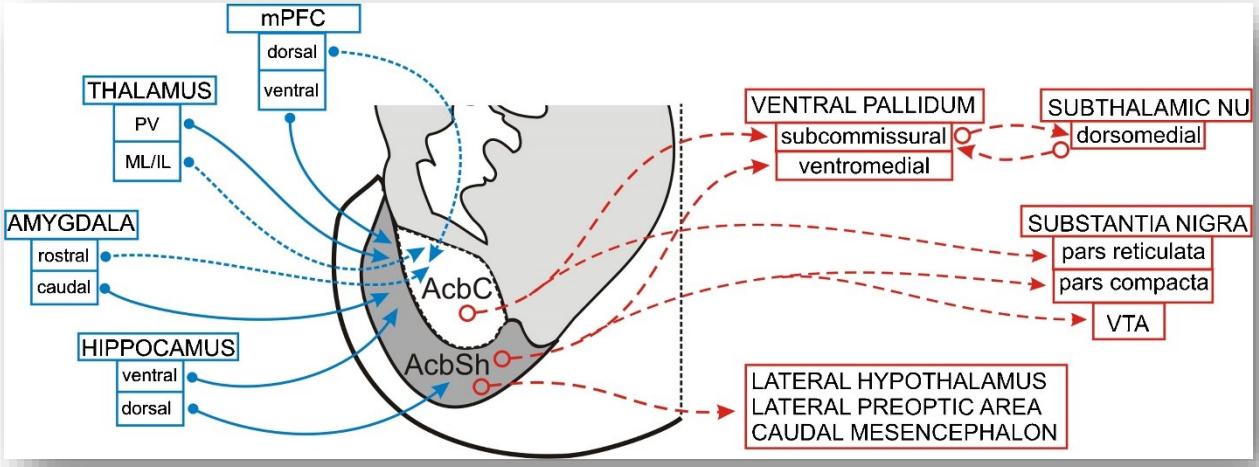

Figure 3. Schematic drawing of the inputs and outputs of the nucleus accumbens core & shell (123)

1.4.2 Medium spiny neurons: neuronal composition of the NAc

The vast majority of cells comprising the NAc are GABAergic medium spiny neurons (MSN) that either express the DA D1 receptor (D1R) along with dynorphin and substance P or express DA D2 receptor (D2R) along with enkephalin (124) in near equal proportions. The remainder of the neuronal composition of the NAc are 1-2% cholinergic interneurons and 1-2% GABAergic interneurons.

Compared to the GABAergic MSNs in the NAcC, intracellularly loaded MSNs in the medial NAcSh are smaller, have fewer, thinner dendrites, and have fewer dendritic spines (96). Likewise, NAcC MSNs have greater density of dendritic spines, branch segments and terminal segments. In the dorsal striatum, D1R MSNs form a large part of the "direct pathway" of the motor signaling in the basal ganglia- forming monosynaptic contacts with the substantia nigra pars reticulata and internal globus pallidus (109). D2R MSNs form part of the "indirect pathway" and send projections to the external globus pallidus (125). Correspondingly, increases in D1R neuronal activity typically elevate movement, whereas increases in D2R neuronal activity reduce movement (126). D1R MSNs are thought to send projections to midbrain structures- VTA primarily- and D2R

towards the ventral pallidum. Although the roles of MSNs in these pathways is well established, the simple dichotomy between the roles of NAc D1R and D2R MSNs is still being studied, much as it is with the basic rate model for the role of basal ganglia direct and indirect pathway function in motor behavior (109). Studies have found that output projections from NAc MSNs do not exclusively segregate based on D1R and D2R subtypes and neither by the direct nor indirect pathway (127). For instance, the ventral pallidum receives close to 50% input from NAc D1R MSNs, indicating considerable anatomical overlap in projection targets of D1R and D2R MSNs (128). In terms of reward and aversion behaviors, it was believed that D1R MSNs relay reward signals, while D2R MSNs convey aversion. However, one study has shown that both subpopulations can act as conduits for encoding both reward and aversion, depending on the pattern of neuronal stimulation (129).

DA transmission and inputs to MSNs may become dysregulated during certain disorders. A core feature of depression, for example, is anhedonia, and DA signaling is essential for hedonic changes associated with this disorder (130). Studies have observed that animal models of depression show differential functioning of the mesolimbic DA system. These observations also reflect a reduction in striatal DA activity also seen in patients diagnosed with depression, compared to controls (131). However, there is still much to be studied about which afferent pathways may induce dysregulations in the DA system.

1.4.3 NAc mediated behaviors

The NAc is targeted by direct glutamatergic inputs from many regions of the brain (109). These excitatory inputs give rise to an array of behavioral functions, but one such example is drug seeking. Glutamate signaling in the NAc has been associated with relapse following drug-seeking extinction (84). However, adaptations in NAc MSN synapses that are caused by drug abuse,

primarily cocaine, can be differential depending on the source of the excitatory input (132). Meanwhile, DA signaling is connected more to compulsive drug use (84). Initially, data showing that all drugs of abuse increase DA release in the NAc suggested that it takes part in instrumental conditioning, as a pleasurable experience leads to the signaling of a reward (109). However, DA input into the NAc specifically encodes incentive salience (133), therefore it is essential to acknowledge that the mechanisms that mediate motivation are separate from those that mediate pleasure.

The NAc is also a critical structure for the circuitry involving the translation of motivation into action. The DA inputs the NAc receives from the VTA onto GABAergic MSNs project both directly and indirectly to the substantia nigra pars reticulata and to the VTA. This basal ganglia-thalamocortical circuitry is essential in the process of reward and aversion learning in mice (89). Contrary to classical views, D1R MSN activation majorly influences reward-based learning, while D2R inactivation influences avoidance behaviors (134), suggesting that D1-D2R functional antagonism does not necessarily ring true for all aspects of reward-related behaviors. Both circuits seem to have oppositional effects on isolation but can also play synergistic roles and this effect may be especially important to drive positive reinforcement learning. Another study relayed that D1R MSNs are required for appetitive aggression, as they demonstrated that selective inhibition of D1R activity in the NAc decreased this behavior (135).

The NAc is significantly linked to several disorders like addiction, depression, stress disorders, anxiety-like behaviors and even overeating (136–138). The NAc is a well-implicated region in the hedonic deficits associated with depression and control of food-motivated behavior (139). One study observed that DIO induced by prolonged feeding of a HFD elicits molecular adaptations in the NAc and anxiodepressive behaviors (140). These molecular adaptations include

higher D2R, BDNF and deltaFosB, but reduced D1R protein expression in the NAc. In animal models of depression, D1R antagonist infusion into the NAc has been shown to rescue deficits in the acquisition of morphine-induced conditioned place preference (136). Contrastingly, NAc DA receptor antagonist injection, which inhibits DA activation in the VTA, was found to induce depressive-like symptoms (141). These findings imply that DA signaling in the NAc may play a significant role in the development and management of the comorbidity of disorders like addiction and depression. In human studies, addiction and depression have been effectively treated using deep brain stimulation of the NAc (142,143). CBT and SSRI treatment in adult studies in humans proved that higher pre-treatment bilateral NAc volume was linked to a higher decline in anxiety-like symptoms (144). Among young people with current anxiety, greater left NAc volume also predicted better pre-to-post CBT and SSRI treatment-related reductions in clinician-rated anxiety symptoms. Moreover, electrophysiological studies have revealed that D1R MSNs to lateral hypothalamus inhibitory signaling is reduced where overeating is promoted (138).

The NAc is also implicated in chronic social defeat stress, as one study found that induction of delta FosB in the NAc was necessary and sufficient for resilience and required for antidepressant to rescue behavioral pathology induced by social defeat (145). The induction of delta FosB was made possible by induction of GluR2 AMPAr subunit, which worked to decrease the responsiveness of NAc neurons to glutamate. There is also evidence to suggest that stress induces modulatory alterations in the NAc (146). In a mouse model of stress-induced anhedonia and passive coping, Pignatelli et colleagues observed that these behaviors are related to increased synaptic strength of excitatory synapses onto D1R MSNs in the NAc from the ventral hippocampus, along with D1R MSN hyperexcitability from lateral hypothalamus projections (147). Their research provided strong evidence that di-synaptic pathways control maladaptive

emotional behavior in the NAc. Potential drugs may target peptides, growth factors and other neuromodulatory factors in the NAc that can change cell firing properties and synaptic transmission and this chemical cocktail could lead to large stress-dependent behavioral alterations (148).

Understanding the mesolimbic system and its implications for obesity and mood disorders is of paramount importance. Thus, the central nervous system and its components are a necessary point of study to fully appreciate the circuitry that mediates these dysfunctions.

1.5 Neuron physiology, activity, and plasticity

1.5.1 Neuronal physiology and activity

The basic components of a neuron are its cell body and branches - dendrites and the axon. At rest, a neuron has intracellular and extracellular concentrations of certain ions, allowing it to maintain an electrochemical equilibrium. Sodium (Na^+) concentrations are much higher extracellularly, at rest, whereas potassium (K^+) ions compose most of the intracellular space, at rest. The lipid bilayer of the neuronal cell membrane acts as a capacitor, while the transmembrane channels act as resistors. Since the membrane of the cell is only permeable to K^+ ions at rest, the K^+ equilibrium potential is typically close to the cell's resting membrane potential (RMP), -75mV (149). Moreover, being that neurons are electrically excitable, they respond to information by producing electrical impulses that spread throughout the cell and along its axon as action potentials. An action potential, therefore, is a rapid sequence of changes measured in voltage across the cell's membrane and down the axon, generated and propagated by changes in cationic gradients (mainly K^+ and Na^+). A neuron's action potential can be defined by its 3 phases: 1) depolarization - at which voltage-gated Na^+ channels open to allow an influx of Na^+ ions. 2) repolarization - where

the flow of K^+ ions out of the cell results in a decrease in membrane potential towards the cell's RMP. Lastly, phase 3) hyperpolarization - the short-term drop in the membrane potential below the normal RMP and the cell's refractory period, in which it cannot fire (150). These components of a neurons' physiology and action allow for the propagation of electrical activity, as well as highly specified communication between neurons via neurotransmitters.

Neurotransmitters are endogenous chemicals where, in the brain, they provide numerous functions through chemical synaptic transmission (151). Several neurotransmitters provide diverse functions throughout the body and brain, namely: glutamate, Gamma-aminobutyric acid (GABA), glycine, DA, norepinephrine, serotonin, and acetylcholine. Glutamate serves as the brains' primary excitatory neurotransmitter, mediating neuroplasticity (152–156). In contrast, GABA (and glycine) are the brain's main inhibitory neurotransmitters, accounting for at least 40% of the brain's inhibitory signaling (151). DA, a neuromodulator, is essential in several brain functions, including reward, emotion, learning, motor control, and executive functions (151). Disturbances in neurotransmission are implicated in psychological disorders such as depression and may even be present in obesity (157,158).

Given the essential nature of neurotransmission, it is important to understand its mechanisms on a cellular scale. Glutamatergic excitatory neurotransmission, the most common type in the mammalian nervous system, acts primarily through AMPAr, a key determinant of synaptic plasticity and strength. When glutamate binds to the receptor, the pore opens, allowing an influx of Na^+ ions (and K^+ efflux) thus permitting depolarization of the cell (159). Interestingly, AMPAr can also allow the influx of calcium (Ca^{2+}) ions, which is important for some Ca^{2+} dependent signaling. However, it is important to note that AMPAr generally govern fast synaptic transmission, and another glutamatergic receptor type, NMDAr, has high Ca^{2+} permeability and,

as a result, controls intracellular signaling and synaptic plasticity (160). NMDAr signaling is thought to stand at the basis of memory formation, inducing cellular processes like LTP (or long-term potentiation) and LTD (long-term depression).

1.5.2 Synaptic plasticity: long-term potentiation & depression

The NMDAr is an ionotropic receptor and controls a ligand-gated ion channel (161). It also acts as a coincidence detector. For the ion channel to open, two essential steps must occur: 1) either glycine or serine must bind NMDAr on the postsynaptic neuron, thereafter two glutamate molecules must also bind, and 2) another glutamate receptor (such as AMPAr) must be activated to depolarize the cell. This will dislodge the magnesium ($Mg2^{+}$) and zinc ions blocking the receptor and thus allow the influx of Ca^{2+} through the channel (160,162). If the depolarization is large enough, the NMDAr will be completely dislodged or unblocked, allowing $Ca2^{+}$ to pass more easily. This influx will recruit more AMPAr to the postsynaptic site, thereby encouraging more depolarization and neuronal growth. This change in synaptic strength serves as the basis of synaptic plasticity. The effect, LTP, can last up to years (160). Other processes, like short-term plasticity and even excitotoxicity occur via similar mechanisms.

LTP has primarily been studied in the mammalian hippocampus, a region of significant importance for memory formation and retrieval. In animal studies, LTP can be induced via a stimulating electrode and a train of high-frequency stimulation (100Hz) in, for example, the afferent pathway of the hippocampus. The postsynaptic depolarization response, which can evoke a field excitatory postsynaptic potential (fEPSP), can be recorded some distance away - in, for example, activated granule cells (163). This may reveal a long-lasting elevation of synaptic strength between the pre and postsynaptic cells. The effect can be phrased as,*" neurons that wire together, fire together,"* and can be defined in more detail by *Hebb's law*. In contrast, there is a

complementary process termed LTD, in which synaptic efficacy may be reduced or depressed, following a train of low-frequency stimulation (1Hz).

Given that synaptic plasticity is studied foremost in the context of memory and within the hippocampus, it is also important to note that other brain structures like the amygdala, lateral hypothalamus and even striatum undergo the LTP and LTD process (164–166). And although stress may impair the LTP process (167), the effect of other disorders, such as obesity, on synaptic plasticity have not been as widely studied. In 2003, Gerges and colleagues showed that in the obese Zucker rat (a genetic model of obesity), various forms of activity-dependent synaptic plasticity are impaired, compared to lean rats, and that these effects have primarily presynaptic origins (168). Understanding the effects of metabolic dysfunction, such as inflammation, on the brain and on processes of synaptic plasticity will be one of the focuses of this study.

1.5.3 Methods of study

There are various methods and tools used to study neuronal activity and processes, one such being electrophysiology. The very first electrophysiology experiments were undertaken in the 1950s and enabled scientists Alan Hodgkin and Andrew Huxley to observe membrane potential in squid giant axons and describe the ionic mechanisms underpinning the action potential (169,170). Today, techniques such as patch clamp electrophysiology - an intracellular method of observing or manipulating a single neuron's activity - and extracellular field recordings - observing large field potentials that may induce LTP - are just two examples of electrophysiology that descend from these first studies.

Another observational method of cellular activity is immunohistochemistry (IHC), a combination of immunology and histology. One gene in particular, the immediate early gene c-fos, is typically used to express rapid changes in neuronal activity, which triggers the production

of the transcription factor c-FOS. With the help of IHC methods, the c-fos protein product may be easily identified, allowing for the use of c-fos detection to map groups of neurons that exhibit variations in their activity.

Neuronal activity is of paramount interest in the study of the central nervous system and how various disorders may affect its activity. Given the importance of brain activity, especially in the midbrain and limbic cortical circuitry of both healthy and disordered individuals, it is paramount to investigate its processes during neuroinflammatory duress.

1.6 Obesity-induced neuroinflammation

Peripheral inflammation, induced by poor diet and metabolic dysregulation, may lead to inflammation in the brain. This may occur when the blood brain barrier (BBB) exhibits reduced integrity and elevated permeability, which favors neuroimmune activation, immune cell infiltration, and mood disorders (67). In DIO, chronic low-grade inflammation disrupts the BBB in part due to endothelial dysfunction, leading to neuroinflammation and elevated oxidative stress (171). Peripheral immunological challenges, like acute LPS secretion or injection, along with the breakdown of the BBB, allows for the entrance of bone marrow-derived immune cells into the brain and supports depressive behaviors, all which can be enhanced by DIO (67,172,173). The BBB is not only a physical barrier but also operates as a regulatory interface between the CNS and immune system. The two systems connect in a myriad of ways, and inflammatory signals cross the BBB to initiate and/or propagate neuroinflammation. The BBB secretes neuroinflammatory substances and properties like cytokines (174). Transporters of the BBB are impacted during states of injury, Alzheimer`s disease and in inflammatory processes. Secretions from the immune system along with cytokines from cells that constitute the BBB are constitutive and inducible. The CNS

and immune system together may modulate transport systems, and during states of disease may even disrupt the BBB (174). Saturated fatty acids themselves, in the context of HFD, may also stimulate neuroinflammation. Elevated levels of free fatty acids in the circulation are one consequence of DIO (175,176). These fatty acids may increase BBB permeability, particularly in the hypothalamus (and its subregions- arcuate nucleus, paraventricular nucleus), a region linked to the regulation of caloric intake. One study showed that after just a few days of HFD feeding, hypothalamic subregions that control feeding behavior expressed a disruption in the BBB and immune cell migration (177). As a result of cytokine circulation, the BBB can also selectively transport several cytokines such as Il-1, Il-1ra, Il-6, TNFα, ciliary neurotrophic factor and adipokines, in one direction. Numerous experiments have shown that cytokines can cause extensive damage to the BBB when they pass through it directly (178). In murine models, several cytokines, such as TNFα, can directly cross the BBB, often within thirty minutes following injection (179). Different mechanisms by which this happens may occur: a saturable influx transport, retrograde axonal transport system (IL-1 and -, IL-6, and TNFα) or circumventricular organs, regions of the brain where the BBB is imperfect and cytokines can cross by simple diffusion (GDNF, glial cell-derived neurotrophic factor) (180). In the case of LPS, it does not cause chronic inflammation and so the effects are likely due to the crossing of peripheral immune signals into the brain.

Increased levels of glucose and saturated fatty acids may cause neuroinflammation along with its components- microglia activation, mitochondrial dysfunction, neuronal death, and impairments in synaptic plasticity, as shown in animal and in vitro models (181). Neuroinflammation can be characterized by the activation of microglia, the brain's resident

immune cells. In an active state, microglia secrete proinflammatory cytokines (TNFα, Il-1b, Il-6), which perpetuates neuroinflammation (181). One study observed that obesity was associated with altered microglia morphology, where reduced synapses in the PFC occurred (182). Other cells constituting the brain parenchyma, including astrocytes, oligodendrocytes and neurons also change their structure and function in response to proinflammatory signals from the circulation (183). Together, inflammatory signals in the brain produce an environment in which there are less neurotrophic and more pro-apoptotic factors, which in the long-term may induce neurodegeneration (182,183).

De Souza and colleagues were the first to show that high-fat feeding increases the levels and expression of proinflammatory cytokines and transcription factor NFkB in the hypothalamus (184). Thereafter, several additional studies showed that a HFD can lead to hypothalamic inflammation and can impair normal signaling related to feeding and metabolism (43,185–187). After eight weeks, a diet heavy in saturated fats causes higher hypothalamic inflammation than a diet high in unsaturated fats (188). In animal models of Alzheimer's disease, increased hippocampal inflammation induced by DIO or high-calorie diets has been associated with learning and memory deficiencies as well as exacerbated cognitive deterioration (189–191). Additionally, some studies have shown that DIO activates neuroinflammatory pathways with negative effects on the functional cerebral cortex (192,193). The reward system may also be damaged by neuroinflammation. Studies show that DIO individuals have reduced D2R availability in the striatum, which may interfere with motivational and reward systems (194). High adiposity may also be a risk factor for those susceptible to Parkinson's disease, as it is known that DA signaling is impaired in this disease (195).

1.6.1 LPS as an inducer of acute peripheral and neural inflammation

Another mechanism by which DIO induces inflammation relies on a HFDs capacity to modulate the gut microbiome. The gut barrier can permeabilize as a result of the ensuing changes in microbiota populations, increasing the flow of bacterial endotoxins into the bloodstream (181). *Escherichia coli* LPS, otherwise known as endotoxins, are produced in gut microbiota and are well-studied contributors to systemic inflammation (67,196). Proinflammatory cytokines are produced by classically activated macrophages (M1) after LPS secretion or injection (197). In both human and murine models, peripheral LPS administration has been shown to significantly increase levels of proinflammatory cytokines- such as TNFTNFα, Il-6, Il-1b, and more (196,198). It has been widely established that LPS can induce sickness behaviors in both preclinical and clinical investigations, and it is believed that these symptoms are induced by increased cytokine action in the brain (196). Findings from human neuroimaging demonstrate increased microglia activity following LPS treatment, which results in depressive-like sickness behavior (67,196). Studies with intracerebral LPS have demonstrated that microglia can adopt an intrinsic activation/M1 phenotype following its application (197). One study displayed that LPS from gut gram-negative bacteria may circulate in the bloodstream and activate microglia in the NAc (199). Another study showed that two daily intraperitoneal injections of LPS activate microglia, particularly in the hippocampus (200). LPS treatment causes "classic" activation of microglia in preclinical paradigms studying neurodegeneration in rodents (197,201). For example, bearing that inflammation plays a large role in the development of Parkinson's disease, a disease model induced by LPS has been widely accepted and utilized for studying the pathogenesis of Parkinson's as well as testing its treatments (201). Overall, LPS has been proven an effective inducer of acute inflammation in both the periphery and the brain.

Concerning acute systemic inflammation induced by an LPS "bomb" and a multifaceted and chronic systemic inflammation induced by DIO, the similarities and differences in the rise in neuroinflammation by these stimuli is less clear. Implicated in both is the circulation of proinflammatory cytokines, the fracturing of the BBB and the activation of microglia (25,197)). In an LPS inflammatory model, there is a specific stimulus associated with a pathogen that seems to have a homogenous effect in producing inflammation in animal models. As previously stated, a DIO inflammatory model does not occur alongside an infection. Therefore, an injection of LPS that does cause site-specific injury and inflammation poses an interesting argument for how different this model of inflammation is from a naturally occurring DIO. Lastly, given the longer time-scale in DIO models of neuroinflammation and possible compensation mechanisms, there is likely a more heterogenous and variable response in the rise of neuroinflammation.

1.6.2 How neuroinflammation affects neuronal activity and synaptic plasticity

According to several studies, cytokines and mechanical injury are just two examples of insults that cause the neuronal expression of NFkB to increase (Carson et al., 2006). A family of inducible transcription factors- NFkB- controls a wide range of genes involved in various immunological and inflammatory response pathways, including cellular responses to stimuli including cytokines (20). Conditional deletion of NFkB using Nes-Cre, which is selective for neuronal cells like neurons, astrocytes, and oligodendrocytes, partially reduces experimental autoimmune encephalomyelitis or EAE production, proving that NFkb also operates in the CNS to regulate inflammation (20). Zhang and colleagues observed that IKKB/NFkB forced activation in the hypothalamus disrupts central insulin and leptin signaling and activities (202). Significant

protection from obesity and glucose intolerance is provided by site- or cell-specific reduction of IKKB, whether it occurs generally throughout the brain, regionally in the mediobasal hypothalamus, or specifically in hypothalamic agouti-related peptide-AGRP)-expressing neurons (202). The same study saw that following 12 weeks of HFD the body weight of AGRP/IKKBlox/lox mice was 13.1% lower than that of controls, indicating that AGRP neurons are primarily in charge of the neuronal effects of IKK/NFkB on body weight homeostasis.

In terms of synaptic plasticity, the length of the inflammation can differentially affect LTP (203). Acute inflammation temporarily reduces LTP, whereas chronic LPS exposure affects LTP for a longer amount of time in slices of animals that have previously received an adjuvant injection (203). Even after stopping the LPS therapy, this impact endures. Hippocampal slices treated with Il-1b prevented LTP in the CA1 (204), CA3 (205) and a later study saw a similarly negative impact on LTP in the dentate gyrus (206). In this instance, it was hypothesized that the cytokine's inhibitory influence on calcium channel activity was responsible for the Il-1b-induced alteration. In transgenic mice models of Alzheimer's disease, an increase in Il-1b expression has also been linked to a deficit in LTP (207). However, it is important to note that Il-1b's effects on LTP are dose-dependent.

1.6.3 Neuroinflammation in the nucleus accumbens

Studying neuroinflammation in the NAc is necessary to elucidate the link between DIO and mood disorders. Multiple lines of evidence suggest that individuals with depression, primarily atypical or immuno-metabolic depression, have a neuroimmune etiology, hence, prolonged immune activation in DIO may result in mood disorders (139). Decarie-Spain and colleagues investigated whether anxiodepressive behavior brought on by DIO is accompanied by neuroinflammatory reactions in the NAc. They looked at how two isocaloric HFDs, rich with either

palm oil (saturated) or olive oil (monounsaturated), affected the immunological activation of mice, their anxiolytic behavior and energy metabolism. They also sought to ascertain if the neuroinflammation induced by a HFD and elevated by the (IKKB)-NFκB pathway contributes to anxiety, depression, and the desire for pleasurable foods. They discovered that a saturated HFD increases weight gain, metabolic dysfunction, inflammation, as well as anxiety-like and despair behaviors. In addition, reactive gliosis was shown to be sparked by the saturated HFD, and NFκB transcriptional activity was revealed to be stimulated in the NAc. Likewise, the viral inhibition of the IKKB/NFkB pathway in the NAc reduced inflammation and rescued anxiodepressive behaviors, along with suppressing compulsive sucrose-seeking in DIO mice.

Alongside this study, Hryhorczuk and colleagues looked at how chronic consumption of HFDs rich in either saturated or monounsaturated fat affected DA-dependent behaviors and signaling (208). They observed that a saturated, but not monounsaturated HFD reduced sensitivity of the rewarding effects of amphetamine. Specifically, they saw that saturated fats reduced the ability of amphetamine to elicit a place preference, suppressed locomotor-sensitizing effects, attenuated D1 receptor-induced locomotor responses, and induced neuroadaptations in the NAc at the same time as reducing D1R signaling and increasing AMPAr activity. Chronic consumption of saturated HFD inhibited DA-dependent behaviors independent of obesity or changes in weight, but an isocaloric diet rich in monounsaturated fats proved protective.

In humans, some studies have displayed a relationship between NAc cell density and microstructure, which can be a measure of neuroinflammation, and weight gain and changes in eating behaviors, in both adolescents and adults (209–212). For example, Rapuano and colleagues collected data on waist circumference and NAc cell density in a cohort of children as well as assessing the link between diet and waist circumference after two years. They observed a

reciprocal relationship between baseline waist circumference and BMI percentile and increased cellularity (gliosis) in the NAc at the two-year measure, where cellularity was measured via anatomical scanners from a single vendor of MRI scanners. At this follow-up, it was also seen that NAc cell density mediated the association between diet and waist-circumference percentile. Overall, the data demonstrated that diet mediates inflammation in the NAc, which can further contribute to bad dieting and weight gain.

These studies, among others, provide evidence of the role DA signaling in the NAc may impact downstream motivational and reward behaviors. Here, we will look at how neuroinflammation may affect this signaling and determine to what degree DIO may influence this circuitry.

2. Research Question, Objectives and Hypothesis

2.1 Research Question

The overarching theme of this project is to unearth the mechanisms underlying the link between DIO and depression. According to previous studies in the lab, saturated, but not monounsaturated fats induce obesity and immunometabolic dysfunction (139,213–215). This ultimately leads to neuroinflammation, reduced mesolimbic DA tone and function, anhedonia, anxiodepressive behaviors and increased compulsive sucrose-seeking. More specifically, these studies observed that alleviating HFD-induced neuroinflammation in the NAc- via knockdown of NFkB predominantly in neurons- rescued neuroinflammation-induced behavioral deficits. Furthermore, the change in DA tone onto the NAc may occur due to modulation of D1R-signaling by DIO. Increases in phospoDARP32 phosphorylation of THr75 during DIO is implicated in a decrease in D1R MSN excitability (208). Chemogenetic activation of D1R MSNs via a Cre-dependent excitatory DREADD resulted in a restoration of anhedonia, as measured by a sucrose

preference test as well as rescued behavioral despair, as measured by a forced swim task, in mice fed a saturated HFD (see Appendix 3).

Overall, this recent data points to a possible mechanism by which DIO may lead to behavioral changes. Saturated HFD intake results in DIO and metabolic dysfunction, ultimately inducing neuroinflammatory signals like glial reactivity and cytokine release. This translates to D1R MSNs displaying increased NFkB and BDNF signaling, leading to reduced excitability (perhaps via increased GABA signaling onto D1R MSNs (216)) and output modulation which will result in behavioral changes like anhedonia and anxiodepressive behavior.

So, our question is: how does neuroinflammation (induced by LPS and DIO) affect neuronal activity in the nucleus accumbens? How does it affect D1R MSN activity? Are its effects different in the NAcC and NAcSh subregions?

2.2 Overall Objectives

Here, we want to elucidate how neuroinflammation, both acutely and chronically, affects neuronal activity in the NAc.

Specific objectives

1. Determine if an acute LPS challenge changes NAc extracellular field responses
2. Determine if an acute LPS challenge changes NAc D1R neuronal activity
3. Determine if chronic DIO changes NAc D1R neuronal activity

2.3 Global Hypothesis

LPS and DIO will lead to neuroinflammation that will modulate general neuron activity and decrease D1R neuronal activity in the NAc.

Specific Hypothesis

1. An acute LPS challenge will induce changes in LTP.
2. An acute LPS challenge will reduce D1R neuronal activity in the NAc.
3. A chronic DIO challenge will reduce D1R neuronal activity in the NAc.

3. Materials and Methods

3.1 Animals and diets

All procedures involving the use of animals were approved by the CRCHUM Animal Care Committee in accordance with Canadian Council on Animal Care guidelines. Male C57Bl/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) and D1R^{Cre} mice (C57Bl/6 background; in-house colony) were housed in a reverse 12h light-dark cycle (lights off at 10h) with ad libitum access to water and food. Colony-derived heterozygous D1R^{Cre} mice and wildtype littermates were weaned at P21-23 and genotyping of transgenic mice was performed by PCR analysis from ear biopsies.

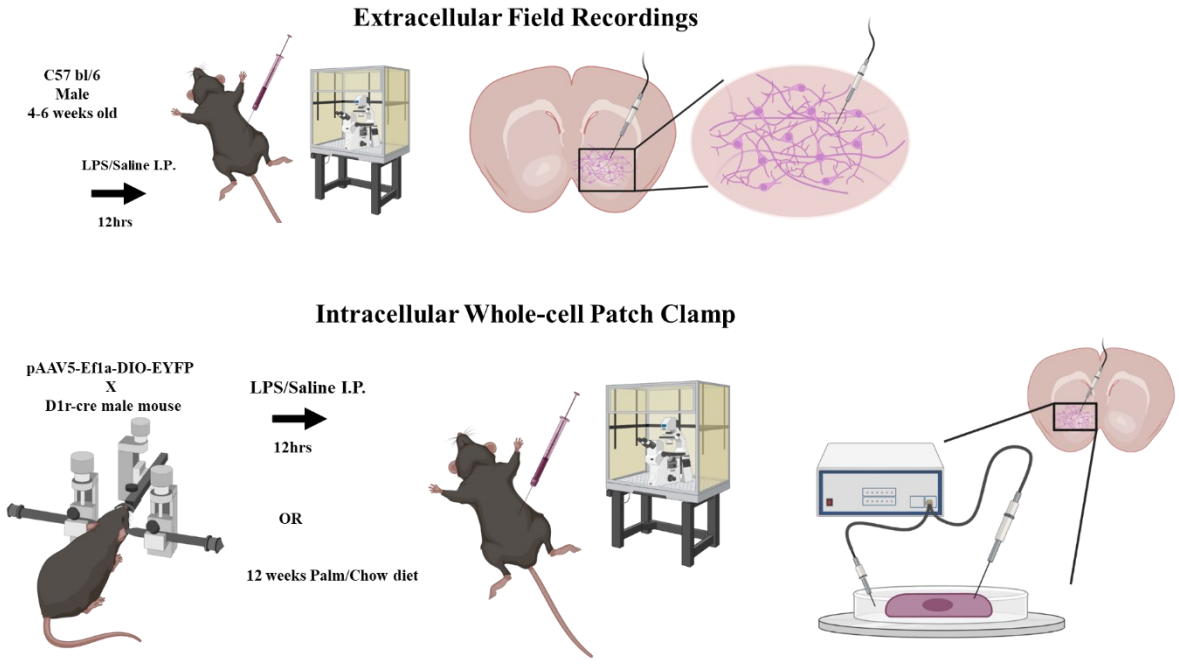

Figure 4. Timeline and Experimental Design

3.1.1 Acute LPS experiments

For extracellular field experiments, male C57Bl6 mice, aged 4-6 weeks, were singly housed upon arrival and for all remaining experiments (Figure 4). The mice were fed a standard irradiated chow diet (containing 16.8% kcal soybean oil) and used for experimentation after one week of habituation.

For whole-cell patch-clamp experiments, D1R-cre reporter mice underwent viral stereotaxic surgery at 8 weeks and thereafter were singly housed (Figure 4). Mice were used for experimentation at least two weeks following surgery.

In previous experiments I contributed to (see Appendix 1), mice displayed anxiety- and sickness-like behaviors 12 hours after LPS (0.83mg/kg) administration without showing significant psychomotor effects of LPS. Therefore, we chose the same dose and timing for the present electrophysiology, IHC, and behavior experiments.

3.1.2 Chronic high-fat feeding experiments

$D1R^{Cre}$ mice underwent viral stereotaxic surgery at 8 weeks and were singly housed. The mice were given free access to one of two diets: a 50% kcal palm oil saturated high-fat diet ("Palm"; modified AIN-93G purified rodent diet with 50% Kcal from fat derived from palm oil, Dyets) or standard chow. Food intake and body weights were measured progressively. All other measures were taken after 12 weeks on the diets.

3.2 Chemical reagents

LPS from Escherichia coli (L-4516, serotype 0127:B8) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and aliquoted for single freeze-thaw use. LPS was dissolved in phosphate-buffered saline (PBS) and diluted to a final concentration of 0,83 mg/kg on testing days.

3.3 Cytokine ELISA

Blood plasma was collected from $D1R^{Cre}$ mice following 12 weeks on Palm or chow diet. Enzyme-linked immunosorbent assays (ELISA) were performed for CRP, TNFα and IL-6 cytokines. The measurements and analyses were carried out at the Cellular Physiology core facility of the CHUM Research Center. Mouse AlphaLISA assay kits (AL504 for IL6, AL505 for TNF) from PerkinElmer (Waltham, MA) and an Envision 2104 plate reader from the same supplier were used to assess plasma levels of the corresponding analyte using half-area white 96-well microplates. A standard curve was run on each plate containing samples (in duplicate). Omnibead calibrators (PerkinElmer) were utilized to ensure the proper operation of the assay and instrument. Plasma mouse CRP levels were determined using an ELISA kit (ab157712) from ABCAM (Waltham, MA) according to the manufacturer's protocol. Standard curves and samples were done in duplicate and plates were red using an Envision 2104 plate reader (PerkinElmer) at 450 nm. Recoveries were >85%.

3.4 Stereotaxic Surgery and AAV delivery

Mice were anesthetized with isoflurane and placed into a mouse ultraprecise stereotaxic instrument (Kopf, Inc.) with Bregma and Lambda in the same horizontal plane. 1.0 ul of Cre-inducible AAV5/EF1a-DIO-eYFP (Addgene plasmid # 27056; http://n2t.net/addgene:27056 ; RRID:Addgene_27056) was delivered bilaterally into the NAc Core ? of $D1R^{Cre}$ mice (AP:+1.5 mm. ML:±1.3 mm, DV: −4.3 mm, relative to bregma and skull surface) using a 2.0 μL NeuroSyringe (Hamilton, Reno, NV, USA). AAV5 titers were 3.7–6 × 1012 viral molecules/ml. Mice were individually housed following surgery. High-fat fed $D1R^{Cre}$ mice received diet two weeks post-surgery.

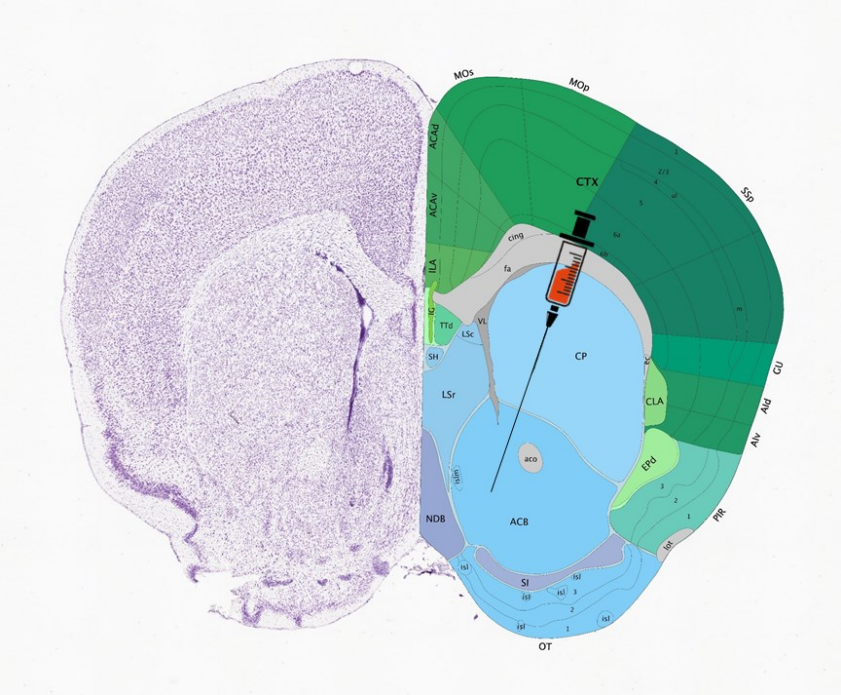

Figure 5. NAc injector placement

3.5 Behavioral Testing

To assess the behavioral effects of acute inflammation, mice were injected IP with LPS (0,83 mg/kg) or vehicle (saline). 12 hours post-injection, the D1R^{Cre} mice underwent the light/dark box (LDB) apparatus. The LPS dose was chosen based on a report showing it is the minimal effective dose to induce anxiety and depressive-like behavior (217). Control mice received an IP injection of vehicle (endotoxin-free saline solution).

3.5.1 Light/Dark Box

The LDB was used to assess anxiety-like behavior. The apparatus (Med Associates, Inc.) consisted of an illuminated compartment made of transparent plastic and a dark compartment made of black plastic, covered by a lid (both 13.7 cm X 13.7 cm X 20.3 cm). The two boxes were separated by a partition wall, with an opening at the bottom to allow the animal to pass freely between

compartments. The number of entries and time spent in the lit compartment of the box were measured by an overhead video camera connected to a PC with Ethovision XT software (Med Associates, Inc.) for a period of five minutes.

3.6 Immunohistochemistry (IHC)

To evaluate the changes in activity of D1R MSNs in the NAcC and NAcSh following LPS treatment, we performed c-fos and cre recombinase IHC, revealed by Vectastain Elite ABC HRP kit using DAB (3, 3'-diaminobenzidine) peroxidase (HRP) substrate (with nickel) and purchased from Vector Laboratories (CA, USA). Between 30-45 minutes after LDB behavior testing, $D1R^{Cre}$ mice were trans-cardially perfused with 1x PBS, then 10% formalin. The brains were dissected and following 4 hours of exposure to formalin, were stored overnight in 20% sucrose, then frozen. Following slicing of 30um NAc sections, slices were washed, and after blocking (2 hours in 3% NGS, 0,1% Triton, and 1x PBS), slices were incubated with primary antibodies overnight at 4°C (3% NGS, 0,1% Triton, 1x PBS, and GPig mAb cre recombinase, 1:500; Synaptic systems). On day 2, slices were washed, blocked, and treated with secondary antibody (Gt anti-GPig Alexa 488, 1:1000) for 2h at room temp. Later, slices were again washed and incubated with primary antibodies overnight at 4°C (3% NGS, 0,1% Triton, 1x PBS, and Rb mAb c-Fos 1:500; Cell Signaling 2250S). On day 3, slices were washed and treated with secondary antibody (Gt anti-Rb Alexa 568; 1:1000) for 2h at room temp. Sections were mounted and imaged with a Zeiss fluorescent microscope (Carl Zeiss AG).

3.7 Electrophysiology

3.7.1 Slice preparation

Animals were deeply anesthetized using isoflurane and trans-cardially perfused with ice-cold, oxygenated (95% O2/5% CO2) N-methyl-d-glucamine (NMDG) containing recovery solution (in

mM: 119,9 NMDG, 2,5 KCL, 25 NaHCO3, 1,0 Cacl2-2H2O, 2,0 MgCl2-6H2O, 1,4 NaH2PO4-H2O, 20 Glucose). Brains were rapidly dissected and washed in ice cold, oxygenated NMDG. 300 um coronal slices were taken in ice cold, oxygenated NMDG solution with Leica VT 1200S vibratome. Slices were transferred to 32° oxygenated NMDG solution in a recovery chamber for 12 min before being stored at room temperature oxygenated artificial cerebrospinal fluid (ACSF) (in mM: 130 NAcl, 2,5 KCl, 1,25 NaH2PO4-H2O, 1,3 MgSO4, 2,0 Cacl2, 10 Glucose (287-295 mOsm), 26 NaHCO3. Slices rested for at least 1 h prior to recording. For electrophysiological recording, slices were moved to a recording stage perfused with oxygenated ACSF at a rate of 200 ml/hr at 30°C using an inline heater.

3.7.2 Whole-cell patch-clamp recordings

For excitatory current whole-cell recordings, the pipette solution included: in mM, 130 KGluconate, 10 Hepes, 5 KCL, 5 NAcl, 4 ATP-Mg, 0,3 GTP-Na, 10 Na2-Creatine-PO4. For inhibitory current whole-cell recordings, the pipette solution included: in mM, 130 CsCl, 10 NAcl, 10 Hepes, 1 EGTA, 0,1 CaCl2, 10 PCr, 4 ATP-Mg, 0,4 GTP-Na, 5 Qx-314 (Lidocaine). Electrophysiological signals were recorded using an Axopatch 700B amplifier (Molecular Devices), low-pass filtered at 2–5 kHz, and analyzed offline on a PC with pCLAMP programs (Molecular Devices). Excitatory (EPSC) and inhibitory (IPSC) postsynaptic currents were measured by whole-cell voltage clamp recordings, and membrane potential rates were measured by whole-cell current clamp recordings from D1R-expressing neurons in brain slices. Recording electrodes had resistances of 2.5–5 MΩ when filled with the K-gluconate or CsCl internal solution. Only cells with a stable holding current less than +/- 100 pA and an access resistance <20 MΩ were included. Furthermore, cells were rejected if the holding current and access resistance changed more than 20% during recording.

Solutions containing drugs were perfused for at least 5 minutes. EPSCs were recorded with gabaAr blocker picrotoxin (60 uM working solution in <1 % DMSO) and IPSCs were recorded with glutamate receptor blocker DNQX (10 uM working solution in H2O).

3.7.3 Extracellular Field Recordings

Coronal slices (300 μm) containing the NAc were prepared as previously described. Extracellular fEPSPs were recorded in current clamp mode with a pipette filled with ACSF placed in the medial NAcC. Monosynaptic EPSCs were elicited by stimulation of afferences with a tungsten concentric bipolar electrode (World Precision Instruments). Baseline synaptic transmission was recorded for 20 minutes, followed by rapid theta burst stimulation (TBS) and subsequent post-stimulation recording for 40 minutes. TBS was executed for 10s and consisted of 4 pulses at 100 Hz repeated at 5 Hz intervals. Signals were amplified using a Multiclamp 700B amplifier (Molecular Devices) and digitized using the Digidata 1440 (Molecular Devices). Data was recorded (pClamp 10.2, Molecular Devices) for offline analysis. The magnitude of LTP was quantified by normalizing and averaging the slope of fEPSPs following LTP induction relative to the last 5 min of baseline.

3.8 Statistical Analysis

All data are expressed as mean ± SEM and analyzed using unpaired t-tests. Data were analyzed using GraphPad Prism 8 (San Diego,CA, USA). Criteria for statistical significance was set at $p \leq 0.05$.

4. Results

4.1 Acute LPS challenge affects NAcC neuronal activity

In our first experiment, we examined how LPS changes the overall synaptic plasticity and activity of the NAc. Field responses were only recorded in the NAcC (Figure 6A) as we could not obtain a field response in the NAcSh. We observed that LPS-injected mice showed increases in fEPSP slope, post theta-burst stimulation, while fEPSP slope was unchanged in vehicle-treated mice (Figure 6B) and that stimulation-induced changes in fEPSP slope were significantly different between groups ($p = 0.04$; $t = 2{,}27$, $df = 12$) (Figure 6C).

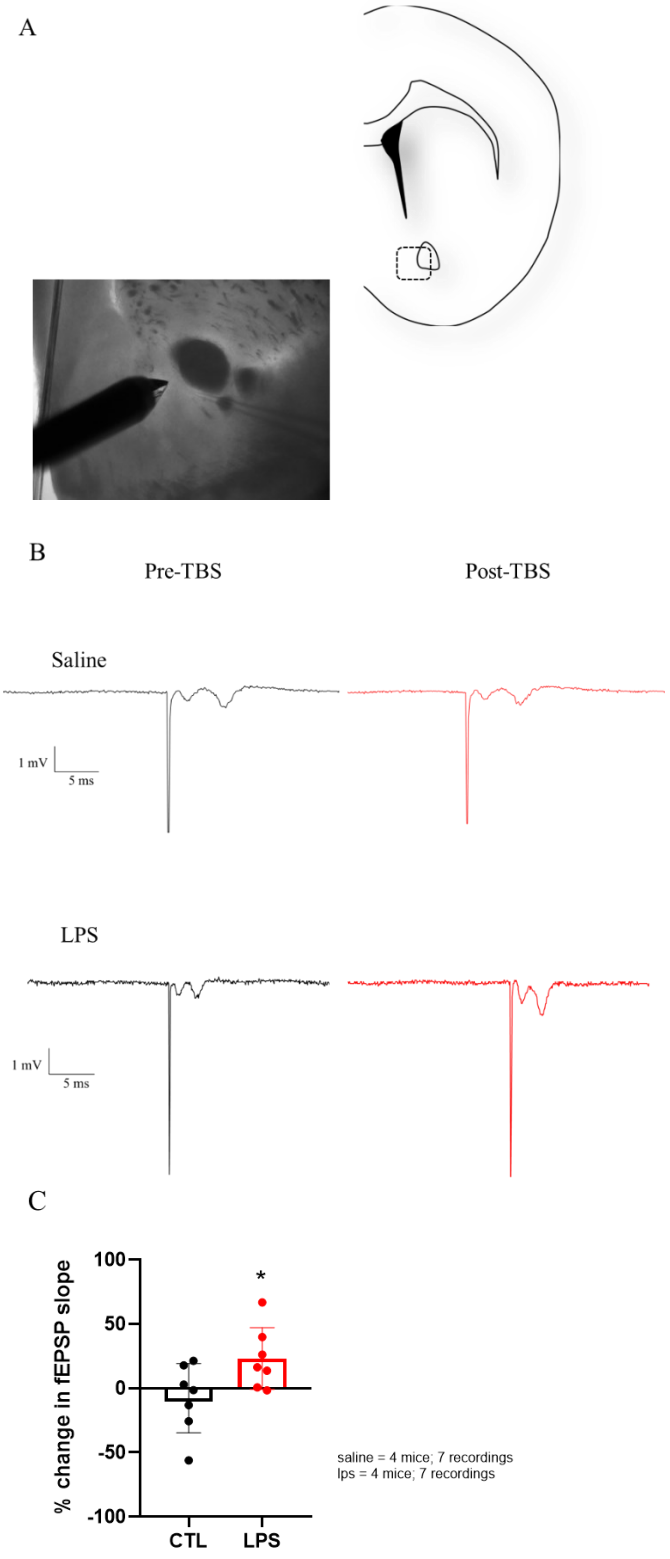

Figure 6. LPS induced changes in LTP-synaptic plasticity of the nucleus accumbens core (NAcC) Extracellular field recordings were performed in 4-6-week-old male C57 bl/6 mice. The figure shows: **(A)** Representative image of the placement of the stimulating (left) and recording electrode (right) in the NAcC. **(B)** Example fEPSP traces, pre (black) and post (red) TBS stimulation. **(C)** LPS-injected mice exhibited significantly larger positive change in fEPSP slope following LTP induction compared to controls. All data are expressed as mean ± SEM and analyzed using unpaired t-tests; * $p<0.05$. The number of field recordings are included in the figure.

4.2 Acute LPS challenge reduces EPSC event frequency and IPSC peak amplitude of D1R MSNs of the NAcC and NAcSh

Next, we set out to determine if an acute LPS challenge alters single unit activity of NAc D1R MSNs. D1R MSNs were marked by injecting a Cre-dependent AAV5-YFP-DIO virus into the NAc of $D1R^{cre}$ mice that labels the cell body. This viral approach allowed us to differentiate between D1R and D2R MSN subtypes as well as the other minority of neurons in the NAc as previously described (129) . For these recordings, we bath applied picrotoxin, a GABAa receptor blocker, to isolate excitatory currents. In the NAcC, D1R MSNs of LPS-injected mice displayed significantly decreased event frequency of spontaneous EPSCs compared to controls ($p=0.01$; $t=2,76$, $df=15$) (Figure 7B, C). These changes were not accompanied by significant changes in the peak amplitude of EPSCs nor in the resting membrane potential of D1R MSNs in the NAcC. In the NAcSh, D1R MSN EPSC event frequency was also significantly reduced ($p=0.05$; $t=2,05$, $df=20$) (Figure 7D). Again, there was no statistically significant effect of LPS on either peak amplitude of EPSCs nor RMP in D1R MSNs of the NAcSh.

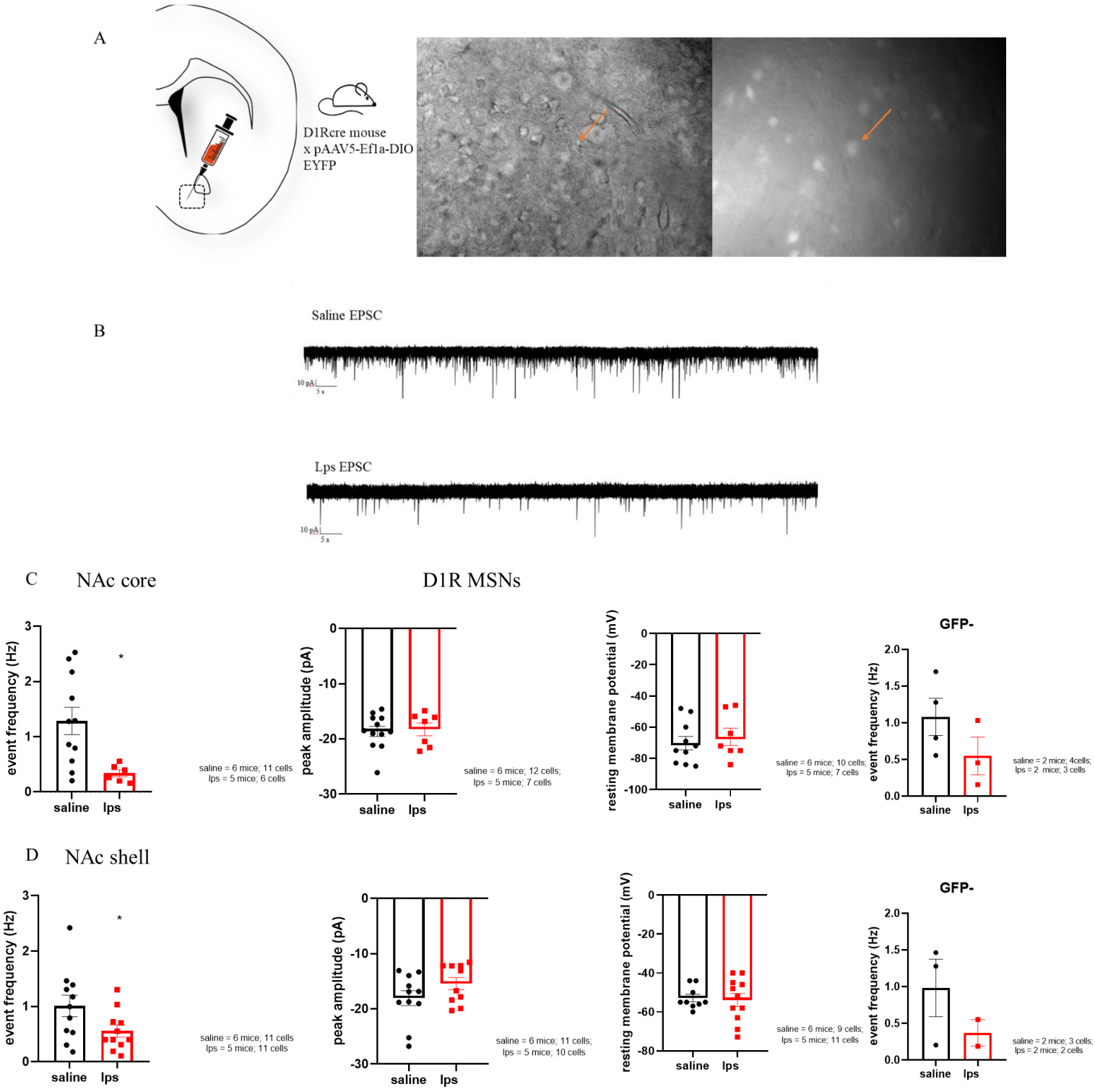

Figure 7. LPS induced changes in D1R neuron EPSCs of the NAcC and NAcSh
Whole-cell patch clamp electrophysiology was performed on D1R cre mice. The figure shows: **(A)** Representative images of patched cells in brightfield and GFP. **(B)** Example EPSC traces. **(C)** Plots show D1R neurons of the NAcC exhibit significantly reduced event frequency in response to LPS; this has no effect on the peak amplitude of EPSCs nor RMP of the cells. GFP- cell event frequency of the NAcC displayed reduced tendency in response to LPS. **(D)** Plots show D1R neurons of the NAcSh exhibit significantly reduced event frequency in response to LPS; this has no effect on the peak amplitude of EPSCs nor RMP of the cells. GFP- cell event frequency of the NAcSh displayed no differences in response to LPS. Data are either expressed as mean ± SEM and analyzed using unpaired t-test; * $p<0.05$ or using non-parametric Mann Whitney test. The number of mice and neurons are indicated in the figure.

We next sought to determine whether inhibitory inputs change in response to acute LPS injection. In a separate group of mice, we used patch pipettes with a CsCl solution to record spontaneous IPSCs onto D1R MSNs of the NAc. DNQX, a glutamate receptor blocker, was bath applied to isolate inhibitory currents. We found that LPS did not induce statistically significant changes in the event frequency of IPSCs onto D1R MSNs (Figure 8B, C). However, we found that the peak amplitude of IPSCs were significantly reduced in both the NAcC ($p=0.04$; $t=2,19$, $df=23$) and NAcSh ($p= <0.01$; $t=4,69$, $df=10$) following LPS injection, suggesting LPS induces alterations in post-synaptic GABA receptor availability and/or function (Figure 8B, C).

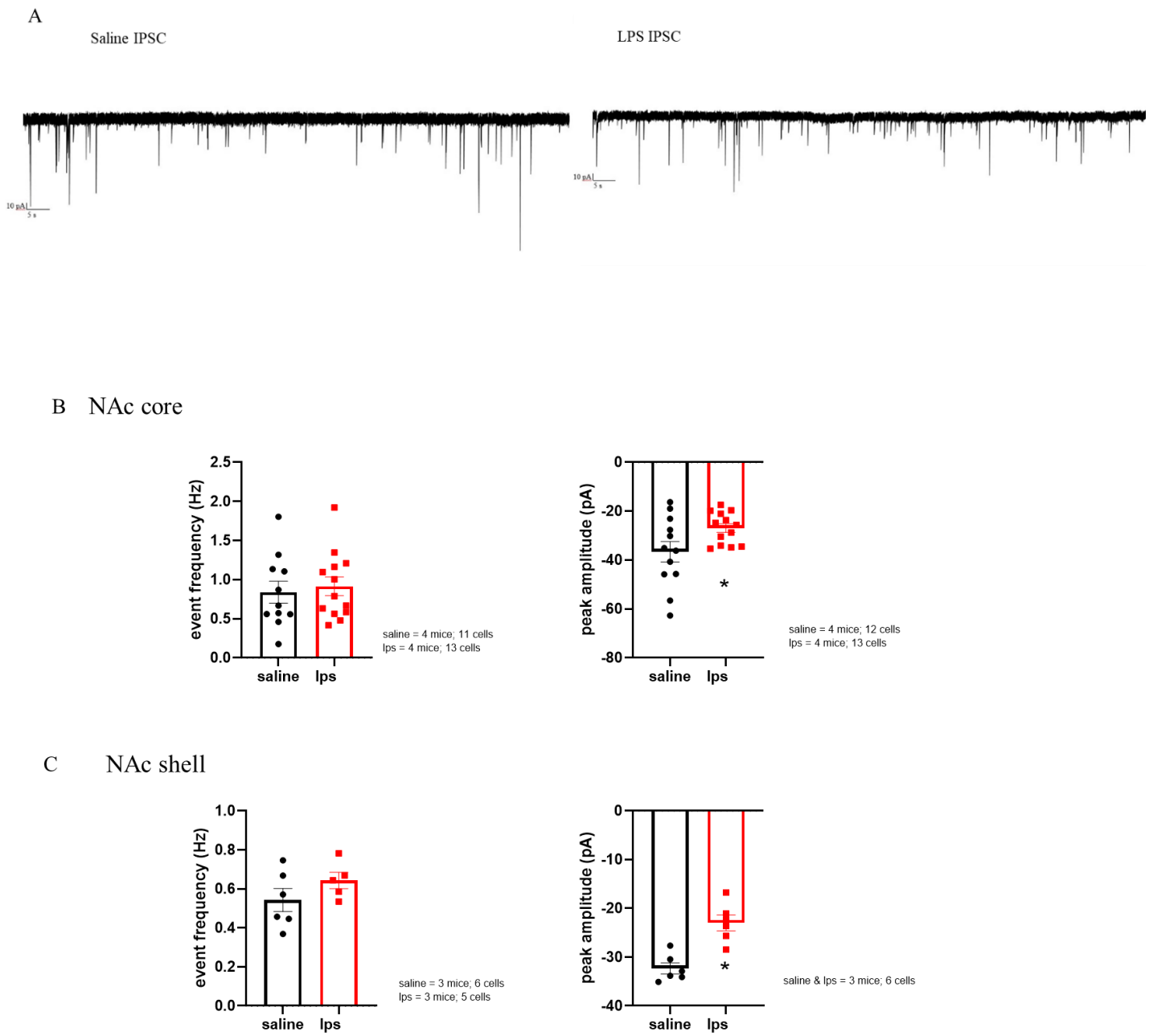

Figure 8. LPS induced changes in D1R neuron IPSCs of the NAcC & NAcSh
Inhibitory post-synaptic potentials were measured in D1R neurons. The figure shows: **(A)** Example IPSC traces. **(B, C)** D1R neurons of both the NAcC and NAcSh exhibit significantly reduced peak amplitude in response to LPS. The event frequency was not changed in either subregion. All data are expressed as mean ± SEM and analyzed using unpaired t-test; * $p<0.05$. The number of mice and neurons are included in the figure.

4.3 Evaluation of LDB and cFos responses

To corroborate and extend our electrophysiology findings, we used an IHC approach to determine if LPS was associated with a reduction in cFos induction in D1R MSNs. Prior to sacrificing mice, we first introduced a challenge by testing them in the light-dark box (LDB). While there were only 3 mice per group in this analysis, we found that there was a tendency for LPS injection to reduce light zone entry ($p=0.30$) and a similar pattern for time and distance in the LDB test (Figure 9A). Importantly, we found statistically significant decreases in these values under the same LPS experimental conditions we previously used (Appendix 1).

Figure 9B shows representative images of cFos (red), cre (green) and DAPI staining in saline and LPS injected mice for NAcC. LPS-injected mice exhibited a trend toward reduced proportion of D1R+ cells that are cFos+ ($p=0.10$) and a trend toward increased proportion of cFos+ cells that are GFP- ($p=0.10$) (Figure 9C). There were not significant differences in cFos expression between mice in the NAcSh (Figure 9D).

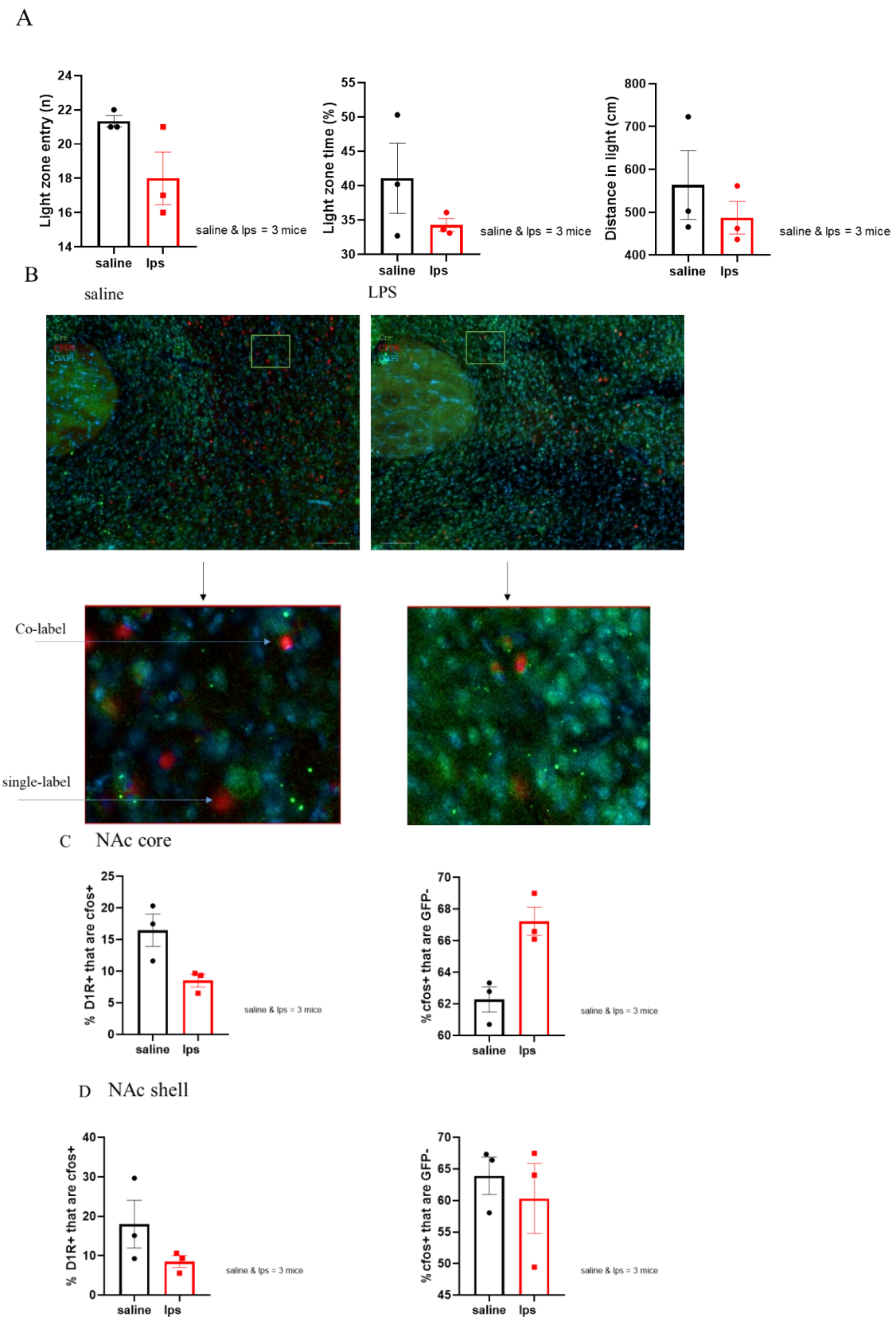

Figure 9. LPS decreases the proportion of cFos+ D1R MSN inthe NAcC and NAcSh

Immunohistochemistry was performed in D1Rcre mice. The figure shows: **(A)** Plots show light zone entry, light zone time, and distance moved in light in LDB test. **(B)** representative images of cFos (red), cre (green) and dapi (blue) staining in the NAcC. **(C)** plots show % D1R+ cells that are cFos+ and % cofs+ that are D1R+ in NAcC and NAcSh. All data are expressed as mean ± SEM and analyzed using non-parametric Mann Whitney test; * $p<0.05$. The number of mice and neurons are included in the figure.

4.4 Chronic, saturated high-fat diet did not change EPSC inputs onto D1R MSNs of the NAc

Following the LPS experiments, we subjected a new cohort of $D1R^{cre}$ mice to either a chow diet or a saturated (palm oil) HFD ("Palm"). Two weeks prior to the dietary intervention, mice underwent viral stereotaxic surgery to inject the AAV5 cre-inducible virus to express GFP in D1R MSNs of the NAc. Following diet onset, body weight was measured throughout, and food intake data was collected for the last 4 weeks. As expected, mice on the palm diet showed increases in body weight ($p=<0.01$; $t=4,842$, $df=12$) (Fig. 10A) and food intake (Fig. 10B). During the last week of diet, blood was collected, and plasma isolated for measures of proinflammatory cytokines. Palm mice had significant increases in plasma TNFα levels ($p<0.01$) (Figure 10C), and despite the bimodal distribution of CRP ($p= 0.08$) and IL6 ($p= 0.11$) levels, there were trends for an increase in the level of circulating cytokines (Figure 10D, E).

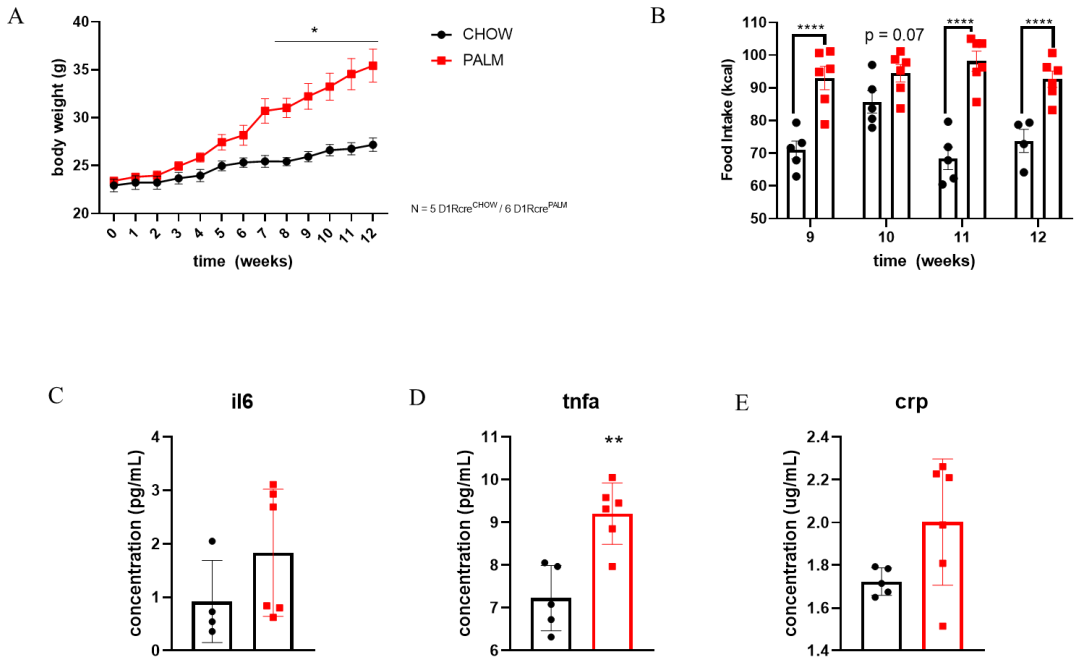

Figure 10. 12 weeks on Palm diet elicited metabolic changes in D1R^{cre} mice

(A) Body weight, **(B)** food intake and **(C-E)** ELISA measures of proinflammatory cytokine levels of chow and palm fed D1Rcre mice after 12 weeks on diet. Data are either expressed as mean ± SEM and analyzed using unpaired t-test or using non-parametric Mann Whitney test; * $p<0.05$, ** $p<0.01$. The number of mice and neurons are included in the figure.

Slice whole cell patch-clamp experiments began at week 12, in which spontaneous EPSCs were recorded from D1R MSNs of the NAcC and NAcSh. We found no statistically significant changes in the event frequency, peak amplitude or RMP of D1R MSNs of the NAcC and NAcSh (Figure 11A, B).

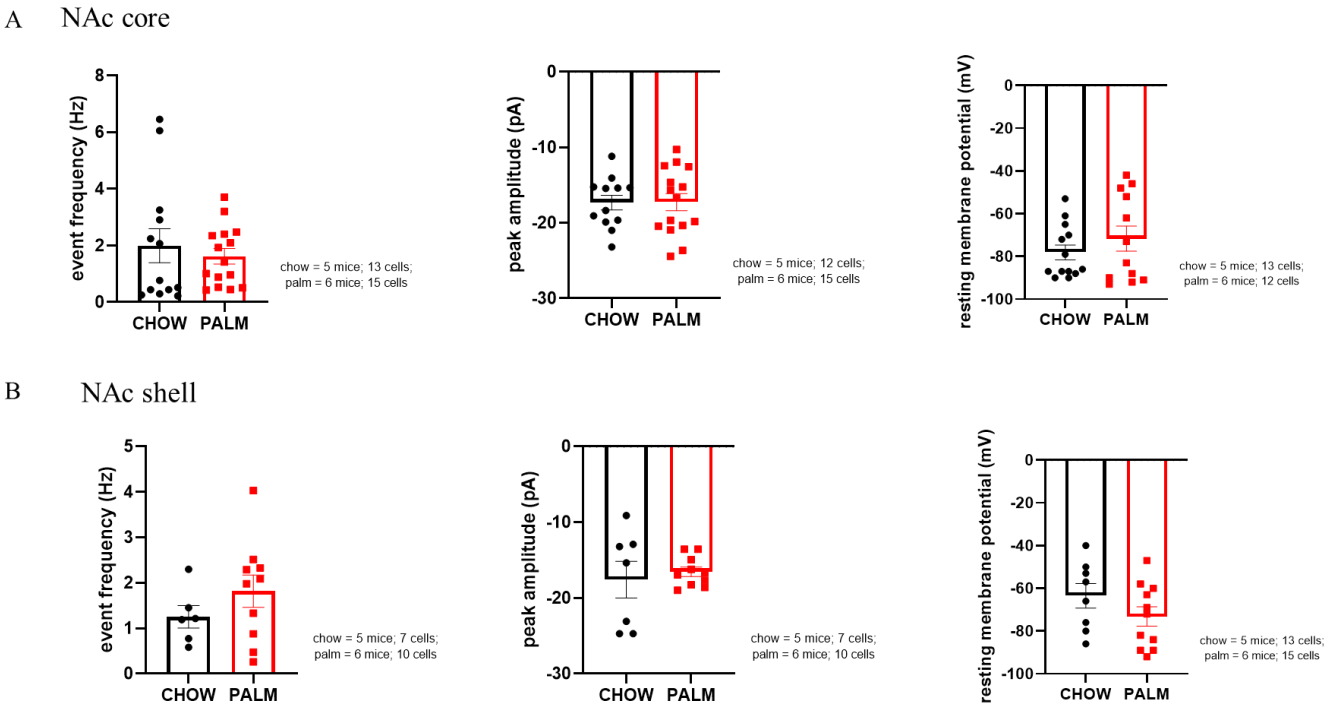

Figure 11. 12 weeks on Palm diet did not induce changes in EPSC inputs on D1R MSNs
Whole-cell patch clamp electrophysiology was performed on D1R^{cre} mice. **(A, B)** Plots show the event frequency, peak amplitude and RMP of D1R neurons of the NAcC and NAcSh. All data are expressed as mean ± SEM and analyzed using unpaired t-test; * $p<0.05$. The number of mice and neurons are included in the figure.

5. Discussion

Summary

In this study, we found that LPS administration previously shown to induce systemic inflammation at this dose changes NAc synaptic plasticity and D1R neuronal activity on an acute scale. Measuring fEPSPs, we were able to determine that in control conditions, the NAcC produces minimal changes in fEPSP slope, while acute LPS increases post-stimulation fEPSP slope. Using whole-cell patch clamp electrophysiology, we found that acute LPS significantly decreases the event frequency of D1R MSN EPSCs in the NAcC and NAcSh. This had no effect on the peak amplitude of EPSCs nor RMP of the cells. We also observed that LPS significantly reduces the peak amplitude of D1R MSN IPSCs in both the NAcC and NAcSh, whereas the event frequency of IPSCs remained unchanged. In the chronic Palm-fed mouse, we found that EPSCs displayed no significant differences from chow fed mice in both the NAcC and NAcSh.

5.1 Assessing the impact of an acute LPS challenge on synaptic plasticity of the NAc

Here, we showed that LPS produces acute effects on synaptic plasticity of the NAcC (Figure 6). The observed changes may indicate increases in LTP during instances of acute systemic inflammation. This could mean that acute LPS treatment increases overall neuronal activity in the NAcC, or it's a possible compensatory mechanism by which neuronal circuitry may overshoot to restore basal activity. We chose to measure the fEPSP slope, which is a measure of the linear regression of the rising phase, because a field recording represents a time derivative of extracellular current sources and thus is more appropriate to measure than amplitude (218). Interestingly, in response to TBS, control mice displayed little to no increases in fEPSP slope. Rather, TBS more often produced either little change or a sustained decrease in fEPSP slope, or LTD. This phenomenon could be due to the irregular stimulation method used in these experiments in which

no specific fibers or afferences were targeted in the NAcC. Our method of stimulation may also explain why we were not able to produce a fEPSP in the NAcSh. Namely, we opted to cut coronal sections and stimulate generally in the NAcC to garner a postsynaptic effect that was not specific to any single afferent and would allow us to observe synaptic plasticity in the NAc without bias. Other research studies typically cut parasagittal slices, to target specific fibers. For example, studies researching synaptic plasticity in the NAc may target dopaminergic afferences from the VTA (219) or efferences projecting from NAc to the hippocampus (220). However, it is also not uncommon for stimulation of NAc MSNs to produce variable changes in plasticity. Ji and colleagues (2012) found that NAc D1R and D2R antagonists produce opposite effects on synaptic plasticity (221). Furthermore, one study found similar results in which paired action potentials and EPSPs produced both LTP and LTD in the NAc in almost equal proportions (222). These studies are in accordance with what we have shown here, where the NAc exhibited variable changes in fEPSP slope, in the control condition.

Given that LPS produces systemic inflammation that is well known to elicit a neuroinflammatory response that can affect neuroplasticity (223), our hypothesis was that synaptic plasticity would be impaired after injection. We were surprised to observe that LPS produced a significantly larger positive change in fEPSP slope than the control condition. In terms of plasticity, it could be that inflammatory mediators may promote neuronal activity, perhaps as a way to rescue basal activity and may indicate an increase in LTP. This is contradictory to what is observed in other brain regions such as the hippocampus. Specifically, a recent study in rats showed that in response to LPS, the hippocampus exhibited decreased LTP (224). That being said, to our knowledge, no other studies have assessed LTP in the NAc in the context of acute inflammation. Therefore, the effect we have observed may pertain to the region studied. Another

possible reasoning for this effect is that D1R and D2R MSNs may have opposed, but equalizing actions on synaptic plasticity. These two MSN subtypes` postsynaptic actions depend on concerted excitatory inputs from distinct glutamatergic afferents (225). If, at baseline, these MSN populations of the NAc produce a net effect in terms of signal transmission, it may be that further stimulation does not change a system already in equilibrium- hence the lack of LTP in control conditions, and variable LTP responses in other studies. Furthermore, it is possible that in control conditions, a maximum potentiation has already been met and therefore, TBS produces no increases in fEPSP slope. If LPS is indeed disrupting this signaling, TBS may rescue this LPS-induced impairment, and therefore display a larger post-stimulation fEPSP slope. However, given the forthcoming EPSC patch-clamp data, indicating a decrease in frequency from both D1-D2R MSN populations, this equalizing of actions hypothesis may not be as simple as previously described.

Given the limitations in this part of the study, further investigation into LPS induced changes in synaptic plasticity of the NAc should target specific afferences. It may be effective to target fibers projecting from the VTA, as DA input to the NAc originates from this region. This may also allow us to garner responses from both the NAcC and NAcSh. It could also be useful to apply high-frequency stimulation (HFS), rather than TBS, as it is more commonly utilized in field electrophysiology experiments. Although TBS is more physiologically relevant, HFS, being a stronger stimulation method, could produce more prominent LTP effects.

5.2 *Assessing the impact of an acute LPS challenge on the activity of D1R MSNs of the NAcC and NAcSh*

Here, we attempted to discover how peripheral LPS injection affects D1R MSNs of both the NAcC and NAcSh. In both the NAcC and NAcSh, we saw robust reductions in the event

frequency of EPSCs compared to controls (Figure 7). These results confirmed that on an acute scale, systemic inflammation may induce a disruption in at least one subtype of DA receptor expressing neurons of the NAc, and possibly in GFP- neurons, although the n was low and underpowered. This observation tells us that excitatory presynaptic inputs on multiple cell types may be impaired in response to acute inflammation, and this response is not as homogenous as previously thought. Previous data in our lab has shown that 12 hours after LPS injection, mice no longer exhibit LPS-induced sickness behavior, but do display significantly elevated proinflammatory cytokine levels along with NAc-mediated behavioral deficits (see Appendix 1). These behavioral changes include anxiety-like and depressive-like behaviors in elevated-plus maze, LDB and 3 chamber social interaction tests. These changes can also be linked to D1R and D2R MSNs in the NAc as they are the main cell type of the NAc. They are known to mediate reward-based and aversive behaviors, and rescue of inflammatory markers in the NAc has been shown to reverse these behavioral impairments (139). Furthermore, prior research demonstrated that acute LPS injection led to depressive-like behaviors as well as changes in AMPAr protein levels. Specifically, one study saw decreased GluR1 in the mPFC and VTA whereas the NAc displayed increased GluR2 expression (226). This increase in NAc GluR2 levels could be indicative of a compensatory mechanism by which impaired MSNs attempt to restore signal transmission. If the reduction in EPSC frequency is sensed by the postsynaptic neuron via a homeostatic mechanism, perhaps this could lead to an increase in GluR2 levels as observed in the aforementioned study. However, it is important to note that this study observed these effects 24 hours after two repeated LPS injections.

Furthermore, it was interesting to see that acute LPS has differential effects (△Mean: -0.63 ± 0.20) in the NAcC and NAcSh (△Mean: -0.45 ± 0.22), although this effect was not statistically

significant. Given that the NAcC receives largely homogenous afferent inputs (101), it may explain why LPS affects the subregions differentially. Precisely, although both subterritories are strongly innervated by diverse glutamatergic inputs, the NacC also receives large GABAergic inputs while the NAcSh receives more heterogeneous neuromodulatory inputs (101). Therefore, the abundance of GABAergic activity and lack of diversity in the NAcC could explain why we have observed a differential decrease in the frequency of EPSCs on postsynaptic neurons of the NAcC and the NAcSh. Moreover, it is well known that the NAc mediates depressive-like and anxiety-like behaviors and as previously mentioned, we have recently demonstrated that acute inflammation by LPS injection leads to changes in these NAc mediated behaviors (see Appendix 1). It has also been shown that the NAcC could play a more important role in controlling behavioral outputs mediated by the NAc (156). While the NAcSh plays a role in facilitating hippocampus dependent appetitive learning, the NAcC was shown to play an integrative role in learning as well as limbic associated behaviors via its dual connection with both the basal amygdala and hippocampus (156). Therefore, the effect of acute LPS-induced inflammation could be different between subregions since the NAcC has been shown to play a greater role in limbic behaviors, which have themselves been demonstrated to change in response to LPS.

Along these lines of separation, classical views express that D1R and D2R MSNs of the NAc play opposing effects on behavior. So, we were interested in knowing if LPS induced effects on D2R as well. To this end, we began measuring EPSCs onto GFP negative (GFP-) cells within the vicinity of D1R (GFP+) MSNs, as 95% of total cells in the NAc express either subtype. We also accounted for the possibility of patching non D1/D2R cells and thus discriminated between different cell types by morphology. However, as this study was primarily focused on D1R MSNs, the overall number of measured GFP- cells was low, and thus we did not observe differences in

EPSC parameters in LPS or control conditions. However, we cannot discount that with a higher n, it may be that excitatory presynaptic input onto GFP- cells are also impaired by LPS and this needs further study (Figure 7).

Given that the NAcC is more GABAr dense and less diverse in terms of neuroactive substances, it gives credence to the idea that acute inflammation may also cause dysfunction to GABAergic signaling. In fact, we saw that LPS does not induce changes in IPSC event frequency, but does reduce peak amplitude in IPSC waveforms, in both subregions (Figure 8). One study observed that LPS injection induces not only microglia activation, but deficits in GABAergic signaling in the hippocampus that leads to prolonged memory impairments (200). These deficits included a significant decrease in the peak amplitude of evoked IPSCs of CA1 hippocampal pyramidal cells. However, these GABAergic synaptic impairments were only observed 4-6 days after LPS administration. This discrepancy in the temporal differences of LPS induced effects may be due to the regions studied. The hippocampus, which is well-known to mediate memory formation and retrieval, may be affected over a longer timescale. Contrarily, the NAc, which is responsible for mediating more acute behaviors, may be affected on a shorter timescale. Thus, we demonstrated that acute systemic inflammation induces rapid GABAergic synaptic impairments in the NAc.

Here, we observed similar LPS-induced decreases in IPSC peak amplitude in both NAcC (△Mean: 9,660 ± 4,416) and NAcSh (△Mean: 9,343 ± 1,992) (Figure 8). We theorize that LPS' impairment on the size of IPSC waves may correlate with deficits in MSN activity of both subregions, since cells in these subregions are primarily GABAergic. Moreover, the NAcC is known to receive more GABAergic projections than the NAcSh.

We also wondered if the change in EPSC event frequency is related to the change in IPSC peak amplitude and whether these changes are directly or indirectly related to neuroinflammation.

One review provided evidence that neural mechanisms may function to maintain homeostasis in synaptic activity and may produce local synaptic adaptations to adjust and bring balance between excitatory and inhibitory activities (227). This may occur through homeostatic synaptic plasticity. There is also evidence to suggest that on the presynaptic neuron, homeostatic modulation of the probability to release a neurotransmitter can occur within individual dendritic branches (228). This phenomenon may prevent the saturation of the synapse by reducing release probability when postsynaptic activation is already elevated. Therefore, we can rationalize the results wherein LPS induces the reduction of event frequencies of EPSCs as a presynaptic effect, while the reduction in peak amplitude of IPSCs is a postsynaptic effect. This may be a compensatory process by which D1R MSNs attempt to maintain baseline homeostasis between excitatory and inhibitory signaling during times of neuroinflammation.

We also decided to validate our electrophysiology findings using an IHC test. Here, we utilized a cFos and cre double staining method and manually counted their expression both individually and together, after LPS injection (Figure 9). This way, we could directly measure D1R MSN activation. In a first cohort, we found very little cFos expression in both NAc subregions, so we decided to challenge a second cohort of mice seen here prior to sacrifice, using the LDB test. This greatly enhanced NAc cFos expression and allowed us to determine what differences may exist between the two groups. This may have occurred because, at baseline, NAc MSNs are not robustly active, but following an anxiety behavior test, we theorize that the NAc D1R MSN population may be more active and thus express higher levels of cFos. The LDB test allowed us to not only stimulate the activation of D1R MSNs, but to measure the effect of acute LPS on anxiety-like behavior. Although statistically insignificant, LPS injected mice displayed a tendency

(p=0.30) towards a decrease in number of entries into the light zone, while there were no changes in percent time spent in the light zone nor distance moved in the light zone.

Quantification of cFos activity in D1R MSNs revealed that LPS decreases these cells` activity, notably in the NAcC (Figure 9). Precisely, using a non-parametric test, we observed a trend toward a decrease in %D1R MSNs that are cFos-positive (p=0.10) (Figure 9C). Cfos+ D1R MSNs in the NAcSh, were not changed by LPS. Despite the low n in this experiment, these results are in accordance with our previous electrophysiology data. As previously shown, following LPS administration, we demonstrated a decrease in the event frequency of EPSCs onto D1R MSNs of the NAc which may explain decreased activation (less cFos) of D1R MSNs, using IHC. In contrast, Frenois et al., demonstrated that LPS induced no change in cFos expression of the NAcC at 6 and 24 hours, while NAcSh exhibited higher cFos expression at those time points (229). This discrepancy between their results and our own may be because we stimulated NAc D1R neurons prior to sacrifice with an LDB test. Moreover, their study did not discern between different cell types in the NAc, while we specifically counted D1R neurons in our results. It is also likely that at our 12-hour time point, expression of cFos may produce differential counts than their 6- and 24-hour points. We also found that the % cFos-positive that are GFP-negative trend higher in the NAcC (Figure 9C). Here, we demonstrate that LPS may induce increased activity and cFos expression in non D1R cells. We hypothesize that these cells may include non-neuronal cells, such as microglia. One review expressed that proinflammatory effects have been suggested for the expression of cFos in microglia, and that LPS specifically has been shown to induce its expression in glial cells, specifically microglia (230). However, this trend is potentially in contrast to the GFP-EPSC data that showed a tendency to be reduced in event frequency. This discrepancy needs to be addressed in future experiments.

Given the limitations in these experiments, there are many possible avenues we can venture in future investigations of LPS induced changes in NAc neurons. Firstly, we would like to add more mice and non-GFP neurons into our whole-cell patch-clamp experiments. This may help us detect possible changes and/or differences between D1R and D2R neurons. Secondly, we would like to measure IPSCs and EPSCs in the same cells, to better characterize LPS induced changes within one cell. Lastly, we would include a higher number of mice in our IHC experiments to reduce variability in anxiety tests along with cFos counts in D1R MSNs and GFP- cells.

5.3 Assessing the impact of a chronic Palm diet challenge on the activity of D1R-expressing neurons of the NAcC and NAcSh

Here, we assessed how a saturated, high fat ("Palm") diet may affect excitatory inputs onto D1R MSNs of the NAc, using whole-cell patch clamp electrophysiology. Our initial results indicated that the Palm diet was effective in significantly increasing body weight and kilocalories of food intake (Figure 10A, B). Next, we measured concentrations of proinflammatory cytokine levels during the last week on diet and found that Palm-fed mice displayed significant increases only in TNFα concentrations, while Il-6 and CRP levels remained unchanged from chow-fed mice (Figure 9C-E). However, following a 12-week diet, we found that Palm-fed mice displayed no significant changes in EPSC frequency, peak amplitude nor RMP in either NAcC or NAcSh (Figure 11). These observations may indicate that there was too much variability in inflammatory markers, and thus no significant changes in neuronal excitability between groups.

In previous studies, Palm-fed mice have exhibited significantly increased CRP, TNFα, Il-1b, corticosterone concentrations as well as increased glucose and insulin levels during tolerance

testing, compared to control mice (139). These impacts of a saturated, high-fat diet on multiple metabolic parameters and inflammatory markers suggest that the mice are indeed DIO.

Mice from a previous cohort displayed behavioral deficits- anxiety, depressive and despair-like behavior- as measured by, sucrose preference test and forced swim task (Appendix 3). So, it is important to contextualize the findings within the original question, concerning the interaction between DIO and depression. For those mice, it is evident that interactions between DIO and D1R MSN inhibition was able to induce behavioral deficits, providing further evidence for the association between diet and behavior. As our DIO mouse model in this current study did not illicit any changes in EPSC recordings, it is difficult to determine the extent to which DIO interacted with depressive-like behavior. However, since the Palm-fed mice exhibited elevated body weight and plasma cytokines, it is possible that they may have exhibited atypical and/or immuno-metabolic depression, often associated with DIO. This current cohort of mice should have also been subjected to behavioral analysis. Given the variation in inflammatory markers here, perhaps despair and anxiety-like behavioral tests would have given us further reason to believe that these animals are exhibiting low-grade systemic inflammation. Given that animal models of both immuno-metabolic depression and DIO have been previously described to exhibit reduced BDNF and increased NFκB expression, perhaps it would have been prudent to measure these markers as well.

In a preliminary experiment, Palm-fed mice also exhibited reduced cFos expression in D1R MSNs following a forced swim task (see Appendix 2). Moreover, Hryhorczuk and colleagues showed that phosphoDARPP32 (Thr75) protein levels were increased in D1R MSNs of Palm-fed mice (208). All these observations served as the basis for our current experiment. However, the cohort of mice produced in our experiments displayed variable markers for DIO. Although Palm-

fed mice showed significantly elevated body weight and food intake compared to controls, cytokine concentrations in these mice were inconsistent. That is, CRP and Il-6 levels were statistically unchanged in comparison to chow-fed mice.

Our electrophysiology results demonstrated that in both the NAcC and NAcSh, Palm-fed mice had no significant differences in EPSC event frequency, peak amplitude and RMP. Despite this, there are possible rationales for these results. Firstly, the variation in inflammatory markers of Palm-fed mice may affect D1R neuronal activity. If there is less neuroinflammation and thus less microglia recruitment to the NAc in mice with lower cytokine levels, it may be that these mice are more similar to chow-fed mice in terms of neuronal activity in the NAc. In one study, researchers saw that in sprague-dawley rats, high-fat diet fed animals showed reduced sEPSC frequency in D1-like neurons and increased sEPSC frequency in D2-like neurons in the NAc (231). These animals showed significantly increased body weight levels along with elevated inflammatory markers, like Il-1b. Interestingly, they also saw that D2-like neurons were significantly elevated in high-fat fed animals. Overall, their more homogenous observations in DIO and inflammatory markers and higher numbers of animals may explain their significant discoveries. In our study, we also noticed, although not statistically significant, a motif in which NAcSh D1R MSNs of Palm-fed mice could be more hyperpolarized (p = 0.197). Given this possibility, with more mice that meet a certain inflammatory threshold, we should continue studying the membrane polarization of D1R MSNs in these sub territories.

Although underlying pathology may be aggravated in humans before the development of obesity, this is not likely the case in in-bred mice. Thus, our mice studies allow us to suggest causality between DIO and synaptic alterations. Obesity in rodent models correlates with microglia activation and impaired synaptic function (232). One study showed that hippocampal

proinflammatory cytokine production was correlated with loss of synaptic protein expression following 3 months of HFD (232). However, the interaction and possible impairment between microglia and neurons in DIO should be subject to further study before defining it as directly related. Underlying pathologies in DIO such as metabolic deficits, could very well also be responsible for observed changes in neuronal activity.

Additionally, our prior data was primarily from male mice, so for the case of simplicity, we continued with males. However, sex differences may play a role in these observed changes. Hwang et al., 2012 described that male DIO mice models were more vulnerable overall to impairments caused by HFD, including weight gain, metabolic and learning deficits, and hippocampal synaptic plasticity (233). Thus, it would be beneficial in future cohorts to observe DIO changes in female cohorts as well.

In future investigations, we will pursue adding more mice to our high-fat studies. We will make sure to include mice that not only display changes in body weight and food intake, but require them to meet a certain threshold in proinflammatory cytokine markers. We may also explore adding more markers such as corticosterone, as Palm-diet may also affect stress levels. Lastly, along with studying EPSCs onto D1R MSNs of the NAc, we will incorporate measuring the rheobase of these cells, especially in the NAcSh. The rheobase of a neuron is the amount of current amplitude that is needed to depolarize the membrane potential to garner an action potential. We would do this by injecting a depolarizing current in steps until the neuron fires. This would allow us to measure how "excitable" a cell is, and with more mice, it may be that D1R MSNs of Palm-fed mice could display differences in output from chow-fed mice. Measuring non-GFP neurons in the vicinity of GFP+ D1R MSNs may also help uncover the role of D2R MSNs during DIO. It is also imperative to mention the differential effects between an acute LPS model and a chronic DIO

model. Using a dose of LPS previously described to induce significant increases in inflammatory markers, it may help explain the more invariant effects on EPSCs in LPS treated mice versus the lack thereof in DIO mice. LPS as an inflammatory insult may not be a perfect model to recreate peripheral inflammation associated to obesity. Therefore, the observations made in LPS conditions should be treated with caution in relation to DIO.

Conclusion

Here, we discovered that acute systemic inflammation changes NAcC synaptic plasticity as well as EPSC and IPSC inputs onto D1R MSNs of both the NAcC and NAcSh. We observed that the effects of acute LPS injection were sub-territory dependent. On a chronic saturated, high-fat diet, D1R MSNs expressed no significant differences in Palm-fed mice as compared to controls. In summary, we saw that many factors, such as stimulation methods for extracellular field recordings, number of mice and inflammatory markers for the intracellular patch-clamp experiments play paramount roles in the outcome of the results. Moving forward, we will incorporate different methods in our experiments. For measuring synaptic plasticity, we may stimulate different afferences and apply HFS stimulation. For acute inflammatory patch-clamp experiments, we will add more mice and measure non-GFP neuronal activity to acquire a more wholesome view of how acute LPS impacts NAc neuronal activity. And lastly, for chronic high-fat patch-clamp experiments, we will incorporate more DIO mice who meet certain inflammatory criteria, measure non-GFP neuronal activity and measure rheobase changes induced by Palm diet.

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Appendix -1

Central activation of the fatty acid sensor GPR120 suppresses microglia reactivity and alleviates sickness-and anxiety-like behaviors

Shingo Nakajima[1*], Geneviève Demers[1,2*], Arturo Israel Machuca-Parra[1], Zahra Dashtehei Pour[1,2], Diane Bairamian[1,2], Khalil Bouyakdan[1], Alexandre Fisette[1], Anita Kabahizi[1,2], Josephine Robb[1], Demetra Rodaros[1], Cyril Laurent[1,4], Guillaume Ferreira[5], Nathalie Arbour[1,4], Thierry Alquier[1,3*], Stephanie Fulton[1,2*]

1- Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Departments of: 2-Nutrition, 3-Medicine, 4-Neuroscience, Université de Montréal, Montréal QC H3T1J4, Canada. 5- Nutrition and Integrative Neurobiology Unit, UMR 1286 INRA-Université de Bordeaux, Bordeaux, France.
* Equal contribution

Corresponding author: stephanie.fulton@umontreal.ca (S. Fulton)

Short running title: GPR120 and neuroinflammation

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Conflict of interest
The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abstract (250 words)

G protein-coupled receptor 120 (GPR120) is a sensor for long-chain fatty acids including omega-3 polyunsaturated fatty acids (n-3 PUFAs) known for beneficial effects on inflammation, metabolism, and mood. GPR120 is reported to mediate the anti-inflammatory and insulin-sensitizing effects of n-3 PUFAs in peripheral tissues. The aim of this study was to determine the impact of GPR120 stimulation on microglial reactivity, neuroinflammation and sickness- and anxiety-like behaviors by acute pro-inflammatory insults. We found higher GPR120 mRNA expression in microglia as compared to neurons and astrocytes in both murine and human isolated neural cells, and *in situ* hybridization revealed GPR120 expression in microglia in mouse brain. In a manner similar to or exceeding n-3 PUFAs, GPR120 agonism (Compound A, CpdA) strongly attenuated lipopolysaccharide (LPS)-induced proinflammatory marker expression in primary mouse microglia, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and inhibited nuclear factor-κB translocation to the nucleus. Central administration of CpdA to adult mice blunted LPS-induced hypolocomotion and anxiety-like behavior and reduced TNF-α, IL-1β and IBA-1 (microglia marker) mRNA in the nucleus accumbens (NAc), a brain region modulating anxiety and motivation and implicated in neuroinflammation-induced mood deficits. GPR120 agonist pre-treatment attenuated NAc microglia reactivity and alleviated sickness-like behaviors elicited by central injection TNF-α and IL-1β. These findings suggest that microglial GPR120 contributes to neuroimmune regulation and behavioral changes in response to acute infection and elevated brain cytokines. GPR120 may participate in the protective action of n-3 PUFAs at the neural and behavioral level and offers potential as treatment target for neuroinflammatory conditions.

Keywords: free fatty acid receptor, Ffar4, endotoxemia, nucleus accumbens, anxiety, sickness behaviors, locomotion, neuropharmacology, behavioral neuroimmunology

Highlights

- GPR120 is enriched in murine and human microglia
- Central GPR120 agonism decreases microglia activation and anxiety- and sickness-like

behaviors in response to LPS

- GPR120 agonism attenuates nucleus accumbens microglia reactivity and behavioral responses to central cytokine administration

Introduction

Neuroinflammatory responses emanating from systemic immune activation contribute to mood and emotional disturbances and sickness-like behaviors such as psychomotor slowing. Microglia play a central role in neuroinflammation and their activation can be triggered by immunometabolic threats provoked by poor diet and obesity [1, 2]. Several reports demonstrate that high-fat feeding or administration of lipopolysaccharide (LPS), a gut-derived bacterial toxin, provokes microglial reactivity and cytokine expression in various brain regions including the hypothalamus, hippocampus, amygdala and nucleus accumbens (NAc) [3-5]. The NAc is a structure regulating motivated behavior and mood that is well-implicated in the pathophysiology of anxiety and depression [6]. We previously found that a saturated, but not a monounsaturated, high-fat diet trigger glial reactivity and inflammatory indices in the NAc that contribute to anxiety- and depressive-like behaviors in mice, effects prevented by a NAc viral intervention blocking nuclear factor-κB (NFκB) activation [4].

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have central actions that mitigate neuroinflammation in part by suppressing microglial cytokine and chemokine production. Dietary n-3 PUFAs supplementation protect against the pro-inflammatory effects of LPS, diet-induced obesity, neural injury, and chronic stress in both peripheral tissues and brain [7, 8]. G-protein coupled receptor 120 (GPR120), also known as free fatty acid receptor 4 (FFAR4), mediates the action of n-3 PUFAs such as α-linolenic acid (ALA; 18:3n-3), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3), on gut hormone secretion and systemic inflammation [9, 10]. GPR120 is expressed in the intestine and lung, among other peripheral tissues, and is particularly enriched in immune cells. Pharmacological GPR120 stimulation was shown to suppress macrophage-mediated adipose tissue inflammation and improve insulin resistance through the inhibition of NFκB [11]. We previously reported GPR120 mRNA expression in mouse brain and ability of intracerebroventricular (ICV) GPR120 agonist infusions to suppress feeding, food reward and anxiety-like behavior caused by a saturated high-fat diet [12]. Despite these results, the cellular expression profile of brain GPR120 and its contribution to microglial reactivity, neuroinflammation and associated behavioral deficits in response to acute neuroimmune challenges remains unclear. The present study set out to determine: (1) GPR120 expression profile in mouse and human microglia, astrocytes and neurons, (2) the capacity of a GPR120 agonist as compared to several long-chain unsaturated fatty acids to moderate the pro-inflammatory responses in primary microglia from the forebrain and specifically the NAc, and (3) the influence of central GPR120 agonism on sickness-like anxiodepressive behaviors and

neuroinflammatory responses in the NAc produced by acute systemic LPS or central cytokine administration.

Materials and Methods

Animals

All experiments were approved by the Institutional Animal Care Committee of the CRCHUM in accordance with the standards of the Canadian Council on Animal Care. Eight to ten-week-old C57Bl/6J male mice from Jackson Laboratories (Bar Harbor, Maine, USA) were used for gene expression and behavioral tests. All animals were maintained in an environmentally controlled room (22–24ºC) on reverse light/dark cycle (light phase 10:00 pm to 10:00 am) with *ad libitum* access to standard chow and water.

Chemicals and reagents

LPS from *Escherichia coli* (L-4516, serotype 0127:B8), oleic acid (OA; O1008), alpha-linolenic acid (ALA, L2376), eicosapentaenoic acid (EPA, E2011), and docosahexaenoic acid (DHA, D2534) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and aliquoted for single freeze-thaw use. Mouse recombinant TNF-α and IL-1β were purchased from R&D systems Inc. (Minneapolis, MN, USA). Papain and DNase I were purchased from Worthington Biochemical corp. (Lakewood, NJ, USA). Compound A (CpdA) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Cell culture reagents were purchased from ThermoFisher Scientific (Waltham, MA, USA) unless specified.

Primary cultures of microglia, neurons and astrocytes

Primary microglia were prepared from forebrain or NAc of C57BL/6 pups at postnatal day 1-3 according to previous reports [13-15]. Briefly, after removal of meninges, the forebrain or NAc was dissected with a scalpel. Suspensions were treated with an enzymatic solution containing papain (9 U/ml), DNase (200 U/ml), glucose (5 mg/ml), cysteine (0.2 mg/ml), and bovine serum albumin (0.2 mg/ml) for 15 min at 37°C in 5% CO_2. Debris were removed by the passing with 70 µm cell strainer. Mixed glial cells were cultured in T75 flask and maintained in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% heat-iNActivated fetal bovine serum (FBS) and 1% of antibiotics (Penicillin G (10,000 U/ml)-Streptomycin Sulfate (10,000 µg/ml) at 37°C in 5% CO_2. Media was replaced with fresh medium at 7 days in vitro, and then the culture was maintained until reaching astrocyte confluence (10-14 days). Subsequently, microglia start

growing on top of a single layer of astrocytes. The culture medium containing primary microglia were transferred to poly-$_L$-lysine (PLL)-coated 12 mm coverslips or 24-well plates for a maximum of five days prior to treatment. To evaluate the expression of GPR120 in different neural cell types, primary astrocytes and neurons derived from the procedures above were also cultured. Primary neurons were cultured in PLL-coated 24-well plates in Neurobasal-A medium containing, 2% B-27 supplement, 1% Glutamax, and 1% antibiotics solution for 7 days in vitro.

Human neural cells

Fetal brain tissue (17-21 weeks) was obtained after written informed consent (University of Washington, Seattle, Washington, USA, STUDY00000380). These studies were approved by the Centre Hospitalier de l'Université de Montréal (CHUM) ethics boards (BH07.001, HD07.002). Astrocytes and microglia were isolated from fetal brain tissue as previously described [16, 17] and cultured in DMEM containing 10% FBS. Neurons were collected as negative fraction after removal of MHC class I (glial cells) and CD235a (red blood cells) expressing cells using Miltenyi beads. Cells were cultured in DMEM F12 without red phenol containing 2% MACS® NeuroBrew® (Miltenyi Biotec), penicillin 100U/mL, streptomycin 100 µg/mL, and 20 mM HEPES [17].

Culture treatments

Free fatty acids (FFAs) (OA, ALA, EPA, and DHA) and CpdA were dissolved in ethanol for stock solution at 100 mM and diluted in the culture media to a final concentration of 10 µM in 0.1% ethanol. FFAs concentration was selected according to our previous studies [18]. The CpdA dose selected for culture experiments was based on that used in cultured macrophages [11]. LPS was dissolved in phosphate-buffered saline (PBS) and diluted to a final concentration of 100 ng/mL. Primary microglia were cultured in the DMEM without FBS and antibiotic for 24 h before the treatment, as FFAs and CpdA may rapidly react with albumin, serum-free medium was used for the test. Microglial cells were pre-treated 1 h with FFAs or CpdA (10 µM) before adding LPS (100 ng/ml) or a cytokine mixture (TNF-α + IL-1β, each 50 ng/ml). After 6 hours incubation, supernatants were harvested for ELISA and cells were processed for RNA extraction.

Stereotaxic surgery

Mice were individually housed one week prior to ICV cannula implantation. Animals were anesthetized with isoflurane (3% induction; 1–2% maintenance) and positioned in an Ultraprecise Mouse stereotaxic apparatus (Kopf Instruments). A single ICV guide cannula (C315GS-5-SP, 5 mm, 26 gauge, Plastics One) was implanted into the right cerebral ventricle using stereotaxic

coordinates (+0.5 mm caudal and +1 mm lateral; -2.0 mm ventral from dura). The cannula was secured to the skull with cyanoacrylate glue and dental cement and closed with an adapted dust cap (Dummy cannula: C315DCS-5-SPC, 5 mm, Plastics One). Correct positioning of the cannula was verified seven days after surgery by the drinking response elicited by injection of angiotensin II (20 ng/μL; Sigma). Behavioral testing was performed at the end of the light cycle. Mice were decapitated under isoflurane anesthesia for the collection of brains and these were stored at -80^{o}C. For histochemical analysis, mice were anesthetized by intraperitoneal (IP) pentobarbital injection and then were perfused with cold PBS and 10% neutral buffered formalin. Brains were post-fixed with 10% neutral buffered formalin overnight followed by an increasing sucrose gradient.

Procedures for behavioral assessment

To assess the behavioral effects of central GPR120 agonism in the context of acute inflammation, mice received ICV CpdA (10 μg in 2 μl 16% DMSO) or vehicle daily during three consecutive days. On day three, one cohort of mice was euthanized 2 h following LPS (0.83 mg/kg, IP) injection for gene expression studies whereas behavioral testing was carried out 12 h after intraperitoneal LPS or ICV cytokine mixture (each 50 ng in 2 μl 0.2% BSA in saline) in a separate cohort. The CpdA dose was based on Oh *et al*. and adapted for ICV administration [11, 19]. Control mice received an IP injection of vehicle (endotoxin-free saline solution). LPS and cytokine doses chosen were based on reports of the minimal effective dose to elicit anxiety- and depressive-like behavior [20-22].

Elevated-plus Maze

The elevated-plus maze (EPM) served as the first test of sickness and anxiety-like behavior and was performed as reported [23]. Briefly, each mouse was placed in the center of the maze facing an open arm opposing the experimenter. Distance travelled, the proportion of time spent in the open arms, and the number of entries to the open arms were measured by an overhead video camera connected to a PC with Ethovision XT software (Med Associates, Inc.) for a period of five minutes.

Light/dark box task

The light/dark box (LDB) was employed as a secondary test of sickness and anxiety-like behavior. The apparatus (Med Associates, Inc.) consisted of an illuminated compartment of transparent plastic walls and a dark compartment with black walls, covered by a lid (both 13.7 cm X 13.7 cm

X 20.3 cm). The two boxes were separated by a partition wall, with an opening at the bottom to allow the animal to pass freely between compartments. Number of entries and time spent in the lit compartment of the box were measured by an overhead video camera connected to a PC with Ethovision XT software (Med Associates, Inc.) for a period of five minutes.

Three chambers social interaction test

The three-chamber social interaction test (3CT) was used to assess general sociability and interest in social novelty as an inference of anxiodepressive behavior. The rectangular apparatus (40 cm X 60 cm X 23 cm) contains three connected compartments divided by opaque Plexiglas walls. An unfamiliar male stimulus mouse was placed under an open-wire cup on one side of the chamber whereas an empty wire cup was placed on the opposing side. Experimental mice were introduced to the chamber center. Distance travelled, the proportion of time spent, the number of entries in the stimulus chamber and the average time spent in the stimulus mouse zone were analyzed by an overhead video camera connected to a PC with Ethovision XT software (Med Associates, Inc.) for a period of five minutes.

Quantitative PCR

For cell culture experiments, TRIzol (Invitrogen) was directly added into the wells. For animal experiments, fresh frozen brains were microdissected using a cryostat and brain nuclei collected with a tissue punch according to the Paxinos and Franklin's mouse brain atlas coordinates. Total RNA was extracted using TRIzol. RNA concentration was quantified and 1000 ng of total RNA was reverse-transcribed by M-MuLV reverse transcriptase (Invitrogen) with random hexamers following the manufacturer's protocol. Quantitative gene expression was measured from 1:5 cDNA dilutions. RT-qPCR were performed using the QuantiFast SYBR Green PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's guidelines on a Corbett Rotor-Gene 6000. Quantitative real-time PCR for GPR120 (*Ffar4*), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), C-C motif chemokine ligand 2 (CCL2), ionized calcium binding adaptor molecule 1 (Iba-1), and 18S (reference gene) were carried out using specific primers (sequences in Table 1). Each PCR reaction was performed in triplicate and relative gene expression was calculated using the ΔΔCT method using BACT (for human) or 18S (for mouse) as the housekeeping gene.

Cytokine release

After treatment, microglial cell culture media was collected and immediately frozen. Murine TNF-α, IL-1β, IL-6 and CCL2 was measured using the antibodies and reference standards contained in R&D Systems (Minneapolis, MN, USA) enzyme-linked immunoabsorbent assay (ELISA) Duokits according to the manufacturer's protocol.

Immunochemistry

Microglia cultures were fixed with 4% paraformaldehyde. Primary antibody for rabbit anti-IBA-1 (1:1000, FUJIFILM Wako Chemicals U.S.A. Co., VA) or rabbit anti-NFκB p65 (1:250, Santa Cruz Biotechnology, Inc., TX) in blocking solution (0.2% Triton-X 100 and 10% normal goat serum in PBS) was applied overnight at 4°C. After washing with PBS several times, secondary antibody (Goat anti-mouse IgG Alexa568 or Goat anti-rabbit IgG Alexa488, 1:500) in blocking solution was applied for 1 h at room temperature.

Slide-mounted blain slices (14 μm) were treated with EDTA (pH 6.0) and boiled for 10 min for antigen retrieval (Ghatak and Combs, 2014). Slices were incubated with a blocking solution (0.3% Triton-X 100 and 3% normal goat serum in PBS) for 1 h. Primary antibody for rabbit anti-IBA-1 (1:500) was applied to brain slice overnight at 4°C. After washing with PBS several times, secondary antibody (Goat anti-rabbit IgG Alexa488, 1:500) in blocking solution was applied for 2 h at room temperature.

Cell cultures and brain slices were washed with PBS followed by the application of mounting media containing Dapi (Vectashield, Vector Laboratories, Inc., Newark, CA, USA). The Z-stack images were captured with Zeiss AxioImager 2 (Carl Zeiss AG, Jena, Germany) and analyzed with ImageJ/Fiji. For morphological analysis, the 16-bit images were converted to binary images after determining threshold at equivalent levels for all samples. The noise reduction by despeckle and fix cell shape by -close command were performed (Young and Morrison, 2018). Cell length, area, and circularity were assessed. For NFκB analysis, the intensity of nuclear and cytoplasmic signals was measured followed by calculating the ratio of nuclear to cytoplasmic area.

In situ hybridization (RNAScope®)

Slide-mounted brain slices (14 μm) were baked at 60°C for 30 min. Slices were dehydrated with ethanol and endogenous peroxidase action was removed by a 5 min H_2O_2 treatment. Tissues were boiled in antigen retrieval reagent for 15 min, and then digested with protease III at 40°C for 30 min in the HybEZ™ II Oven (ACD Bio). The detection of mouse Ffar4 (ACD Bio. Cat. 447041) and mouse Tmem119 (ACD Bio. Cat. 472901-C2) mRNA expression in NAc was performed with RNAscope Multiplex Fluorescent V2 Assay according to manufacturer's protocol. The Z-stack

images were captured with Zeiss AxioImager 2 (Carl Zeiss AG, Jena, Germany) and processed with ImageJ/Fiji.

Statistical analyses

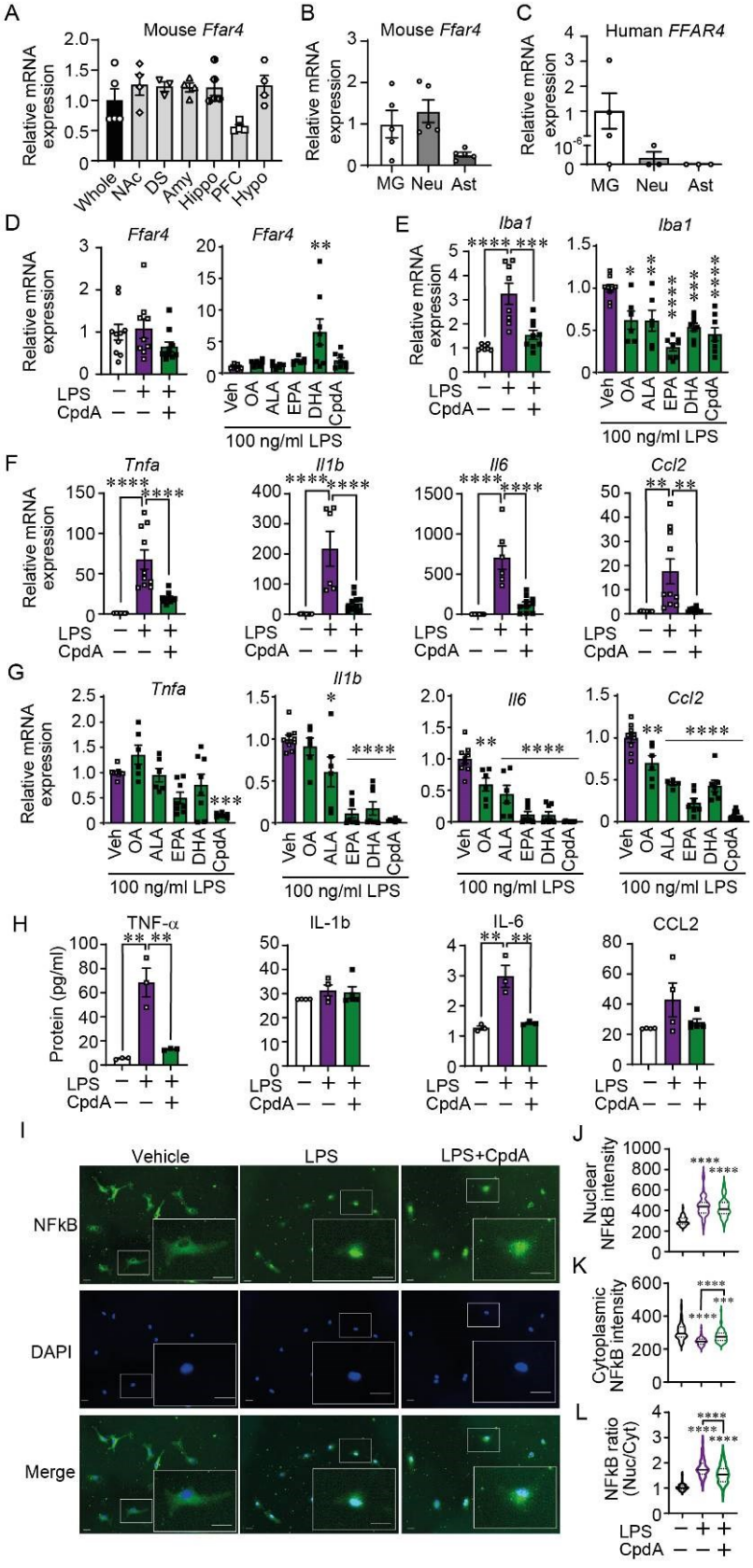
All data are expressed as mean ± SEM. Data were analyzed using GraphPad Prism 9 (San Diego, CA, USA). Between-group comparisons were made with a one-way ANOVA with Sidak post-hoc tests. Criteria for statistical significance was set at $p \leq 0.05$.

Results

GPR120 activation attenuated LPS-induced cytokine production in primary microglia

We first set out to determine the gene expression profile of GPR120 in different regions of the mouse brain. Similar levels of GPR120 mRNA were expressed in the NAc, dorsal striatum (DS), amygdala (Amy), hippocampus (Hippo) and hypothalamus, with lower expression in the prefrontal cortex (PFC) (Fig. 1A). In cultured mouse brain cells, GPR120 mRNA was highly enriched in microglia and neurons derived from newborn mouse pups compared to astrocytes (Fig. 1B). Although GPR120 mRNA in human fetal microglia was variable, expression was markedly higher herein as compared to astrocytes and neurons (Fig. 1C). GPR120 levels were not affected by LPS and/or GPR120 agonist (CpdA) treatments in primary microglia; however, DHA application increased GPR120 mRNA in LPS-treated microglia (Fig. 1D). To identify the role of GPR120 in microglia, we tested the anti-inflammatory properties of GPR120 activation on endotoxin-challenged microglia. Pretreatment with CpdA or n-3 and n-9 unsaturated FFAs blunted LPS-induced Iba-1 mRNA expression (Fig. 1E). GPR120 agonism reduced LPS-induced expression of TNF-α, IL-1β, IL-6, and Ccl2 (Fig. 1F). DHA and EPA showed a stronger anti-inflammatory effect compared to OA (a n-9 fatty acid) in LPS-treated microglia (Fig. 1G). These changes translated to reduced secretion of TNF-α and IL-6 levels in the culture medium (Fig. 1H). NFκB is a major transcription factor for the induction of cytokine responses in immune cells. Upon activation, NFκB translocates to the nucleus to regulate gene transcription [26]. LPS increased NFκB nuclear localization (Fig. 1I, 1J); however, CpdA recovered decreases in cytosolic NFκB (Fig. 1K) and reduced the ratio of nuclear/cytoplasmic NFκB (Fig. 1L).

Figure 1


A
Mouse Ffar4
Relative mRNA expression
Whole NAc DS Amy Hippo PFC Hypo
B
Mouse Ffar4
MG Neu Ast
C
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MG Neu Ast
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LPS
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100 ng/ml LPS
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Ccl2
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NFkB ratio (Nuc/Cyt)

Figure 1. Central GPR120 expression and microglial anti-inflammatory function

(A) Distribution of GPR120 (Ffar4) mRNA in mouse brain (Whole: Whole brain, NAc: nucleus accumbens, DS: dorsal striatum, Amy: amygdala, Hippo: hippocampus, PFC: prefrontal cortex, Hypo; hypothalamus) (n=3-5/group). **(B)** GPR120 mRNA expression in primary murine microglia (MG), neurons (Neu), and astrocytes (Ast) (n=5). **(C)** GPR120 mRNA expression in human microglia (MG), neurons (Neu), and astrocytes (Ast) (n = 3-4). Effect of GPR120 agonism on **(D)** Ffar4 and **(E)** Iba-1 mRNA expression on LPS-stimulated primary microglia (n=6-11/group). Proinflammatory cytokine mRNA expression on LPS-stimulated primary cultured microglia pre-treated with **(F)** CpdA or **(G)** unsaturated FAs (OA; oleic acid, ALA; α-linolenic acid, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid). **(H)** Cytokine protein levels in culture medium (n = 3-5/group). **(I)** Representative image of NFκB translocation after LPS-treatment with or without CpdA pre-treatment. NFκB intensity in nuclear **(J)** and cytoplasmic **(K)** compartments **(L)** Ratio of nuclear/cytoplasmic NFκB (n=63, from 3 cover slips). Data are expressed as the mean ± SEM. One-way ANOVA with post-hoc Sidak multiple comparison test; * p≤ 0.05, ** p≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001 vs Veh LPS.

Central GPR120 agonism attenuated LPS sickness-and anxiety-like behaviors and inflammatory indices in the nucleus accumbens

We next evaluated if central administration of CpdA could abrogate LPS-induced decreases in locomotion and anxiety-like behavior using two well-established behavioral tests (Fig. 2A). Three days of ICV CpdA pre-treatment completely prevented systemic LPS-induced hypolocomotion (distance travelled) in the EPM test (Fig. 2B); however, LPS and CpdA did not significantly alter the proportion of time spent (Fig. 2C) and the number of entries in the open arms (Fig. 2D). Mice also demonstrated sickness-like behaviors in the light-dark box task following LPS injection as shown by a reduction of the total distance traveled, time spent and number of entries in the lit compartment (Fig. 2E-G). All of the these effects were blunted by CpdA suggesting that central GPR120 activation has protective actions on sickness behaviors elicited by peripheral immune activation (Fig. 2E-G).

As there is heterogeneity in microglia numbers and activity patterns between different brain areas [27], we also investigated the capacity of GPR120 stimulation to alleviate inflammation in the NAc, a region in which inflammatory insults can contribute to locomotor activity, anxiodepressive behavior and increased food-motivated behavior [4, 28]. Expression of the microglial marker Iba-1 in the NAc was increased by LPS-injection, an effect attenuated by CpdA (Fig. 2H) in a manner

resembling results in primary microglia. Furthermore, CpdA pretreatment significantly prevented LPS-induced increases in TNF-α and IL-1β, but not IL-6 mRNA levels, in the NAc (Fig. 2I-K).

Figure 2

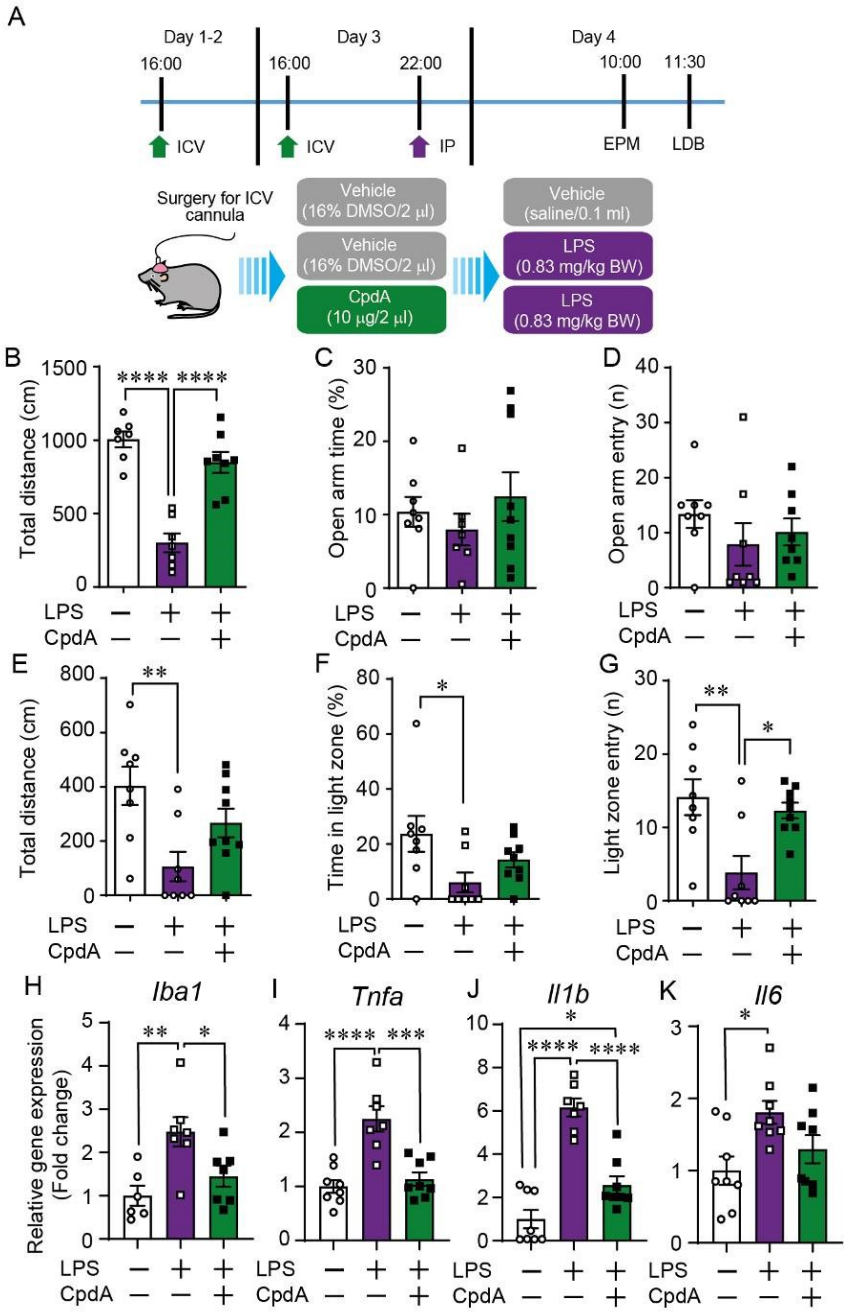


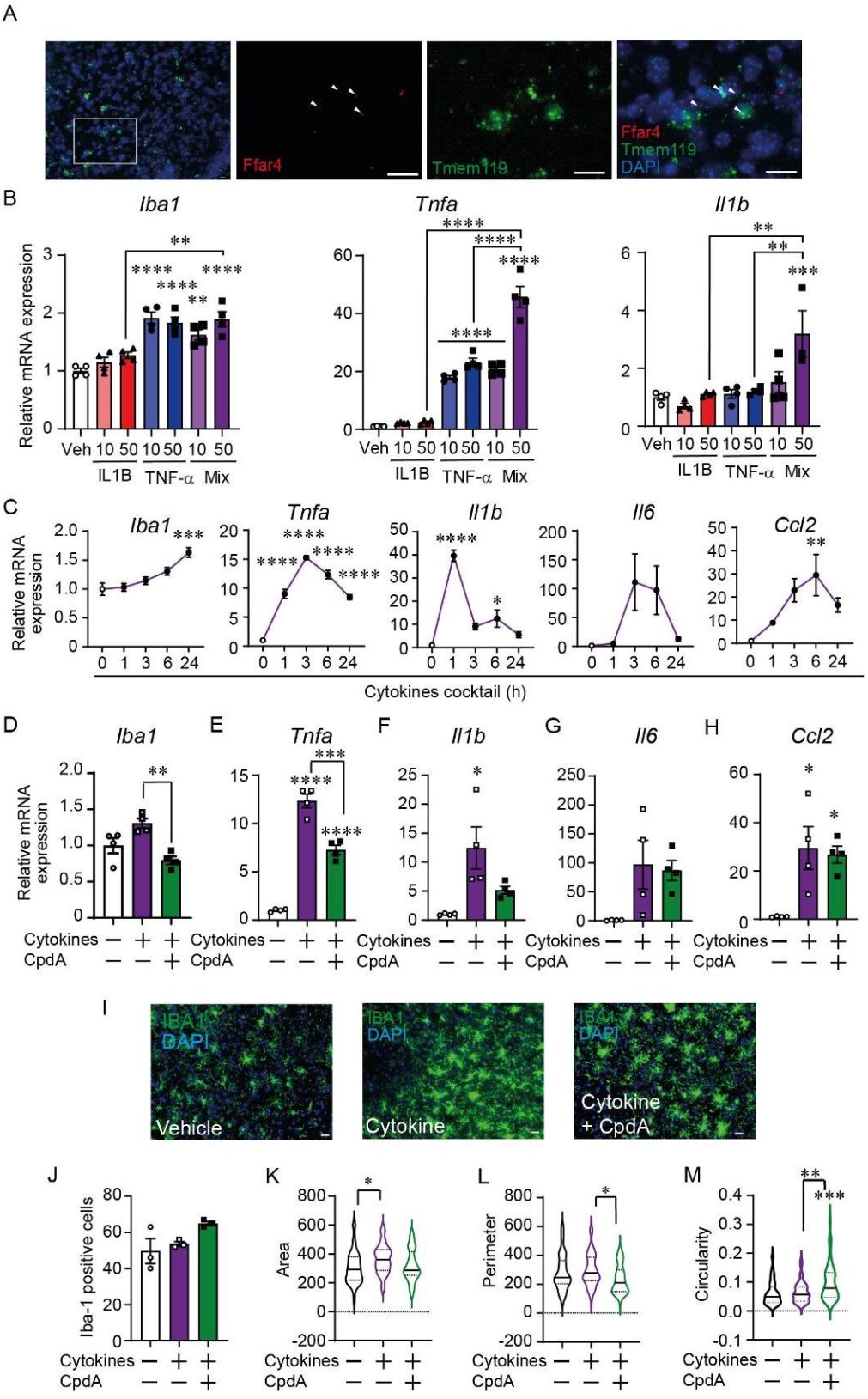
Figure 2. Central GPR120 agonism reduces LPS-induced hypolocomotion and anxiety-like behavior

(A) Experimental design of *in vivo* study 1 **(B)** Total distance travelled, **(C)** time spent and **(D)** number of entries made into open arms of the elevated plus maze (EPM) test. **(E)** Total distance travelled **(F)** time spent and **(G)** number of entries in the light compartment of the light-dark box test. Effect of GPR120 agonism on LPS-induced **(H)** Iba-1, **(I)** TNF-α, **(J)** IL-1β, and **(K)** IL-6 mRNA expression in the nucleus accumbens (NAc). n=7-8/group. Mean ± SEM; One-way ANOVA with post-hoc Sidak multiple comparison test;* p≤ 0.05, ** p≤ 0.01, **** p ≤ 0.0001.

Anti-inflammatory actions of GPR120 activation in nucleus accumbens microglia

Similar to the effects of peripheral LPS, increasing proinflammatory cytokine levels in the brain triggers neuroinflammation and sickness-like behaviors. Neuroinflammatory responses in the NAc have been linked to reduced locomotion and enhanced anxiodepressive-behavior [4, 28]. Thus, we next sought to determine if GPR120 stimulation protects against the activation of microglia derived from the NAc in response to application of TNF-α and IL-1β. These two cytokines are required for the innate immune response and known to be upregulated in the brain in response to systemic LPS, stress and obesity and to contribute to blunted affective states [2, 29]. First, RNAscope i*n situ* hybridization revealed GPR120 co-expression with the microglial marker Tmem119 in the NAc (Fig. 3A). To evaluate the anti-inflammatory effect of GPR120 agonism on cytokine-induced neuroinflammation in the NAc, we cultured primary microglia derived from the NAc. Co-treatment with TNF-α and IL-1β increased Iba-1, TNF-α, and IL-1β mRNA expression more so than either cytokine alone (Fig. 3B). The cytokine mix increased proinflammatory cytokine expression in a time- and cytokine-dependent manner (Fig. 3C). CpdA attenuated increases in Iba-1, TNF-α, and IL-1β mRNA expression (Fig. 3D-F) without affecting IL-6 and Ccl2 expression (Fig. 3G-H). Next, we assessed NAc microglial morphology via immunohistochemistry on slices derived from mice treated with the ICV cytokine mix with or without 3 days pre-treatment with CpdA (Fig. 3I). The number of IBA-1 positive cells did not significantly change (Fig. 3J), however microglial cell area increased by the cytokine mix and this effect was prevented by CpdA treatment (Fig. 3K). Moreover, CpdA pre-treatment significantly reduced cell perimeter (Fig. 3L) and increased cell circularity compared to vehicle and cytokines (Fig. 3M).

Figure 3


A
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Tnfa
Il1b
Il6
Ccl2
Relative mRNA expression
Cytokines cocktail (h)
D
Iba1
E
Tnfa
F
Il1b
G
Il6
H
Ccl2
Relative mRNA expression
Cytokines
CpdA
I
IBA1
DAPI
Vehicle
Cytokine
Cytokine + CpdA
J
Iba-1 positive cells
K
Area
L
Perimeter
M
Circularity

Figure 3. GPR120 agonism attenuates nucleus accumbens microglial activation by cytokines

(A) GPR120 expression in NAc microglia detected by Tmem119 RNAscope *in situ* hybridization in adult mouse brain **(B)** The effect of IL-1β and/or TNF-α application on Iba-1, TNF-α, and IL-1β mRNA expression in primary microglia derived from NAc. **(C)** Time course of cytokine -induced proinflammatory and microglial marker mRNA expression. Effect of GPR120 agonist pre-treatment on cytokine-induced **(D)** Iba-1, **(E)** TNF-α, **(F)** IL-1β, **(G)** IL-6, and **(H)** Ccl2 mRNA expression (n=4). **(I)** Representative images of NAc microglia in slice preparations derived from mice injected ICV with the TNF-α and IL-1β mixture with/without ICV CpdA pre-treatment. **(J)** Number of IBA-1 positive cells in each image (n = 3) **(K)** Cell area, **(L)** cell perimeter, and **(M)** circularity (n=41-47 microglia/n=3 brain). Mean ± SEM; One-way ANOVA with post-hoc Sidak multiple comparison test; * p≤ 0.05, ** p≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001 vs Veh.

Central GPR120 agonism attenuated sickness-like behaviors by cytokines

We next examined if ICV CpdA could diminish sickness-like behaviors produced by central administration of the cytokine mix (Fig. 4A). There was a trend for reduced locomotor activity following ICV cytokine administration in the EPM (P = 0.15, Fig. 4B) and LDB (P = 0.13, Fig. 4E), however, anxiety-like behavior as inferred by a decrease in the proportion of time spent in open arms in response to cytokine treatment alone was not observed in the EPM (Fig. 4C, 4D) and LDB (Fig. 4F, 4G). We next submitted mice to a social interaction test to measure sociability and anxiodepressive behavior. Central cytokine injection reduced total distance travelled in the 3CT and this effect was prevented in mice pre-treated with CpdA (Fig. 4H). The cytokine mix also reduced the number of entries in the stimulus mouse zone which was also blocked by CpdA (Fig. 4I). However, the average duration (time in mouse zone/entry number) was increased by cytokine treatment and blocked in mice pre-treated with CpdA (Fig. 4J).

Figure 4

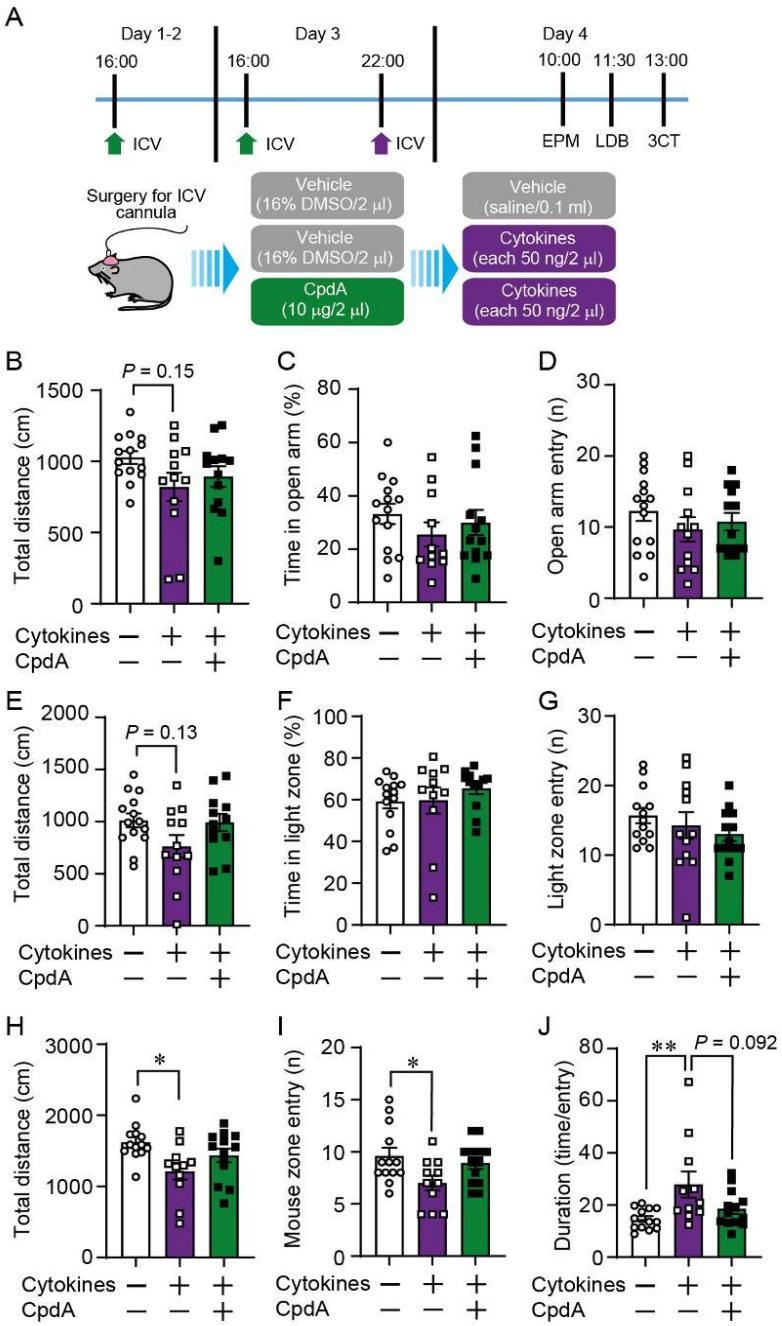

Figure 4. Central GPR120 agonism protects against central cytokine-induced behavioral responses

(A) Experimental design of *in vivo* study 2 **(B)** Total distance travelled, **(C)** time spent and **(D)** number of entries made in open arms of the elevated plus maze (EPM) test. **(E)** Total distance travelled, **(F)** time spent and **(G)** number of entries made in the light compartment of the light-dark box (LDB) test. **(H)** Total distance travelled, **(I)** number of entries in mouse zone, **(J)** duration of

time spent in mouse zone in the 3-chamber (3-CT) social interaction test. n=11-14/group. Mean ± SEM; One-way ANOVA with post-hoc Sidak multiple comparison test;* p≤ 0.05, ** p≤ 0.01.

Discussion

The present study provides the first evidence that brain GPR120 is relatively enriched in microglial neural cells in both mouse and human and that its activation in murine microglia can significantly improve acute neuroimmune responses. GPR120 agonism was effective in abrogating LPS-induced inflammation in primary microglia in a manner that resembled or exceeded the effects of n-3 fatty acids. Further, central GPR120 agonist pre-treatment was effective in attenuating sickness- and anxiety-like behaviors triggered by systemic LPS and central TNF-α and IL-1β administration. Together, these results suggest that brain GPR120 could be a valuable target to mitigate neuroinflammation associated with systemic infections and suggest a potential for its use in the treatment of neuroinflammatory conditions.

PUFAs and their metabolites including eicosanoids and prostaglandins are immune modulators in peripheral tissues and the CNS [30-32]. However, the receptor signaling pathways involved in fatty acid modulation of neuroinflammation remain elusive. Previous work showed that GPR120-positive microglia increase after focal cerebral ischemic injury and GPR120 activation by DHA rescues ischemic injury and neuroinflammation by the Japanese Encephalitis virus [33, 34]. The present study supports and broadens these neuroprotective properties of GPR120. First, we demonstrate that GPR120 mRNA is enriched in microglia cells as compared to other neural cell types in mouse and human brain, similar to observations in a human RNAseq dataset [35]. Revealing functional outputs of microglial GPR120, we discovered that GPR120 agonism strongly diminishes LPS-induced cytokine expression and secretion in primary forebrain microglia in a manner similar to or greater than the effects of n-3 PUFA application. In addition, GPR120 agonism reduced NFκB nuclear translocation induced by LPS in primary microglia, consistent with its anti-inflammatory signaling actions in peripheral macrophages [11].

As there is heterogeneity in microglial gene expression and activity patterns between different brain areas [27], we also investigated the influence of GPR120 stimulation in microglia derived solely from the NAc, a region in which inflammatory insults can contribute to locomotor activity, anxiodepressive behavior and increased food-motivated behavior [4, 28]. The protective actions of GPR120 agonism also extended to the reactivity of NAc microglia in response to combined TNF-α and IL-1β application. Consistent with these findings, we previously found that n-3 supplementation prevents saturated high-fat diet induced increases in glial reactivity in the NAc [8]. Moreover, the blockade of inflammatory responses in NAc by adenoviral inhibition of

IKKβ/NFκB and the inhibition of central TNF-α action recovers anxiodepressive behaviors in diet-induced obese and diabetic mice, respectively [4, 36].

We also investigated the capacity of central GPR120 agonism to attenuate behavioral responses to acute inflammatory interventions. To this end, we found that suppression of locomotor activity caused by LPS was blocked by GPR120 agonist pretreatment in both behavioral tasks. However, behavioral changes typically characterizing anxiolytic actions were only observed in one of the two tests employed after LPS. Specifically, while GPR120 stimulation reduced light compartment entries and time spent in the LDB, it did not significantly reverse LPS-induced decreases in open arm entries and time in the EPM task. The alleviation of sickness-like behaviors by GPR120 agonist pre-treatment coincided with a considerable reduction in proinflammatory marker (Iba-1, TNF-α, IL-1β, and IL-6) expression in the NAc. The NAc was pinpointed because of previous findings highlighting the contribution of neuroinflammation in this region in anxiodepressive behavior by prolonged high-fat feeding. Elevated IL-1β and TNF-α expression in the brain is one of the hallmarks of rodent depressive-like behavior by LPS [37, 38]. In addition, ICV injection of these proinflammatory cytokines induces sickness and anxiodepressive-behavior [22, 39, 40].

In the behavioral experiments following cytokine administration, combined TNF-α and IL-1β injection failed to significantly modulate sickness- and anxiety-like behaviors in the EPM and LDB test, although there was a trend for less distance travelled by cytokines that was absent in mice pre-treated with the GPR120 agonist. Nevertheless, locomotion was moderately diminished by cytokines in the social interaction test and this effect was not observed in mice pre-treated with CpdA. Cytokine administration also reduced entries into the stimulus mouse zone of the 3CT test, which was absent in the CpdA condition. The duration of time spent near the stimulus mouse unexpectedly increased with cytokine administration, but as this was reversed by CpdA we speculate that the sickness-related actions of the cytokines were at play here to perhaps trigger additional sluggishness and weariness when mice entered the stimulus mouse zone. Collectively, these behavioral results are in line with our earlier findings showing central infusions of a GPR120 agonist abrogates anxiety-like behavior in diet-induced obese mice [12]. The longer duration of ICV agonist infusions and inflammatory nature of the diet manipulation may explain the clearer anxiolytic effects of GPR120 stimulation observed in that study. That the behavioral impact of central cytokines was weaker than that obtained with LPS in the current study may well be due to dosing and/or the more robust influence peripheral immune activation can have on the CNS via enhanced production of local inflammatory mediators, not limited to IL-1β and TNF-α, by

endothelial cells, perivascular macrophages, microglia and astrocytes at the blood brain barrier [41-44].

Comorbidity of metabolic and psychiatric diseases have been tied in part to the immunometabolic consequences of poor diet and obesity development [2]. GPR120 is a promising anti-obesity and -diabetes target because it mediates fatty acid signaling in the periphery to regulate insulin and glucagon-like peptide 1 [9]. Along with our previous findings showing that prolonged central GPR120 activation can inhibit anxiety in high-fat fed mice [12], our current results suggest that brain GPR120 agonism may offer a promising strategy for attenuating neuroimmune responses and sickness behaviors caused by endotoxemia via its capacity to restrain microglial reactivity and coincident cytokine synthesis and release. Thus, in addition to its glycaemia regulating properties, GPR120 activation could be used as an intervention to protect against neuroinflammation and associated behaviors caused by poor diet and increases in pro-inflammatory gut-derived bacteria. In agreement, peripheral GPR120 activation was also shown to reduce prostaglandin D2-microglia-provoked neuroinflammation and contribute to memory function [45]. n-3 PUFA deficiency induces anxiety-like behavior and anhedonia with the low-frequency stimulation induced a long-term depression of evoked excitatory postsynaptic potential in NAc [46]. In clinical studies, it has demonstrated that lower n-3 PUFA links with emotion and cognition and potential of n-3 PUFA supplementation to improve these brain dysfunction [47], however, it remains unclear which mechanisms link plasma n-3 PUFAs levels and depression [48]. While more needs to be uncovered regarding how central GPR120 contributes to the neurobehavioral effects of modulating dietary n-3 PUFA, GPR120-based pharmacotherapies may offer a new opportunity for successful treatment of metabolic, psychiatric and neurological diseases encompassing microglial activation and neuroinflammation.

Conclusion

Taken together, this work demonstrates the enrichment of GPR120 in microglial cells and its capacity to reduce inflammation induced by LPS and cytokines. These findings identify GPR120 as a promising target for the prevention of microglia-mediated neuroinflammation and protection from inflammation-induced sickness behaviors (Fig. 5). Collectively, this work implicates GPR120 targeting in moderating immune response following systemic inflammation and potentially offers an innovative therapeutic approach for neuroinflammation-associated diseases such as anxiety

and depression. Further studies will be required to elucidate the role of GPR120 activation in the resolution of obesity and associated metabolic inflammation.

Figure 5

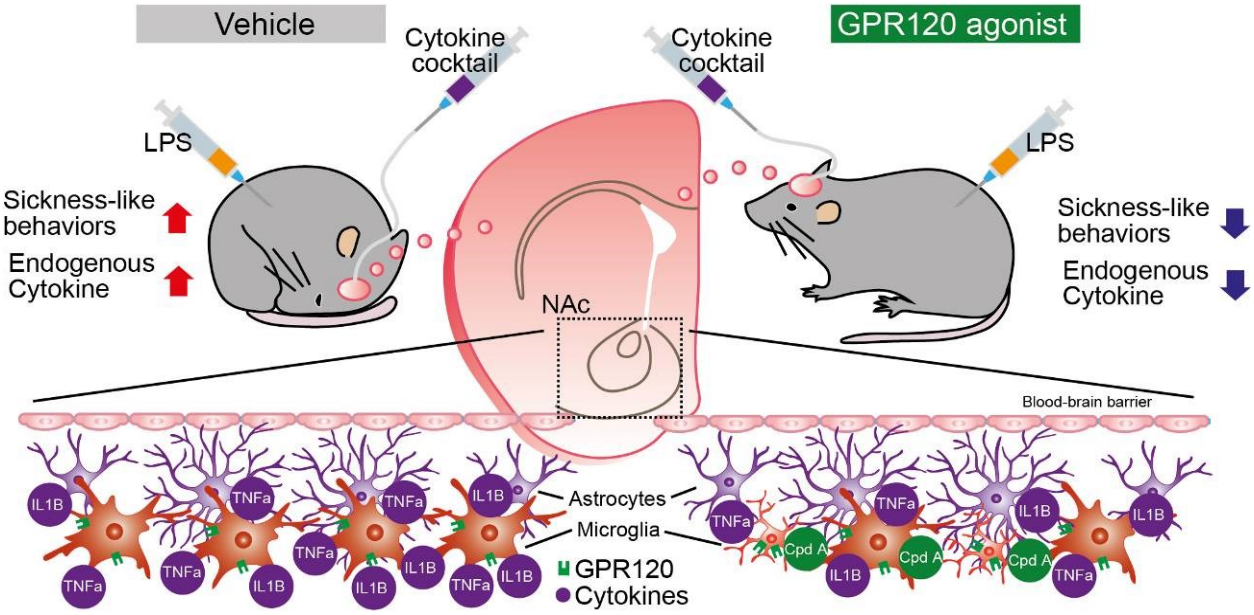

Figure 5 Illustrating the potential action of GPR120 agonism on the regulation of neuroinflammation and sickness-like behaviors.

GPR120 expression is enriched in microglia. GPR120 agonism attenuated LPS or cytokines-induced microglial activation including endogenous cytokine production. Sickness-like behaviors caused by LPS injection (i.p.) or cytokine cocktail infusion (i.c.v.) was rescued by central GPR120 agonist infusion.

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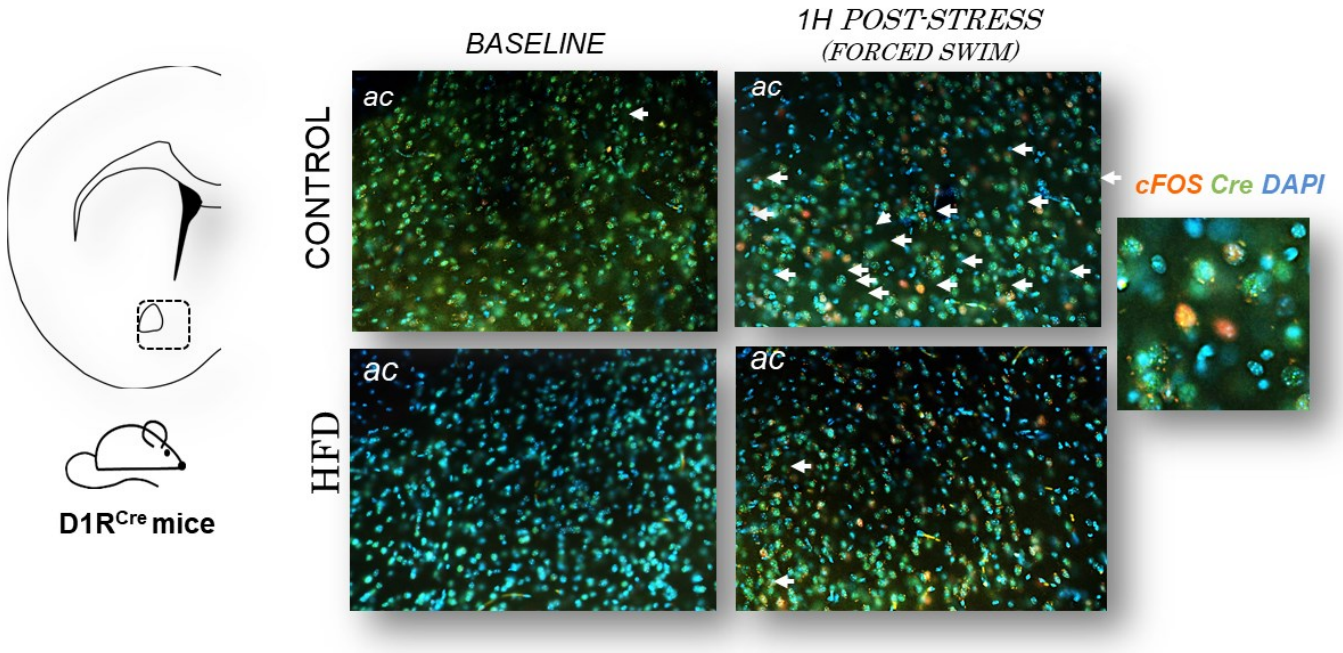
Table 1: Primer sequences

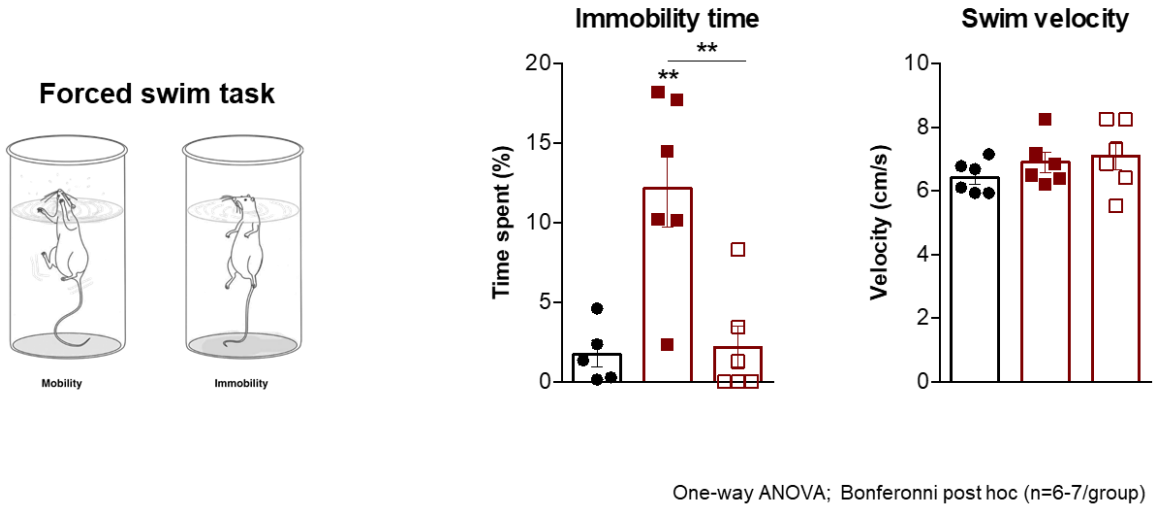
Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')
h-BACT	TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA	CTA GAA GCA TTT GCG GTG GAC GAT GGA
m-18S	TAG CCA GGT TCT GGC CAA CGG	AAG GCC CCA AAA GTG GCG CA
h-FFAR4	TGG AGA TGC ACA TTG TTT GGA GA	AGC CTC CAA GTG GTG GAG TGA
m-Ffar4	TTT ACA GAT CAC GAA AGC ATC GC	GTG CGG AAG AGT CGG TAG TC
m-Iba-1	GGA TTT GCA GGG AGG AAA AG	TGG GAT CAT CGA GGA ATT G
m-IL-1β	GAC CCC AAA AGA TGA AGG GCT	ATG TGC TGC TGC GAG ATT TG
m-IL-6	CAG AGT CCT TCA GAG AGA TAC	AGC TTA TCT GTT AGG AGA GC
m-TNF-α	CAC GCT CTT CTG TCT ACT G	AAG ATG ATC TGA GTC TGA GG
m-Ccl2	ATT GGG ATC ATC TTG CTG GT	CCT GCT GTT CAC AGT TGC C

Appendix 2

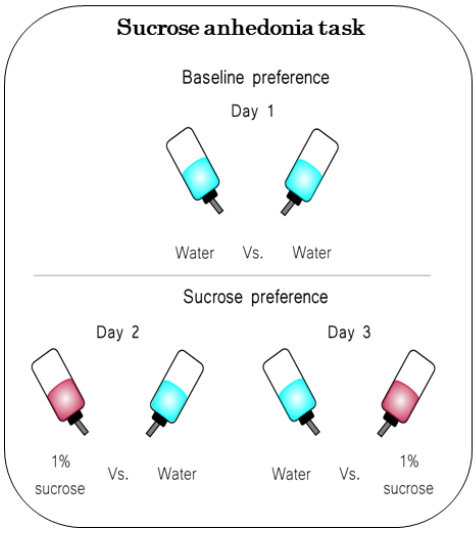


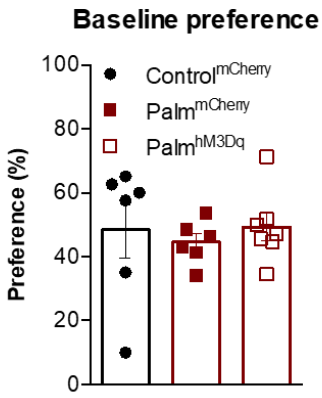
Saturated high-fat diet may decrease c-fos in nucleus accumbens D1-MSNs
Immunohistochemistry was performed in D1R^{cre} mice. This figure shows: representative images of cFos (red), cre (green) and dapi (blue) staining in the NAc at baseline and following a forced swim task in Palm fed mice.

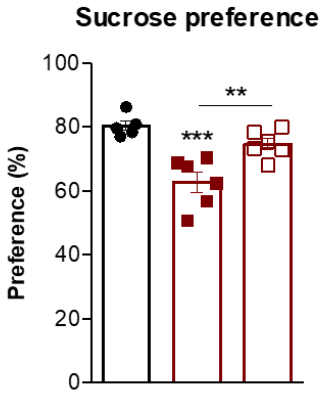
Appendix 3



Activation of D1-MSN rescues behavioral despair provoked by saturated high-fat feeding
Behavioral tests were performed in D1R^{cre} mice. This figure shows: Representative image of mice in the forced swim task. Plots show the immobility time and swim velocity of control mice, Palm fed mice, and Palm fed mice after D1-MSN activation.







Activation of D1-MSN rescues anhedonia provoked by saturated high-fat feeding
Behavioral tests were performed in D1R^{cre} mice. This figure shows: Representative image of sucrose anhedonia task paradigm. Plots show the baseline preference and sugar preference of control mice, Palm fed mice, and Palm fed mice after D1-MSN activation.