

UC Berkeley

UC Berkeley Electronic Theses and Dissertations

Title

Elucidating a Novel Peptidergic Circuit in the Nucleus Accumbens Medial Shell

Permalink

<https://escholarship.org/uc/item/9cf270fk>

Author

Aisenberg, Erin Elizabeth

Publication Date

2024

Peer reviewed|Thesis/dissertation

Elucidating a Novel Peptidergic Circuit in the Nucleus Accumbens Medial Shell

By

Erin Elizabeth Aisenberg

A dissertation submitted in partial satisfaction of the
requirements for the degree of
Doctor of Philosophy
in
Neuroscience
in the
Graduate Division
of the
University of California, Berkeley

Committee in Charge:

Professor Helen Bateup, Chair
Professor Diana Bautista
Professor Marla Feller
Professor Stephan Lammel

Spring 2024

ABSTRACT

Elucidating a Novel Peptidergic Circuit in the Nucleus Accumbens Medial Shell

By

Erin Elizabeth Aisenberg

Doctor of Philosophy in Neuroscience

University of California, Berkley

Professor Helen Bateup, Chair

Neuromodulators are key signaling molecules in the nervous system that regulate neuronal activity by modulating intrinsic excitability, synaptic transmission, and plasticity. The largest known class of neuromodulators are neuropeptides. Traditionally, neurons have been defined primarily by the neurotransmitter they release (i.e. glutamatergic or GABAergic); however, recent single cell RNA sequencing studies have revealed that many neurons also express genes encoding for one or more neuropeptides and/or neuropeptide receptors. Gastrin-releasing peptide (GRP), a bombesin-like neuropeptide, is one such neuropeptide that has mainly been studied in the spinal cord in the context of itch behavior. Our lab previously showed that *Grp* mRNA is also expressed in the midbrain, in about a third of dopamine (DA) neurons. My research goal was to further explore these *Grp*-expressing neurons and determine whether a functional GRP/GRPR sub-circuit exists within the basal ganglia.

I first used fluorescent in situ hybridization (FISH) to probe for *Grpr*, which encodes gastrin releasing peptide receptor (GRPR), in the targets of the *Grp*-expressing DA neurons. I found that *Grpr* was expressed by specific populations of neurons in the striatum and nucleus accumbens medial shell (NAc MSh). This complementary expression pattern suggests that GRP/GRPR signaling may define novel peptidergic sub-circuits within the basal ganglia. For the purpose of this project, I chose to focus on the *Grpr*-expressing neurons in the NAc MSh. I performed additional FISH experiments to genetically characterize the *Grpr*-expressing cells in the context of the basal ganglia. I determined that the *Grpr*⁺ cells appeared to be a small subpopulation of GABA-ergic indirect pathway striatal projection neurons (iSPNs). Morphology analyses supported their SPN identity.

Next, I performed whole-cell recordings on these cells to determine their electrophysiological properties and intrinsic excitability using a GRPR-eGFP reporter mouse. While genetically and morphologically the GRPR⁺ cells appeared to be SPNs, their electrophysiological properties were distinct from other types of SPNs. Mainly, a majority of GRPR⁺ cells were spontaneously active at baseline, they were more depolarized, had higher membrane resistance, and had wider action potentials than

SPNs. Their properties were also distinct from neighboring cholinergic interneurons in the NAc MSh. The higher intrinsic excitability and activity of GRPR+ cells was confirmed with FISH where I found that *Grpr*+ cells were approximately twice as likely to be *Fos*+ compared to *Grpr*- cells.

I next investigated whether there were other sources of GRP into the NAc mSh, in addition to the previously identified *Grp*+ DA neurons from the ventral tegmental area. I injected a retrograde tracer into the NAc MSh and probed for expression of *Grp* mRNA in synaptically connected neurons using FISH. I found that a significant number of the *Grp*+ inputs into the NAc MSh arise from glutamatergic neurons projecting from the hippocampus as well as the basolateral amygdala.

After identifying receptor expression and mapping the GRP inputs to the NAc MSh, I characterized how the GRPR-expressing cells in the NAc MSh respond to GRP both in slice and in vivo. In slice, GRP wash-on led to depolarization and an approximately 140% increase in membrane resistance, effects that could be blocked with a GRPR-specific antagonist. GRP injections in vivo induced *Fos* expression selectively in *Grpr*+ cells in the injected hemisphere but not the un-injected hemisphere.

Having characterized the GRPR-expressing cells in the NAc MSh, I probed the function of GRP/GRPR signaling in the circuit. To do so, I generated *Grpr* knock-out (KO) mice and ran them through behavioral tasks known to involve the NAc MSh: progressive ratio lever pressing, accelerating rotarod, and sucrose preference. I also ran the mice through the open field to assess general locomotor activity and spontaneous behaviors. In the open field, mice lacking *Grpr* travelled significantly less total distance and displayed different behavioral syllable usage identified by motion sequencing (MoSeq), suggesting that disruption of GRPR-signaling globally affects mouse behavior patterns. I saw no consistent changes in the progressive ratio task, indicating that global disruption of GRP-GRPR signaling does not affect motivation. In the accelerating rotarod, however, *Grpr* KO mice had better performance, but showed no differences in learning, indicating that GRP-GRPR signaling is involved in motor coordination. In the sucrose preference test, I found that *Grpr* KO mice displayed less of a preference, indicating that GRP signaling may be involved in detecting positive valence and “liking” sensation.

Overall, I have identified and characterized a novel subpopulation of iSPNs in the NAc MSh defined by their expression of GRPR. Furthermore, I mapped the inputs to the NAc MSh that express *Grp* and have shown both in slice and in vivo that GRPR+ cells in the NAc MSh respond to GRP. Finally, I have shown how disruption of GRP/GRPR signaling leads to behavioral changes in NAc MSh-dependent behaviors. Together, my dissertation research has characterized a novel peptidergic circuit in the NAc MSh defined by GRP/GRPR expression that is functionally relevant in NAc MSh-dependent behavior.

Acknowledgements

There are numerous individuals that have helped and supported me both scientifically and personally during my PhD, without whom this work would not have been possible.

Firstly, I would like to thank Helen for taking a chance on me and accepting me into your lab five years ago. I really appreciate you letting me work on a project that was outside of the main focus of your lab and guiding me along the way. Your mentorship, guidance, and attention to scientific rigor helped me develop into a better scientist. Thank you for believing in me more than I believed in myself and trusting me to work on a project I was truly passionate about.

I would also like to thank my thesis committee members, Diana Bautista, Marla Feller, and Stephan Lammel. Thank you for your guidance and support over the years, both scientifically as well as how to navigate the challenges of graduate school. My project would not have turned into what it did without your valuable input.

Thank you to Dr. Paul Slesinger for hiring me to be your lab manager right after college even though I did not have any of the relevant skills. Thank you for taking the time to personally mentor me. The projects I had the opportunity to work on in your lab started my interest in neuropeptides and helped me tremendously during my PhD.

Thank you to all of the past and present members of the Bateup Lab. In particular, I would not be here if it were not for Dan Kramer. Thank you, Dan, for mentoring me as a rotation student and starting the Grp project. Thank you to Katie Cording for teaching me how to patch, generally being a great lab mate, and always listening and offering wise words when I needed scientific advice or a pep talk. Thank you to Atehsa for not only helping me quantify my numerous in situs, but more importantly, for being a great friend. Thank you for being there for me and supporting me when I needed it the most and for listening to me endlessly vent about things that were probably my fault and/or things that I was not going to do anything about. I will always have fond memories of our walks home.

Thank you to Julia and Sonali. Thank you for being amazing roommates and close friends. You have always been there for me these past six years. Thank you for making HT a welcoming and fun place to come home to after many days of failed experiments. Thank you for venting with me and helping me overcome personal and scientific challenges. Thank you for listening to my talk about quitting graduate school with no real intention of quitting and never complaining about my 4 AM alarm. From taking our quals the first week of quarantine and our iconic post-quals photoshoot to just generally hanging out in the living room, I cannot imagine better roommates or a better support system in graduate school. I cannot believe we are all almost finished, and I am so excited to follow and see what you both do next.

Mom, Dad, Dan, and Matt, I truly cannot thank you enough for everything. For my whole life you have been my biggest cheerleaders. You have always believed in me and supported me. Thank you for always accepting and loving me and for understanding that I could not always be there for everything, but somehow my experiments conveniently

never got in the way of trips around the world to see Rafa. Your unconditional love and support gave me the strength I needed to succeed.

Finally, thank you to Kamran. Thank you for always being there for me, near or far, these past 4.5 years. Thank you for always helping me with my figures, both the serious ones and the stupid ones for lab meeting that I am sure no one else thought were funny—I know I should have learned how to do some of this stuff by now. From Berkeley to Boston to now Palo Alto, you always had my back. You listened to me vent, offered me scientific and emotional support, consoled me when I cried over my numerous failed experiments, and always encouraged me and made me almost believe that I could actually do it. Thank you for always loving me and having my back. I am excited for what the future holds and to finally be in the same place again.

Table of Contents

Acknowledgements.....	i
Chapter 1: Neuropeptides and the Basal Ganglia	1
Neuropeptides in the brain	1
The basal ganglia	2
Dorsal striatum	2
Organization	2
Cell types.....	3
Inputs and outputs.....	4
Behavior/function	4
Nucleus accumbens	4
Cell types.....	4
Inputs and outputs.....	5
Behavior/function	6
Striatal-associated behaviors used in this thesis.....	7
Accelerating rotarod	7
Progressive ratio lever pressing	8
Sucrose preference test	9
Neuropeptides in the nucleus accumbens medial shell.....	9
Kappa opioid receptor/dynorphin	9
Mu opioid receptor/beta-endorphin	10
Delta opioid receptor/enkephalin.....	10
Nociceptin receptor/nociceptin	11
Other neuropeptides in the NAc MSh	11
Gastrin releasing peptide.....	11
Dissertation research questions	12
Figures.....	14
Chapter 2: Characterizing a Novel Peptidergic Circuit in the Nucleus Accumbens Medial Shell Defined by Gastrin Releasing Peptide Receptor	17
Introduction.....	17
Results.....	19
Gastrin releasing peptide receptor is expressed in striatal neurons	19

Gastrin releasing peptide receptor-expressing neurons in the nucleus accumbens medial shell are morphologically similar to neighboring striatal projection neurons	20
Gastrin releasing peptide receptor+ neurons in the nucleus accumbens medial shell have high intrinsic excitability	20
Multiple inputs to the nucleus accumbens medial shell express Gastrin releasing peptide mRNA	21
Gastrin releasing peptide depolarizes and increases the excitability of Gastrin releasing peptide receptor-expressing neurons in the nucleus accumbens medial shell	22
Gastrin releasing peptide injection in vivo increases Fos expression in the nucleus accumbens medial shell	22
Gastrin releasing peptide receptor knockout mice were tested in striatal-associated behavioral assays	23
Gastrin releasing peptide receptor knockout mice move significantly less in the open field and have differential usage of behavioral syllables	23
Gastrin releasing peptide receptor knockout mice show no differences in operant conditioning	24
Gastrin releasing peptide receptor knockout mice show enhanced performance on the accelerating rotarod	24
Gastrin releasing peptide receptor knockout mice show reduced sucrose preference	24
Discussion	25
Grpr-expressing neurons are a novel subtype of iSPNs in the NAc MSh	25
Both glutamatergic and dopaminergic inputs into the NAc MSh express Grpr	26
GRP-GRPR signaling affects striatal associated behaviors	27
Limitations and future directions	28
Methods	29
Mice	29
Fluorescent in situ hybridization	29
Immunohistochemistry	30
Confocal microscopy	30
Neurobiotin-filled neuron reconstruction for Sholl analysis	30
Sparse viral injections for spine density quantification	31
Electrophysiology	32
Stereotaxic injections	33

Behavioral experiments.....	34
Quantification and statistical analysis	36
Figures.....	37
Figure 1	37
Figure 2	39
Figure 3	41
Figure 4	43
Figure 5	45
Figure 6	47
Figure 7	49
Figure 8	50
Figure 9	52
Figure 10	53
Figure 11	54
Figure 12	55
Figure 13	57
Figure 14	59
Figure 15	60
Chapter 3: Summary and Future Directions	61
Thesis summary	61
Future directions	62
References	66

Chapter 1: Neuropeptides and the Basal Ganglia

Neuropeptides in the brain

Neuropeptides are the largest and most diverse known class of neuromodulators, which regulate brain states and modify neural activity and vascular tone [1, 2]. A common standard criteria for a molecule to be classified as a neuropeptide was outlined by Burbach in 2011 and requires: translation of a neuropeptide precursor protein in the endoplasmic reticulum, cleavage of the neuropeptide precursor into a neuropeptide product during passage through the rough endoplasmic reticulum-Golgi apparatus complex where it is packed into dense core vesicles, transport and storage of dense core vesicles within the source neuron, secretion of the neuropeptide product upon specific activity leading to exocytosis of the dense core vesicles, and finally diffusion as a means of clearance after binding to a receptor [3].

Neuropeptides are composed of short chains of amino acids that are synthesized from larger precursor peptides that are made in the nucleus [4, 5]. The processing of precursor peptides can vary across tissues [6]. One such example is somatostatin which is produced in the 14-amino acid form in the hypothalamus, but the 28-residue form in the pancreas and intestine [6]. The precursor peptides are cleaved in the Golgi apparatus and then the final peptide product is packaged into dense core vesicles [4, 7]. Compared to synaptic vesicles which transport classical neurotransmitters and are 30-40 nanometers and clear, dense core vesicles are 80-200 nanometers in diameter and have an electron-dense core [8]. The dense core vesicles carrying the packaged neuropeptides can be released at the dendrites or travel down the axon where they are released either at the synaptic cleft or along the axon [9, 10]. Additionally, while synaptic vesicles release their contents within a millisecond following calcium influx, dense core vesicles take more than 50 milliseconds to release their contents [8].

While many neuropeptides in the brain have excitatory or inhibitory effects akin to classical neurotransmitters, there are many key differences. Firstly, the concentration of neuropeptides packaged in the dense core vesicles, and eventually released, is significantly less than the amount of neurotransmitter that is packaged and released from synaptic vesicles [9]. One likely reason for this is that neuropeptide receptors have a 1000-fold greater binding affinity, in the nanomolar range, compared to those of classic neurotransmitters [9, 11]. Importantly, neuropeptides rarely exist on their own in neurons and are mainly present in neurons with at least one classical neurotransmitter [12]. Neurons that contain at least one classical neurotransmitter in addition to one or more neuropeptides, therefore can communicate via both fast (2-5 ms) and slow (100-500 ms) neurotransmission given the differences in release dynamics and signaling through ionotropic (neurotransmitters) and metabotropic receptors (neurotransmitters and neuropeptides) [9]. This allows for more complex and nuanced signaling. In contrast to classical neurotransmitters, neuropeptides are released in response to high frequency burst firing [9, 13]. Once released, neuropeptides act via volume transmission and diffuse

throughout the extracellular space, often travelling long distances (μm to mm) beyond the synaptic cleft [9, 11, 13].

Once released, neuropeptides act on specific metabotropic neuropeptide receptors that are G-protein coupled (GPCRs) [11, 14-16]. GPCRs contain seven transmembrane domains and can be subdivided into three main families, G_q , $G_{i/o}$, and G_s with different downstream pathways and effects on the postsynaptic cell. $G_{i/o}$ -coupled receptors have inhibitory effects through the inhibition of cyclic AMP production, G_s -coupled receptors have excitatory effects through the stimulation of cyclic AMP, and G_q -coupled receptors also have modulatory effects due to the release of intracellular calcium stores [17]. Genes encoding GPCRs are also conserved across species [18]. Neuronal signaling through GPCRs accounts for the slower component of neurotransmission and modulates the fast synaptic transmission from neurotransmitters binding to ionotropic receptors [15, 18-21]. Upon binding to their receptors, neuropeptides are not re-uptaken into the releasing neuron or degraded, but rather can persist for minutes and their action is halted by diffusion [7, 22, 23].

While it is well-established that many GABAergic neurons in the brain, especially in the cortex, express neuropeptides such as vasoactive intestinal peptide, somatostatin, neuropeptide Y, and substance P, recent advances in single-cell RNA sequencing (scRNA-seq) have revealed that neuropeptides may be far more prevalent than initially assumed [24]. In particular, a recent scRNA-seq study found that 98% of mouse cortical neurons express at least one neuropeptide precursor or neuropeptide receptor gene [24]. This highlights the previously underestimated prevalence of neuropeptides and neuropeptide signaling in the brain.

The basal ganglia

The basal ganglia are a group of subcortical brain structures that work together to modulate motor control, motor learning, habit formation, and reward [25-37]. The principle input structure of the basal ganglia is the striatum [38]. The striatum can be subdivided into the dorsal and ventral striatum with the dorsal striatum being important for motor function and the ventral striatum for motivation and reward [25-37]. Interestingly, there is no clear boundary between the dorsal and ventral striatum on the basis of cytoarchitecture, myeloarchitecture, or chemoarchitecture [39]. For this reason, Voorn et al. (2004) has suggested that the medial-lateral axis may be equally important and that a more accurate description of the functional organization of the striatum is a graded axis between dorsolateral-to ventromedial [39].

Dorsal striatum

Organization

The dorsal striatum has been canonically divided into two major regions: the dorsomedial striatum (DMS) and the dorsolateral striatum (DLS) [40]. The DMS corresponds to the caudate in humans and DLS the putamen [40]. Additionally, the DMS receives inputs from the prefrontal and associative cortices, while the DLS mainly receives sensorimotor cortical inputs [40-43]. As such, it has been suggested that the DMS is important for early

stages of motor learning and goal-directed actions, while the DLS is important for learned motor actions and motor habits [40].

Cell types

The striatum consists of five main cell types: two projection neuron subtypes termed striatal projection neurons (SPNs), previously named medium spiny neurons given their medium size and high dendritic spine density, which make up 90-95% of all striatal neurons [44-47], as well as three types of interneurons, fast-spiking parvalbumin (PV), low threshold somatostatin, and tonically active cholinergic interneurons that make up the remaining 5-10% of striatal neurons (Fig 1) [48, 49].

SPNs are a class of GABAergic neurons that can be further subdivided into two main classes: direct pathway (dSPNs) and indirect pathway (iSPNs) neurons that express different genetic markers and have different downstream outputs [38, 47, 50, 51]. Markers for dSPNs include dopamine D1 receptors, dynorphin and substance P and for iSPNs include dopamine D2 receptors, adenosine 2A receptors, and enkephalin. Additionally, these markers are highly conserved across species [52-54]. Electrophysiologically, both populations have low baseline activity and are driven to spike by excitatory inputs; however, iSPNs have higher intrinsic excitability [55].

Fast-spiking PV interneurons are a subclass of striatal GABAergic interneuron similar in size to SPNs, but lacking dendritic spines [56, 57]. They have complex dendritic arborizations whereby 4-8 dendrites arborize into second and third order branches and have dense axonal collaterals extending 300 μ m [58]. A majority of SPNs receive connections from one or more PV interneurons [58-60]. These interactions hyperpolarize the SPNs [59, 60]. While PV interneurons are not active at baseline, they are highly excitable and can reach a firing frequency of over 400 Hz [47].

The second subclass of GABAergic interneurons are the somatostatin-expressing low threshold spiking interneurons [48]. They are similar in size to the other GABAergic neurons in the striatum, but compared to SPNs and PV interneurons, they are relatively depolarized [48, 61]. Their dendritic branching is not complex, but their dendritic arbors can extend long distances and synapse onto the dendrites of SPNs [61, 62].

The final main subclass of interneuron in the striatum is the tonically active cholinergic interneurons. Their long processes and large soma allow them to coordinate activity across large areas of the striatum [63, 64]. They are spontaneously active and encode information through pauses in firing [65-67]. Unlike the other classes of striatal interneurons, which synapse directly on SPNs, cholinergic interneurons disinhibit incoming dopamine axons [68-70]. There is little evidence of direct connections between cholinergic interneurons and SPNs [47]. Interestingly, cholinergic interneurons can also express dopamine D2 receptors [63].

The cells of the dorsal striatum can be further divided into two different compartments: the patch and the matrix [71]. A majority of the striatum (85-95%) is rich in neurons containing calbindin and considered the matrix, while the patches, or striosomes, are

interspersed and are characterized by high expression of mu opioid receptors [56, 72]. While both patch and matrix contain dSPNs and iSPNs, patches contain more dSPNs than iSPNs and neurons within each compartment only communicate with other neurons within the same compartment [73]. Generally, the patches receive more limbic inputs while the matrix is more sensorimotor [74, 75].

Inputs and outputs

Both classes of SPNs receive and integrate a variety of inputs including glutamatergic inputs from the cortex and thalamus, as well as local inhibition from interneurons and other SPNs (Fig. 2) [38]. In addition, the striatum also receives a large number of dopaminergic inputs from midbrain neurons in the substantia nigra pars compacta (SNc) [32]. Additionally, there are feedback loops within the basal ganglia where the external segment of the globus pallidus sends GABAergic projections back to the striatum [76-80]. This is the most prominent source of inhibitory input into the striatum [79].

dSPNs “directly” innervate the substantia nigra pars reticulata (SNr), while iSPNs go through the globus pallidus and subthalamic nucleus before reaching the SNr [32, 38, 47]. Through the different output pathways, the excitatory inputs from the cortex are able to provide both inhibitory and excitatory regulation of the basal ganglia [32]. Interestingly, the outputs of the striatum are topographically organized with dorsolateral SPNs innervating more dorsolateral populations in the SNr and more ventromedial SPNs projecting to more ventromedial portions of the SNr [81]. In addition to projecting out of the striatum, approximately 15% of SPNs synapse onto neighboring SPNs, mostly onto other SPNs of the same subtype, although some iSPNs synapse onto dSPNs [60, 82]. However, recent advances in technology and circuit tracing techniques have shown that the indirect and direct pathway segregation may not be as straightforward as originally thought [83, 84].

Behavior/function

Cannonicly it is thought that the activity of the direct and indirect pathways have opposing effects on motor behaviors with the direct pathway facilitating movement and the indirect pathway suppressing motor output [32]. Movement is thought to arise from pauses in GABAergic inhibition in the SNr due to activation of the direct pathway [32]. Excitatory inputs from the cortex activate GABAergic dSPNs which inhibit the GABAergic neurons in the SNr [85, 86]. More recent studies have challenged this and shown that there is activation of both output pathways during action initiation [87]. The current theory proposes that the striatum determines the appropriate action by incorporating sensorimotor, cognitive, and emotional information from the cortex [25, 88, 89].

Nucleus accumbens

Cell types

The nucleus accumbens (NAc), sometimes referred to as the ventral striatum, can be subdivided into three parts: the core, the lateral shell, and the medial shell (MSH) (Fig. 1) [47, 90]. The NAc sits below the dorsal striatum, with the core surrounding the anterior commissure and the shell surrounding the core. The cell types in the striatum and NAc

are largely similar, but the distributions and cellular properties vary slightly and the cells have different inputs and outputs [47].

Similar to the dorsal striatum, SPNs are the main subtype in the NAc core, although they are slightly smaller in size [91]. Additionally, the inputs to these SPNs are quite different compared to the dorsal striatum inputs onto SPNs. The NAc core primarily receives inputs from the insula, ventral tegmental area, and ventral pallidum [92-94]. Compared to the other ventral striatum regions, the NAc core is most similar to the dorsal striatum in terms of general anatomical characteristics, cell types, organization, and segregation of iSPNs and dSPNs [47].

In contrast to the NAc core, the NAc MSh is quite distinct from the dorsal striatum. The two main cell types are still dSPNs and iSPNs, which are equally distributed, however the cell bodies are smaller and have fewer spines than both the core and the dorsal striatum [47, 91]. The SPNs in the NAc MSh are also the most active SPNs in the striatum with baseline firing rates of 1.5-3 Hz, in particular the iSPNs [82, 95-97]. Additionally, in contrast to the dorsal striatum, the division between dSPNs and iSPNs is less clear, with 5-30% of cells containing both types of dopamine receptors [98-100]. A third type of dopamine receptor, D3Rs, are also expressed in the NAc MSh [101, 102].

While the SPNs in the NAc MSh still make up 90-95% of all of the cells in the region, the proportion of interneurons making up the other 5-10% of cells differs from the dorsal striatum [47]. There are almost no PV interneurons in the NAc MSh [103]. In turn, there are more of the other two interneuron populations expressing somatostatin and acetylcholine [47].

The NAc MSh also shows differences related to the patch and matrix distinctions of the dorsal striatum. Both areas still exist, but the patches are far more prevalent compared with the matrix [47]. Accordingly, there is significantly greater expression of mu opioid receptors in the NAc MSh [104].

On the lateral side of the NAc core lies the NAc lateral shell (LSh). While the cellular properties and distributions of the NAc MSh and LSh are quite similar, they have differential connectivity and inputs. In particular, neurons from the laterodorsal tegmentum synapse onto dopamine neurons projecting to the LSh, leading to reward signaling [105]. Additionally, projections from the LSh innervate the lateral ventral tegmental area and disinhibit dopamine neurons [106, 107].

Inputs and outputs

While SPNs are present throughout the dorsal and ventral striatum in approximately equal proportion, the inputs and outputs of these SPNs differ greatly. These different circuits lead to diverse functions between not only the dorsal striatum, but also between the NAc core and MSh, even though the cell types are largely the same. While both the NAc core and MSh receive inputs from the anterior cortex, hippocampus, thalamus, amygdala, ventral pallidum, lateral hypothalamus, dorsal raphe, and ventral tegmental area, the relative contributions of these inputs and subregion specificity differs [108]. The NAc MSh

also receives additional inputs from the septum [108]. With the exception of the inputs from the ventral tegmental area which are dopaminergic and the serotonergic inputs from the dorsal raphe, the other inputs to the NAc are predominantly glutamatergic [47]. In total, a recent brain mapping study found that 57 unique brain regions project to the NAc core, 55 to the NAc lateral shell, and 72 to the NAc MSh [109]. Additionally, Li et al. (2018) found no differences between the inputs to dSPNs compared to iSPNs in the NAc core and MSh [108]. Electron microscopy studies have shown, however, that the dopaminergic inputs to the NAc MSh synapse onto the dendritic shafts of SPNs unlike in the dorsal striatum where they preferentially synapse onto the dendritic spines [110].

Given that similar to the dorsal striatum, the NAc also contains two types of SPNs expressing different dopamine receptors, it was falsely assumed that in the NAc the type of dopamine receptor expressed defined the output pathway [111, 112]. With recent technological advances, it has become clear that the output pathways of SPNs in the NAc are not coded by the subfamily of dopamine receptor they express [99]. The SPNs from the NAc ultimately project to and influence the thalamus through the ventral pallidum or the ventral mesencephalon [112]. While there is a direct pathway that only D1 SPNs appear participate in that goes through the ventral mesencephalon and then to the thalamus, both D1- and D2-SPNs project to the ventral pallidum and inhibit cells that either project directly to the thalamus or through the ventral mesencephalon [112]. Thus, D1- and D2-SPNs can each inhibit or disinhibit the thalamus (Fig. 3) [99]. These findings highlight the ventral pallidum as an important region in determining the effect of NAc outputs.

Behavior/function

The NAc has been implicated in a variety of behaviors including appetitive and aversive responses, feeding, social interaction, reinforcement learning, motivation, and reward [33-37]. The behaviors mediated by the NAc can be divided based on the diverse inputs to the NAc MSh.

The dopaminergic inputs from the ventral tegmental area have classically been associated with reward [113-116]. More specifically, the dopaminergic inputs to the NAc are thought to encode reward prediction error, the difference between the expected and received reward [114, 115]. When an unexpected reward is received, midbrain dopamine neurons elicit phasic firing patterns which are inhibited by adverse events [117, 118]. However, aversive stimuli have also been found to cause dopamine release and phasic firing [119-123]. Therefore, the current theory is that the dopaminergic projection from the ventral tegmental area to the NAc plays a critical role in motivational control and salience [114, 124-126]. The opposing control of reward and aversion associated with the NAc has been tied to differential inputs and outputs of the NAc MSh and LSh [107, 127]. More specifically, the dopaminergic projections to the NAc MSh have been found to encode aversive-predicting stimuli, while dopaminergic terminals in all other NAc subregions are inhibited by aversive stimuli [127]. Along these lines, it is thought that addiction “hijacks” the mesolimbic dopamine system by increasing dopamine concentrations in the NAc as

well as the ventral tegmental area [128-131]. Additionally, drugs of abuse have also been shown to change synaptic plasticity in the NAc [128].

The amygdala to NAc inputs have been shown to be important in fear salience detection [132-135]. Interestingly, the NAc core has been more implicated in fear, compared with the shell [136, 137]. Aversive conditioned stimuli can evoke dopamine release in the NAc [138, 139]. The glutamatergic inputs synapse in close proximity to the dopamine varicosities, thus influencing the SPNs by modulating the dopaminergic afferents [140, 141]. Additionally, the amygdala to NAc circuit has been found to regulate the persistence of fear extinction [142]. While the amygdala alone has been shown to be necessary for acquisition and consolidation of fear extinction, it is specifically the projection to the NAc that allows for retrieval of the fear extinction memory [143, 144]. Thus, the NAc has been suggested to be critical for fear extinction [145-147]. Corriua et al. (2016) found that specific activation of this circuit is able to decrease long-term fear in rats [142].

The hippocampus also provides significant inputs to the NAc [108]. There is strong evolutionary drive to remember the context of both rewarding and fearful experiences [148]. The hippocampal input to the NAc MSh is important for driving NAc MSh activity to help regulate goal-directed behaviors [149, 150]. Hippocampal activity itself has been found to be altered by contextual features of rewarding stimuli [151, 152]. Additionally, cocaine, a rewarding stimulus, has been found to strengthen the hippocampus to NAc connection [153]. LeGates et al. (2018) found that high frequency stimulation of the hippocampus induced long-term synaptic potentiation in the NAc as well as persistent contextual reward behavior [148]. Silencing these synapses disrupted the formation of only the contextual reward-related memories, but not the primary reward processing itself [148]. Additionally, these hippocampal to NAc connections have been found to be sensitive to chronic stress [148]. Specifically, LeGates et al. (2018) using a chronic multimodal stress paradigm where the mice were stressed daily for 10-14 days and found that chronic stress not only induced anhedonia, or the inability to experience the rewarding aspects of pleasurable stimuli, but also weakened the excitatory synaptic inputs from the hippocampus to NAc [148]. Together, these results implicate the hippocampal inputs to the NAc as an important integrator of reward and aversion pathways.

Another important input into the NAc is from the lateral hypothalamus [47]. Importantly, these inputs provide orexin and melanin-concentrating hormones to the NAc MSh [154, 155]. This connection provides important metabolic and motivational information to the NAc MSh [47].

Striatal-associated behaviors used in this thesis

Accelerating rotarod

The accelerating rotarod is a common test to measure motor coordination and learning [156-159]. Mice are trained to walk on a rotating rod that increases in velocity at a constant rate for a set period of time (generally 5 minutes). Performance is measured by recording the velocity at which the mice are unable to stay on the rod or when they rotate

backwards. Initial performance on the task is a measure of general coordination and performance over the course of the 12 trials is a measure of motor learning. Changes in performance on the accelerating rotarod task have been linked to the basal ganglia [160-162]. *In vivo* electrophysiological recordings of the striatum during the task have shown that neuronal activity in the striatum is highly correlated with performance [160, 163]. In particular, the dorsomedial striatum is thought to be important in early training, while the dorsolateral striatum is activated after extensive training [161].

While the accelerating rotarod has canonically been associated with the dorsal striatum, a previously underappreciated role of the ventral striatum has become apparent [164-166]. Selective ablation of iSPNs in the NAc impaired rotarod learning and genetic manipulation of genes selectively in the NAc have led to changes in rotarod performance and learning [164-166]. This is consistent with a theory that learning involves both the dorsal and ventral striatum and the interaction between the two [81]. Additionally, manipulations of both subtypes of SPN have been found to impact rotarod performance [161, 162, 164, 167, 168]. Thus, performance on different aspects of the rotarod involves both SPN populations across the dorsal and ventral striatum.

Progressive ratio lever pressing

The progressive ratio lever pressing task is a common test to measure motivation [169-171]. The task requires animals to press a lever in order to receive a reward. The number of lever presses that yield a reward increases progressively throughout the course of the task, as the name implies. The maximum number of times an animal is willing to press the lever for a single reward is termed the breakpoint and used as a proxy for motivation. The ventral striatum has been implicated in this task [172, 173]. Drugs of abuse, including cocaine and amphetamine, have been shown to increase the breakpoint [174, 175]. Excitotoxic lesions to the NAc were found to increase the breakpoint [171]. Interestingly, lesions to the ventral pallidum, the main downstream structure of the NAc decreased the breakpoint [176]. Breakpoint was unaffected in animals with lesions to the medial or lateral dorsal striatum [171].

More recent studies have examined differential contributions of SPN subtypes on breakpoint in the progressive ratio task [177-180]. These studies have yielded mixed results. Overexpressing the dopamine D2 receptor in the striatum (dorsal and ventral) during development was shown to impair motivation [181], while over-expression in adulthood resulted in increased motivation [182]. A chemogenetic study that inhibited the NAc to ventral pallidum projection also increased the breakpoint [178]. Soares-Cunha et al. (2016) used optogenetics to selectively activate either D1- or D2-expressing SPNs in the NAc and found that activation of both pathways increased breakpoint. Inhibition of D2-expressing SPNs, however was associated with decreased breakpoint [180]. Together, these studies implicate the NAc as an important regulator of motivation as assessed using the progressive ratio test, but also highlight that more research is needed to determine the contributions of SPN subtypes.

Sucrose preference test

The sucrose preference test is a reward-based test, used as a measure of anhedonia, or the decreased ability to experience pleasure [183-187]. Rodents are born with a natural interest in sweet foods and solutions and thus a reduction in this preference is considered anhedonia [183, 187, 188]. The sucrose preference test involves providing mice with two water bottles, one containing standard water and the other containing sucrose water. Mice are first habituated to the bottle setup as well as exposed to the sucrose. For the test itself, mice are individually housed and the percentage of total liquid consumed that is the sucrose solution is recorded [183].

Exposure to chronic stress has been shown to reduce or abolish sucrose preference in mice, but can be restored with selective serotonin reuptake inhibitors [148]. The loss of sucrose preference after chronic stress has been hypothesized to be due to a change in hedonic state where the sucrose becomes less rewarding [184]. The NAc MSh has been implicated in the sucrose preference test. It has been shown that when the dopaminergic inputs to the NAc MSh are lesioned in mice, the generation of sucrose preference is delayed [189]. Additionally, using optogenetics to stimulate the infralimbic to NAc MSh glutamatergic inputs, resulted in a sustained increase in preference for sucrose [190]. Mice displaying a reduction in sucrose preference after exposure to chronic stress also display SPN hypertrophy in the NAc MSh, which can be reversed with antidepressants along with the decreased sucrose preference [191].

Neuropeptides in the nucleus accumbens medial shell

It has recently become clear that one aspect that makes the NAc MSh particularly distinct from the other regions of the striatum is the role endogenous neuropeptides play in modulating signals to amplify or dampen behavior [47]. The most prominent neuromodulators known to regulate the NAc MSh are the endogenous opioids [47]. There are four pairs of endogenous opioids and their associated receptors in the NAc MSh: mu opioid receptors and Beta-Endorphin (BEND), delta opioid receptors (DOPR) and enkephalins (ENK), kappa opioid receptors (KOPR) and dynorphins (DYN), and nociceptin receptors (NOPR) and nociceptin [47]. Notably, all four of these neuropeptide receptors are $G_{i/o}$ -coupled and have an inhibitory effect through their activation of G-protein inward rectifying potassium (GIRK) channels [47]. While the endogenous opioids have the ability to signal both synaptically and through volume transmission, they generally appear to do the latter given the high expression on their receptors outside of the synapse [192-196].

Kappa opioid receptor/dynorphin

Generally, in the brain, the KOPR/dynorphin system is known to be important in aversive signaling in the context of stress. More specifically, KOPR activation is important for conditioned place avoidance, shock avoidance, odor avoidance, social defeat, and learned helplessness in the forced swim model [197-203]. In the NAc MSh, KOPRs appear to act mostly presynaptically, modulating glutamatergic, dopaminergic, and serotonergic inputs [47]. Glutamatergic inputs to the NAc MSh are thought to be important

for conveying cognitive and saliency information, and recordings have shown that in the presence of KOPR activation, postsynaptic EPSPs are reduced, implicating KOPR in regulating glutamate transmission [204]. Additionally, KOPRs have been found to differentially interact with D1 and D2 SPNs in the NAc MSh biasing signaling towards D1 or D2 SPN activity [82]. In addition to regulating glutamatergic transmission in the NAc MSh, KOPRs also modulate the dopaminergic terminals [205-210]. One specific example was by Carlezon and colleagues (1998) who were able to show that the rewarding effects of cocaine in the NAc were negatively regulated by overexpression of CREB, which is regulated by KOPR [205].

KOPR/dynorphin signaling in the NAc MSh is also believed to be involved in appetitive behaviors [47]. KOPR stimulation in the anterior NAc MSh is implicated in positive reactions to sweet sucrose, while KOPR stimulation in the posterior NAc MSh reduced these positive reactions [211]. Similar results were seen with optogenetic stimulation of dynorphinergic neurons in the dorsal vs ventral NAc MSh, generating either a real time place preference or avoidance [100].

Mu opioid receptor/beta-endorphin

MOPRs are associated with motivated behaviors, and many drugs of abuse, including heroin, morphine, oxycodone, and fentanyl, amongst others, act as highly potent MOPR agonists [47]. MOPRs augment many behaviors such as analgesia, food intake and social investigation, but importantly are not needed for baseline behavior, indicating that they act as a state-dependent neuromodulator that is recruited to enhance behavioral responses to stress [212-215]. MOPR stimulation in the NAc MSh is sufficient to drive food intake as well as other types of motivated behaviors including drug seeking, pair-bond formation in prairie voles, sexual behavior, and operant conditioning for food rewards [216-223].

Delta opioid receptor/enkephalin

Similar to the other opioids, DOPR stimulation leads to analgesic responses to pain [224-226]. Additionally, it induces anxiolytic states by which animals spend more time in the open arms of the elevated plus maze and in the center of the open field arena [225]. In contrast to MOPRs, DOPR activity appears necessary for basal function as knockout mice show anxiety-like phenotypes [225]. Additionally, similar to the other opioids, DOPR signaling plays a role in food intake [216, 218, 227]. DOPR signaling in the NAc also has also been found to have a protective nature to repeated stress such as social defeat [228, 229]. In one such study, Henry et al., (2018) quantified *Penk* mRNA levels by *in situ* in the amygdala of mice after repeated social defeat and found that mRNA levels only decreased in mice that were not resilient to repeated stress [229].

In addition to expression on SPNs, DOPRS are also expressed on cholinergic interneurons [98, 230]. Additionally, in the patch regions of the dorsal striatum, DOPRs reduce lateral GABAergic inhibition from iSPNs [230].

Nociceptin receptor/nociceptin

The least is known about the role of NOPR and nociceptin signaling in the NAc MSh. Nociceptin is an anxiolytic opioid that helps facilitate stress reduction both acutely and chronically [231-236]. While genetically similar to KOPR, NOPR activation does not induce conditioned place preference or avoidance [237]. One suggested explanation for this is that it may act as a general dampening signal that reduces the value of stimuli [47]. Interestingly, when it is locally infused into the NAc MSh, nociceptin enhances food intake in rats, similar to both MOPR and DOPR [238].

Other neuropeptides in the NAc MSh

While the opioids are the most studied neuropeptides in the NAc MSh, there is emerging research on other neuropeptides that modulate behaviors in the NAc MSh [47]. The hypothalamus contains many neuropeptide-expressing neurons (orexin, melanin-concentrating hormone, and agouti-related peptide) and also projects to the NAc MSh, bringing many peptides into the NAc MSh through its projections [47]. Additionally, there are local sources of neuropeptides including Substance P which is expressed in dSPNs and somatostatin in the low threshold spiking interneurons [47]. Substance P appears to interact with MOPR as mice lacking the Substance P receptor, neurokinin 1, mice do not have a morphine conditioned place preference, but have a robust cocaine place preference [239]. Additionally, Substance P stimulation has been shown to reduce EPSPs and enhance dopamine release [240]. While studies looking at neuropeptides in the NAc MSh are still limited, the peptides that have been studied show that they can enhance dopamine transmission (Cholecystokinin and Neuropeptide Y), enhance appetitive behaviors (Neuropeptide Y, Melanin-concentrating hormone, Corticotropin-releasing factor, Orexin, and Oxytocin), and enhance aversive motivated behaviors (Oxytocin and Corticotropin-releasing factor) [241-249].

Gastrin releasing peptide

Gastrin releasing peptide (GRP) is a 27-amino acid bombesin-like neuropeptide that signals through a G_q-coupled GPCR (gastrin releasing peptide receptor, GRPR, also called BB2), which has mainly been studied in the spinal cord in the context of itch and pain [250, 251]. In the brain, GRPR expression and GRP-GRPR signaling has been reported in the amygdala [252, 253], paraventricular thalamus [254], and auditory cortex [255]. In each of these regions, GRP wash-on led to depolarization of GRPR-expressing cells [251, 254, 255].

In the spinal cord, GRP-GRPR signaling is the molecular basis of itch [251, 256-258]. Here, GRP is produced by excitatory interneurons in the dorsal horn of the spinal cord that activate third-order GRPR-expressing interneurons in the dorsal horn whose excitation activates spinobrachial projection neurons [259-261]. Ablation of GRPRs both globally and locally in the spinal cord causes strongly reduced responses to pruritogens, substances that when introduced to the skin lead to itch, implying that glutamate signaling alone is not sufficient to cause itch [256, 262]. Pagani et al. (2019) used optogenetics to

demonstrate that GRP release in the dorsal horn required repetitive burst-like activation, establishing GRP signaling as the spinal gating mechanism for itch [251].

In the amygdala, *Grp* is highly enriched in the lateral nucleus where it excites inhibitory interneurons that contain GRPR on their cell surface [252]. Global ablation of GRPR significantly reduced this inhibition, thus enhancing synaptic long-term potentiation in the cortico-amygdala synapses, leading to prolonged fear memory for auditory and contextual cues [252]. Additionally, acute stress has been found to increase GRP expression in the amygdala [253]. In a separate study, mice lacking GRP were found to freeze longer in response to both contextual and auditory fear conditioning paradigms [253]. Together, these results indicate that the GRP-GRPR signaling in the amygdala is important for suppressing excessive fear memories [252, 253].

GRPR has also been found in the paraventricular thalamus [254]. While a specific behavioral role for GRP signaling in this region has not been explored, low nanomolar concentrations of GRP led to significant depolarization and rhythmic bursting or tonic firing of GRPR-expressing neurons [254]. Furthermore, Hermes et al. (2013) found that this response was due to suppression of a Ba^{2+} -sensitive inward rectifying potassium conductance [254]. Interestingly, these results were replicated by Pagani et al. (2019) in the dorsal horn population, providing evidence that Ba^{2+} -sensitive inward rectifying potassium channels mediate GRP-induced depolarization [251].

In the auditory cortex, GRPR is expressed in vasoactive intestinal peptide-expressing interneurons [255]. Interestingly, in the auditory cortex, GRP-GRPR signaling was found to enhance auditory fear memories [255]. This is opposite of what was seen in the amygdala where GRP was found to suppress fear memories, including auditory fear memories [252, 253]. A key difference between these studies, however, was that Melzer et al. (2021) knocked out *Gpr* only in the auditory cortex [255] whereas, the two studies looking at GRP-GRPR signaling in the amygdala globally knocked out *Grpr* [252] or *Grp* [253].

Dissertation research questions

Recent technological advances, particularly in scRNA-seq have highlighted that inter-neuronal chemical neuronal signaling is more complex than just classical neurotransmitters and their receptors. Rather, many neurons express RNA for one or more neuropeptide precursors or receptors, indicating they can likely signal and respond using neuropeptide signaling in addition to their classical neurotransmitter. One such scRNA-seq study was performed previously in the lab and identified a population of midbrain dopamine neurons that expressed *Grp* mRNA [263]. The primary goal for my dissertation was to further investigate this population and determine if GRP had a functional role in mesolimbic circuits, and, if so, what role. Specifically, I sought to address the following questions: 1) are there GRP receptors in the targets of these midbrain dopamine neurons? 2) what is the identity of the *Grpr*-expressing neurons in the NAc MSh and what are their electrophysiological properties? 3) are there other *Grp*⁺ inputs to the NAc MSh and from where? 4) what are the effects of GRP in slice and *in vivo* on the

Grpr⁺ neurons? These questions will be addressed in the subsequent chapter. Finally, I will provide concluding remarks on the implications of this research and future directions.

Figures

Figure 1

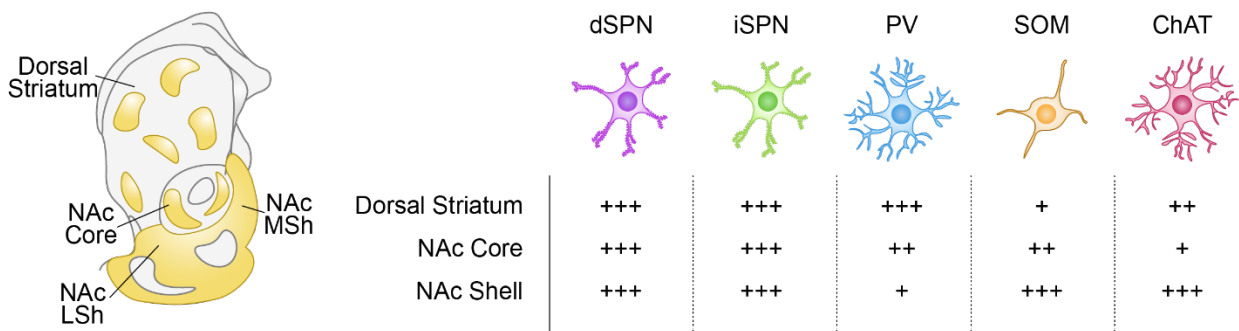


Figure 1: Striatal subregions and cell types.

Schematic illustrating the striatal subdivisions, cell types, and their relative proportions. Left, coronal section of the striatum showing the main regions: dorsal striatum, nucleus accumbens core (NAc Core), nucleus accumbens medial shell (NAc MSh), and nucleus accumbens lateral shell (NAc LSh). Patch regions are shaded yellow and the matrix is in gray. Right, the five major cell types in the striatum: direct pathway striatal projection neurons (dSPNs), indirect pathway SPNs (iSPNs), parvalbumin/fast-spiking interneurons (PV), somatostatin/low-threshold spiking interneurons (SOM), and cholinergic/tonically active interneurons (ChAT). +'s indicate relative proportions across regions: +++ high relative expression, ++ average relative expression, + low relative expression. Figure adapted from Castro and Bruchas, 2019 [47].

Figure 2

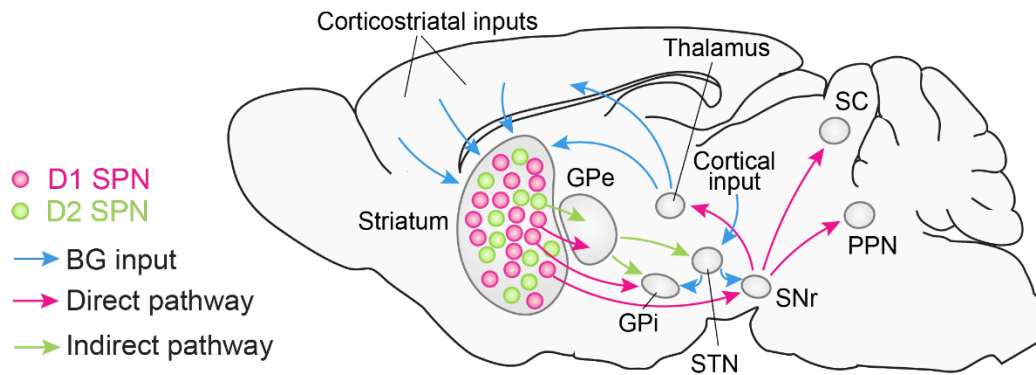


Figure 2: Dorsal striatum circuits.

Schematic illustrating the indirect and direct pathways of the dorsal striatum. Arrows indicate corticothalamic inputs (blue) to the dorsal striatum and direct (magenta) and indirect (green) output pathways. Globus pallidus external segment (GPe), globus pallidus internal segment (GPi), subthalamic nucleus (STN), substantia nigra pars reticulata (SNr), superior colliculus (SC), pedunculopontine nucleus (PPN). Figure adapted from Gerfen and Bolam, 2016 [32].

Figure 3

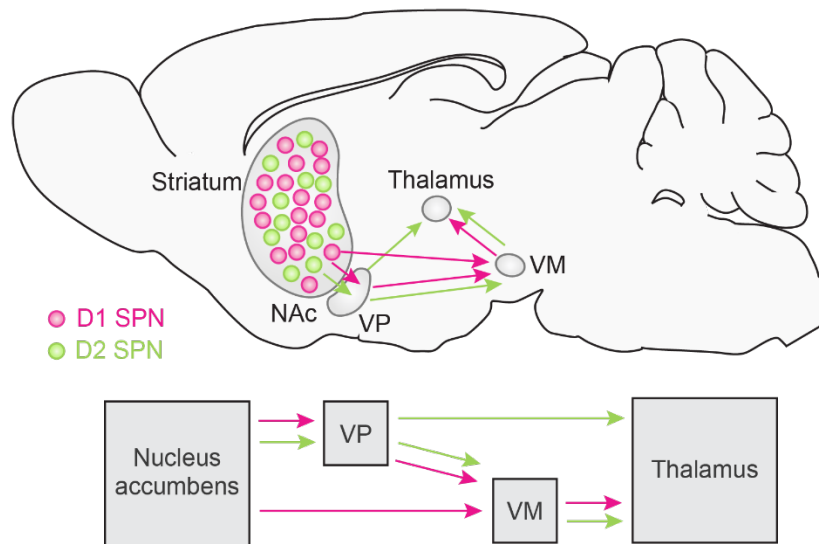


Figure 3: Nucleus accumbens output pathways.

Schematic illustrating the output pathways of striatal projection neurons (SPNs) in the nucleus accumbens (NAc). Arrows indicate projections of both D1 SPNs (magenta) and D2 SPNs (green). Both D1 and D2 SPNs can participate in direct and indirect pathways to inhibit or disinhibit the thalamus. Nucleus accumbens (NAc), ventral pallidum (VP), ventral mesencephalon (VM).

Chapter 2: Characterizing a Novel Peptidergic Circuit in the Nucleus Accumbens Medial Shell Defined by Gastrin Releasing Peptide Receptor

Introduction

Neuromodulators play a critical role in nervous system activity via their regulation of neuronal intrinsic excitability, synaptic transmission, and plasticity. The largest family of known neuromodulators are neuropeptides—small short-chain polypeptides that act via metabotropic G-protein-coupled receptors (GPCRs) [47]. Compared to classical neurotransmitters, neuropeptides lead to diverse effects on the neurons they act on and regulate numerous physiological functions such as eating, sleeping, sexual behaviors, stress response, and pain perception [254]. Additionally, neuropeptides have distinct machinery for their synthesis and transport and cannot be synthesized or recycled in axons [7]. It has recently become appreciated that many neurons co-express genes for both neurotransmitters and one or more neuropeptides [24, 263]. In particular, a recent single-cell RNA-sequencing (scRNA-seq) study found that 98% of mouse cortical neurons express at least one neuropeptide or neuropeptide receptor gene [24]. As such, neuropeptides are poised to make a major contribution to brain function and behavior; however, an understanding of their breadth, scope and mechanisms of action is currently limited.

One such neuropeptide is Gastrin-releasing peptide (GRP), a bombesin-like neuropeptide that signals through a G_q -coupled GPCR (gastrin releasing peptide receptor, GRPR, also called BB2), which has mainly been studied in the spinal cord in the context of itch and pain [251]. While the functions of Grp within the brain are not well understood, a few studies have demonstrated that Grp can alter the activity of GRPR-expressing neurons. For example, in the lateral nucleus of the amygdala, *Grp* provides negative feedback regulating fear. In this region, Grp excites the GABAergic interneurons that express GRPR, increasing their inhibition of the principle neurons expressing *Grp* [252]. Additionally, GRP wash-on in slices from the paraventricular thalamic nucleus leads to membrane depolarization with rhythmic bursts or tonic firing [254]. In the auditory cortex, *Grpr* is expressed in vasoactive intestinal polypeptide (VIP) neurons and GRP release and binding recruits disinhibitory cortical microcircuits and enhances auditory fear memories [255].

A recent study from our lab performed scRNA-seq of midbrain dopamine (DA) neurons and found that 30% of midbrain DA neurons in the ventral tegmental area (VTA) the substantia nigra pars compacta (SNc) express the mRNA for *Grp* [263]. Additionally, *Grp* has been identified as the most differentially expressed genetic marker of VTA DA neurons compared to the SNc DA neurons [264]. More specifically, when looking at the projection targets of the *Grp*⁺ neurons, Kramer et al. (2018) found two populations, one that resides in the ventral SNc and project to the dorsal medial striatum (DMS) and the other that is in the medial VTA and targets the nucleus accumbens medial shell (NAc MSh) [263]. These findings suggest that Grp may be co-released by DA and may be a novel, functionally relevant signaling molecule in the striatum.

The striatum is the main input structure of the basal ganglia and integrates glutamatergic inputs from the cortex and thalamus, DA from the midbrain, GABAergic inhibition from multiple sources, and acetylcholine from cholinergic interneurons, among others [38]. GABAergic striatal projection neurons (SPNs) are the major output neuron of the striatum, comprising 90-95% of the neuronal population [265]. SPNs can be divided into direct-pathway and indirect-pathway neurons based on their primary projections to either the substantia nigra pars reticulata and globus pallidus internal segment (SNr/GPi) or globus pallidus external segment (GPe), respectively (Gerfen, 2000). Direct pathway SPNs typically express type 1 DA receptors (D1) while indirect pathway neurons generally express type 2 DA receptors (D2) [266]. The dorsal striatum (DS) is important for motor functions including action selection and motor learning, while the ventral striatum (NAc) controls motivated behaviors and reward learning [267]. In the DS, bulk activation of direct pathway SPNs (dSPNs) promotes movement and which bulk activation of indirect pathway SPNs (iSPNs) inhibits [268, 269]. Additionally, during movement, both pathways are active and must work in a coordinated manner [32, 270]. The NAc also contains direct- and indirect-pathway SPNs. In the NAc, the direct-pathway SPNs have canonically been thought to directly project to and inhibit the VTA, promoting motivated behavior through disinhibition of VTA GABA neurons that synapse onto VTA DA neurons, which project back to the NAc [112]. The indirect-pathway SPNs first project to and inhibit the ventral pallidum (VP). VP neurons then project to the VTA and ultimately the thalamus, resulting in inhibition of motivated behavior [112].

With increasing use of cell type-specific approaches, it has become clear that our canonical divisions of the striatum: D1 vs D2 SPNs, dorsal vs ventral striatum, indirect vs direct pathway are oversimplified and there is likely further complexity to discover within these circuits [99, 271]. While neuropeptide signaling has been known to occur in the NAc through opioid receptors, these receptors are all $G_{i/o}$ -coupled, and the opioids themselves are synthesized within the NAc [47]. In contrast, the *Grp*⁺ DA neurons identified by Kramer et al. (2018) would bring in neuropeptides from outside the striatum and are hypothesized to increase the excitability of GRPR-expressing neurons [263].

The goal of this study was to assess a possible role of GRP-GRPR signaling in the NAc MSh. We found that *Grpr* is expressed in the NAc MSh mainly in iSPNs. While the genetic profile of *Grpr*⁺ neurons in the NAc MSh was consistent with an iSPN identity, we found that they have unique electrophysiological properties. Specifically, a majority of *Grpr*⁺ cells are spontaneously active at baseline and they are hyper-excitable. We also found that in addition to the *Grp*⁺ DA neurons, the NAc MSh receives glutamatergic *Grp* inputs from the hippocampus and amygdala. Additionally, we show that GRP activates and increases the excitability of GRPR neurons in the NAc MSh both in slice and *in vivo*. Finally, we show that GRPR knockout leads to significant behavioral alternations, including reduced movement in the open field and increased motor coordination. These findings identify a previously uncharacterized cell type in the NAc MSh and map out a functional GRP-GRPR circuit that mediates striatal associated behaviors.

Results

Gastrin releasing peptide receptor is expressed in striatal neurons

Our lab previously discovered two subpopulations of *Grp*-expressing dopamine neurons. One population is located in ventral substantia nigra pars compacta (SNc) and projects to the dorsomedial striatum (DMS). The other is in the ventral tegmental area (VTA) and projects to the NAc mSh [263]. To investigate whether GRP may be a functionally relevant neuromodulator in the nigrostriatal and mesolimbic dopamine circuits, we used fluorescent *in situ* hybridization (FISH) to probe for expression of mRNA encoding the GRP receptor (*Grpr*) in the DMS and NAc MSh (Fig. 1a). Indeed, we found *Grpr* expression in subpopulations of cells in the DMS and NAc MSh (Fig. 1b).

The striatum is composed of two types of SPNs as well as a diverse set of interneurons. To determine the identity of *Grpr*-expressing cells, we used multi-plex FISH with markers for known striatal cell types (Fig. 1c-l). In the NAc mSh, we found that the vast majority of *Grpr*⁺ cells were positive for *Gad2* mRNA (96.29%) (Fig. 1e), and no *Grpr*⁺ cells were *Chat*⁺ (Fig. 1i). This suggests that *Grpr*⁺ cells are GABA-ergic and do not express the primary marker for cholinergic interneurons, the most prevalent interneuron type in the NAc MSh. In the DS, we found similar expression patterns (Fig. 1d,f,h,k,l). Here, 92.66% of *Grpr*⁺ cells were *Gad2*⁺ (Fig. 1f) compared to only 1.90% co-expressing *Chat* mRNA (Fig. 1k). To determine whether the *Grpr*⁺ cells were SPNs or a type of GABAergic interneuron, we probed for *Ppp1r1b1*, which encodes the SPN-marker DARPP-32 (Fig. 1g,h,j,l). Nearly all *Grpr*-expressing cells co-expressed *Ppp1r1b1* in the NAc MSh (93.76%) (Fig. 1j) and DS (75.52%) (Fig. 1l), suggesting they were likely SPNs.

To determine the specific SPN identity of the *Grpr*⁺ cells, we probed for mRNA encoding dopamine D1 (*Drd1*) and D2 (*Drd2*) receptors (Fig. 2a,f), which are typically expressed in direct pathway SPNs (dSPNs) and indirect pathway SPNs (iSPNs), respectively. The majority of *Grpr*⁺ cells in the NAc MSh expressed *Drd2* mRNA (77.26%) (Fig. 2c), suggesting that they could be iSPNs. However, there was a small subpopulation of *Grpr*⁺ cells in the NAc MSh that expressed *Drd1* (26.71%) (Fig. 2d). Multiple studies have reported that a substantial proportion (5-30%) of SPNs in the NAc MSh express both *Drd1* and *Drd2* [98-100]; therefore, we examined whether the *Grpr*⁺/*Drd1*⁺ population in the NAc MSh were dSPNs or SPNs co-expressing both DA receptors (Fig. 2f). We found that 61.51% of *Grpr*⁺ cells in the NAc MSh expressed *Drd2*, but not *Drd1*, 14.10% expressed *Drd1* only, and 12.61% expressed both *Drd1* and *Drd2* (Fig. 2f). Given the preferential expression of *Drd2* in *Grpr*⁺ cells, we probed for the iSPN marker *Penk* (Fig. 2b), and found that it was expressed by the majority (67.38%) of *Grpr*⁺ cells in the NAc MSh (Fig. 2e). We also examined the co-localization between *Drd2* and *Penk* mRNA and found that 63.12% of *Grpr*⁺ cells in the NAc MSh expressed both markers, while 22.17% just expressed *Drd2* and 4.26% only expressed *Penk* (Fig. 2g). Together this analysis indicates that *Grpr*⁺ cells in the NAc MSh are likely to be GABA-ergic SPNs of the indirect pathway. That said, we note that only a small percentage of all *Ppp1r1b1* (1.54%), *Drd2* (3.94%), or *Penk*-expressing (2.79%) cells in the MSh were *Grpr* positive, indicating that *Grpr*⁺ cells are a select subpopulation of iSPNs in the NAc MSh (Fig. 2h-j).

We next examined the identity of the *Grpr*⁺ cells in the DS that we identified in Fig. 1b. While we noted that similar to the NAc MSh population, a majority of *Grpr*⁺ cells in the NAc MSh expressed SPN markers (Fig. 1g-l), the SPN sub-type classification was less clear in the DS. *Drd1* mRNA was expressed in 43.28% of *Grpr*⁺ cells, while *Drd2* mRNA was expressed in 32.74% of DS *Grpr*⁺ cells (Fig. 2k,m,n). We also found that *Penk* mRNA was found in 38.03% of *Grpr*⁺ cells in the DS, consistent with the proportion expressing *Drd2* mRNA (Fig. 2l,n,o). Together this suggests that *Grpr* is expressed in both types of SPNs in the DS.

Given the more consistent iSPN identity of *Grpr*⁺ cells in the NAc MSh and the fact that *Grp* was previously identified as the top differentially expressed marker in VTA compared to SNc dopamine neurons [264], we decided to focus further analysis on the *Grpr*-expressing cell population in the NAc MSh, which receives inputs from the VTA.

Gastrin releasing peptide receptor-expressing neurons in the nucleus accumbens medial shell are morphologically similar to neighboring striatal projection neurons

To further study the properties of GRPR-expressing cells in the NAc MSh, we obtained a transgenic mouse line that fluorescently labels GRPR⁺ cells with GFP. *Grpr*::eGFP mice (Gensat) have previously been shown to express eGFP exclusively in *Grpr*⁺ cells [251]. We bred the *Grpr*::eGFP mice to a mouse line that expresses tdTomato under control of the *Drd1* promoter (D1-tdTomato) [272] to enable visualization of dSPNs (Fig. 3a). We found that consistent with our ISH results, only ~5% of eGFP⁺ cells in the NAc MSh were tdTomato⁺, implying that the eGFP⁺ cells in the NAc MSh are likely iSPNs (Fig. 3c,d). By contrast, 81.21% of eGFP⁺ cells in the DS were D1-tdTomato positive (Fig. 3b,d).

To characterize the morphology of GRPR⁺ cells in the NAc MSh, we filled cells with neurobiotin through a patch pipette and performed cellular reconstructions of eGFP⁺ and eGFP⁻ cells (Fig. 3e-f). We found no differences in dendritic complexity as measured by sholl analysis (Fig. 3g). Additionally, we observed no differences in the number of primary dendrites or total dendrite length in eGFP⁺ vs eGFP⁻ cells (Fig. 3h-i). We also examined dendritic spine number in cells transduced with an mCherry virus and confirmed that GRPR⁺ cells do have spines, and have a similar spine density as neighboring SPNs (Fig. 3j-l). This analysis confirms that GRPR⁺ cells in the NAc MSh are a subpopulation of SPN.

Gastrin releasing peptide receptor⁺ neurons in the nucleus accumbens medial shell have high intrinsic excitability

We next characterized the functional and physiological properties of GRPR-expressing neurons in the NAc MSh using the *Grpr*::eGFP;D1-tdTomato reporter mouse (Fig. 4a-c). We used whole-cell electrophysiology to record from *Grpr*-eGFP⁺ neurons and compared them to neighboring *Grpr*-eGFP⁻ neurons in the MSh (Fig. 4d-e). We also recorded from genetically identified dSPNs (D1-tdTomato⁺, Fig. 4f), presumed iSPNs that were GRPR negative (D1-tdTomato⁻; *Grpr*-eGFP⁻, Fig. 4g), and ChAT⁺ cells using a ChAT-Cre mouse [273] bred with the Ai9 tdTomato Cre reporter line [274] (Fig. 4h). Interestingly, we found that the baseline physiological properties of *Grpr*-eGFP⁺ cells differed significantly from

other SPNs in the NAc MSh. Approximately 75% of the *Grpr*-eGFP⁺ cells were spontaneously active at baseline, which was not characteristic of eGFP⁻ SPNs (Fig. 4d-g). We also found that compared to neighboring cell populations, *Grpr*-eGFP⁺ were significantly more depolarized (Fig. 4i), had higher membrane resistance (Fig. 4j), and had significantly wider action potentials (Fig. 4k). While the ChAT-Cre⁺ cells also tended to be spontaneously active at baseline (53%, Fig. 4h), their membrane potential, membrane resistance, and action-potential half-width were all significantly different from *Grpr*-eGFP⁺ cells (Fig. 4i-k). Additionally, with increasing positive current injection, the ChAT cells were able to continue to fire action potentials, while the *Grpr*-eGFP⁺ cells went into depolarization block with +60 pA current injection (Fig. 4l). This, together with the fact that we did not find any co-localization between *Chat* and *Grpr* mRNA (see Fig. 1i), rules out the possibility that GRPR⁺ cells are cholinergic interneurons.

Given the high excitability of *Grpr*-eGFP⁺ cells in our slice recordings, we asked whether they may be more active *in vivo*. To assess this, we performed FISH in naïve wild-type mice and probed for *Grpr*, *Fos*, and *Drd2* mRNA. We assessed the percentage of *Grpr*⁺ cells in the NAc MSh that were *Fos*⁺ under baseline conditions, using *Fos* as a proxy for neuronal activity. We found that *Drd2*⁺/*Grpr*⁺ cells were nearly twice as likely to be *Fos*⁺ compared to neighboring *Drd2*⁺/*Grpr*⁻ cells (18.73% compared to 8.60% *Fos*⁺) (Fig. 4m-n). Together, this indicates that in the NAc MSh, *Grpr*⁺ cells are a subpopulation of iSPNs that are intrinsically more excitable and more likely to be activated *in vivo*.

Multiple inputs to the nucleus accumbens medial shell express Gastrin releasing peptide mRNA

While it was previously established that *Grp*⁺ dopamine neurons project to the NAc MSh from the VTA [263], the NAc MSh receives inputs from a number of other brain regions including the hippocampus, amygdala, thalamus, and entorhinal cortex, amongst others [108]. We noted that several of these areas appeared to express *Grp* mRNA according to the Allen Brain Atlas (<https://portal.brain-map.org/>). Therefore, we asked whether other inputs into the NAc might also have the potential to co-release GRP in addition to the previously established dopaminergic inputs. To investigate this, we injected retrobeads into the NAc MSh and performed FISH for *Grp* in the main brain regions that send projections to the NAc MSh (Fig. 5a,b). Notably, we found a high percentage of retrobead⁺ cells that were *Grp*⁺ in the VTA, hippocampus (subiculum and CA1), and basolateral amygdala (Fig. 5c,d). We found little to no expression of *Grp* in other regions projecting to the NAc MSh, including the lateral entorhinal cortex, anterior piriform cortex, and thalamus, amongst others (Fig. 5c,d). This suggests that a select group of afferents to the NAc MSh could have the capacity to co-release GRP.

We summed together all of the *Grp*⁺ inputs quantified and found that the main sources of *Grp* into the NAc MSh were from the subiculum (51%), CA1 (34%), VTA (6%), and basolateral amygdala (5%) (Fig. 6d-h). We further probed these four regions for *Vglut* (excitatory neurons) and *Gad2* (inhibitory neurons) and found that in CA1, the subiculum, and the basolateral amygdala, nearly all of the *Grp* inputs were glutamatergic (Fig. 5i-k).

This suggests that not only dopaminergic inputs, but several different glutamatergic inputs to the NAc MSh may have the ability to use GRP as a neuromodulator.

Gastrin releasing peptide depolarizes and increases the excitability of Gastrin releasing peptide receptor-expressing neurons in the nucleus accumbens medial shell

After establishing that several brain regions send *Grp*⁺ inputs to the NAc MSh, we next sought to determine how GRP affects the functional properties of GRPR⁺ cells. To do this, we washed on GRP and measured membrane potential and resistance in *Grpr*-eGFP⁺ cells using whole-cell electrophysiology. We found that 300 nM GRP caused an approximately 6 mV depolarization in *Grpr*-eGFP⁺ cells and an average 140% increase in membrane resistance (Fig. 6a,c,d), consistent with the effects of GRP in other cell types [251, 254, 255]. Importantly, these changes were blocked in the presence of the GRPR-specific antagonist, DPDMB (Fig. 6b,e,f). As a control, we performed the same experiment in D2-GFP⁺ cells in D2-GFP mice [275] (Gensat) (the majority of which are not expected to express GRPR, see Fig. 2i) and did not detect any change in membrane potential or resistance (Fig. 6g-i). D2-GFP⁺ cells did show a slight decrease in membrane resistance in the presence of the antagonist DPDMB (Fig. 6i).

We also compared the baseline membrane potential and resistance of *Grpr*-eGFP⁺ and D2-GFP⁺ cells with and without DPDMB, prior to the addition of GRP. We found a small decrease in the membrane resistance of *Grpr*-eGFP⁺ cells treated with DPDMB compared to untreated cells, which did not occur in D2-GFP⁺ cells (Fig. 7a-d). This suggests that GRPR either has basal activity that can be blocked with DPDMB or that there may be tonic GRP-GRPR signaling in the slice preparation.

Gastrin releasing peptide injection in vivo increases Fos expression in the nucleus accumbens medial shell

After establishing how GRPR⁺ cells in the NAc MSh respond to GRP at a single-cell level in brain slices, we next sought to examine whether GRP has functional effects *in vivo*, as well as any non-cell autonomous effects. We injected 3 μ M GRP unilaterally into the NAc MSh of anesthetized wild-type (WT) mice and harvested the brains 45 minutes later (Fig. 8a). We then performed FISH and probed for *Drd2*, *Grpr*, and *Fos* mRNA and compared the NAc MSh of the injected hemisphere to the uninjected hemisphere (Fig. 8b-c). We found an increase in the total number of *Fos*⁺ cells in the GRP-injected hemisphere compared to the control, implying an increase in active cells upon GRP injection (Fig. 8d). The total number of *Grpr*⁺ cells was unchanged, as expected (Fig. 8e). Importantly, *Fos* was selectively induced in *Grpr*⁺ cells as we found an increase in the total number of *Grpr*⁺ cells that were *Fos*⁺ as well as an increase in the proportion of *Grpr*⁺ cells that were *Fos*⁺ (Fig. 8f-g). Given that *Fos* is a proxy for neuronal activity, these data imply that GRP increases the activity of *Grpr*⁺ cells in the NAc MSh. We also observed that while the percentage of *Fos*⁺ cells that were *Grpr*⁺/*Drd2*⁺ increased, the percentage *Fos*⁺ cells that were *Grpr*⁻/*Drd2*⁺ did not change, showing that the GRP-induced activation was selective to GRPR-expressing cells and did not induce larger network-level activation (Fig. 8h-i).

To verify that this effect was induced by GRP and not due to the injection itself, we repeated the above experiment (Fig. 8), but injected sterile saline instead of GRP. Once again, we performed FISH and probed for *Drd2*, *Grpr*, and *Fos* mRNA and compared the NAc MSh of the injected hemisphere to the un-injected hemisphere (Fig. 9a-b). While we saw an increase in the total number of *Fos*⁺ cells in the saline-injected hemisphere, there was no increase in the total number of *Grpr*⁺ cells or the *Grpr*⁺ cells that were *Fos*⁺ (Fig. 9c-e). Additionally, we saw no increase in the percentage of *Grpr*⁺ cells that were *Fos*⁺ (Fig. 9f). Together, these results indicate that while saline injection can acutely increase neuronal activity in the NAc MSh, it does not preferentially activate *Grpr*⁺ cells.

Gastrin releasing peptide receptor knockout mice were tested in striatal-associated behavioral assays

Given that GRP-GRPR signaling had a strong effect on modulating the excitability of a subset of NAc MSh neurons, we tested whether deletion of *Grpr* affected striatal-dependent behaviors. To generate *Grpr* knock-out (KO) mice, we obtained *Grpr^{fl}* mice [276] and bred them to CMV-Cre mice [277] to induce constitutive deletion of *Grpr* throughout the body. We performed PCR genotyping from tail samples to test for the presence of the floxed allele, the LoxP scar, and an internal region inside *Grpr* exon 2, the exon being deleted. Figure 11 shows PCR results identifying mice with exon 2 deletion and wild-type (WT) controls. *Grpr* KO mice and littermate controls were then run through the open field test, a progressive ratio lever-pressing task, and the accelerating rotarod (Fig. 10). The open field was used to assess general locomotor behavior and analyzed using DeepLabCut [278]. Additionally, we identified behavioral syllables using MoSeq [279]. Following open field testing, the mice began food restriction to achieve between 90-95% of their initial body weight for operant conditioning. In operant chambers, mice were trained to press a lever with daily fixed ratio sessions where a single lever press delivered a food pellet reward. Mice then underwent a single two-hour progressive ratio session to assess motivation. Motivation is canonically associated with the NAc MSh and this task has been shown to be sensitive to manipulation of iSPN activity in the NAc MSh [178]. Finally, after a break of at least five days and return to ad libitum food, the mice were run on the accelerating rotarod to assess motor coordination and learning [280].

Gastrin releasing peptide receptor knockout mice move significantly less in the open field and have differential usage of behavioral syllables

We first assessed the effects of *Grpr* deletion on general locomotor behavior using the open field test. Mice were placed in the open field arena for one hour under red light while an overhead camera recorded their movements. *Grpr* KO mice travelled significantly less distance over the course of the one-hour session compared to littermate controls (Fig. 12a-b). We also assessed time spent in the center of the open field arena, as a proxy for avoidance behavior, and found no differences between *Grpr* KO and control mice (Fig. 12c). Using hierarchical clustering, we found that the top 22 most commonly used syllables fell into three main groups (Fig 12d). Analysis of syllable usage between groups identified significant differences in forward and backward movement as well as turning syllables. In particular, *Grpr* KO mice, on average, had increased usage of static syllables

(0) and decreased use of syllables related to forward motion (2, 5, 10, 11, 12, 17, 19, 20) (Fig. 12e). No differences were observed in the grooming syllables (Fig. 11e). Additionally, a trained decoder was able to distinguish between *Grpr* KO and control littermates based on their different syllable usage (Fig. 12f). Together, this suggests that disruption of GRPR-signaling globally affects mouse behavior patterns.

Gastrin releasing peptide receptor knockout mice show no differences in operant conditioning

To examine how *Grpr* deletion impacts motivation, we tested the mice on a progressive-ratio (PR) lever pressing task. Prior to training, mice were food restricted to 90-95% of their body weight. Importantly there were no differences between WT and KO mice in their starting weight, response to food restriction, or weight on the day of the PR task (Fig. 13a-c). The mice were trained to press the levers for a food pellet reward in daily sessions with a fixed-ratio of 1 paradigm until they successfully received 20 or more rewards in a one-hour session on two consecutive days. No differences were seen in the number of days it took mice to learn the task (Fig. 13d). During the 2-hour PR session, no differences were seen in the total number of lever presses (Fig. 13e), breakpoint (the maximum number of times a mouse is willing to press the lever for a single reward) (Fig. 13f), the number of head entries (Fig. 13g), or the ratio of head entries per lever press (Fig. 12h). The average interval between presses was also not different per mouse (Fig. 13i). Notably, WT mice seemed to increase their frequency of pressing sooner than KO mice as the ratio increased (Fig. 13j). Together, this suggests that disruption of GRPR-signaling does not lead to changes in motivation as assessed by a PR task.

Gastrin releasing peptide receptor knockout mice show enhanced performance on the accelerating rotarod

To examine how *Grpr* deletion impacts striatal-dependent motor coordination and motor learning, we tested the mice on the accelerating rotarod with three trials per day across four consecutive testing days. This task is known to require plasticity in striatal circuits, involving both the dorsal and ventral striatum [160-162, 165, 166, 280]. To make sure weight would not be a confounding factor, we verified that there was no difference in starting weight at the beginning of the rotarod (Fig. 14a). We found that *Grpr* KO mice had overall enhanced performance on this task (Fig. 14b), which was already apparent on the first trial (Fig. 14c). We saw no differences in the maximum performance or the learning rate (Fig. 14d-e). This is notable given that previous studies have found that altered gene expression specifically in the ventral striatum can lead to enhanced rotarod performance [165, 166]. Together, these results suggest that GRPR signaling plays a role in motor coordination, but not motor learning. Importantly, differences in weight could not explain the differential performance, as no difference was seen in weight between groups at the start of the rotarod (Fig. 14a).

Gastrin releasing peptide receptor knockout mice show reduced sucrose preference

Finally, we ran a subset of the *Grpr* KO and control mice in the sucrose preference test (Fig. 15a). This test is a measure of hedonia, or the ability to experience pleasure, and

has been canonically associated with the NAc MSh [189-191]. We found that *Grpr* KO mice had a significantly decreased preference for sucrose (Fig. 15c), indicating anhedonia, which is the loss of pleasure from something that should be rewarding. Importantly, we saw no difference in the starting weight between genotypes, indicating that weight was not a confound (Fig. 15b). These results suggest that GRPR signaling plays a role in hedonic behavior and reward value processing.

Discussion

Neuromodulators play a critical role in nervous system activity via their regulation of neuronal intrinsic excitability, synaptic transmission, and plasticity [1, 2]. It has recently become appreciated that many neurons co-express genes for both neurotransmitters and one or more neuropeptides [24, 263]. As such, neuropeptides are poised to make a major contribution to brain function and behavior; however, an understanding of their breadth, scope and mechanisms of action is currently limited. Here we have identified a novel role for the neuropeptide GRP and its receptor GRPR in mediating the excitability and activity of neurons in the nucleus accumbens. Specifically, we find that *Grpr*⁺ neurons in the NAc MSh are a subpopulation of iSPNs with distinct electrophysiological properties. We mapped the *Grp*-expressing inputs into the NAc MSh and showed that in addition to the previously identified population from the VTA, the NAc MSh receives significant *Grp* inputs from glutamatergic neurons in the hippocampus and amygdala. Furthermore, we show both in slice and *in vivo* that the *Grpr*⁺ neurons in the NAc MSh can be excited by GRP. Finally, we eliminate GRP-GRPR signaling by generating a KO mouse that shows behavioral alterations, suggesting that disruption of GRPR signaling globally affects behavioral patterns, including striatal-associated behaviors. Together, these experiments identify and characterize a new SPN subtype and circuit in the NAc MSh with a functional role in motor coordination and behavior.

Grpr-expressing neurons are a novel subtype of iSPNs in the NAc MSh

Similar to the DS, the NAc MSh is mainly composed of two main types of SPNs that make up 95% of the cells in the region [265]. These SPNs can be further divided into indirect- and direct-pathway based on the type of dopamine receptor they express and their output pathway to other basal ganglia structures [38, 47, 50, 51]. In the NAc MSh, the dSPNs express dopamine D1 receptors, dynorphin, and substance P and have canonically been thought to promote motivated behavior while the iSPNs express dopamine D2 receptors, adenosine 2A receptors, and enkephalin and generally inhibit it [52, 53, 112]. The other 5% of cells in the NAc MSh are local interneuron populations, with the two main interneurons in the NAc MSh being tonically active cholinergic interneurons and low-threshold spiking interneurons [47, 49]. In comparison to the DS, SPNs in the NAc MSh are smaller, slightly less spiny and more active than their DS counterparts [91, 96]. Additionally, between 5-30% of SPNs in the NAc MSh express both types of dopamine receptors [98-100].

When characterizing the *Grpr*⁺ cells that we identified in the NAc MSh, we found that they co-expressed many canonical iSPN markers including *Drd2*, *Penk*, *Gad2*, and *Ppp1r1b*.

We also found they had spines and their morphology was similar to neighboring cells in the NAc MSh. Yet, when we performed whole-cell patch clamp recordings, the *Grpr*-eGFP+ cells appeared quite different from all other examined cell types in the NAc MSh. While iSPNs in the NAc MSh are considered the most depolarized and excitable cells at baseline [47, 82, 95-97], *Grpr*-eGFP+ cells were both more depolarized and had an approximately 160% greater membrane resistance than *Grpr*-negative iSPNs. We found parallel results *in vivo* when we probed for *Fos* mRNA in naïve mice, and found that *Grpr*+ iSPNs were twice as likely to be *Fos*+ than *Grpr*- iSPNs in the NAc MSh. Additionally, we found that approximately 75% of *Grpr*-eGFP+ cells were spontaneously active at baseline, suggesting that they could be a type of tonically active cholinergic interneurons [47, 281]. However, *Grpr*+ cells did not express *Chat* mRNA, which encodes the synthetic enzyme for acetylcholine and is a marker of these cells [282]. In addition, *Grpr*-EGFP+ cells had distinct physiological across all measurements compared to ChAT+ cells. While the ChAT-Cre recordings were from a different mouse strain, given the *in situ* and spine data we can convincingly rule out the possibility that GRPR+ cells are cholinergic interneurons. These experiments therefore identify a novel hyper-excitable iSPN subtype in the NAc MSh that expresses *Grpr*.

Both glutamatergic and dopaminergic inputs into the NAc MSh express Grpr

While this study was initially based on our lab's previous identification of a subpopulation of midbrain dopamine neurons that expressed *Grp* and project to the NAc MSh [263], many other brain regions are also known to project to the NAc MSh [108]. We therefore combined retrograde tracing with fluorescent *in situ* hybridization and found that in addition to the previously identified inputs from the VTA, additional *Grp*-expressing inputs come into the NAc MSh from the hippocampus and amygdala and are glutamatergic. This raises the possibility of co-release of GRP with both dopamine or glutamate. Additionally, while neuropeptide signaling has been known to occur in the NAc MSh through opioid receptors, these receptors are all $G_{i/o}$ -coupled, and the opioids themselves are synthesized within the NAc [47]. In contrast, the *Grp*+ inputs we identified come into the NAc MSh from other brain regions.

GRPR is a G_q -coupled receptor therefore leads to an increase in intracellular calcium when activated [250]. Previous studies that have examined GRP-GRPR signaling in other brain regions have found that GRP does in fact excite GRPR-expressing neurons in the auditory cortex, paraventricular thalamus, lateral amygdala, and spinal cord [251, 252, 254, 255]. While we found that the *Grpr*+ neurons in the NAc MSh were already spontaneously active at baseline, when we washed on GRP in slice, we found that the *Grpr*+ neurons depolarized even further and increased the membrane resistance by 140%, effects that could be blocked by a GRPR-specific antagonist. Additionally, we noted that the presence of the antagonist alone led to a decrease in baseline membrane resistance, raising the possibility that there is basal GRP-GRPR activity in slice. Furthermore, we saw similar effects *in vivo* when we injected GRP into the NAc MSh of wildtype mice and found a significant increase in the number and percentage of *Grpr*+ cells that were *Fos*+. Together these experiments map out a GRPR-GRP circuit in the

NAc MSh whereby GRP comes into the NAc MSh from glutamatergic and dopaminergic neurons from the hippocampus, amygdala, and VTA and excites GRPR+ cells, which are GABAergic.

GRP-GRPR signaling affects striatal associated behaviors

GRP-GRPR signaling in other brains regions has been shown to be important in a variety of different behaviors. In the basolateral amygdala, GRPR KO mice showed enhanced long-term fear memories [252]. In the spinal cord, GRP-GRPR signaling is the basis of itch [251, 283]. More recently, GRPR KO in the auditory cortex resulted in decreased auditory fear memories [255]. Given that GRP-GRPR signaling is sufficient to modulate behaviors in other brains regions, we generated GRPR KO mice to test whether this signaling impacted striatal-associated behaviors.

We first ran GRPR KO mice in the open field test to assess general locomotion and used MoSeq [279] to identify behavioral syllables in an unbiased manner. In the open field test we found significant differences in total distance travelled as well as in many of behavioral syllables. Importantly, we saw no changes in grooming syllables, but rather in lateral movement and turning. These results suggest that globally eliminating GRP-GRPR signaling affects general mouse behavioral patterns. Thus, even though GRP is a neuromodulator, eliminating its ability to signal is sufficient to induce behavioral changes.

In addition to the open field test, we ran the mice through an operant conditioning protocol as well as the accelerating rotarod, tests that have been associated with dorsal and ventral striatum [160-162, 165, 166, 178, 182, 280]. The NAc MSh is known to be important in motivation and, more specifically, iSPNs have been shown to inhibit motivation [99]. Given this, we used a progressive ratio lever pressing task that has previously been shown to be sensitive to manipulations of NAc MSh iSPNs to assess motivation [178]. In this study, the authors used chemogenetics to inhibit iSPNs in the NAc MSh and found this caused an increase in motivation. This is consistent with studies showing that iSPNs inhibit motivation [47, 112, 267]. By eliminating GRP-GRPR signaling, we expect to reduce the excitability of a small subpopulation of iSPNs, but not silence the cells themselves. We saw that disruption of GRP-GRPR signaling lead to no changes in motivation. This indicates that GRP-GRPR signaling is not necessary for motivated behaviors

Additionally, we assessed the role of GRP-GRPR signaling in motor coordination and motor learning using the accelerating rotarod. While the accelerating rotarod has previously been thought to be mainly associated with the DS, recent studies have demonstrated an underappreciated role of the ventral striatum in motor coordination and leaning assessed by this task [165, 166, 284]. One such study found that *Nlgn3* deletion increases rotarod performance due to a deficit in inhibition onto dSPNs [165]. Thus, a manipulation that reduces the activity of iSPNs, as is the case with GRPR deletion, would be expected to have the same net effect. Indeed, this is what we saw when we ran our GRPR KO mice on the accelerating rotarod. GRPR deletion led to a significant increase in motor coordination, that was apparent from even the first trial. Notably, we saw no

differences in motor learning. The increased rotarod performance also provides evidence that the decrease in lever pressing in the operant conditioning is not due to some gross motor deficit.

Finally, we assessed the role of GRP-GRPR signaling in assessing reward and hedonic state using the sucrose preference test. We saw that GRPR KO mice displayed a decreased sucrose preference. While this is commonly seen after mice have been exposed to chronic stress, neither KO or control mice had been specifically exposed to stress and were littermates. Interestingly, however, endogenous opioid receptor activation, specifically in the NAc MSh, has been shown to play a role in “liking” of sweet rewards such as sucrose [47]. Activation of kappa opioid receptors in the NAc MSh has been found to both increase and decrease “liking” reactions to sucrose [211]. In the case of GRPR, when ablated, it appears to lead to deficits in detecting the positive valence or the “liking” of sucrose, a naturally rewarding substance.

In summary, we demonstrate that disruption in GRP-GRPR signaling globally has a significant effect on general mouse behavior as well as on tasks that are known to be associated with the dorsal and ventral striatum. Future studies should examine NAc MSh-specific KO of GRPR to confirm that the decrease in motivation and increased motor coordination are due to the identified mesolimbic circuit.

Limitations and future directions

This study identified and characterized a previously undescribed subpopulation of iSPNs in the NAc MSh that express *Grpr*. We present several experiments where we characterize these cells genetically, electrophysiologically, and probe a possibly behavioral role of the identified circuit. The experiments lay the groundwork for further characterization of the role of not only these specific cells, but also GRP-GRPR signaling in the striatum in general. Moreover, the larger role of neuropeptide circuits and signaling within the basal ganglia as well as more generally in the brain, warrant exploration. We noted that although it was beyond the scope of the present study, *Grpr*⁺ cells also exist in the DS. Given the unique electrophysiological properties of the GRPR⁺ cells in the NAc MSh, the GRPR⁺ in the DS warrant their own characterization. Additionally, while we identified the *Grp*⁺ inputs into the NAc MSh and noted that the *Grpr*⁺ cells themselves were GABAergic, we did not confirm that the *Grpr*⁺ cells project and inhibit the ventral pallidum. Recent evidence has indicated that unlike the DS, the canonical pathways in the NAc are oversimplified and both D1- and D2-SPNs can participate in indirect and direct pathways depending on their downstream target from the ventral pallidum [99]. Therefore, the outputs of these *Grpr*⁺ cells should be examined in future studies. Additionally, while we provide an initial behavioral assessment of mice lacking GRPR, future studies should examine more behaviors as well as a NAc MSh-specific deletion. One such direction could be examined are tasks that assess context-induced reinstatement of drugs of abuse. These have been associated specifically with the ventral subiculum to NAc MSh projections, where we see a majority of the *Grp* inputs to the NAc

MSh [285, 286]. Finally, given the G_q -coupling, a possible role of GRP in synaptic plasticity should be examined.

Overall, our study identifies a new cell type in the NAc MSh and characterizes the associated circuit demonstrating a role of neuromodulation in the NAc MSh through neuropeptide signaling.

Methods

Mice

Animal experiments were performed in accordance with protocols approved by the University of California, Berkeley, Institutional Animal Care and Use Committee (protocol #: AUP-2016-04-8684-2). Both male and female mice were used across all experiments with the exception of behavior experiments where only male mice were used to generate littermate WT and *Grpr* KO controls. Mice were postnatal day (P) 40-90 unless otherwise stated. The following mouse lines were used in this study: C57BL/6J, The Jackson Laboratory strain #000664, *Grpr::eGFP* (GENAT [MMRRC #036178-UCD]), *Drd2-eGFP* (GENSAT [MMRRC #000230-UNC]), *Drd1a*-tdTomato (The Jackson Laboratory strain #016204) [272], CMV-Cre (The Jackson Laboratory strain #006054) [277], *Grpr^{fl}* (The Jackson Laboratory strain # 033148) [276], ChAT-IRES-Cre (The Jackson Laboratory strain # 06410) [273], Ai9 (The Jackson Laboratory strain # 07909) [274]. *Grpr* KO mice were generated by breeding CMV-Cre mice to *Grpr^{fl}* mice to knock out *Grpr* in all cells.

Fluorescent in situ hybridization

Fluorescent *in situ* hybridization was performed to identify and characterize *Grpr*-expressing cells in the NAc MSh and dorsal striatum as well as their *Grp*-expressing inputs. Brains were harvested, flash-frozen in OCT mounting medium (Fisher Scientific #23-730-571) on dry ice and stored at -80 °C for up to 6 months. 16 μ m-thick coronal sections were collected using a cryostat, mounted directly onto 75x25mm Superfrost® Plus glass slides (VWR #48311-703) and stored at -80°C for up to 1 month. *In situ* hybridization was performed according to protocols provided with the RNAscope® Multiplex Fluorescent Reagent Kit (ACD #320850) and RNAscope® Multiplex Fluorescent Reagent Kit V2 (ACD #323100). *Grpr* mRNA was visualized with a probe in channel 2 (ACD #317871-C2), *Grp* mRNA in channel 2 (ACD #317861-C2), *Drd2* mRNA in channel 1 (ACD #406501), *Drd1* mRNA in channel 3 (ACD #461901-C3), *Chat* mRNA in channel 1 (ACD #408731), *Ppp1r1b* mRNA in channel 3 (ACD #405901-C3), *Fos* mRNA in channel 3 (ACD #316921-C3), *Gad2* mRNA in channel 1 (ACD #439371), *Slc1717* mRNA in channel 1 (ACD #481851). Sections were imaged using an Olympus FluoView 3000 confocal microscope equipped with 405, 488, 561, and 640 nm lasers and a motorized stage for tile imaging. Z stack images captured the entire thickness of the section at 1-2.5 μ m steps for images taken with 20x (Olympus #UCPLFLN20X) or 10x air objectives (UPLXAPO10X). Additional images were acquired on a Zeiss LSM 710 AxioObserver with Zeiss 10x, 20x, and 63x objectives housed in the Molecular Imaging Center at UC Berkeley. Images were analyzed using FIJI. Cells were considered positive

for *Grpr* if they contained two or more fluorescent puncta within the cellular boundary created by DAPI. All images for a single dataset were acquired on the same microscope.

Immunohistochemistry

Male and female mice (P30-P90) were deeply anesthetized by isoflurane and transcardially perfused with ice cold 1 x PBS (~5–7 ml) followed by 4% paraformaldehyde (PFA) solution (Electron Microscopy Sciences: 15713) in 1 x PBS (~5–10 ml) using a peristaltic pump (Instech). The brains were removed and post-fixed by immersion in 4% PFA in 1 x PBS solution overnight at 4 °C. Brains were suspended in 30% sucrose in 0.1 M PB for cryoprotection. After brains descended to the bottom of the vial (typically 24–28 hr), 30 µm coronal sections of the NAc MSh were cut on a freezing microtome (American Optical AO 860), collected into serial wells, and stored at 4 °C in 1 x PBS containing 0.02% (w/v) sodium azide (NaN₃; Sigma Aldrich).

Free-floating sections were washed with gentle shaking, 3 × 5 min in 1 x PBS followed by 1 hr incubation at RT with BlockAid blocking solution (Life Tech: B10710). Primary antibodies were applied at 4 °C in 1 x PBS containing 0.25% (v/v) Trinton-X-100 (PBS-Tx) for overnight to ensure penetration of the antibody throughout the slice. Sections were then washed with cold 1 x PBS-Tx 3 × 10 min and incubated for 2 hr at RT with secondary antibodies in PBS-Tx. Sections were washed in cold 1 x PBS 3 × 10 min, mounted on SuperFrost slides (VWR: 48311–703), and coverslipped with Prolong Gold antifade mounting media (Life Tech: P36935).

We used a primary antibody against GFP (1:5000, Abcam 13970) and an Alexa Fluor 488 goat anti-chicken secondary antibody (1:500 Invitrogen, A-11039).

Confocal microscopy

Images of 30 µm sections processed for immunohistochemistry were acquired using an Olympus FluoView 3000 confocal microscope equipped with 405, 488, 561, and 640 nm lasers and a motorized stage for tile imaging. Z-stack images captured the entire thickness of the section at 1–2 µm steps for images taken with a 20X air (Olympus #UCPLFLN20X) or a 10X air Olympus #UPLXAPO10X) objective.

Neurobiotin-filled neuron reconstruction for Sholl analysis

Male and female mice were deeply anesthetized by isoflurane, transcardially perfused with ice cold ACSF using a peristaltic pump (Instech) and decapitated. 275-µm-thick coronal midbrain slices were prepared on a vibratome (Leica VT1000 S) in oxygenated ice-cold choline-based external solution (pH 7.8) containing 110 mM choline chloride, 25 mM NaHCO₃, 1.25 mM NaHPO₄, 2.5 mM KCl, 7 mM MgCl₂, 0.5 mM CaCl₂, 25 mM glucose, 11.6 mM sodium ascorbate, and 3.1 mM sodium pyruvate. Slices were recovered in ACSF at 34°C for 15 min and then kept at room temperature (RT). All solutions were continuously bubbled with 95% O₂ and 5% CO₂. For whole cell recordings, 3–5 mΩ borosilicate glass pipettes (Sutter Instrument: BF150-86-7.5) were filled with a potassium-based internal solution (pH 7.4) containing 135 mM KMeSO₄, 5

mM KCl, 5 mM HEPES, 4 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM phosphocreatine, 1 mM EGTA, and 4 mg/ml neurobiotin (Vector laboratories #SP-1120).

275 μ m slices containing *Grpr*-eGFP $\pm$ NAc MSh neurons filled with neurobiotin-containing internal solution (4 mg/ml) during patch-clamp experiments were fixed in 4% paraformaldehyde solution (Electron Microscopy Sciences: 15713) in 1 x PBS for 24–48 hr at 4 °C. With continuous gentle shaking, slices were washed in 1X PBS 3 $\times$ 5 min and incubated with BlockAid blocking solution (Life Tech: B10710) for 1 hr at RT. Streptavidin Alexa Fluor 633 conjugate (1:750, Invitrogen: S21375) was applied overnight at 4 °C in 1 x PBS containing 0.50% (v/v) Triton-X-100 (PBS-Tx). The following day, slices were washed in 1 x PBS 3 $\times$ 10 min, mounted on SuperFrost slides (VWR: 48311–703) with the cell-containing side of the slice facing up, and coverslipped with Prolong Gold antifade mounting media (Life Tech: P36935).

Filled cells in the mounted sections were imaged on an Olympus FluoView 3000 confocal microscope at 20x magnification with 2X digital zoom for a final magnification of 40X. 1 μ m steps were used to acquire a z-stack spanning the entirety of the neurobiotin-filled cell body and dendritic arbor. 3D reconstruction of the cells was done using IMARIS 9.2.1 software (Bitplane) with automated filament tracing and manual editing. The mask generated by the automated filament tracing algorithm was continuously cross-referenced with the original z-stack image to ensure accuracy. Spurious segments created by the automated filament tracer due to background noise were removed, while processes with incomplete reconstruction were manually edited to incorporate missing segments.

Sparse viral injections for spine density quantification

Neonatal (P0) or P28–30 *Grpr*::eGFP were injected bilaterally with 250 nL (neonates) or 100 nL (adults) AAV5-hSyn-mCherry (Addgene 114422-AAV5) diluted 1:200 to label neurons in the NAc MSh. Neonatal mice were cryoanesthetized and injected bilaterally at 200 nL/min. Injections were targeted to the NAc MSh, with coordinates approximately 0.75 mm lateral to midline, 1.0 mm posterior to bregma, and 3.0 mm ventral to the head surface. For adult injections, mice were briefly anesthetized with 3% isoflurane (Piramal Healthcare – PIR001710) and oxygen. Mice were mounted on a stereotaxic frame (Kopf instruments – Model 940) with stabilizing ear cups and a nose cone delivering constant 1.5% isoflurane in medical oxygen. Viruses were injected using a pulled glass capillary at a rate of 100 nl/minute. Following injection, the capillary remained in place for 1 minute per every minute spent injecting to allow the tissue to recover and prevent virus backflow up the injection tract upon retraction. Coordinates from Bregma for adult injections were as follows: M/L $\pm$ 1.00 mm, A/P +1.90 mm, D/V -4.50 mm. All mice were perfused at P56. Mice were perfused and brains were post-fixed with 4% paraformaldehyde overnight, then sectioned at 80 μ m. Sections were blocked for 1 hr at RT in BlockAid (ThermoFisher, B10710) and incubated for 16 hr at 4°C with antibodies against RFP (1:500, Rockland (VWR) RL600-401-379) and GFP (1:500, Abcam 13970). Sections were washed 3 $\times$ 10 min in PBS-Tx and incubated for 2 hr at RT with Alexa Fluor 488 and

Alexa Fluor 546 secondary antibodies (1:500, Invitrogen, A-11039 and A-11001). Sections were washed 3×10 min in 1x PBS and mounted onto slides using Prolong Gold antifade mounting media (Life Tech: P36935).

Dendrites were imaged on an Olympus FluoView 3000 scanning confocal microscope at 60x magnification with 2.5x digital zoom for a final magnification of 150x. Images were deconvoluted in the CellSens4 software using a built-in advanced maximum likelihood algorithm. Dendritic spine reconstructions were generated using the filament tracer feature in Imaris 9.3.1. 1-9 dendrites were reconstructed per cell, with the detection parameters for thinnest spine diameter set to $1.5 \mu\text{m}$ and the maximum spine length set to $3.5 \mu\text{m}$. After automatic detection of spines, the digital reconstruction was compared to the original z-stack image to ensure accuracy. Detected spines were manually reviewed by the experimenter and added or removed as needed. Spine density of each dendrite was calculated by dividing the number of spines on that dendrite by the total dendrite length. The values of all dendrites quantified for a given neuron were averaged and the average spine density per dendrite is reported.

Electrophysiology

Mice (P30-90) were perfused transcardially with ice-cold ACSF (pH=7.4) containing (in mM): 127 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, and 25 glucose, bubbled continuously with carbogen (95% O₂ and 5% CO₂). Brains were rapidly removed and coronal slices ($275 \mu\text{m}$) were cut on a VT1000S vibratome (Leica) in oxygenated ice-cold choline-based external solution (pH=7.8) containing (in mM): 110 choline chloride, 25 NaHCO₃, 1.25 NaHPO₄, 2.5 KCl, 7 MgCl₂, 0.5 CaCl₂, 25 glucose, 11.6 sodium ascorbate, and 3.1 sodium pyruvate. Slices were recovered in ACSF at 34°C for 15 min and then kept at RT before recording.

Recordings were made with a MultiClamp 700B amplifier (Molecular Devices) at RT using 3-5 MOhm glass patch electrodes (Sutter, BF150-86-7.5). Data were acquired using ScanImage software, written and maintained by Dr. Bernardo Sabatini (<https://github.com/bernardosabatini/%20SabalabAcq>). Traces were analyzed in Igor Pro (Wavemetrics). Recordings with a series resistance > 30 MOhms were rejected.

Current-clamp recordings

Current clamp recordings were made using a potassium-based internal solution (pH=7.4) containing (in mM): 135 KMeSO₄, 5 KCl, 5 HEPES, 4 Mg-ATP, 0.3 Na-GTP, 10 phosphocreatine, and 1 ETGA. No synaptic blockers were included and no holding current was applied to the membrane. For intrinsic excitability experiments, positive current steps (1s, +10 to +100 pA) were applied in 10 pA intervals to generate an input-output curve. Membrane potential was averaged over a 300 ms period between 50-350 ms of the 2 s sweep prior to current injection. Membrane resistance was calculated from voltage-clamp RC checks prior to switching to current-clamp. A cell was considered spontaneously active at baseline if it fired at least one action potential not during the 1 s period of current injection during the first 2 minutes after breaking in. Action potential half

width was calculated by determining the peak after a spike crossing of 0 mV and then calculating the width at half of the difference between peak and the baseline immediately prior to the spike.

GRP wash-on

Membrane potential changes upon bath application of 300 nM GRP (Anaspec AS-24214) were monitored in current clamp mode. 5 s current clamp recordings were recorded every 2 s. Prior to GRP wash-on, membrane potential was recorded for 10 minutes at baseline, and membrane potential was averaged over a 300 ms window between 50-350 ms of the 2 s sweep. 300 nM GRP was then bath applied for 10 minutes. At the conclusion of GRP wash-on, we briefly switched back to voltage-clamp to perform 5 RC checks to calculate membrane resistance and confirm that series resistance was stable and <30 MOhms. GRP was then washed-out. The baseline membrane potential was calculated as the average over the two minutes prior to GRP wash-on and the membrane potential following GRP application was averaged over the last two minutes of wash-on. Baseline membrane resistance was calculated from the initial voltage-clamp RC check. Wash-on experiments in the presence of the GRPR antagonist, 1 μ M DPDMB (Bachem H-3042) were identical, except the slices were pre-incubated in DPDMB for at least 15 minutes prior to recording and DPDMB was present in the ACSF for the duration of the recordings.

Stereotaxic injections

Retrobead injections

P50-90 wild-type male and female mice were used for retrograde labeling experiments. Red retrobeads IX (Lumafluor) were diluted 1:3 in sterile saline. Beads were unilaterally injected using a pulled glass pipette. The following coordinates from bregma were used to inject 350 nL into the NAc MSh: M/L + 0.65 mm, A/P +1.90 mm, D/V -4.50 mm. To allow for sufficient labeling, mice were sacrificed 14 days post injection. Brains were harvested, flash-frozen in OCT mounting medium (Fisher Scientific #23-730-571) on dry ice and stored at -80 °C for up to 6 months. 16 μ m-thick coronal sections were collected using a cryostat, mounted directly onto 75x25mm Superfrost® Plus glass slides (VWR #48311-703) and stored at -80°C for up to 1 month. Prior to sectioning the posterior part of the brain, the injection site was validated to ensure that retrobeads were contained in the NAc MSh. After confirming, the rest of the brain was sectioned and fluorescent *in situ* hybridization was performed on all sections containing brain regions with retrobead+ cells.

GRP injections

Male and female mice (P30-P90) underwent stereotaxic surgery to inject GRP into the NAc MSh. 800 nL of 3 μ M GRP or sterile saline were injected unilaterally into the left NAc MSh. The stereotaxic coordinates used were: + 0.65 mm, A/P +1.90 mm, D/V -4.50 mm. 45 minutes after the end of the injection, mice were sacrificed and their brains harvested and flash frozen for *in situ* hybridization.

Behavioral experiments

Behavior studies were carried out in the dark phase of the light cycle under red lights (open field) or white lights (rotarod and operant conditioning). Sucrose preference was carried out in the room where the mice are normally housed. Mice were habituated to the behavior testing room for at least 30 minutes prior to testing, and at least 24 hours elapsed between sessions. All behavior equipment was cleaned between each trial with 70% ethanol. Equipment was rinsed with diluted soap and water at the end of each day. Only male mice were used in behavior experiments and behavior experiments began when the mice were P50-60. Experimenters were blinded to genotype during behavioral testing.

Genotyping to validate KO

I designed a genotyping strategy to confirm the genotype of *Grpr* KO and WT control mice. The strategy involved primer pairs for the presence of the floxed or wt allele, the LoxP scar, and an internal region of *Grpr* exon 2. The following primer sets were used: *Grpr* floxed (WT- forward: 5' GGAGAGAGGTATAGAGGGGC 3', WT-R: 5' ACATGTAGGTTGCAGGGGAT 3', MUT-F 5' GGGTTATTGTCTCATGAGCGG 3'), LoxP scar (Forward 5' AGACAGCTCTATCACGGTCC 3', Reverse 5' GAGTTCAGTTCCCAGCAACC 3'), *Grpr* Exon 2 (Forward 5' TTTCTGACCTCCACCCCTTC 3', Reverse 5' CACGGGAAGATTGTAGGCAC 3'). With this strategy, I expected the following bands for *Grpr* KO mice: *Grpr* floxed (no band), CMV-Cre (521 bp internal control), LoxP scar (298 bp), *Grpr* Exon 2 (no band) and WT mice: *Grpr* floxed (573 bp wt band), CMV-Cre (521 bp internal control and 180 bp band), LoxP scar (no band), *Grpr* Exon 2 (206 bp).

Open field

Exploratory behavior in a novel environment and general locomotor activity were assessed by a 60 min session in an open field chamber (40 cm L x 40 cm W x 34 cm H) made of transparent plexiglass. The mouse was placed in the bottom right hand corner of the arena and behavior was recorded using an overhead monochrome camera (GS3-U3-41C6NIR-C) and a 16 mm wide angle lens (LM16HC) placed on top of the arena from a height of 50 cm. Data was analyzed using DeepLabCut [278, 287] and MoSeq [288].

Processing of open field data

To extract the body part (keypoint) coordinates from the video recordings, we used DeepLabCut (DLC) 2.3.4 [278, 287]. 14 body parts including nose, head, left ear, right ear, left forelimb, right forelimb, spine 1, spine 2, spine 3, left hindlimb, right hindlimb, tail 1, tail 2, and tail 3 were manually labelled on a small subset of the video frames. A DLC model was then trained using the annotated frames to label these 14 body parts for all videos recorded. The distance travelled and center entries were calculated with the coordinate of body part tail 1.

Analysis of open field data

Discrete behavior syllables were extracted with Keypoint-MoSeq 0.4.4 [288]. Syllable usage and transition data were obtained using built-in functions in Keypoint-MoSeq package. Decoding analysis was done with customized Python 3.9 script. Code available upon request/Github.

Rotarod

The accelerating rotarod test was used to examine motor learning. Mice were trained on a rotarod apparatus (Ugo Basile, 47650) for four consecutive days. Three trials were completed per day with a 5 min break between trials. The rotarod was accelerated from 5-40 revolutions per minute (rpm) over 300 s for trials 1-6 (days 1 and 2), and from 10-80 rpm over 300 s for trials 7-12 (days 3 and 4). On the first testing day, mice were first acclimated to the apparatus by being placed on the rotarod rotating at a constant 5 rpm for 60 s and returned to their home cage for 5 minutes prior to starting trial 1. Latency to fall, or to rotate off the top of the rotarod barrel, was measured by the rotarod stop-trigger timer.

Operant conditioning

Motivated behavior was assessed in an operant conditioning lever pressing task using a progressive ratio reinforcement schedule with a food pellet reinforcer. Animals were food restricted to ~90-95% of their body weight before commencing training in operant conditioning chambers (Med Associates #ENV-307A). The chambers contained a single retractable lever on the left side of the food receptacle and a house light on the opposite end of the chamber. Each session began with illumination of the house light and the presentation of the left lever. The session ended with retraction of the lever and with the house light turning off. The lever remained present throughout the session. Mice were weighed before each session and habituated to the experiment room for at least 30 minutes.

During the first training session, following two days of food restriction, a food pellet (14 mg regular “chow” pellet, Bio-Serve CAT #F05684) was delivered on a random time schedule (RT), with a reinforcer delivered on average every 60 seconds for a total of 15 minutes with no levers present. On subsequent days, mice underwent 60 minute fixed ratio (FR1) sessions where mice were presented with only the left lever. Each lever press was rewarded with a single pellet reward. During initial FR1 sessions, crushed pellets were placed on the lever at the beginning of the task and each time 10 minutes elapsed without a lever press to prime the mice to press the levers. Mice underwent daily FR1 sessions until they had two consecutive sessions with 20 or more lever presses. After earning 20 rewards the first time, the levers were no longer primed with crushed pellets. The mice then moved on to the progressive ratio (PR) session the following day. The PR session was 2 hours long and the number of presses required for a reward increased with each reward. The number of lever presses to earn a reward were as follows: 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 22, 24, 28, 32, 36, 40, 44, 48, 52, 56, 64, 72, 80, 88, 96, 104, 112, 120, 128, 136.

Sucrose preference test

Grpr KO and WT mice were assessed for normal hedonic behavior by using the sucrose preference test. Three days prior to the sucrose preference test, the water source in the home cage was switched from a Hydro Pac to a water bottle containing the contents of the Hydro Pak. After three days of acclimating to the water bottles, a second water bottle was added to the home cage containing 5% sucrose in Hydro Pac water. Mice had access to both bottles for 24 hours. After 24 hours, mice were individually housed, again with two bottles, one with 5% sucrose and the other with standard water. Mice were kept singly housed for another 24 hours. The amount of water and sucrose consumed during this 24 hour period was measured. Sucrose preference was calculated by taking the total volume of sucrose consumed and dividing it by the total liquid consumed in the 24-hour period. To avoid any confounds due to side-specific preferences, the bottles' location was randomly assigned (front or back) for each mouse. All experiments were conducted on weekends, ensuring a quiet environment for the tested animals.

Quantification and statistical analysis

GraphPad Prism 9 was used to perform statistical analyses. All datasets were first analyzed using D'Agostino and Pearson normality test, and then parametric or non-parametric two-tailed statistical tests were employed accordingly to determine significance. If the variances between two groups were significantly different, a Welch's correction was applied. Significance was set as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. P values were corrected for multiple comparisons. Statistical details including sample sizes for each experiment are reported in the figure legends.

Figures
Figure 1

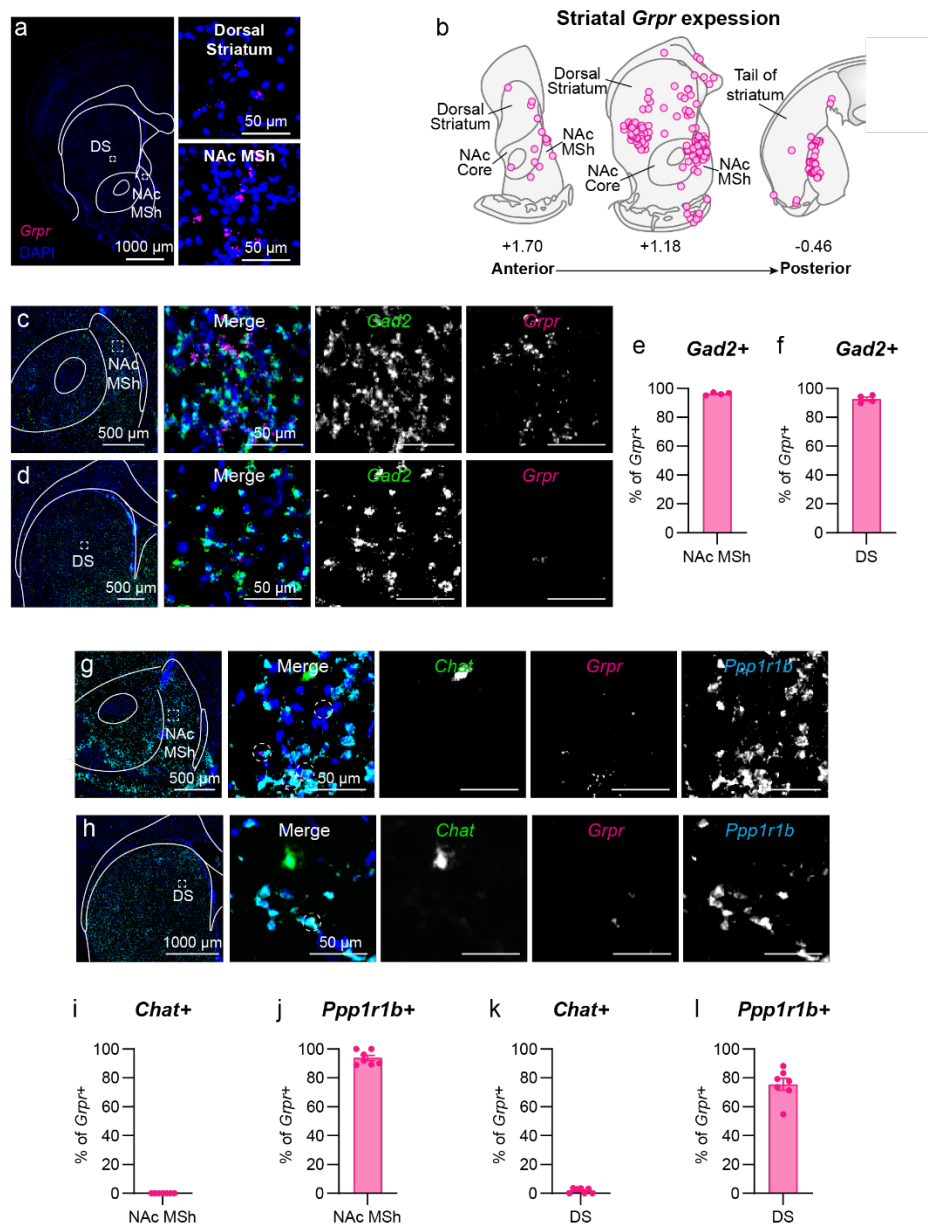


Figure 1: *Grpr* is expressed in striatal projection neurons.

(a) Representative image of a coronal brain section (A/P +1.18) from a wild-type mouse. *In situ* hybridization (ISH) for *Grpr* in magenta. Nuclei are labeled in blue with DAPI. Insets show higher magnification images for dorsal striatum (DS) and nucleus accumbens medial shell (NAc MSh). **(b)** Summary schematic of *Grpr* expression across the A/P axis of the dorsal striatum and NAc MSh. Each dot represents the location of a *Grpr*⁺ cell. Dots are summed across 3 wild-type mice. **(c-d)** Representative image of the **(c)** NAc MSh and **(d)** DS. *In situ* hybridization for *Gad2* in green and *Grpr* in magenta. **(e-f)** Mean $\pm$ SEM of percentage of *Grpr*⁺ cells in the **(e)** NAc MSh and **(f)** DS that are *Gad2*⁺ in wild-type mice. (n= 4 mice). **(g-h)** Representative image of **(g)** NAc MSh and **(h)** DS. *In situ* hybridization for *Chat* in green, *Grpr* in magenta, and *Ppp1r1b* in cyan. Dashed circles outline *Grpr*⁺ cells. **(i-j)** Mean $\pm$ SEM of percentage of *Grpr*⁺ cells in the NAc MSh of wild-type mice that are **(i)** *Chat*⁺ (n=7 mice) or **(j)** *Ppp1r1b*⁺ (n=7 mice). **(k-l)** Mean $\pm$ SEM of percentage of *Grpr*⁺ cells in the DS of wild-type mice that are **(k)** *Chat*⁺ (n=7 mice) or **(l)** *Ppp1r1b*⁺ (n=7 mice). *In situ* quantifications done by Atehsa Sahagun.

Figure 2

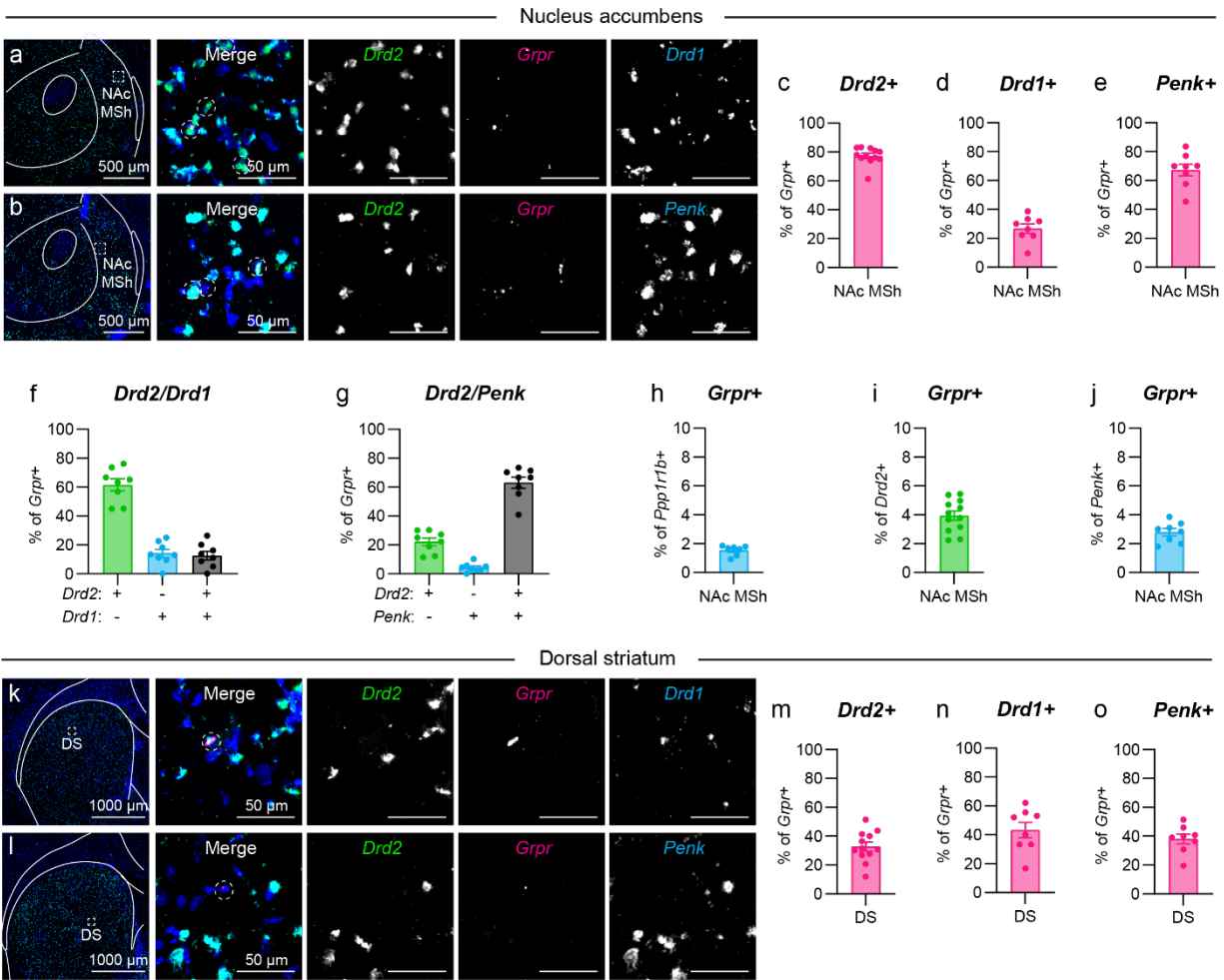


Figure 2: *Grpr* is expressed in sub-populations of dSPNs and iSPNs.

(a-b) Representative image of NAc MSh. **(a)** *In situ* hybridization for *Drd2* in green, *Grpr* in magenta, and *Drd1* in cyan. Dashed circles outline *Grpr*⁺ cells. **(b)** *In situ* hybridization for *Drd2* in green, *Grpr* in magenta, and *Penk* in cyan. Dashed circles outline *Grpr*⁺ cells. **(c-e)** Mean $\pm$ SEM of percentage of *Grpr*⁺ cells in the NAc MSh of wild-type mice that are **(c)** *Drd2*⁺ (n=12 mice), **(d)** *Drd1*⁺ (n=8 mice), or **(e)** *Penk*⁺ (n=8 mice). **(f)** Mean $\pm$ SEM of percentage of *Grpr*⁺ cells in the NAc MSh that are *Drd2*⁺/*Drd1*⁻ (green), *Drd2*⁻/*Drd1*⁺ (blue), and *Drd2*⁺/*Drd1*⁺ (black) in wild-type mice. (n= 8 mice). **(g)** Mean $\pm$ SEM of percentage of *Grpr*⁺ cells in the NAc MSh that are *Drd2*⁺/*Penk*⁻ (green), *Drd2*⁻/*Penk*⁺ (blue), and *Drd2*⁺/*Penk*⁺ (black) in wild-type mice. (n= 8 mice). **(h-i)** Mean $\pm$ SEM of percentage of **(h)** *Ppp1r1b*⁺ (n=7 mice), **(i)** *Drd2*⁺ (n=12 mice), or **(j)** *Penk*⁺ (n=8 mice) cells in the NAc MSh that are *Grpr*⁺ in wild-type mice. **(k-l)** Representative image of the dorsal striatum (DS). **(k)** *In situ* hybridization for *Drd2* in green, *Grpr* in magenta, and *Drd1* in cyan. Dashed circles outline *Grpr*⁺ cells. **(l)** *In situ* hybridization for *Drd2* in green, *Grpr* in magenta, and *Penk* in cyan. Dashed circles outline *Grpr*⁺ cells. **(m-o)** Mean $\pm$ SEM of percentage of *Grpr*⁺ cells in the DS of wild-type mice that are **(m)** *Drd2*⁺ (n=12 mice), **(n)** *Drd1*⁺ (n=8 mice), or **(o)** *Penk*⁺ (n=8 mice). *In situ* quantifications done by Atehsa Sahagun.

Figure 3

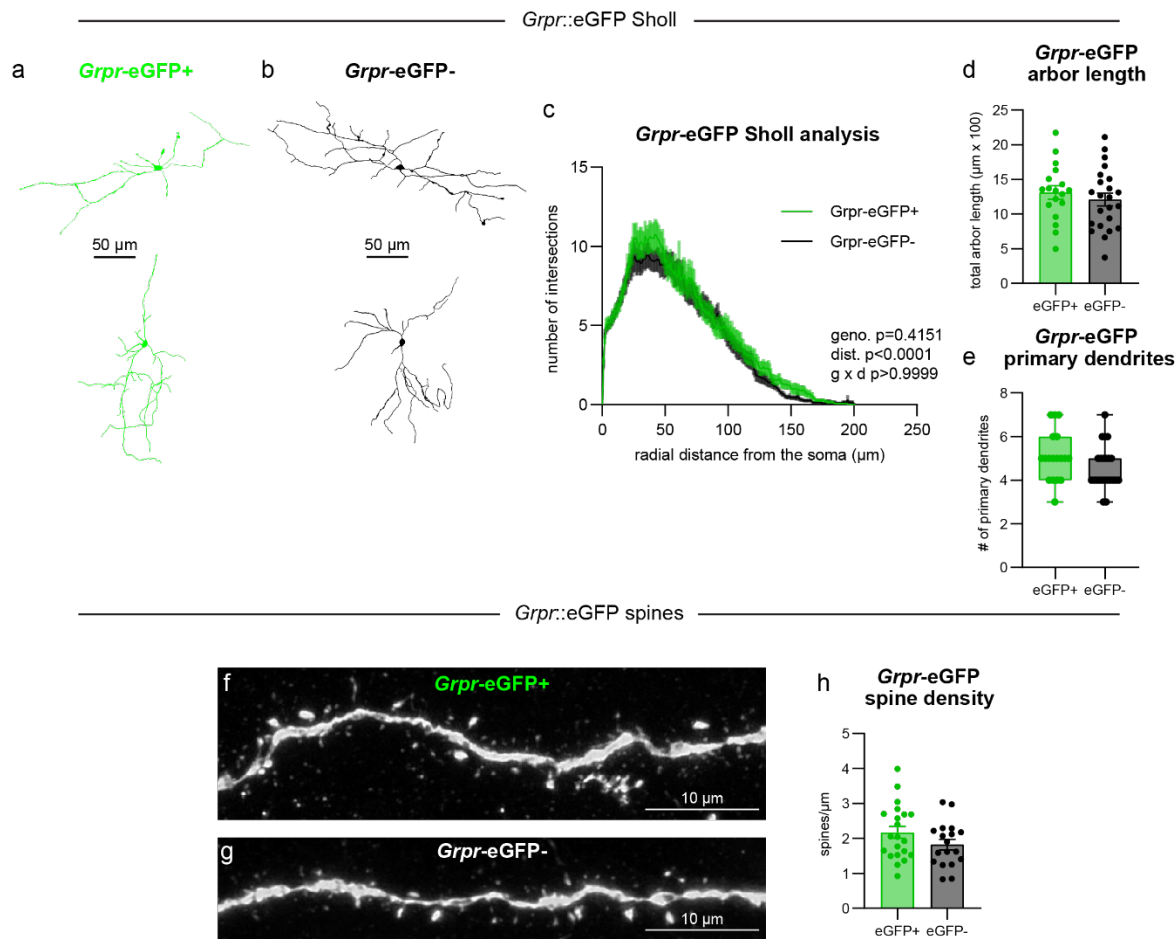


Figure 3: GRPR-expressing neurons in the nucleus accumbens medial shell are morphologically similar to neighboring striatal projection neurons.

(a) Representative coronal brain section (A/P +1.70) from a *Grpr::eGFP*;Drd1-tdTomato mouse that was used to identify GRPR+ cells for whole-cell recordings. (b) Zoom-in of the DS from j. (c) Zoom-in of the NAc MSh from j. (d) Mean $\pm$ SEM percent of eGFP+ cells that are tdTomato+ in *Grpr::eGFP*;Drd1-tdTomato mice in the DS (magenta) and NAc MSh (green). (n=6 mice). (e-f) Reconstructions of the dendrites and cell body of nucleus accumbens medial shell (NAc MSh) (e) *Grpr*-eGFP+ neurons and (f) *Grpr*-eGFP- neurons. (g) Sholl analysis of NAc MSh neuron dendritic arbors. Dark colored lines are the mean, lighter color shading is the SEM. *Grpr*-eGFP+ in green: n=18 neurons from 8 mice, *Grpr*-eGFP- in black: n=23 neurons from 14 mice. Two-way ANOVA p-values are shown. (h) Mean $\pm$ SEM total dendritic arbor length per cell. *Grpr*-eGFP+ in green: n=18 neurons from 8 mice, *Grpr*-eGFP- in black: n=23 neurons from 14 mice; p=0.4452, Welch's two-tailed t-test. (i) Box plots of the number of primary dendrites per neuron for each genotype (boxes represent the interquartile range (25-75%), lines denote the median, whiskers represent min to max values). *Grpr*-eGFP+ in green: n=18 neurons from 8 mice, *Grpr*-eGFP- in black: n=23 neurons from 14 mice; p=0.1151, Welch's two-tailed t-test. (j-k) Representative images of dendrites from a (j) *Grpr*-eGFP+ and (k) *Grpr*-eGFP- neuron in the NAc MSh. (l) Mean $\pm$ SEM spine density in *Grpr*-eGFP+ neurons vs *Grpr*-eGFP- neurons. *Grpr*-eGFP+ in green: n=21 neurons from 11 mice, *Grpr*-eGFP- in black: n=18 neurons from 9 mice; p=0.1357, Welch's two-tailed t-test. For panels d, e, and h, dots represent values from individual neurons.

Figure 4

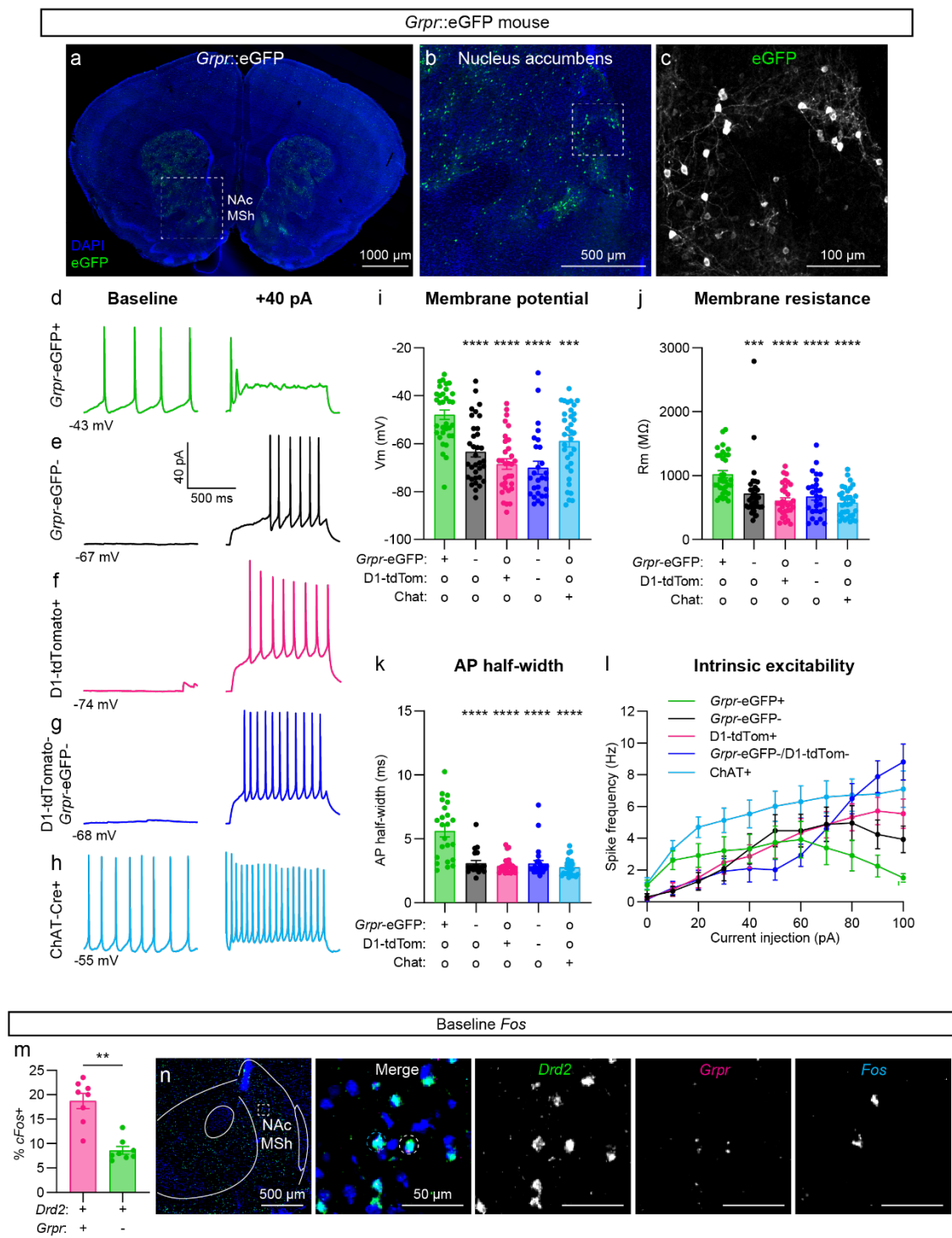


Figure 4: GRPR-expressing neurons in the nucleus accumbens medial shell have high intrinsic excitability.

(a) Representative image of a coronal brain section (A/P +1.18) from a *Grpr::eGFP* mouse that was used to identify GRPR+ cells for whole-cell recordings. eGFP in green, DAPI labeled nuclei in blue. (b) Zoom-in of the nucleus accumbens medial shell (NAc MSh) from panel a. (c) Zoom-in of panel b showing eGFP+ cells in grayscale. (d-h) Representative traces from a (d) *Grpr*-eGFP+ (e), *Grpr*-eGFP- (f), D1-tdTomato+ (g), D1-tdTomato-/Grpr-eGFP- (presumed D2+), and (h) ChAT-Cre+ cell in the NAc MSh recorded at baseline (left) and following +40 pA current injection (right). (i) Mean $\pm$ SEM membrane potential of NAc MSh neurons. *Grpr*-eGFP+ in green: n=33 cells from 15 mice, *Grpr*-eGFP- in black: n=34 cells from 11 mice, D1-tdTomato+ in magenta: n=31 cells from 5 mice, D1-tdTomato-/Grpr-eGFP- in dark blue: n=27 cells from 3 mice, and ChAT-Cre+ in light blue: n=34 cells from 7 mice. ($p < 0.0001$, one-way ANOVA; Holm-Sidak's multiple comparison tests, **** $p < 0.0001$ *Grpr*-eGFP+ vs *Grpr*-eGFP-, **** $p < 0.0001$ *Grpr*-eGFP+ vs D1-tdTomato+, **** $p < 0.0001$ *Grpr*-eGFP+ vs D1-tdTomato-/Grpr-eGFP-, *** $p = 0.0007$ *Grpr*-eGFP+ vs ChAT-Cre+). (j) Mean $\pm$ SEM membrane resistance of NAc MSh neurons. *Grpr*-eGFP+ in green: n=33 cells from 15 mice, *Grpr*-eGFP- in black: n=34 cells from 11 mice, D1-tdTomato+ in magenta: n=31 cells from 5 mice, D1-tdTomato-/Grpr-eGFP- in dark blue: n=27 cells from 3 mice, and ChAT-Cre+ in light blue: n=34 cells from 7 mice. ($p < 0.0001$, one-way ANOVA; Holm-Sidak's multiple comparison tests, *** $p = 0.0002$ *Grpr*-eGFP+ vs *Grpr*-eGFP-, **** $p < 0.0001$ *Grpr*-eGFP+ vs D1-tdTomato+, **** $p < 0.0001$ *Grpr*-eGFP+ vs D1-tdTomato-/Grpr-eGFP-, **** $p < 0.0001$ *Grpr*-eGFP+ vs ChAT-Cre+). (k) Mean $\pm$ SEM action potential (AP) half-width of NAc MSh neurons. *Grpr*-eGFP+ in green: n=23 cells from 10 mice, *Grpr*-eGFP- in black: n=20 cells from 7 mice, D1-tdTomato+ in magenta: n=28 cells from 5 mice, D1-tdTomato-/Grpr-eGFP- in dark blue: n=26 cells from 3 mice, and ChAT-Cre+ in light blue: n=24 cells from 4 mice. ($p < 0.0001$, one-way ANOVA; Holm-Sidak's multiple comparison tests, **** $p < 0.0001$ *Grpr*-eGFP+ vs *Grpr*-eGFP-, **** $p < 0.0001$ *Grpr*-eGFP+ vs D1-tdTomato+, **** $p < 0.0001$ *Grpr*-eGFP+ vs D1-tdTomato-/Grpr-eGFP-, **** $p < 0.0001$ *Grpr*-eGFP+ vs ChAT-Cre+). (l) Input-output curves showing the mean $\pm$ SEM firing frequency of NAc MSh neurons in response to positive current steps of increasing amplitude. *Grpr*-eGFP+ in green: n=23 cells from 10 mice, *Grpr*-eGFP- in black: n=21 cells from 7 mice, D1-tdTomato+ in magenta: n=31 cells from 5 mice, D1-tdTomato-/Grpr-eGFP- in dark blue: n=27 cells from 3 mice, and ChAT-Cre+ in light blue: n=34 cells from 7 mice. (m) Mean $\pm$ SEM percent *Grpr*+/*Drd2*+ (magenta) vs. *Grpr*-/*Drd2*+ (green) cells in the NAc MSh that are *Fos*+. (n=8 mice; ** $p = 0.0078$, Wilcoxon test). (n) Representative image of NAc MSh. *In situ* hybridization for *Drd2* in green, *Grpr* in magenta, and *Fos* in cyan. Dashed circles outline *Grpr*+ cells, white=*Grpr*+/*Drd2*+/*Fos*-, cyan=*Grpr*+/*Drd2*+/*Fos*+. For panels i, j, and k, + indicates the presence of a fluorophore labeling the given cell type, - indicates that the cell was negative for the fluorophore, and o indicates that the tissue did not have a fluorophore that would label the given cell type. *In situ* quantifications done by Atehsa Sahagun.

Figure 5

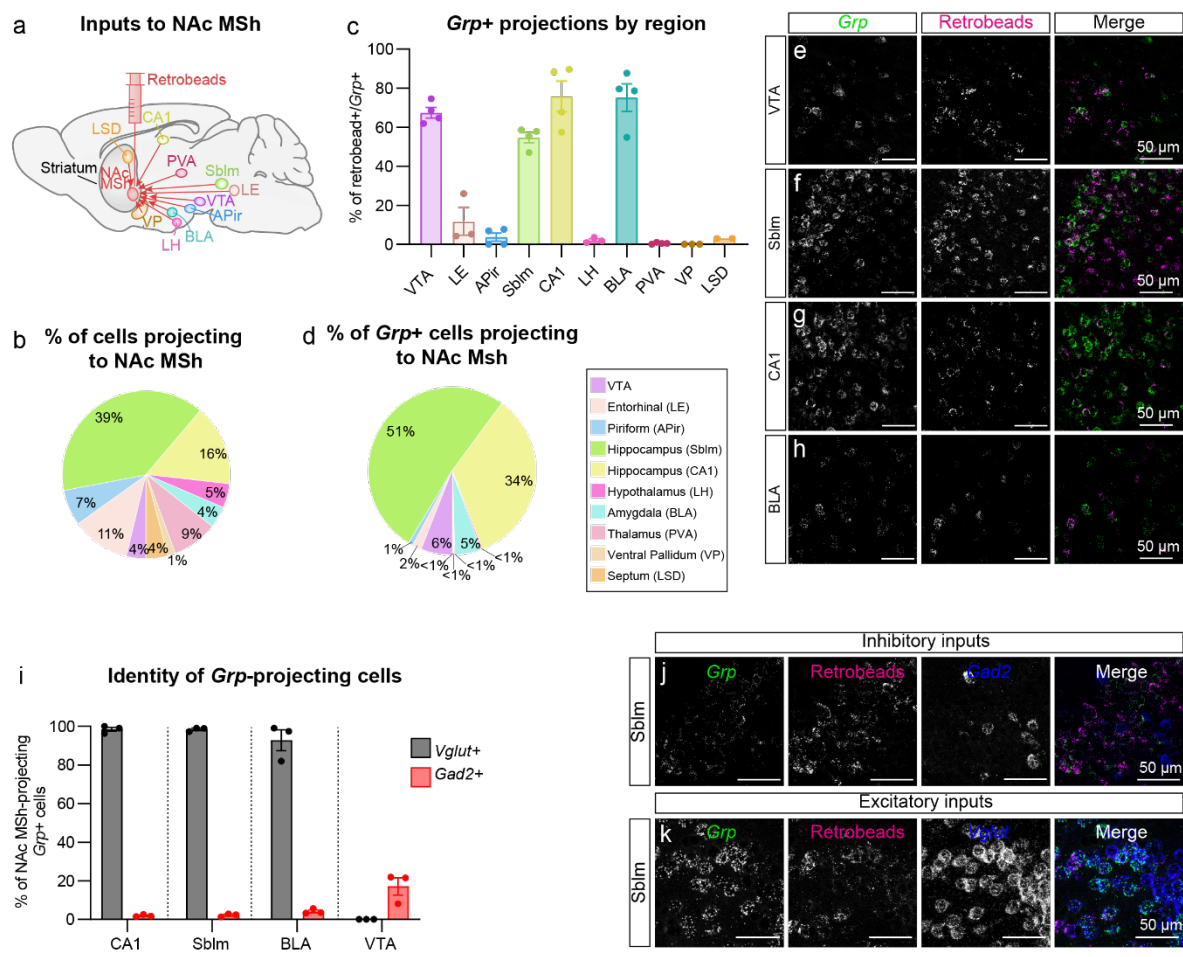


Figure 5: Glutamatergic cells from the hippocampus and amygdala that project to the nucleus accumbens medial shell express *Grp*.

(a) Schematic of retrograde tracing experiment. Red retrobeads were injected into the nucleus accumbens medial shell (NAc MSh) and the listed projection targets were examined for *Grp* mRNA and retrobead co-localization (ventral tegmental area (VTA), lateral entorhinal cortex (LE), anterior piriform (APir), hippocampus (subiculum) (Sblm), hippocampus (CA1), lateral hypothalamus (LH), basolateral amygdala (BLA), paraventricular thalamus (PVA), ventral pallidum (VP), and dorsal lateral septum (LSD)). **(b)** Pie chart displaying the percentage of total retrobead+ cells coming from each region. Retrobead+ cells were summed across all mice (n=4 injections from 3 mice) and then divided by the number of positive cells in each region. **(c)** Percentage of total retrobead+ cells that were *Grp*+ in each of the examined regions. Bars represent mean +/- SEM, dots represent summed values from all slices in that region per 4 unique injection sites from 3 mice. **(d)** Pie chart showing the percentage of total retrobead+/*Grp*+ cells coming from each region, quantified across all mice (n=4 injections from 3 mice). **(e-h)** Representative ISH images from the brain regions indicated showing *Grp*+ cells in green and retrobead+ cells in magenta; **(e)** VTA, **(f)** subiculum, **(g)** CA1, and **(h)** BLA. **(i)** Percentage of retrobead+/*Grp*+ cells that were *Gad2*+ or *Vglut*+ for the four main *Grp*+ inputs into the NAc MSh (mean +/- SEM, dots represent summed value from all slices in that region per 3 unique injection sites from 2 mice). **(j-k)** Representative ISH images of the subiculum showing *Grp*+ cells in green, retrobead+ cells in magenta, and **(j)** *Gad2*+ or **(k)** *Vglut*+ cells in blue.

Figure 6

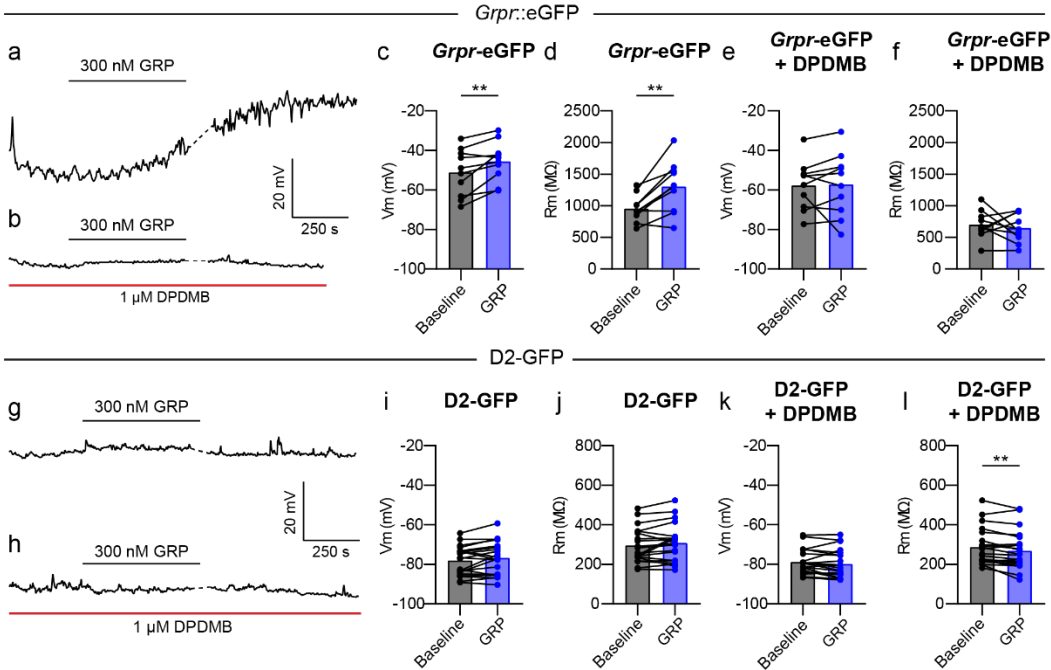


Figure 6: GRP depolarizes and increases the excitability of GRPR-expressing neurons in the nucleus accumbens medial shell.

(a) Representative trace showing the membrane potential over time of a nucleus accumbens medial shell (NAc MSh) *Grpr*-eGFP+ cell. The black bar represents a ten-minute wash-on of 300 nM GRP. (b) Representative trace showing the membrane potential over time of a NAc MSh *Grpr*-eGFP+ cell. 300 nM GRP (black bar) was washed-on for 10 minutes in the presence of 1 μ M DPDMB (red bar, entirety of recording). (c) Membrane potential of *Grpr*-eGFP+ NAc MSh neurons at baseline (black) and after 300 nM GRP wash-on (blue). Baseline value is the average of the two-minute period prior to GRP wash-on and 300 nM GRP value is the average of the final two minutes of 300 nM GRP wash-on; (n=10 cells from 7 mice; **p=0.0059, Wilcoxon test). (d) Membrane resistance of *Grpr*-eGFP+ NAc MSh neurons at baseline (black) and after 300 nM GRP wash-on (blue). Baseline value is the average of five sweeps of VC RC check at the beginning of the recording and just following 300 nM GRP wash-on. (n=10 cells from 7 mice; **p=0.0098, Wilcoxon test). (e) Membrane potential of *Grpr*-eGFP+ NAc MSh neurons at baseline (black) and after 300 nM GRP wash-on (blue) in the presence of 1 μ M DPDMB. Baseline value is the average of two-minute period prior to GRP wash-on and 300 nM GRP value is the average of the final two minutes of 300 nM GRP wash-on; (n=10 cells from 8 mice; p=0.3750, Wilcoxon test). (f) Membrane resistance of *Grpr*-eGFP+ NAc MSh neurons at baseline (black) and after 300 nM GRP wash-on (blue) in the presence of 1 μ M DPDMB. Baseline value is the average of five sweeps of VC RC check at the beginning of recording and just following 300 nM GRP wash-on; (n=10 cells from 8 mice; p=0.6953, Wilcoxon test). (g) Representative trace showing the membrane potential over time of a NAc MSh D2-GFP+ cell. Black bar represents ten-minute wash-on of 300 nM GRP. (h) Representative trace showing the membrane potential over time of a NAc MSh D2-GFP+ cell. 300 nM GRP (black bar) was washed-on in the presence of 1 μ M DPDMB (red bar, entirety of recording). (i) Membrane potential of D2-GFP+ NAc MSh neurons at baseline (black) and after 300 nM GRP wash-on (blue). Baseline value is the average of two-minute period prior to GRP wash-on and 300 nM GRP value is the average of the final two minutes of 300 nM GRP wash-on; (n=20 cells from 20 mice; p=0.1054, Wilcoxon test). (j) Membrane resistance of D2-GFP+ NAc MSh neurons at baseline (black) and after 300 nM GRP wash-on (blue). Baseline value is the average of five sweeps of VC RC check at the beginning of recording and just following 300 nM GRP wash-on; (n=20 cells from 20 mice; p=0.1429, Wilcoxon test). (k) Membrane potential of D2-GFP+ NAc MSh neurons at baseline (black) and after 300 nM GRP wash-on (blue) in the presence of 1 μ M DPDMB. Baseline value is the average of two-minute period prior to GRP wash-on and 300 nM GRP value is the average of the final two minutes of 300 nM GRP wash-on; (n=22 cells from 17 mice; p=0.0501, Wilcoxon test). (l) Membrane resistance of D2-GFP+ NAc MSh neurons at baseline (black) and after 300 nM GRP wash-on (blue) in the presence of 1 μ M DPDMB. Baseline value is the average of five sweeps of VC RC check at the beginning of recording and just following 300 nM GRP wash-on. (n=22 cells from 17 mice; **p=0.0025, Wilcoxon test).

Figure 7

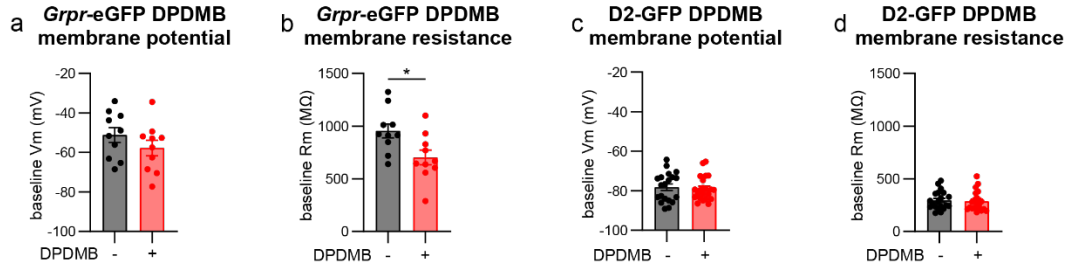


Figure 7: DPDMB decreases the baseline excitability of GRPR-expressing neurons in the nucleus accumbens medial shell.

(a) Mean $\pm$ SEM membrane potential of *Grpr-eGFP*+ nucleus accumbens medial shell (NAc MSh) neurons at baseline without (black) and in the presence of 1 μ M DPDMB (red). Baseline value is the average of two-minute period prior to GRP wash-on; (n=10 cells per condition from 7 mice with no DPDMB and 8 mice with DPDMB; p=0.1968, Mann-Whitney test). (b) Mean $\pm$ SEM membrane resistance of *Grpr-eGFP*+ NAc MSh neurons at baseline without (black) and in the presence of 1 μ M DPDMB (red). Baseline value is the average of five sweeps of VC RC check at the beginning of recording; (n=10 cells per condition from 7 mice with no DPDMB and 8 mice with DPDMB; *p=0.0232, Mann-Whitney test). (c) Mean $\pm$ SEM membrane potential of *D2-GFP*+ NAc MSh neurons at baseline without (black) and in the presence of 1 μ M DPDMB (red). Baseline value is the average of two-minute period prior to GRP wash-on; (no DPDMB: n=20 cells from 20 mice; with DPDMB: n=22 cells from 17 mice; p=0.8129, Mann-Whitney test). (d) Mean $\pm$ SEM membrane resistance of *D2-GFP*+ NAc MSh neurons at baseline without (black) and in the presence of 1 μ M DPDMB (red). Baseline value is the average of five sweeps of VC RC check at the beginning of recording (no DPDMB: n=20 cells from 20 mice baseline, with DPDMB: n=22 cells from 17 mice; p=0.6092, Mann-Whitney test). For all panels, dots represent values for individual neurons.

Figure 8

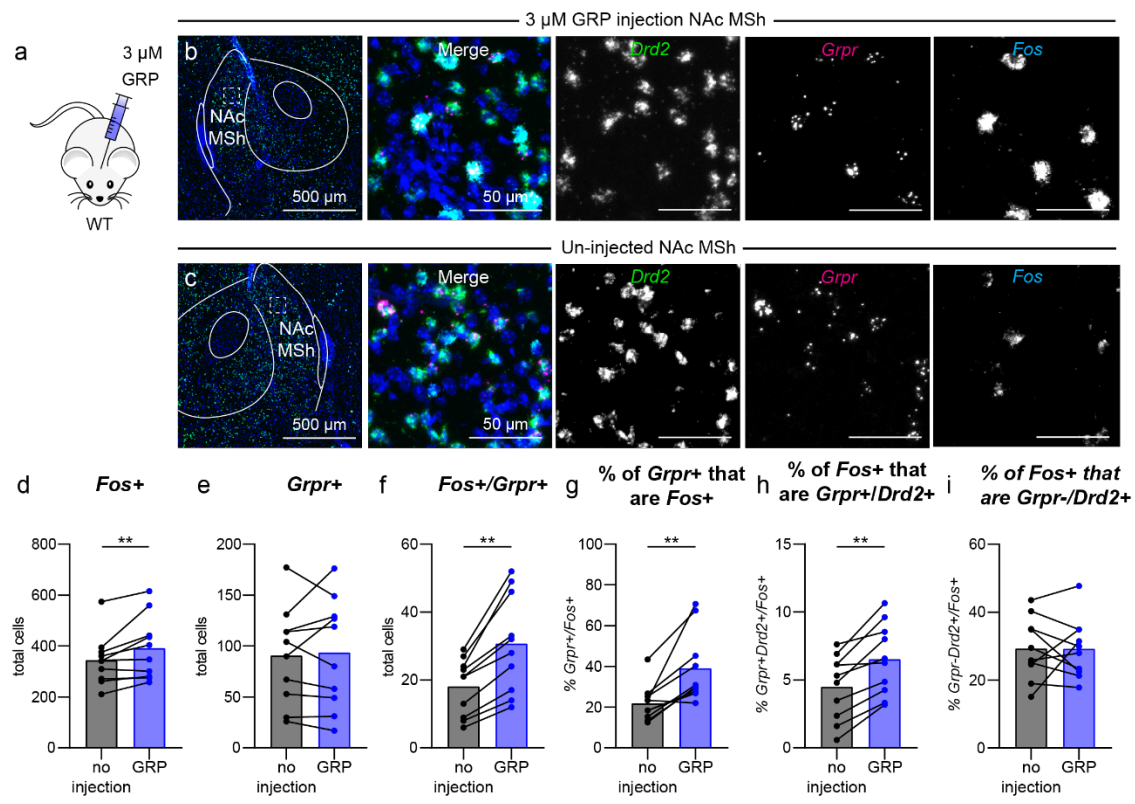


Figure 8: GRP injection *in vivo* increases *Fos* expression in the nucleus accumbens medial shell.

(a) Schematic of the experiment. 3 μ M GRP was injected unilaterally into the nucleus accumbens medial shell (NAc MSh) of wild-type mice. 45 minutes post injection, the mice were sacrificed and brains were harvested for *in situ* hybridization. The NAc MSh from the injected hemisphere was then compared to the un-injected hemisphere. (b-c) Representative images of NAc MSh from the (b) injected hemisphere and (c) un-injected hemisphere. *In situ* hybridization for *Drd2* in green, *Grpr* in magenta, and *Fos* in cyan. Nuclei are labeled with DAPI in blue. (d) Total number of cells in the NAc MSh that were *Fos*⁺ in the un-injected (black) vs 3 μ M GRP-injected hemisphere (blue) summed from two coronal sections per mouse; (n=10 mice; **p=0.0098, Wilcoxon test). (e) Total number of cells in the NAc MSh that were *Grpr*⁺ in the un-injected (black) vs 3 μ M GRP-injected hemisphere (blue) summed from two coronal sections per mouse; (n=10 mice; p=0.9043, Wilcoxon test). (f) Total number of *Grpr*⁺ cells in the NAc MSh that were *Fos*⁺ in the un-injected (black) vs 3 μ M GRP-injected hemisphere (blue) summed from two coronal sections per mouse; (n=10 mice; **p=0.0020, Wilcoxon test). (g) Percentage of *Grpr*⁺ cells in the NAc MSh that were *Fos*⁺ in the un-injected (black) vs 3 μ M GRP-injected hemisphere (blue) summed from two coronal sections per mouse; (n=10 mice; **p=0.0039, Wilcoxon test). (h) Percentage of *Fos*⁺ cells in the NAc MSh that were *Drd2*⁺ and *Grpr*⁺ in the un-injected (black) vs 3 μ M GRP-injected hemisphere (blue) summed from two coronal sections per mouse; (n=10 mice; **p=0.0020, Wilcoxon test). (i) Percentage of *Fos*⁺ cells in the NAc MSh that were *Drd2*⁺ and *Grpr*⁻ in the un-injected (black) vs 3 μ M GRP-injected hemisphere (blue) summed from two coronal sections per mouse; (n=10 mice; p=0.7695, Wilcoxon test).

Figure 9

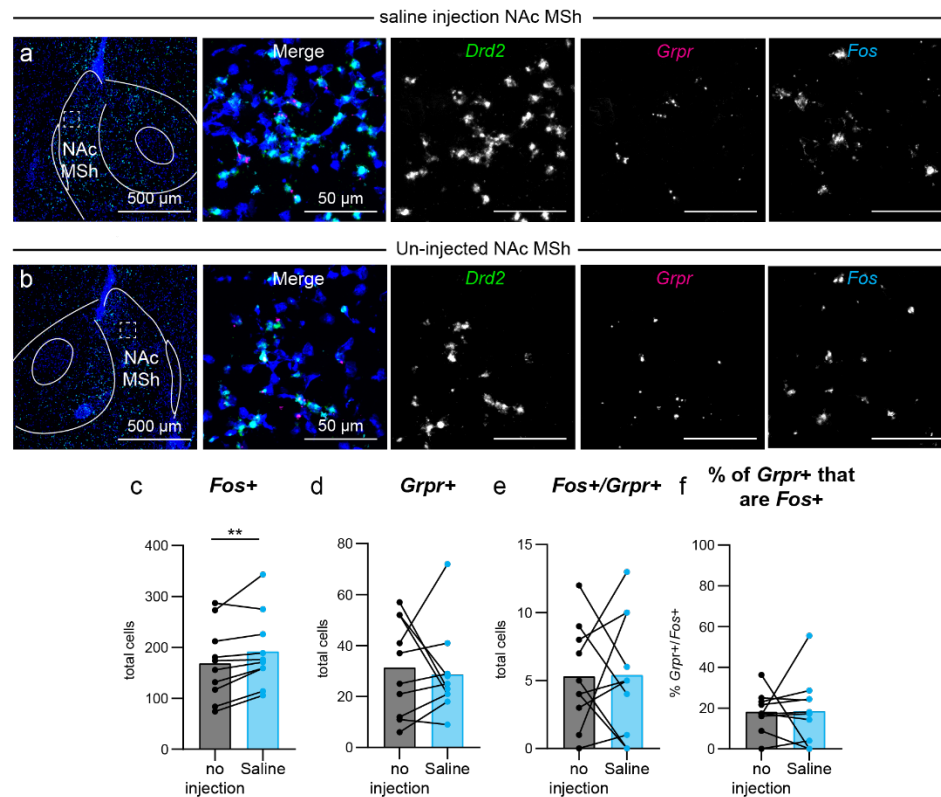


Figure 9: Saline injection into the nucleus accumbens medial shell does not lead to *Fos* induction in *Grpr*-expressing cells.

(a-b) Representative image of nucleus accumbens medial shell (NAc MSh) from the **(a)** saline injected hemisphere and **(b)** un-injected hemisphere. *In situ* hybridization for *Drd2* in green, *Grpr* in magenta, and *Fos* in cyan. Nuclei are labeled with DAPI in blue. **(c)** Total number of cells in the NAc MSh that were *Fos*⁺ in the un-injected (black) vs saline-injected hemisphere (blue) summed from two coronal sections per mouse; (n=10 mice; **p=0.0098, Wilcoxon test). **(d)** Total number of cells in the NAc MSh that were *Grpr*⁺ in the un-injected (black) vs saline-injected hemisphere (blue) summed from two coronal sections per mouse; (n=10 mice; p=0.9414, Wilcoxon test). **(e)** Total number of *Grpr*⁺ cells in the NAc MSh that were *Fos*⁺ in the un-injected (black) vs saline-injected hemisphere (blue) summed from two coronal sections per mouse; (n=10 mice; p=0.9570, Wilcoxon test). **(f)** Percentage of *Grpr*⁺ cells in the NAc MSh that were *Fos*⁺ in the un-injected (black) vs saline-injected hemisphere (blue) summed from two coronal sections per mouse; (n=10 mice; p=0.7695, Wilcoxon test).

Figure 10

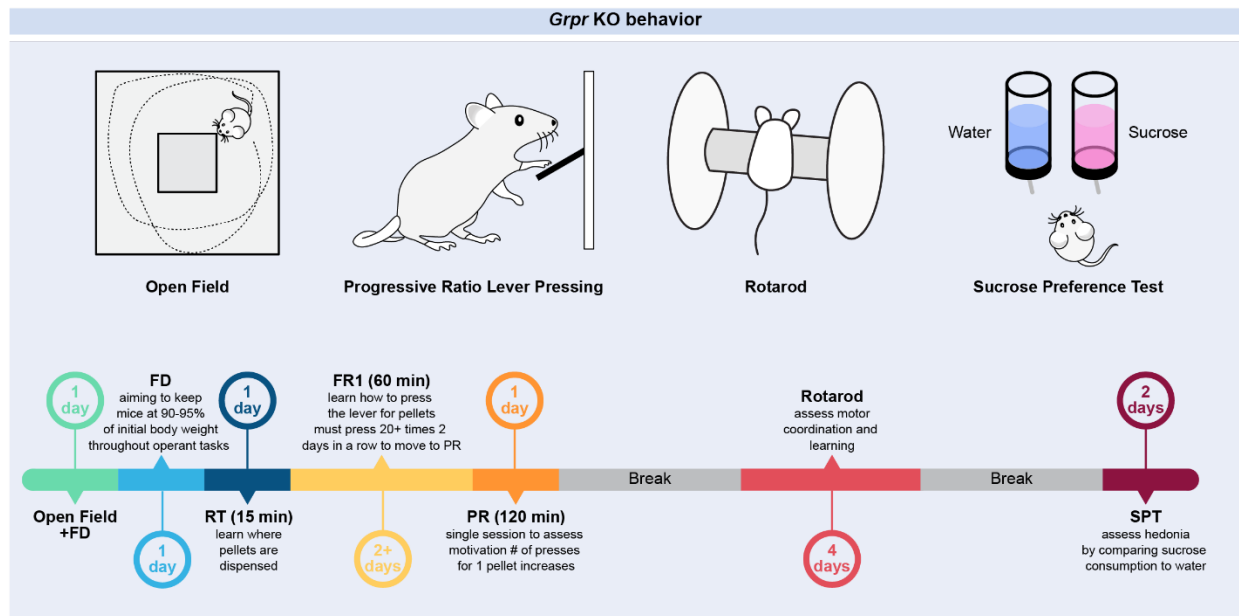


Figure 10: *Grpr* KO mice were tested in striatal-dependent behavior tasks.

Schematic of behavioral paradigm. *Grpr* KO mice and control littermates were run through the open field test, a progressive ratio lever pressing task, and the accelerating rotarod. Mice were first run through the open field followed by two days of food deprivation. Mice then began learning how to press levers for food and were kept at 90-95% original body weight for the duration of operant conditioning. The operant training consisted of a single session of a random trial program where pellets were dispensed at a random interval. Subsequent sessions were 60 minutes of a fixed-ratio of one (FR1), whereby each lever press resulted in a reward. After two subsequent sessions of twenty or more lever presses/rewards in the FR1 paradigm, mice were considered trained and the following day they underwent a two-hour progressive ratio session in which the number of lever presses for a single pellet reward increased over time. Mice were then given a break of at least five days and food restriction ceased before beginning the accelerating rotarod. The accelerating rotarod test consisted of three, 5-minute trials per day for four days. On the first two days the rotarod increased in speed from 5-40 RPM over each trial and on the last two days the rotarod increased from 10-80 RPM over each trial. After a break of at least 4 days, mice underwent the sucrose preference test. They were first acclimated to a single bottle in their home cage for 4 days. Then they were given two bottles in their home cage, one with standard water and another with 5% sucrose for 24 hours. After 24 hours, they were individually housed with two bottles for 24 hours and the percentage of total liquid consumed that was sucrose was measured.

Figure 11

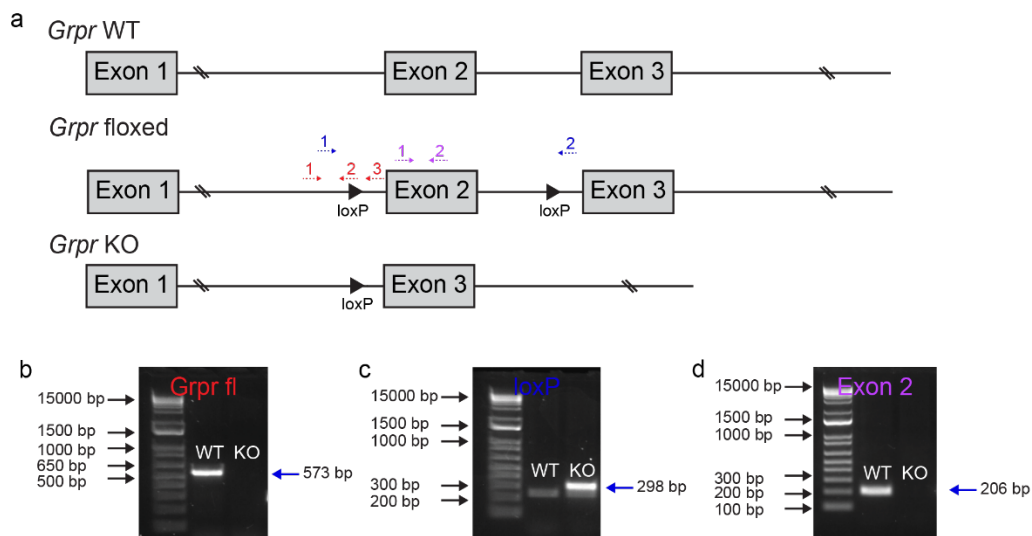


Figure 11: Genotyping strategy for *Grpr* KO mice.

(a) Genotyping strategy for detection of *Grpr* KO and WT mice. *Grpr* floxed mice were bred with CMV-Cre mice to create KOs. A three-polymerase chain reaction (PCR) strategy was used. Primers were designed based on the *Grpr* floxed mice to detect WT and KO mice. Three primers (red) were designed based on the *Grpr* floxed mouse to distinguish between WT, floxed, and KO mice. 1-WT forward, 2-floxed reverse, and 3-WT reverse. WT mice were expected to have a band at 573 bp, floxed mice (not shown or used for behavior) were expected to have a band at 342 bp. KO mice are missing the binding sites for both primers 2 and 3 and therefore are not expected to have a band. Two primers (blue) were designed in regions flanking the loxP sites to detect the loxP scar. 1-loxP forward and 2-loxP reverse. In WT mice the section was too large to amplify so no band was expected, but in the case of KO mice, with the deletion of Exon 2, a 298 bp band was expected. Two primers (purple) were designed within Exon 2 to distinguish between WT and KO mice. 1-Exon 2 forward and 2-Exon 2 reverse. In WT mice, a 206 bp band was expected and in KO mice no band was expected, given that Exon 2 is looped out by Cre. (b) PCR looking for the presence of the floxed or WT allele for *Grpr*. WT mice were expected to have a band at 573 bp and KO mice to have no band. (c) PCR looking for the presence of a loxP scar, indicating successful recombination. WT mice were not expected to have a band as the sequence is too large to be amplified, while KO mice were expected to have a band at 298 bp. (d) PCR for the presence of *Grpr* exon 2. WT mice were expected to have a band at 206 bp, while KO mice were not expected to have a band.

Figure 12

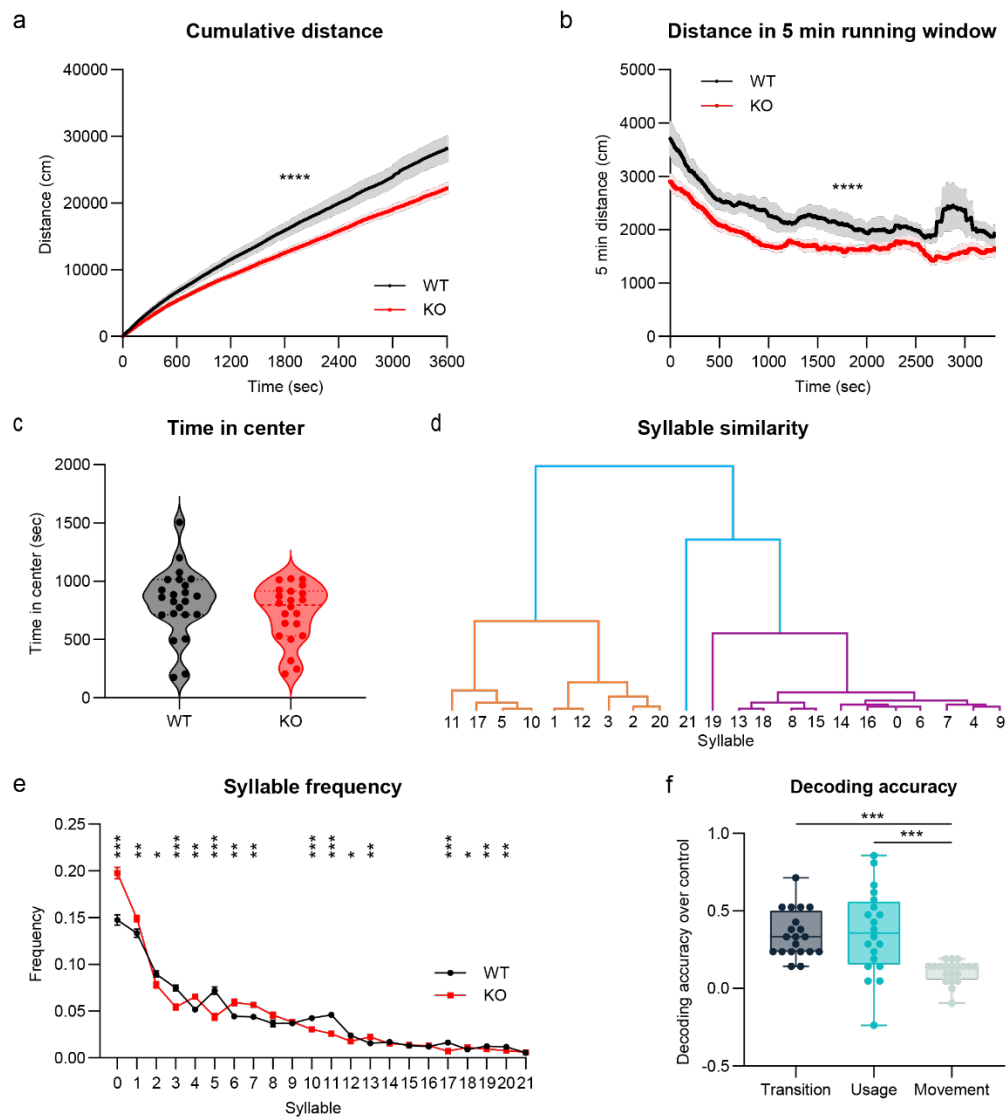


Figure 12: *Grpr* KO mice have reduced locomotor activity and altered behavioral syllable usage in the open field.

(a) Cumulative distance travelled throughout the one-hour session for *Grpr* WT (black) and KO (red) mice. Dark lines are the mean and light shading is the SEM. (n=23 WT and 22 KO mice for all panels). **** $p < 0.0001$, Kolmogorov–Smirnov test. (b) Total distance travelled in five-minute running windows, plotted continuously for *Grpr* WT (black) and KO (red) mice. Dark lines are the mean and light shading is the SEM. **** $p < 0.0001$, Kolmogorov–Smirnov test. (c) Total time spent in the center of the open field during the one-hour session for *Grpr* WT (black) and KO (red) mice. Center was defined as a 20 cm x 20 cm square in the middle of the arena. Mann-Whitney test, $p = 0.3156$. (d) Hierarchical clustering of the 22 most frequent behavioral syllables identified using MoSeq analysis. (e) Frequency of syllable usage for *Grpr* WT (black) and KO (red) mice. Syllable descriptions: 0-static, 1-turn 1, 2-forward-stop, 3-turn 2, 4-rear 1, 5-forward 1, 6-static-backward, 7-turn 3, 8-grooming 1, 9-grooming-rear, 10-forward 2, 11-forward 3, 12-turn-forward, 13-rear 2, 14-turn 4, 15-grooming 2, 16-turn 5, 17-rear-forward 1, 18-grooming-rear 2, 19-turn-forward 3, 20-rear-forward 2, 21-grooming 3. Kruskal-Wallis with Dunn's correction for multiple comparisons, 0: *** $p = 0.000733$, 1: ** $p = 0.005800$, 2: * $p = 0.025667$, 3: *** $p = 0.000733$, 4: ** $p = 0.001375$, 5: *** $p = 0.000733$, 6: ** $p = 0.001257$, 7: ** $p = 0.002200$, 8: $p = 0.134847$, 9: $p = 0.604189$, 10: *** $p = 0.000733$, 11: *** $p = 0.000733$, 12: * $p = 0.010529$, 13: ** $p = 0.003300$, 14: $p = 0.195678$, 15: $p = 0.86410$, 16: $p = 0.803210$, 17: *** $p = 0.000733$, 18: * $p = 0.030112$, 19: ** $p = 0.006050$, 20: ** $p = 0.009646$, 21: $p = 0.663080$. (f) Decoding accuracy for transition, usage, and movement. Decoding was run 20 times with different iterations of training and test data. Control is data with shuffled labels. Plotted values represent the difference between test and control data. Transition, usage, and movement test data were compared to controls using Wilcoxon test, transition: **** $p < 0.0001$, usage: **** $p < 0.0001$, and movement *** $p < 0.0001$. Decoding over control was compared for all 3 variables using a one-way ANOVA with multiple comparisons with Bonferroni correction. One-way ANOVA, **** $p < 0.0001$; transition vs usage $p > 0.9999$, transition vs movement *** $p = 0.0004$, usage vs movement *** $p = 0.002$. Data analyzed by Hongli Wang.

Figure 13

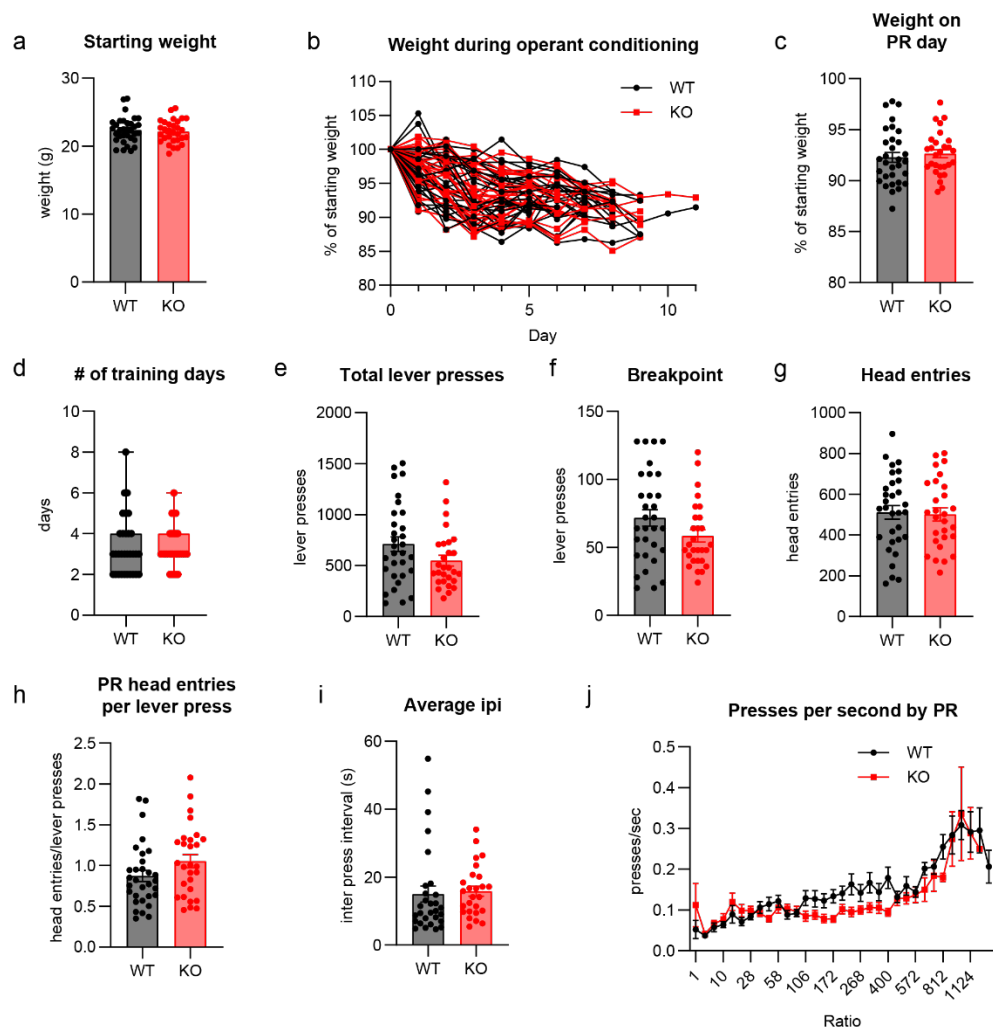


Figure 13: *Grpr* KO mice show no differences in operant conditioning

(a) Mean $\pm$ SEM weight of *Grpr* WT (black) vs *Grpr* KO (red) mice just prior to starting food deprivation. Mann-Whitney test, $p=0.6923$ ($n= 31$ WT and 28 KO mice for all panels). **(b)** Individual lines per mouse of the percentage of starting weight of WT (black) and KO (red) mice for the duration of operant conditioning and food deprivation. **(c)** Mean $\pm$ SEM percentage of starting weight per mouse on the day the mouse completed the progressive ratio task. Mann-Whitney test, $p=0.4714$. **(d)** Box plots of the number of days it took WT (black) and KO (red) mice to reach criterion on the lever pressing task (boxes represent the interquartile range (25-75%), lines denote the median, whiskers represent min to max values). Mice reached criterion after two consecutive days of 20+ lever presses in the fixed-ratio (FR1) sessions. Mann-Whitney test, $p=0.7318$. **(e)** Mean $\pm$ SEM total lever presses in the two-hour progressive ratio session. Mann-Whitney test, $p=0.1131$. **(f)** Mean $\pm$ SEM breakpoint in the two-hour progressive ratio session. Breakpoint was defined as the number of lever presses for a single reward at which the mouse stopped pressing the lever. Welch's two-tailed t-test, $p=0.0837$. **(g)** Mean $\pm$ SEM number of head entries to the location at which the pellets were dispensed in the two-hour progressive ratio session. Welch's two-tailed t-test, $p=0.8418$. **(h)** Mean $\pm$ SEM number of head entries divided by the number of lever presses in the two-hour progressive ratio session. Mann-Whitney test, $p=0.0795$. **(i)** Mean $\pm$ SEM average inter-press interval (ipi) during the two-hour progressive ratio session. Mann-Whitney test, $p=0.0840$. **(j)** Mean $\pm$ SEM of the press rate as a function of the ratio requirement.

Figure 14

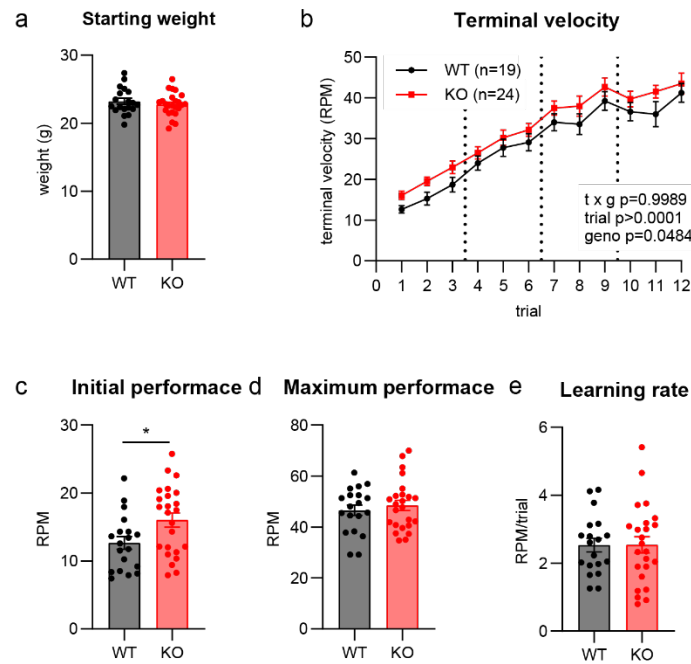


Figure 14: *Grpr* KO mice show enhanced performance on the accelerating rotarod.

(a) Mean $\pm$ SEM weight of *Grpr* WT (black) vs *Grpr* KO (red) mice on Day 1 of rotarod. Mann-Whitney test, $p=0.6940$ ($n= 19$ WT and 24 KO mice for all panels). (b) Rotarod performance for *Grpr* WT (black) and KO mice across all trials, measured as terminal velocity (mean $\pm$ SEM). Two-way repeated measures ANOVA p-values shown in graph. (c) Mean $\pm$ SEM of performance on trial 1 of the rotarod. Mann-Whitney test, $*p=0.0289$. (d) Mean $\pm$ SEM of maximum velocity achieved per-mouse across all twelve trials. Mann-Whitney test, $p=0.8989$. (e) Mean $\pm$ SEM of learning rate, measured as the slope of the linear regression of the performance per mouse across all trials. Mann-Whitney test, $p=0.8893$.

Figure 15

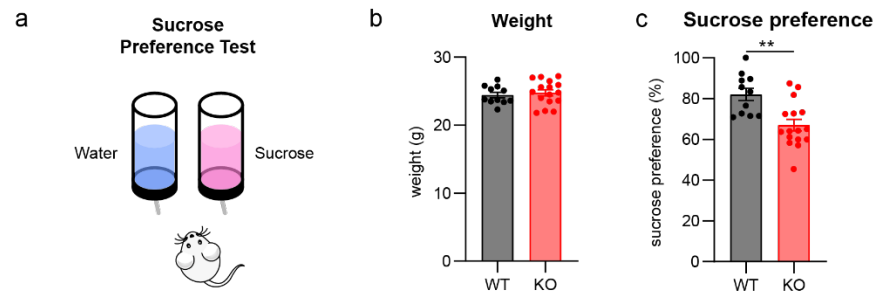


Figure 15: *Grpr* KO mice show decreased sucrose preference.

(a) Schematic of the sucrose preference test. **(b)** Mean $\pm$ SEM weight of *Grpr* WT (black) vs KO (red) mice just prior to sucrose preference test. Mann-Whitney test, $p=0.6016$ ($n=11$ WT and 16 KO mice). **(c)** Mean $\pm$ SEM sucrose preference measured as the percentage of total liquid consumed that was 5% sucrose. Welch's two-tailed t-test, $**p=0.0014$; (n is the same as for **b**).

Chapter 3: Summary and Future Directions

Thesis summary

Neurons have been classically defined by the type of neurotransmitters they express and respond to. Recently, it has become clear that this is oversimplified and chemical transmission between neurons involves more than just classical neurotransmitters. Recent advances in single-cell RNA sequencing have highlighted a potentially significant contribution of neuropeptides[24, 263]. One such neuropeptide is Gastrin releasing peptide (GRP). In this dissertation, I have presented my work following-up on a previous study in the lab, which identified a subpopulation of midbrain dopamine neurons that expresses *Grp* mRNA.

To first probe whether *Grp* in midbrain dopamine neurons might be functionally relevant, I first looked for mRNA for the receptor for GRP, *Grpr*, in the targets of these midbrain dopamine neurons, the dorsal medial striatum and nucleus accumbens medial shell (NAc MSh). Indeed, I found *Grpr* mRNA in both the dorsal striatum and NAc MSh. I next wanted to further characterize these cells and performed multiplex fluorescent *in situ* hybridization for common striatal genetic markers. In the NAc MSh, I found that *Grpr* was mainly expressed in iSPNs. In the dorsal striatum, *Grpr* was also co-expressed with SPN markers, and showed a slight preference for dSPNs, but the SPN subtype was not as consistent as in the NAc MSh. Therefore, for the rest of my dissertation, I chose to focus on the *Grpr* expressing cells in the NAc MSh.

To further study the *Grpr*⁺ cells in the NAc MSh, I obtained a transgenic mouse model that labeled *Grpr*⁺ neurons with eGFP. Using this mouse model, I was able to examine the morphology of these *Grpr*⁺ neurons in the NAc MSh compared to other neurons in the NAc MSh, a majority of which are SPNs. I found that *Grpr*-expressing neurons in the NAc MSh showed no differences in dendritic complexity or spine density compared to neighboring cells in the NAc MSh. Interestingly, however, when I performed whole-cell patch clamp recordings from these cells, I found that even though genetically and morphologically they appeared to be a subpopulation of iSPNs in the NAc MSh, they had quite distinct electrophysiological properties. Approximately 75% of the *Grpr*-expressing cells were spontaneously active at baseline and compared to dSPNs, iSPNs, and ChAT⁺ cells, they were significantly more depolarized and had greater membrane resistance. Consistent with these findings, I performed multiplex fluorescent *in situ* hybridization on naïve wildtype mice and found that cells that expressed both *Drd2* and *Grpr* mRNA were approximately twice as likely to be *Fos*⁺ compared to *Drd2*⁺ cells in the NAc MSh that did not express *Grpr*.

I next wanted to investigate how these cells respond to GRP. First, however, I noted that in addition to the midbrain dopamine neurons many other brain regions also project to the NAc MSh. Therefore, I combined retrograde tracing with fluorescent *in situ* hybridization to map all of the *Grp*-expressing inputs to the NAc MSh. In addition to the previously known dopaminergic population, I identified glutamatergic inputs from the basolateral amygdala and ventral hippocampus that also bring GRP into the NAc MSh. After mapping

the *Grpr*⁺ inputs, I next wanted to look at the functional effect that GRP has on *Grpr*⁺ cells in the NAc MSh, as well as on the NAc MSh more generally. I examined this in two ways. First, I washed on GRP to the NAc MSh while recording from *Grpr*⁺ cells. GRP wash-on further depolarized *Grpr*⁺ cells and increased the membrane resistance approximately 140%. Importantly, these effects were abolished with a GRPR-specific antagonist. Additionally, I performed the same wash-on experiments in D2-expressing cells and saw that GRP had no effect on D2-cells in the NAc MSh. While I previously identified *Grpr*-expressing cells as a subpopulation of iSPNs, *Grpr* was only found to be expressed in less than 5% of *Drd2*⁺ cells in the NAc MSh, therefore there was a low probability that one of the D2⁺ cells we recorded from expressed GRPR. I next examined GRP's effects *in vivo*. To explore GRP's effects in the NAc MSh at the network level, I injected GRP into the NAc MSh of mice and then harvested the tissue for fluorescent *in situ* hybridization. I found that GRP injection induced *Fos* expression in the NAc MSh *Grpr*⁺ cells. No differences in *Fos* expression were seen in *Drd2*⁺/*Grpr*⁻ cells, indicating that GRP-induced activation was selective to GRPR-expressing cells and did not induce larger network-level activation.

Finally, after characterizing the cells and mapping the circuit, I wanted to examine the functional effects of GRP-GRPR signaling in the NAc MSh. To do so, I generated mice lacking GRPR globally and ran them through a variety of tasks known to involve the NAc MSh. In the open field, I saw that *Grpr* knockout (KO) mice travelled significantly less distance than control mice. I also found that *Grpr* KO mice differed in their frequency of usage of behavioral syllables identified with MoSeq. Additionally, although KO mice no showed differences in motivation in operant conditioning, they demonstrated enhanced motor coordination on the accelerating rotarod, and decreased sucrose preference in the sucrose preference test. Together these results indicate that even though GRP is a neuromodulator, eliminating its ability to signal is sufficient to induce gross behavioral changes.

Future directions

The experiments in this dissertation identify and characterize a previously unreported subpopulation of iSPNs in the NAc MSh that express *Grpr*. However, while I have reported on the *Grpr*-expressing cells in the NAc MSh and mapped out a GRP circuit through the NAc MSh, more functional characterization is necessary to fully understand the role of GRP-GRPR signaling in the NAc MSh. Important follow-up experiments stemming from this dissertation include: conditional ablation of GRP-GRPR signaling in only the NAc MSh, examining endogenous GRP in the NAc MSh through evoked-release experiments, exploring a possible role for GRP in synaptic plasticity, and investigating possible co-release of GRP with dopamine and glutamate, amongst others.

For my thesis project, I globally eliminated GRPR expression and then tested mice on behaviors with known contributions of the ventral striatum. A logical next step would, therefore be to repeat these behaviors, as well as others, in mice where GRPR is only eliminated in the NAc MSh. This can be done through viral delivery of CRE to the NAc

MSh to the *Grpr^{fl}* mouse line I used in my dissertation. I showed that eliminating GRP-GRPR signaling is sufficient to induce behavioral changes, implying that GRP signaling is sufficient to influence behavior. However, even with the use of tasks known to involve the NAc MSh, I cannot conclusively attribute the behavioral effects I see to the GRPR neurons in the NAc MSh. Past studies using global and conditional *Grpr* KO mice found opposite effects on auditory fear conditioning [252, 255]. A study focused on the amygdala using a global KO mouse and saw prolonged fear memories for auditory and contextual clues [252]. In contrast, Melzer and colleagues (2021) studied auditory fear conditioning in the auditory cortex and eliminated GRPR only in this region [255]. They saw that eliminating GRP-GRPR signaling only in the auditory cortex, inhibited auditory fear memories [255]. Additionally, these studies highlight how multiple brain regions can modulate the same behavior as Melzer et al. (2021) studied modulation of auditory fear conditioning by GRPR-expressing interneurons in the auditory cortex and Shumyatsky et al. (2002) were studying the GRPR neurons in the amygdala and their role in auditory fear [252, 255].

Here, I tested a few of the behaviors that have been known to be modulated by the NAc MSh. Future studies should test additional behaviors that are thought to involve the NAc MSh. One interesting behavior to pursue would be cocaine conditioned place preference. I have shown that *Grpr* is expressed in SPNs in both the NAc MSh and dorsal striatum. In the NAc MSh it was mostly expressed in iSPNs and in the dorsal striatum, the segregation was less clear, but showed a dSPN preference. This is interesting in the context of cocaine addiction given that the neural mechanisms underlying initial rewarding drug use to compulsive drug use start in the NAc MSh and then move to the dorsal striatum over the course of addiction [289]. Additionally, initial drug use activates D2-expressing cells in the NAc MSh and when drug use becomes habitual and switches to the dorsal striatum, it involves D1-expressing cells [290-292]. Other behaviors that may involve GRP-GRPR signaling in the NAc MSh are those that have been associated with the subiculum to NAc MSh projections. My data showed that a majority of the subiculum inputs to the NAc MSh expressed *Grp*. Behaviors that have been associated with this projection include context-induced reinstatement of heroin use [285] as well as anticipating predictable rewards [286]. A possible role of GRP-GRPR signaling should be examined in these behaviors, amongst others.

Another area that warrants exploration is examining endogenous GRP release. While I showed that neurons expressing *Grp* project to the NAc MSh and that *Grpr*-expressing neurons in the NAc MSh can respond to GRP wash-on and *in vivo* GRP injection, without tools such as sensors that can detect GRP release, it is not known if the concentrations used in this project are physiologically relevant. I did find some evidence for endogenous GRP being present in slice when I saw that the application of a GRPR-specific antagonist led to a decrease in baseline membrane resistance compared to control conditions. Additionally, Pagani et al. (2019) were able to optogenetically evoke GRP release in the spinal cord [251]. While I obtained the *Grp*-CRE mouse they used, it did not sufficiently label the projections to the NAc MSh. An alternative approach that could be used to try to

evoke GRP release in the NAc MSh would be to virally express channelrhodopsin in the ventral subiculum where a large number of cells express *Grp* and project to the NAc MSh and use the stimulation protocol established by Pagani et al. (2019) [251].

iSPNs in the NAc have previously been shown to express endocannabinoid-dependent long-term synaptic depression through activation of group 1 mGluRs [293]. Both group 1 mGluRs and GRPR are Gq-coupled receptors. Therefore, it is possible that GRP-GRPR signaling can also lead to endocannabinoid-dependent long-term depression or have other effects on synaptic plasticity. While not a direct mechanism as the one suggested above, GRP-GRPR signaling has been implicated in synaptic plasticity in the amygdala [252]. Here, GRPR KO mice showed enhanced long-term potentiation due to decreased inhibition onto principle neurons in the amygdala [252].

Here I used multiplex fluorescent *in situ* hybridization to further characterize *Grpr*-expressing neurons in the NAc MSh. While I probed for other striatal cell-type markers, this was not unbiased. Given their unique cellular properties, it would be interesting to do scRNA-seq on *Grpr*-expressing neurons to provide more insight into the genetic makeup of these previously uncharacterized cells in the NAc MSh.

In this dissertation, I identified additional sources of *Grp*-expressing inputs to the NAc MSh in addition to the dopaminergic population previously identified in the lab [263]. While I show that GRP-GRPR signaling is possible in the GRPR-expressing neurons in the NAc MSh and the dopaminergic and glutamatergic signaling in the NAc MSh is well established, an interesting future direction would be to examine the effects of GRP co-release with glutamate and/or dopamine. While the most physiologically relevant iteration of this experiment would be to look at endogenous co-release, a simpler and still informative experiment would be to pair synaptic stimulation of glutamatergic afferents with GRP wash-on in slice with and without the antagonist and record from the GRPR neurons in the NAc MSh in the presence of synaptic blockers to isolate the effects on glutamatergic transmission.

The main focus of this project was on GRP-GRPR signaling in the NAc MSh, however, given the anatomical proximity of the dorsal striatum and NAc MSh, this study provided some preliminary genetic characterization of another population of *Grpr*-expressing SPNs in the dorsal striatum. Given that GRP-GRPR signaling has been shown to be sufficient to modulate behavior in a variety of brain regions, it is worth further investigating this population. Additionally, I found that *Grpr* global KO mice had enhanced motor coordination on the accelerating rotarod. This task has been linked to both the dorsal and ventral striatum, so it is possible that the enhanced motor coordination was due to ablation of GRP-GRPR signaling in the dorsal striatum.

Finally, this study demonstrates one example of how a single neuropeptide and its associated circuit can influence cellular and behavioral properties. This highlights the role that neuropeptides may play more broadly not only in the NAc MSh, but also, more generally in the brain. Increasing evidence shows that their expression is widespread and

their signaling is poised to influence circuits and behaviors. They can also potentially identify subpopulations of neurons, as was the case in this study, that have unique properties. Further scRNA-seq studies and additional analysis of previously published datasets can be used to identify new neuropeptide targets to examine in more depth. One such dataset was generated by Saunders et al. (2018), however, a limitation of this dataset in the context of this study is that they did not sequence cells in the NAc [294].

In conclusion, this dissertation describes a subpopulation of iSPNs in the NAc MSh that express *Grpr* and the associated circuit, with possible functional implications in coordination, reward salience, and motivation. It demonstrates the effects that neuropeptide signaling can have in the NAc MSh and that disruption of this is sufficient to modulate behaviors. In addition, I showed that neuropeptides can serve as important markers of functionally unique subpopulations of neurons. There is much more still to understand about GRP-GRPR signaling in the NAc MSh and the contribution of neuropeptide signaling more broadly, but a better understanding of neuropeptide expression in circuits is poised to lead to a greater understanding of neural circuits and behaviors.

References

1. Nusbaum, M.P., *Neurotransmission: peptide transmitters turn* 36. J Exp Biol, 2017. **220**(Pt 14): p. 2492-2494.
2. Guillaumin, M.C.C. and D. Burdakov, *Neuropeptides as Primary Mediators of Brain Circuit Connectivity*. Front Neurosci, 2021. **15**: p. 644313.
3. Burbach, J.P., *What are neuropeptides?* Methods Mol Biol, 2011. **789**: p. 1-36.
4. Mocchetti, I. and E. Costa, *In vivo studies of the regulation of neuropeptide stores in structures of the rat brain*. Neuropharmacology, 1987. **26**(7B): p. 855-62.
5. Fricker, L.D. and S.H. Snyder, *Enkephalin convertase: purification and characterization of a specific enkephalin-synthesizing carboxypeptidase localized to adrenal chromaffin granules*. Proc Natl Acad Sci U S A, 1982. **79**(12): p. 3886-90.
6. Reisine, T. and G.I. Bell, *Molecular properties of somatostatin receptors*. Neuroscience, 1995. **67**(4): p. 777-90.
7. Russo, A.F., *Overview of Neuropeptides: Awakening the Senses? Headache*, 2017. **57 Suppl 2**(Suppl 2): p. 37-46.
8. Edwards, R.H., *Neurotransmitter release: variations on a theme*. Current biology, 1998. **8**(24): p. R883-R885.
9. Hokfelt, T., T. Bartfai, and F. Bloom, *Neuropeptides: opportunities for drug discovery*. Lancet Neurol, 2003. **2**(8): p. 463-72.
10. Ludwig, M. and Q.J. Pittman, *Talking back: dendritic neurotransmitter release*. Trends Neurosci, 2003. **26**(5): p. 255-61.
11. van den Pol, A.N., *Neuropeptide transmission in brain circuits*. Neuron, 2012. **76**(1): p. 98-115.
12. Hokfelt, T., et al., *Cellular localization of peptides in neural structures*. Proc R Soc Lond B Biol Sci, 1980. **210**(1178): p. 63-77.
13. Fuxe, K. and L.F. Agnati, *Volume transmission in the brain: novel mechanisms for neural transmission*. (No Title), 1991.
14. Hoyer, D. and T. Bartfai, *Neuropeptides and neuropeptide receptors: drug targets, and peptide and non-peptide ligands: a tribute to Prof. Dieter Seebach*. Chem Biodivers, 2012. **9**(11): p. 2367-87.
15. Krishnan, A. and H.B. Schioth, *The role of G protein-coupled receptors in the early evolution of neurotransmission and the nervous system*. J Exp Biol, 2015. **218**(Pt 4): p. 562-71.
16. Siegel, G.J. and R.W. Albers, *Basic neurochemistry: molecular, cellular, and medical aspects*. 1994: Raven Press.
17. Syrovatkina, V., et al., *Regulation, Signaling, and Physiological Functions of G-Proteins*. J Mol Biol, 2016. **428**(19): p. 3850-68.
18. Varoquaux, F. and D. Fasshauer, *Getting Nervous: An Evolutionary Overhaul for Communication*. Annu Rev Genet, 2017. **51**: p. 455-476.
19. Elphick, M.R., O. Mirabeau, and D. Larhammar, *Evolution of neuropeptide signalling systems*. Journal of Experimental Biology, 2018. **221**(3): p. jeb151092.
20. Grimmlikhuijzen, C.J. and F. Hauser, *Mini-review: the evolution of neuropeptide signaling*. Regul Pept, 2012. **177 Suppl**: p. S6-9.
21. Jekely, G., *Global view of the evolution and diversity of metazoan neuropeptide signaling*. Proc Natl Acad Sci U S A, 2013. **110**(21): p. 8702-7.

22. Ludwig, M. and G. Leng, *Dendritic peptide release and peptide-dependent behaviours*. Nat Rev Neurosci, 2006. **7**(2): p. 126-36.
23. Nassel, D.R., *Neuropeptide signaling near and far: how localized and timed is the action of neuropeptides in brain circuits?* Invert Neurosci, 2009. **9**(2): p. 57-75.
24. Smith, S.J., et al., *Single-cell transcriptomic evidence for dense intracortical neuropeptide networks*. Elife, 2019. **8**.
25. Cisek, P. and J.F. Kalaska, *Neural mechanisms for interacting with a world full of action choices*. Annual review of neuroscience, 2010. **33**: p. 269-298.
26. Graybiel, A.M., et al., *The basal ganglia and adaptive motor control*. Science, 1994. **265**(5180): p. 1826-1831.
27. Hikosaka, O., Y. Takikawa, and R. Kawagoe, *Role of the basal ganglia in the control of purposive saccadic eye movements*. Physiological reviews, 2000. **80**(3): p. 953-978.
28. Mink, J.W., *The basal ganglia: focused selection and inhibition of competing motor programs*. Progress in neurobiology, 1996. **50**(4): p. 381-425.
29. Redgrave, P., T.J. Prescott, and K. Gurney, *The basal ganglia: a vertebrate solution to the selection problem?* Neuroscience, 1999. **89**(4): p. 1009-23.
30. Wichmann, T. and M.R. DeLong, *Functional neuroanatomy of the basal ganglia in Parkinson's disease*. Adv Neurol, 2003. **91**: p. 9-18.
31. Yin, H.H. and B.J. Knowlton, *The role of the basal ganglia in habit formation*. Nat Rev Neurosci, 2006. **7**(6): p. 464-76.
32. Gerfen, C.R. and J.P. Bolam, *The neuroanatomical organization of the basal ganglia*, in *Handbook of behavioral neuroscience*. 2016, Elsevier. p. 3-32.
33. O'Connor, E.C., et al., *Accumbal D1R Neurons Projecting to Lateral Hypothalamus Authorize Feeding*. Neuron, 2015. **88**(3): p. 553-64.
34. Dölen, G., et al., *Social reward requires coordinated activity of nucleus accumbens oxytocin and serotonin*. Nature, 2013. **501**(7466): p. 179-84.
35. Smith-Roe, S.L. and A.E. Kelley, *Coincident activation of NMDA and dopamine D1 receptors within the nucleus accumbens core is required for appetitive instrumental learning*. J Neurosci, 2000. **20**(20): p. 7737-42.
36. Kravitz, A.V., L.D. Tye, and A.C. Kreitzer, *Distinct roles for direct and indirect pathway striatal neurons in reinforcement*. Nat Neurosci, 2012. **15**(6): p. 816-8.
37. Berridge, K.C. and M.L. Kringelbach, *Pleasure systems in the brain*. Neuron, 2015. **86**(3): p. 646-64.
38. Gerfen, C.R. and D.J. Surmeier, *Modulation of striatal projection systems by dopamine*. Annu Rev Neurosci, 2011. **34**: p. 441-66.
39. Voorn, P., et al., *Putting a spin on the dorsal-ventral divide of the striatum*. Trends Neurosci, 2004. **27**(8): p. 468-74.
40. Cataldi, S., et al., *Decreased Dorsomedial Striatum Direct Pathway Neuronal Activity Is Required for Learned Motor Coordination*. eNeuro, 2022. **9**(5).
41. Cataldi, S., et al., *Interpreting the role of the striatum during multiple phases of motor learning*. Febs j, 2022. **289**(8): p. 2263-2281.
42. Graybiel, A.M., *Habits, rituals, and the evaluative brain*. Annu Rev Neurosci, 2008. **31**: p. 359-87.
43. Hunnicutt, B.J., et al., *A comprehensive excitatory input map of the striatum reveals novel functional organization*. Elife, 2016. **5**.

44. Cajal, S. and S. Cajal, *Histologie du Systeme Nerveux de l'Homme et des Vertebres (Maloine)*. Paris (1909–1911), 1911.
45. Meredith, G.E., et al., *The structural basis for mapping behavior onto the ventral striatum and its subdivisions*. Brain Structure and Function, 2008. **213**: p. 17-27.
46. Wilson, C.J., et al., *Three-dimensional structure of dendritic spines in the rat neostriatum*. J Neurosci, 1983. **3**(2): p. 383-8.
47. Castro, D.C. and M.R. Bruchas, *A motivational and neuropeptidergic hub: Anatomical and functional diversity within the nucleus accumbens shell*. Neuron, 2019. **102**(3): p. 529-552.
48. Kawaguchi, Y., *Physiological, morphological, and histochemical characterization of three classes of interneurons in rat neostriatum*. J Neurosci, 1993. **13**(11): p. 4908-23.
49. Tepper, J.M., et al., *Heterogeneity and diversity of striatal GABAergic interneurons*. Frontiers in neuroanatomy, 2010. **4**: p. 150.
50. Kravitz, A.V., et al., *Regulation of parkinsonian motor behaviours by optogenetic control of basal ganglia circuitry*. Nature, 2010. **466**(7306): p. 622-6.
51. Tai, L.H., et al., *Transient stimulation of distinct subpopulations of striatal neurons mimics changes in action value*. Nat Neurosci, 2012. **15**(9): p. 1281-9.
52. Anderson, K.D. and A. Reiner, *Extensive co-occurrence of substance P and dynorphin in striatal projection neurons: an evolutionarily conserved feature of basal ganglia organization*. Journal of Comparative Neurology, 1990. **295**(3): p. 339-369.
53. Besson, M., A. Graybiel, and B. Quinn, *Co-expression of neuropeptides in the cat's striatum: an immunohistochemical study of substance P, dynorphin B and enkephalin*. Neuroscience, 1990. **39**(1): p. 33-58.
54. Schiffmann, S.N. and J.-J. Vanderhaeghen, *Adenosine A2 receptors regulate the gene expression of striatopallidal and striatonigral neurons*. Journal of Neuroscience, 1993. **13**(3): p. 1080-1087.
55. Gertler, T.S., C.S. Chan, and D.J. Surmeier, *Dichotomous anatomical properties of adult striatal medium spiny neurons*. Journal of Neuroscience, 2008. **28**(43): p. 10814-10824.
56. Gerfen, C.R., K.G. Baimbridge, and J.J. Miller, *The neostriatal mosaic: compartmental distribution of calcium-binding protein and parvalbumin in the basal ganglia of the rat and monkey*. Proceedings of the National Academy of Sciences, 1985. **82**(24): p. 8780-8784.
57. Kita, H., T. Kosaka, and C. Heizmann, *Parvalbumin-immunoreactive neurons in the rat neostriatum: a light and electron microscopic study*. Brain research, 1990. **536**(1-2): p. 1-15.
58. Koós, T. and J.M. Tepper, *Inhibitory control of neostriatal projection neurons by GABAergic interneurons*. Nature neuroscience, 1999. **2**(5): p. 467-472.
59. Gittis, A.H., et al., *Distinct roles of GABAergic interneurons in the regulation of striatal output pathways*. Journal of Neuroscience, 2010. **30**(6): p. 2223-2234.
60. Planert, H., et al., *Dynamics of synaptic transmission between fast-spiking interneurons and striatal projection neurons of the direct and indirect pathways*. Journal of Neuroscience, 2010. **30**(9): p. 3499-3507.

61. Straub, C., et al., *Principles of synaptic organization of GABAergic interneurons in the striatum*. Neuron, 2016. **92**(1): p. 84-92.
62. Aoki, C. and V.M. Pickel, *Neuropeptide Y in cortex and striatum. Ultrastructural distribution and coexistence with classical neurotransmitters and neuropeptides*. Annals of the New York Academy of Sciences, 1990. **611**: p. 186-205.
63. Chuhma, N., et al., *Dopamine neurons control striatal cholinergic neurons via regionally heterogeneous dopamine and glutamate signaling*. Neuron, 2014. **81**(4): p. 901-912.
64. Ma, Y., et al., *Morphological diversity of GABAergic and cholinergic interneurons in the striatal dorsolateral and ventromedial regions of rats*. Cellular and molecular neurobiology, 2014. **34**: p. 351-359.
65. Aosaki, T., et al., *Responses of tonically active neurons in the primate's striatum undergo systematic changes during behavioral sensorimotor conditioning*. Journal of Neuroscience, 1994. **14**(6): p. 3969-3984.
66. Atallah, H.E., et al., *Neurons in the ventral striatum exhibit cell-type-specific representations of outcome during learning*. Neuron, 2014. **82**(5): p. 1145-1156.
67. Shimo, Y. and O. Hikosaka, *Role of tonically active neurons in primate caudate in reward-oriented saccadic eye movement*. Journal of Neuroscience, 2001. **21**(19): p. 7804-7814.
68. Cacheope, R., et al., *Selective activation of cholinergic interneurons enhances accumbal phasic dopamine release: setting the tone for reward processing*. Cell reports, 2012. **2**(1): p. 33-41.
69. Nelson, A.B., et al., *Striatal cholinergic interneurons Drive GABA release from dopamine terminals*. Neuron, 2014. **82**(1): p. 63-70.
70. Threlfell, S., et al., *Striatal dopamine release is triggered by synchronized activity in cholinergic interneurons*. Neuron, 2012. **75**(1): p. 58-64.
71. Brimblecombe, K.R. and S.J. Cragg, *The striosome and matrix compartments of the striatum: a path through the labyrinth from neurochemistry toward function*. ACS chemical neuroscience, 2017. **8**(2): p. 235-242.
72. Pert, C.B., M.J. Kuhar, and S.H. Snyder, *Opiate receptor: autoradiographic localization in rat brain*. Proceedings of the national academy of Sciences, 1976. **73**(10): p. 3729-3733.
73. Gerfen, C.R. and W.S. Young III, *Distribution of striatonigral and striatopallidal peptidergic neurons in both patch and matrix compartments: an in situ hybridization histochemistry and fluorescent retrograde tracing study*. Brain research, 1988. **460**(1): p. 161-167.
74. Friedman, A., et al., *A corticostriatal path targeting striosomes controls decision-making under conflict*. Cell, 2015. **161**(6): p. 1320-1333.
75. Gerfen, C.R., *The neostriatal mosaic: compartmentalization of corticostriatal input and striatonigral output systems*. Nature, 1984. **311**(5985): p. 461-464.
76. Beckstead, R.M., *A pallidostriatal projection in the cat and monkey*. Brain research bulletin, 1983. **11**(6): p. 629-632.
77. Bevan, M.D., et al., *Selective innervation of neostriatal interneurons by a subclass of neuron in the globus pallidus of the rat*. Journal of Neuroscience, 1998. **18**(22): p. 9438-9452.

78. Kita, H. and S. Kita, *The morphology of globus pallidus projection neurons in the rat: an intracellular staining study*. Brain research, 1994. **636**(2): p. 308-319.
79. Mallet, N., et al., *Dichotomous organization of the external globus pallidus*. Neuron, 2012. **74**(6): p. 1075-1086.
80. Staines, W.A., S. Atmadja, and H. Fibiger, *Demonstration of a pallidostriatal pathway by retrograde transport of HRP-labeled lectin*. Brain research, 1981. **206**(2): p. 446-450.
81. Haber, S.N., J.L. Fudge, and N.R. McFarland, *Striatonigrostriatal pathways in primates form an ascending spiral from the shell to the dorsolateral striatum*. Journal of Neuroscience, 2000. **20**(6): p. 2369-2382.
82. Tejada, H.A., et al., *Pathway-and cell-specific kappa-opioid receptor modulation of excitation-inhibition balance differentially gates D1 and D2 accumbens neuron activity*. Neuron, 2017. **93**(1): p. 147-163.
83. Cazorla, M., et al., *Dopamine D2 receptors regulate the anatomical and functional balance of basal ganglia circuitry*. Neuron, 2014. **81**(1): p. 153-164.
84. Saunders, A., et al., *A direct GABAergic output from the basal ganglia to frontal cortex*. Nature, 2015. **521**(7550): p. 85-89.
85. Chevalier, G., et al., *Disinhibition as a basic process in the expression of striatal functions. I. The striato-nigral influence on tecto-spinal/tecto-diencephalic neurons*. Brain research, 1985. **334**(2): p. 215-226.
86. Deniau, J.M. and G. Chevalier, *Disinhibition as a basic process in the expression of striatal functions. II. The striato-nigral influence on thalamocortical cells of the ventromedial thalamic nucleus*. Brain research, 1985. **334**(2): p. 227-233.
87. Cui, G., et al., *Concurrent activation of striatal direct and indirect pathways during action initiation*. Nature, 2013. **494**(7436): p. 238-242.
88. Balleine, B.W., M.R. Delgado, and O. Hikosaka, *The role of the dorsal striatum in reward and decision-making*. Journal of Neuroscience, 2007. **27**(31): p. 8161-8165.
89. Nambu, A., *Seven problems on the basal ganglia*. Current opinion in neurobiology, 2008. **18**(6): p. 595-604.
90. Záborszky, L., et al., *Cholecystokinin innervation of the ventral striatum: a morphological and radioimmunological study*. Neuroscience, 1985. **14**(2): p. 427-53.
91. Meredith, G.E., et al., *Morphological differences between projection neurons of the core and shell in the nucleus accumbens of the rat*. Neuroscience, 1992. **50**(1): p. 149-62.
92. Ikemoto, S., *Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex*. Brain Res Rev, 2007. **56**(1): p. 27-78.
93. Saunders, B.T., et al., *Dopamine neurons create Pavlovian conditioned stimuli with circuit-defined motivational properties*. Nat Neurosci, 2018. **21**(8): p. 1072-1083.
94. Wright, C.I., A.V. Beijer, and H.J. Groenewegen, *Basal amygdaloid complex afferents to the rat nucleus accumbens are compartmentally organized*. J Neurosci, 1996. **16**(5): p. 1877-93.

95. Ma, Y.Y., et al., *Regional and cell-type-specific effects of DAMGO on striatal D1 and D2 dopamine receptor-expressing medium-sized spiny neurons*. ASN Neuro, 2012. **4**(2).
96. Roitman, M.F., R.A. Wheeler, and R.M. Carelli, *Nucleus accumbens neurons are innately tuned for rewarding and aversive taste stimuli, encode their predictors, and are linked to motor output*. Neuron, 2005. **45**(4): p. 587-97.
97. Taha, S.A. and H.L. Fields, *Encoding of palatability and appetitive behaviors by distinct neuronal populations in the nucleus accumbens*. J Neurosci, 2005. **25**(5): p. 1193-202.
98. Bertran-Gonzalez, J., et al., *Opposing patterns of signaling activation in dopamine D1 and D2 receptor-expressing striatal neurons in response to cocaine and haloperidol*. J Neurosci, 2008. **28**(22): p. 5671-85.
99. Kupchik, Y.M., et al., *Coding the direct/indirect pathways by D1 and D2 receptors is not valid for accumbens projections*. Nature neuroscience, 2015. **18**(9): p. 1230-1232.
100. Al-Hasani, R., et al., *Distinct subpopulations of nucleus accumbens dynorphin neurons drive aversion and reward*. Neuron, 2015. **87**(5): p. 1063-1077.
101. Landwehrmeyer, B., G. Mengod, and J.M. Palacios, *Differential visualization of dopamine D2 and D3 receptor sites in rat brain. A comparative study using in situ hybridization histochemistry and ligand binding autoradiography*. Eur J Neurosci, 1993. **5**(2): p. 145-53.
102. Sokoloff, P., et al., *Localization and function of the D3 dopamine receptor*. Arzneimittelforschung, 1992. **42**(2a): p. 224-30.
103. Kubota, Y., S. Mikawa, and Y. Kawaguchi, *Neostriatal GABAergic interneurons contain NOS, calretinin or parvalbumin*. Neuroreport, 1993. **5**(3): p. 205-8.
104. Mansour, A., et al., *Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study*. J Comp Neurol, 1994. **350**(3): p. 412-38.
105. Lammel, S., et al., *Input-specific control of reward and aversion in the ventral tegmental area*. Nature, 2012. **491**(7423): p. 212-7.
106. Chen, G., et al., *Distinct reward processing by subregions of the nucleus accumbens*. Cell Rep, 2023. **42**(2): p. 112069.
107. Yang, H., et al., *Nucleus Accumbens Subnuclei Regulate Motivated Behavior via Direct Inhibition and Disinhibition of VTA Dopamine Subpopulations*. Neuron, 2018. **97**(2): p. 434-449.e4.
108. Li, Z., et al., *Cell-type-specific afferent innervation of the nucleus accumbens core and shell*. Frontiers in neuroanatomy, 2018. **12**: p. 84.
109. Ma, L., et al., *Brain-Wide Mapping of Afferent Inputs to Accumbens Nucleus Core Subdomains and Accumbens Nucleus Subnuclei*. Front Syst Neurosci, 2020. **14**: p. 15.
110. Zahm, D.S. and J.S. Brog, *On the significance of subterritories in the "accumbens" part of the rat ventral striatum*. Neuroscience, 1992. **50**(4): p. 751-67.
111. Albin, R.L., A.B. Young, and J.B. Penney, *The functional anatomy of basal ganglia disorders*. Trends Neurosci, 1989. **12**(10): p. 366-75.
112. Kupchik, Y.M. and P.W. Kalivas, *The direct and indirect pathways of the nucleus accumbens are not what you think*. Neuropsychopharmacology, 2017. **42**(1): p. 369.

113. Björklund, A. and S.B. Dunnett, *Dopamine neuron systems in the brain: an update*. Trends Neurosci, 2007. **30**(5): p. 194-202.
114. Bromberg-Martin, E.S., M. Matsumoto, and O. Hikosaka, *Dopamine in motivational control: rewarding, aversive, and alerting*. Neuron, 2010. **68**(5): p. 815-34.
115. Schultz, W., *Behavioral dopamine signals*. Trends in neurosciences, 2007. **30**(5): p. 203-210.
116. Lammel, S., B.K. Lim, and R.C. Malenka, *Reward and aversion in a heterogeneous midbrain dopamine system*. Neuropharmacology, 2014. **76 Pt B**(0 0): p. 351-9.
117. Schultz, W., *The phasic reward signal of primate dopamine neurons*, in *Advances in pharmacology*. 1997, Elsevier. p. 686-690.
118. Ungless, M.A., P.J. Magill, and J.P. Bolam, *Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli*. Science, 2004. **303**(5666): p. 2040-2042.
119. Abercrombie, E.D., et al., *Differential effect of stress on in vivo dopamine release in striatum, nucleus accumbens, and medial frontal cortex*. Journal of neurochemistry, 1989. **52**(5): p. 1655-1658.
120. Bassareo, V., M.A. De Luca, and G. Di Chiara, *Differential expression of motivational stimulus properties by dopamine in nucleus accumbens shell versus core and prefrontal cortex*. Journal of neuroscience, 2002. **22**(11): p. 4709-4719.
121. Young, A.M., *Increased extracellular dopamine in nucleus accumbens in response to unconditioned and conditioned aversive stimuli: studies using 1 min microdialysis in rats*. Journal of neuroscience methods, 2004. **138**(1-2): p. 57-63.
122. Mantz, J., A. Thierry, and J. Glowinski, *Effect of noxious tail pinch on the discharge rate of mesocortical and mesolimbic dopamine neurons: selective activation of the mesocortical system*. Brain research, 1989. **476**(2): p. 377-381.
123. Brischoux, F., et al., *Phasic excitation of dopamine neurons in ventral VTA by noxious stimuli*. Proceedings of the national academy of sciences, 2009. **106**(12): p. 4894-4899.
124. Matsumoto, M. and O. Hikosaka, *Two types of dopamine neuron distinctly convey positive and negative motivational signals*. Nature, 2009. **459**(7248): p. 837-841.
125. Berridge, K.C., T.E. Robinson, and J.W. Aldridge, *Dissecting components of reward: 'liking', 'wanting', and learning*. Current opinion in pharmacology, 2009. **9**(1): p. 65-73.
126. Ungless, M.A., E. Argilli, and A. Bonci, *Effects of stress and aversion on dopamine neurons: implications for addiction*. Neuroscience & Biobehavioral Reviews, 2010. **35**(2): p. 151-156.
127. de Jong, J.W., et al., *A Neural Circuit Mechanism for Encoding Aversive Stimuli in the Mesolimbic Dopamine System*. Neuron, 2019. **101**(1): p. 133-151.e7.
128. Lüscher, C. and R.C. Malenka, *Drug-evoked synaptic plasticity in addiction: from molecular changes to circuit remodeling*. Neuron, 2011. **69**(4): p. 650-663.
129. Di Chiara, G. and A. Imperato, *Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats*. Proceedings of the National Academy of Sciences, 1988. **85**(14): p. 5274-5278.
130. Nestler, E.J., *Is there a common molecular pathway for addiction?* Nature neuroscience, 2005. **8**(11): p. 1445-1449.

131. Lüscher, C. and M.A. Ungless, *The mechanistic classification of addictive drugs*. PLoS medicine, 2006. **3**(11): p. e437.
132. Fendt, M. and M.S. Fanselow, *The neuroanatomical and neurochemical basis of conditioned fear*. Neuroscience & Biobehavioral Reviews, 1999. **23**(5): p. 743-760.
133. Martinez, R.C.R., A.R. de Oliveira, and M.L. Brandão, *Conditioned and unconditioned fear organized in the periaqueductal gray are differentially sensitive to injections of muscimol into amygdaloid nuclei*. Neurobiology of learning and memory, 2006. **85**(1): p. 58-65.
134. Martínez-García, F., A. Novejarque, and E. Lanuza, *Two interconnected functional systems in the amygdala of amniote vertebrates*. Brain research bulletin, 2008. **75**(2-4): p. 206-213.
135. Ray, M.H., et al., *The nucleus accumbens core is necessary to scale fear to degree of threat*. Journal of Neuroscience, 2020. **40**(24): p. 4750-4760.
136. Li, S.S. and G.P. McNally, *A role of nucleus accumbens dopamine receptors in the nucleus accumbens core, but not shell, in fear prediction error*. Behavioral neuroscience, 2015. **129**(4): p. 450.
137. Badrinarayan, A., et al., *Aversive stimuli differentially modulate real-time dopamine transmission dynamics within the nucleus accumbens core and shell*. Journal of Neuroscience, 2012. **32**(45): p. 15779-15790.
138. Ikemoto, S. and J. Panksepp, *The role of nucleus accumbens dopamine in motivated behavior: a unifying interpretation with special reference to reward-seeking*. Brain Research Reviews, 1999. **31**(1): p. 6-41.
139. Young, E.A., et al., *Parallel modulation of striatal dopamine synthetic enzymes by second messenger pathways*. European journal of pharmacology, 1998. **357**(1): p. 15-23.
140. Johnson, L., et al., *Input from the amygdala to the rat nucleus accumbens: its relationship with tyrosine hydroxylase immunoreactivity and identified neurons*. Neuroscience, 1994. **61**(4): p. 851-865.
141. Floresco, S.B., et al., *Association basolateral amygdala stimulation evokes glutamate receptor-dependent dopamine efflux in the nucleus accumbens of the anaesthetized rat*. European Journal of Neuroscience, 1998. **10**(4): p. 1241-1251.
142. Correia, S.S., et al., *Amygdala-ventral striatum circuit activation decreases long-term fear*. Elife, 2016. **5**: p. e12669.
143. Herry, C., et al., *Switching on and off fear by distinct neuronal circuits*. Nature, 2008. **454**(7204): p. 600-606.
144. Laurent, V., A.R. Marchand, and R.F. Westbrook, *The basolateral amygdala is necessary for learning but not relearning extinction of context conditioned fear*. Learning & memory, 2008. **15**(5): p. 304-314.
145. Holtzman-Assif, O., V. Laurent, and R.F. Westbrook, *Blockade of dopamine activity in the nucleus accumbens impairs learning extinction of conditioned fear*. Learning & memory, 2010. **17**(2): p. 71-75.
146. Whittle, N., et al., *Deep brain stimulation, histone deacetylase inhibitors and glutamatergic drugs rescue resistance to fear extinction in a genetic mouse model*. Neuropharmacology, 2013. **64**: p. 414-423.

147. Rodriguez-Romaguera, J., F.H. Do Monte, and G.J. Quirk, *Deep brain stimulation of the ventral striatum enhances extinction of conditioned fear*. Proceedings of the National Academy of Sciences, 2012. **109**(22): p. 8764-8769.
148. LeGates, T.A., et al., *Reward behaviour is regulated by the strength of hippocampus–nucleus accumbens synapses*. Nature, 2018. **564**(7735): p. 258-262.
149. Britt, J.P., et al., *Synaptic and behavioral profile of multiple glutamatergic inputs to the nucleus accumbens*. Neuron, 2012. **76**(4): p. 790-803.
150. O'Donnell, P. and A.A. Grace, *Synaptic interactions among excitatory afferents to nucleus accumbens neurons: hippocampal gating of prefrontal cortical input*. Journal of Neuroscience, 1995. **15**(5): p. 3622-3639.
151. Ólafsdóttir, H.F., et al., *Hippocampal place cells construct reward related sequences through unexplored space*. Elife, 2015. **4**: p. e06063.
152. Tryon, V.L., et al., *Hippocampal neural activity reflects the economy of choices during goal-directed navigation*. Hippocampus, 2017. **27**(7): p. 743-758.
153. Sjulson, L., et al., *Cocaine place conditioning strengthens location-specific hippocampal coupling to the nucleus accumbens*. Neuron, 2018. **98**(5): p. 926-934. e5.
154. Baldo, B.A., et al., *Overlapping distributions of orexin/hypocretin-and dopamine- β -hydroxylase immunoreactive fibers in rat brain regions mediating arousal, motivation, and stress*. Journal of Comparative Neurology, 2003. **464**(2): p. 220-237.
155. Diniz, G.B. and J.C. Bittencourt, *The melanin-concentrating hormone as an integrative peptide driving motivated behaviors*. Frontiers in systems neuroscience, 2017. **11**: p. 32.
156. Dunham, N. and T. Miya, *A Note on a Simple Apparatus for Detecting Neurological Deficit in Rats and Mice** College of Pharmacy, University of Nebraska, Lincoln 8*. Journal of the American Pharmaceutical Association (Scientific ed.), 1957. **46**(3): p. 208-209.
157. Hamm, R.J., et al., *The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury*. Journal of neurotrauma, 1994. **11**(2): p. 187-196.
158. Heng, M.Y., P.J. Detloff, and R.L. Albin, *Rodent genetic models of Huntington disease*. Neurobiology of disease, 2008. **32**(1): p. 1-9.
159. Lubrich, C., P. Giesler, and M. Kipp, *Motor behavioral deficits in the cuprizone model: Validity of the rotarod test paradigm*. International Journal of Molecular Sciences, 2022. **23**(19): p. 11342.
160. Costa, R.M., D. Cohen, and M.A. Nicolelis, *Differential corticostriatal plasticity during fast and slow motor skill learning in mice*. Current Biology, 2004. **14**(13): p. 1124-1134.
161. Yin, H.H., et al., *Dynamic reorganization of striatal circuits during the acquisition and consolidation of a skill*. Nature neuroscience, 2009. **12**(3): p. 333-341.
162. Durieux, P.F., S.N. Schiffmann, and A. de Kerchove d'Exaerde, *Differential regulation of motor control and response to dopaminergic drugs by D1R and D2R neurons in distinct dorsal striatum subregions*. The EMBO journal, 2012. **31**(3): p. 640-653.

163. Barnes, T.D., et al., *Activity of striatal neurons reflects dynamic encoding and recoding of procedural memories*. Nature, 2005. **437**(7062): p. 1158-1161.
164. Le Merrer, J., et al., *Balance between projecting neuronal populations of the nucleus accumbens controls social behavior in mice*. Biological psychiatry, 2024. **95**(2): p. 123-135.
165. Rothwell, P.E., et al., *Autism-associated neuroligin-3 mutations commonly impair striatal circuits to boost repetitive behaviors*. Cell, 2014. **158**(1): p. 198-212.
166. Platt, R.J., et al., *Chd8 mutation leads to autistic-like behaviors and impaired striatal circuits*. Cell reports, 2017. **19**(2): p. 335-350.
167. Benthall, K.N., et al., *Loss of Tsc1 from striatal direct pathway neurons impairs endocannabinoid-LTD and enhances motor routine learning*. Cell reports, 2021. **36**(6).
168. Ma, L., et al., *Locomotion activates PKA through dopamine and adenosine in striatal neurons*. Nature, 2022. **611**(7937): p. 762-768.
169. Richardson, N.R. and D.C. Roberts, *Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy*. Journal of neuroscience methods, 1996. **66**(1): p. 1-11.
170. Hodos, W., *Progressive ratio as a measure of reward strength*. Science, 1961. **134**(3483): p. 943-944.
171. Eagle, D.M., et al., *Effects of regional striatal lesions on motor, motivational, and executive aspects of progressive-ratio performance in rats*. Behav Neurosci, 1999. **113**(4): p. 718-31.
172. Robbins, T. and B. Sahakian, *Behavioral effects of psychomotor stimulant drugs: clinical and neuropsychological implications*. Stimulants: neurochemical, behavioral, and clinical perspectives, 1983: p. 301-338.
173. Taylor, J.R. and T.W. Robbins, *6-Hydroxydopamine lesions of the nucleus accumbens, but not of the caudate nucleus, attenuate enhanced responding with reward-related stimuli produced by intra-accumbens d-amphetamine*. Psychopharmacology (Berl), 1986. **90**(3): p. 390-7.
174. Poncelet, M., et al., *The progressive ratio schedule as a model for studying the psychomotor stimulant activity of drugs in the rat*. Psychopharmacology, 1983. **80**: p. 184-189.
175. Risner, M.E. and D.L. Silcox, *Psychostimulant self-administration by beagle dogs in a progressive-ratio paradigm*. Psychopharmacology, 1981. **75**: p. 25-30.
176. Brown, V.J., M.P. Latimer, and P. Winn, *Memory for the changing cost of a reward is mediated by the sublentiform extended amygdala*. Brain research bulletin, 1996. **39**(3): p. 163-170.
177. Olivetti, P.R., et al., *Emerging roles of striatal dopamine D2 receptors in motivated behaviour: implications for psychiatric disorders*. Basic & clinical pharmacology & toxicology, 2020. **126**: p. 47-55.
178. Gallo, E.F., et al., *Accumbens dopamine D2 receptors increase motivation by decreasing inhibitory transmission to the ventral pallidum*. Nature communications, 2018. **9**(1): p. 1-13.
179. Aberman, J.E., S.J. Ward, and J.D. Salamone, *Effects of dopamine antagonists and accumbens dopamine depletions on time-constrained progressive-ratio performance*. Pharmacol Biochem Behav, 1998. **61**(4): p. 341-8.

180. Soares-Cunha, C., et al., *Reappraising striatal D1-and D2-neurons in reward and aversion*. Neuroscience & Biobehavioral Reviews, 2016. **68**: p. 370-386.
181. Simpson, E.H., et al., *Pharmacologic rescue of motivational deficit in an animal model of the negative symptoms of schizophrenia*. Biological psychiatry, 2011. **69**(10): p. 928-935.
182. Trifilieff, P., et al., *Increasing dopamine D2 receptor expression in the adult nucleus accumbens enhances motivation*. Molecular psychiatry, 2013. **18**(9): p. 1025-1033.
183. Liu, M.-Y., et al., *Sucrose preference test for measurement of stress-induced anhedonia in mice*. Nature protocols, 2018. **13**(7): p. 1686-1698.
184. Wulff, A.B., et al., *How sucrose preference is gained and lost: An in-depth analysis of drinking behavior during the sucrose preference test in mice*. Eneuro, 2023. **10**(9).
185. Strekalova, T., et al., *Update in the methodology of the chronic stress paradigm: internal control matters*. Behavioral and Brain Functions, 2011. **7**: p. 1-18.
186. Serchov, T., et al., *Increased signaling via adenosine A1 receptors, sleep deprivation, imipramine, and ketamine inhibit depressive-like behavior via induction of Homer1a*. Neuron, 2015. **87**(3): p. 549-562.
187. Serchov, T., D. van Calker, and K. Biber, *Sucrose preference test to measure anhedonic behaviour in mice*. Bio-protocol, 2016. **6**(19): p. e1958-e1958.
188. Goshen, I., et al., *Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression*. Molecular psychiatry, 2008. **13**(7): p. 717-728.
189. Martínez-Hernández, J., E. Lanuza, and F. Martínez-García, *Lesions of the dopaminergic innervation of the nucleus accumbens medial shell delay the generation of preference for sucrose, but not of sexual pheromones*. Behavioural brain research, 2012. **226**(2): p. 538-547.
190. Hurley, S.W. and R.M. Carelli, *Activation of infralimbic to nucleus accumbens shell pathway suppresses conditioned aversion in male but not female rats*. Journal of Neuroscience, 2020. **40**(36): p. 6888-6895.
191. Bessa, J., et al., *Stress-induced anhedonia is associated with hypertrophy of medium spiny neurons of the nucleus accumbens*. Translational psychiatry, 2013. **3**(6): p. e266-e266.
192. Banghart, M.R. and B.L. Sabatini, *Photoactivatable neuropeptides for spatiotemporally precise delivery of opioids in neural tissue*. Neuron, 2012. **73**(2): p. 249-259.
193. Duggan, A., *Neuropeptide spread in the brain and spinal cord*. Progress in Brain Research, 2000. **125**: p. 369-380.
194. Svingos, A.L., C.L. Clarke, and V.M. Pickel, *Cellular sites for activation of δ -opioid receptors in the rat nucleus accumbens shell: relationship with Met5-enkephalin*. Journal of Neuroscience, 1998. **18**(5): p. 1923-1933.
195. Svingos, A.L., E.E. Colago, and V.M. Pickel, *Cellular sites for dynorphin activation of κ -opioid receptors in the rat nucleus accumbens shell*. Journal of Neuroscience, 1999. **19**(5): p. 1804-1813.
196. Svingos, A.L., et al., *Ultrastructural immunocytochemical localization of μ -opioid receptors in rat nucleus accumbens: extrasynaptic plasmalemmal distribution and*

- association with *Leu5-enkephalin*. *Journal of Neuroscience*, 1996. **16**(13): p. 4162-4173.
197. Barr, G.A., S. Wang, and S. Carden, *Aversive properties of the κ opioid agonist U50, 488 in the week-old rat pup*. *Psychopharmacology*, 1994. **113**: p. 422-428.
 198. Bruchas, M.R., et al., *Selective p38 α MAPK deletion in serotonergic neurons produces stress resilience in models of depression and addiction*. *Neuron*, 2011. **71**(3): p. 498-511.
 199. Donahue, R.J., et al., *Effects of acute and chronic social defeat stress are differentially mediated by the dynorphin/kappa-opioid receptor system*. *Behavioural pharmacology*, 2015. **26**(7 0 0): p. 654.
 200. Land, B.B., et al., *The dysphoric component of stress is encoded by activation of the dynorphin κ -opioid system*. *Journal of Neuroscience*, 2008. **28**(2): p. 407-414.
 201. Land, B.B., et al., *Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking*. *Proceedings of the National Academy of Sciences*, 2009. **106**(45): p. 19168-19173.
 202. McLaughlin, J.P., et al., *Social defeat stress-induced behavioral responses are mediated by the endogenous kappa opioid system*. *Neuropsychopharmacology*, 2006. **31**(6): p. 1241-1248.
 203. Tang, A. and R. Collins, *Behavioral effects of a novel kappa opioid analgesic, U-50488, in rats and rhesus monkeys*. *Psychopharmacology*, 1985. **85**: p. 309-314.
 204. Hjelmstad, G.O. and H.L. Fields, *Kappa opioid receptor inhibition of glutamatergic transmission in the nucleus accumbens shell*. *Journal of Neurophysiology*, 2001. **85**(3): p. 1153-1158.
 205. Carlezon Jr, W.A., et al., *Regulation of cocaine reward by CREB*. *Science*, 1998. **282**(5397): p. 2272-2275.
 206. Chefer, V., et al., *Kappa-opioid receptor activation prevents alterations in mesocortical dopamine neurotransmission that occur during abstinence from cocaine*. *Neuroscience*, 2000. **101**(3): p. 619-627.
 207. Chefer, V.I., et al., *Kappa opioid receptors on dopaminergic neurons are necessary for kappa-mediated place aversion*. *Neuropsychopharmacology*, 2013. **38**(13): p. 2623-2631.
 208. Ehrich, J.M., et al., *Kappa opioid receptor-induced aversion requires p38 MAPK activation in VTA dopamine neurons*. *Journal of Neuroscience*, 2015. **35**(37): p. 12917-12931.
 209. Ehrich, J.M., P.E. Phillips, and C. Chavkin, *Kappa opioid receptor activation potentiates the cocaine-induced increase in evoked dopamine release recorded in vivo in the mouse nucleus accumbens*. *Neuropsychopharmacology*, 2014. **39**(13): p. 3036-3048.
 210. Heidbreder, C.A., et al., *Increased responsiveness of mesolimbic and mesostriatal dopamine neurons to cocaine following repeated administration of a selective κ -opioid receptor agonist*. *Synapse*, 1998. **30**(3): p. 255-262.
 211. Castro, D.C. and K.C. Berridge, *Opioid hedonic hotspot in nucleus accumbens shell: mu, delta, and kappa maps for enhancement of sweetness "liking" and "wanting"*. *Journal of Neuroscience*, 2014. **34**(12): p. 4239-4250.

212. Contet, C., et al., *Dissociation of analgesic and hormonal responses to forced swim stress using opioid receptor knockout mice*. Neuropsychopharmacology, 2006. **31**(8): p. 1733-1744.
213. Gavériaux-Ruff, C., et al., *Inflammatory pain is enhanced in delta opioid receptor-knockout mice*. European Journal of Neuroscience, 2008. **27**(10): p. 2558-2567.
214. Papaleo, F., et al., *Decreased motivation to eat in μ -opioid receptor-deficient mice*. European Journal of Neuroscience, 2007. **25**(11): p. 3398-3405.
215. Weibel, R., et al., *Mu opioid receptors on primary afferent nav1. 8 neurons contribute to opiate-induced analgesia: insight from conditional knockout mice*. PloS one, 2013. **8**(9): p. e74706.
216. Bakshi, V.P. and A.E. Kelley, *Feeding induced by opioid stimulation of the ventral striatum: role of opiate receptor subtypes*. Journal of Pharmacology and Experimental Therapeutics, 1993. **265**(3): p. 1253-1260.
217. Peciña, S. and K.C. Berridge, *Opioid site in nucleus accumbens shell mediates eating and hedonic 'liking' for food: map based on microinjection Fos plumes*. Brain research, 2000. **863**(1-2): p. 71-86.
218. Ragnauth, A., M. Moroz, and R.J. Bodnar, *Multiple opioid receptors mediate feeding elicited by mu and delta opioid receptor subtype agonists in the nucleus accumbens shell in rats*. Brain research, 2000. **876**(1-2): p. 76-87.
219. Zhang, M. and A.E. Kelley, *Opiate agonists microinjected into the nucleus accumbens enhance sucrose drinking in rats*. Psychopharmacology, 1997. **132**: p. 350-360.
220. Cui, Y., et al., *Targeted expression of μ -opioid receptors in a subset of striatal direct-pathway neurons restores opiate reward*. Nature neuroscience, 2014. **17**(2): p. 254-261.
221. Hanlon, E.C., et al., *Increases in food intake or food-seeking behavior induced by GABAergic, opioid, or dopaminergic stimulation of the nucleus accumbens: is it hunger?* Psychopharmacology, 2004. **172**: p. 241-247.
222. Resendez, S.L., et al., *μ -Opioid receptors within subregions of the striatum mediate pair bond formation through parallel yet distinct reward mechanisms*. Journal of Neuroscience, 2013. **33**(21): p. 9140-9149.
223. Wiskerke, J., et al., *μ -Opioid receptors in the nucleus accumbens shell region mediate the effects of amphetamine on inhibitory control but not impulsive choice*. Journal of Neuroscience, 2011. **31**(1): p. 262-272.
224. Corder, G., et al., *Endogenous and exogenous opioids in pain*. Annual review of neuroscience, 2018. **41**: p. 453-473.
225. Filliol, D., et al., *Mice deficient for δ -and μ -opioid receptors exhibit opposing alterations of emotional responses*. Nature genetics, 2000. **25**(2): p. 195-200.
226. Pradhan, A.A., et al., *δ -Opioid receptor agonists inhibit migraine-related hyperalgesia, aversive state and cortical spreading depression in mice*. British journal of pharmacology, 2014. **171**(9): p. 2375-2384.
227. Bodnar, R.J., et al., *General, μ and κ opioid antagonists in the nucleus accumbens alter food intake under deprivation, glucoprivic and palatable conditions*. Brain research, 1995. **700**(1-2): p. 205-212.

228. Alongkronrusmee, D., T. Chiang, and R.M. van Rijn, *Involvement of delta opioid receptors in alcohol withdrawal-induced mechanical allodynia in male C57BL/6 mice*. Drug and alcohol dependence, 2016. **167**: p. 190-198.
229. Henry, M.S., et al., *Delta opioid receptor signaling promotes resilience to stress under the repeated social defeat paradigm in mice*. Frontiers in Molecular Neuroscience, 2018. **11**: p. 100.
230. Banghart, M.R., et al., *Enkephalin disinhibits mu opioid receptor-rich striatal patches via delta opioid receptors*. Neuron, 2015. **88**(6): p. 1227-1239.
231. Griebel, G., G. Perrault, and D.J. Sanger, *Orphanin FQ, a novel neuropeptide with anti-stress-like activity*. Brain research, 1999. **836**(1-2): p. 221-224.
232. Jenck, F., et al., *Orphanin FQ acts as an anxiolytic to attenuate behavioral responses to stress*. Proceedings of the National Academy of Sciences, 1997. **94**(26): p. 14854-14858.
233. Köster, A., et al., *Targeted disruption of the orphanin FQ/nociceptin gene increases stress susceptibility and impairs stress adaptation in mice*. Proceedings of the National Academy of Sciences, 1999. **96**(18): p. 10444-10449.
234. Reinscheid, R. and O. Civelli, *The orphanin FQ/nociceptin knockout mouse: a behavioral model for stress responses*. Neuropeptides, 2002. **36**(2-3): p. 72-76.
235. Devine, D.P., et al., *Nociceptin/orphanin FQ content is decreased in forebrain neurones during acute stress*. Journal of neuroendocrinology, 2003. **15**(1): p. 69-74.
236. Nazzaro, C., et al., *Swim stress enhances nociceptin/orphanin FQ-induced inhibition of rat dorsal raphe nucleus activity in vivo and in vitro: role of corticotropin releasing factor*. Neuropharmacology, 2010. **58**(2): p. 457-464.
237. Devine, D.P., et al., *The novel neuropeptide orphanin FQ fails to produce conditioned place preference or aversion*. Brain research, 1996. **727**(1-2): p. 225-229.
238. Stratford, T.R., M.R. Holahan, and A.E. Kelley, *Injections of nociceptin into nucleus accumbens shell or ventromedial hypothalamic nucleus increase food intake*. Neuroreport, 1997. **8**(2): p. 423-426.
239. Murtra, P., et al., *Rewarding effects of opiates are absent in mice lacking the receptor for substance P*. Nature, 2000. **405**(6783): p. 180-183.
240. Kombian, S.B., et al., *Substance P depresses excitatory synaptic transmission in the nucleus accumbens through dopaminergic and purinergic mechanisms*. Journal of neurophysiology, 2003. **89**(2): p. 728-737.
241. Marshall, F., et al., *Cholecystokinin modulates the release of dopamine from the anterior and posterior nucleus accumbens by two different mechanisms*. Journal of neurochemistry, 1991. **56**(3): p. 917-922.
242. Sørensen, A.T., et al., *Hippocampal NPY gene transfer attenuates seizures without affecting epilepsy-induced impairment of LTP*. Experimental neurology, 2009. **215**(2): p. 328-333.
243. Brown, C.M., D.V. Coscina, and P.J. Fletcher, *The rewarding properties of neuropeptide Y in perifornical hypothalamus vs. nucleus accumbens*. Peptides, 2000. **21**(8): p. 1279-1287.

244. Georgescu, D., et al., *The hypothalamic neuropeptide melanin-concentrating hormone acts in the nucleus accumbens to modulate feeding behavior and forced-swim performance*. Journal of Neuroscience, 2005. **25**(11): p. 2933-2940.
245. Pecina, S., J. Schulkin, and K. Berridge, *Nucleus accumbens CRF increases cue-triggered motivation for sucrose reward: paradoxical positive incentive effects in stress*. BMC Biol, 2006. **4**(1): p. 8.
246. Thorpe, A. and C. Kotz, *Orexin A in the nucleus accumbens stimulates feeding and locomotor activity*. Brain research, 2005. **1050**(1-2): p. 156-162.
247. Cheng, J.Y., S.W. Zhang, and F.D. Tai, *Effects of nucleus accumbens oxytocin and its antagonist on social approach behavior*. Behavioural pharmacology, 2016. **27**(8): p. 672-680.
248. Bosch, O.J., et al., *Oxytocin in the nucleus accumbens shell reverses CRFR2-evoked passive stress-coping after partner loss in monogamous male prairie voles*. Psychoneuroendocrinology, 2016. **64**: p. 66-78.
249. Chen, Y.-W., et al., *Corticotropin-releasing factor in the nucleus accumbens shell induces swim depression, anxiety, and anhedonia along with changes in local dopamine/acetylcholine balance*. Neuroscience, 2012. **206**: p. 155-166.
250. Kroog, G.S., et al., *The Gastrin-releasing Peptide Receptor Is Rapidly Phosphorylated by a Kinase Other Than Protein Kinase C After Exposure to Agonist (*)*. Journal of Biological Chemistry, 1995. **270**(14): p. 8217-8224.
251. Pagani, M., et al., *How gastrin-releasing peptide opens the spinal gate for itch*. Neuron, 2019. **103**(1): p. 102-117. e5.
252. Shumyatsky, G.P., et al., *Identification of a signaling network in lateral nucleus of amygdala important for inhibiting memory specifically related to learned fear*. Cell, 2002. **111**(6): p. 905-918.
253. Goto, F., et al., *Gastrin-releasing peptide regulates fear learning under stressed conditions via activation of the amygdalostratial transition area*. Molecular Psychiatry, 2022. **27**(3): p. 1694-1703.
254. Hermes, M.L., et al., *Gastrin-releasing peptide acts via postsynaptic BB2 receptors to modulate inward rectifier K⁺ and TRPV1-like conductances in rat paraventricular thalamic neurons*. J Physiol, 2013. **591**(7): p. 1823-39.
255. Melzer, S., et al., *Bombesin-like peptide recruits disinhibitory cortical circuits and enhances fear memories*. Cell, 2021. **184**(22): p. 5622-5634. e25.
256. Sun, Y.-G. and Z.-F. Chen, *A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord*. Nature, 2007. **448**(7154): p. 700-703.
257. Albisetti, G.W., et al., *Dorsal horn gastrin-releasing peptide expressing neurons transmit spinal itch but not pain signals*. Journal of Neuroscience, 2019. **39**(12): p. 2238-2250.
258. Sun, S., et al., *Leaky gate model: intensity-dependent coding of pain and itch in the spinal cord*. Neuron, 2017. **93**(4): p. 840-853. e5.
259. Goswami, S.C., et al., *Itch-associated peptides: RNA-Seq and bioinformatic analysis of natriuretic precursor peptide B and gastrin releasing peptide in dorsal root and trigeminal ganglia, and the spinal cord*. Molecular pain, 2014. **10**: p. 1744-8069-10-44.
260. Huang, J., et al., *Circuit dissection of the role of somatostatin in itch and pain*. Nature neuroscience, 2018. **21**(5): p. 707-716.

261. Mu, D., et al., *A central neural circuit for itch sensation*. Science, 2017. **357**(6352): p. 695-699.
262. Sun, Y.-G., et al., *Cellular basis of itch sensation*. Science, 2009. **325**(5947): p. 1531-1534.
263. Kramer, D.J., et al., *Combinatorial expression of Grp and Neurod6 defines dopamine neuron populations with distinct projection patterns and disease vulnerability*. Eneuro, 2018. **5**(3).
264. Viereckel, T., et al., *Midbrain gene screening identifies a new mesoaccumbal glutamatergic pathway and a marker for dopamine cells neuroprotected in Parkinson's disease*. Scientific Reports, 2016. **6**(1): p. 35203.
265. Kemp, J.M. and T.P.S. Powell, *The structure of the caudate nucleus of the cat: light and electron microscopy*. Philosophical Transactions of the Royal Society of London. B, Biological Sciences, 1971. **262**(845): p. 383-401.
266. Gerfen, C.R., *Molecular effects of dopamine on striatal-projection pathways*. Trends in neurosciences, 2000. **23**: p. S64-S70.
267. Voorn, P., et al., *Putting a spin on the dorsal-ventral divide of the striatum*. Trends in neurosciences, 2004. **27**(8): p. 468-474.
268. Kravitz, A.V., S.F. Owen, and A.C. Kreitzer, *Optogenetic identification of striatal projection neuron subtypes during in vivo recordings*. Brain research, 2013. **1511**: p. 21-32.
269. Bateup, H.S., et al., *Distinct subclasses of medium spiny neurons differentially regulate striatal motor behaviors*. Proceedings of the National Academy of Sciences, 2010. **107**(33): p. 14845-14850.
270. Cox, J. and I.B. Witten, *Striatal circuits for reward learning and decision-making*. Nature Reviews Neuroscience, 2019. **20**(8): p. 482-494.
271. Puighermanal, E., et al., *Functional and molecular heterogeneity of D2R neurons along dorsal ventral axis in the striatum*. Nature communications, 2020. **11**(1): p. 1957.
272. Ade, K.K., et al., *An Improved BAC Transgenic Fluorescent Reporter Line for Sensitive and Specific Identification of Striatonigral Medium Spiny Neurons*. Front Syst Neurosci, 2011. **5**: p. 32.
273. Rossi, J., et al., *Melanocortin-4 receptors expressed by cholinergic neurons regulate energy balance and glucose homeostasis*. Cell Metab, 2011. **13**(2): p. 195-204.
274. Madisen, L., et al., *A robust and high-throughput Cre reporting and characterization system for the whole mouse brain*. Nature neuroscience, 2010. **13**(1): p. 133-140.
275. Gong, S., et al., *A gene expression atlas of the central nervous system based on bacterial artificial chromosomes*. Nature, 2003. **425**(6961): p. 917-925.
276. Yu, Y.-Q., et al., *Molecular and neural basis of contagious itch behavior in mice*. Science, 2017. **355**(6329): p. 1072-1076.
277. Schwenk, F., U. Baron, and K. Rajewsky, *A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells*. Nucleic Acids Res, 1995. **23**(24): p. 5080-1.
278. Mathis, A., et al., *DeepLabCut: markerless pose estimation of user-defined body parts with deep learning*. Nature neuroscience, 2018. **21**(9): p. 1281-1289.

279. Wiltschko, A.B., et al., *Revealing the structure of pharmacobehavioral space through motion sequencing*. *Nature neuroscience*, 2020. **23**(11): p. 1433-1443.
280. Cording, K.R. and H.S. Bateup, *Altered motor learning and coordination in mouse models of autism spectrum disorder*. *Frontiers in cellular neuroscience*, 2023. **17**.
281. Zhou, F.M., C.J. Wilson, and J.A. Dani, *Cholinergic interneuron characteristics and nicotinic properties in the striatum*. *Journal of neurobiology*, 2002. **53**(4): p. 590-605.
282. Bolam, J., B. Wainer, and A. Smith, *Characterization of cholinergic neurons in the rat neostriatum. A combination of choline acetyltransferase immunocytochemistry, Golgi-impregnation and electron microscopy*. *Neuroscience*, 1984. **12**(3): p. 711-718.
283. Majumdar, I.D. and H.C. Weber, *Biology of mammalian bombesin-like peptides and their receptors*. *Current Opinion in Endocrinology, Diabetes and Obesity*, 2011. **18**(1): p. 68-74.
284. Hulbert, S.W., et al., *A novel Chd8 mutant mouse displays altered ultrasonic vocalizations and enhanced motor coordination*. *Autism Research*, 2020. **13**(10): p. 1685-1697.
285. Bossert, J.M., et al., *Role of projections from ventral subiculum to nucleus accumbens shell in context-induced reinstatement of heroin seeking in rats*. *Psychopharmacology*, 2016. **233**: p. 1991-2004.
286. Martin, P.D. and T. Ono, *Effects of reward anticipation, reward presentation, and spatial parameters on the firing of single neurons recorded in the subiculum and nucleus accumbens of freely moving rats*. *Behavioural brain research*, 2000. **116**(1): p. 23-38.
287. Nath, T., et al., *Using DeepLabCut for 3D markerless pose estimation across species and behaviors*. *Nat Protoc*, 2019. **14**(7): p. 2152-2176.
288. Weinreb, C., et al., *Keypoint-MoSeq: parsing behavior by linking point tracking to pose dynamics*. *bioRxiv*, 2023.
289. Everitt, B.J. and T.W. Robbins, *From the ventral to the dorsal striatum: devolving views of their roles in drug addiction*. *Neuroscience & Biobehavioral Reviews*, 2013. **37**(9): p. 1946-1954.
290. Anderson, S. and R. Pierce, *Cocaine-induced alterations in dopamine receptor signaling: implications for reinforcement and reinstatement*. *Pharmacology & therapeutics*, 2005. **106**(3): p. 389-403.
291. Lobo, M.K. and E.J. Nestler, *The striatal balancing act in drug addiction: distinct roles of direct and indirect pathway medium spiny neurons*. *Frontiers in neuroanatomy*, 2011. **5**: p. 41.
292. Smith, J.L., et al., *Deficits in behavioural inhibition in substance abuse and addiction: a meta-analysis*. *Drug and alcohol dependence*, 2014. **145**: p. 1-33.
293. Grueter, B.A., G. Brasnjo, and R.C. Malenka, *Postsynaptic TRPV1 triggers cell type-specific long-term depression in the nucleus accumbens*. *Nature neuroscience*, 2010. **13**(12): p. 1519.
294. Saunders, A., et al., *Molecular diversity and specializations among the cells of the adult mouse brain*. *Cell*, 2018. **174**(4): p. 1015-1030. e16.