Neuropeptidergic modulation of the nucleus accumbens and its contribution to motivated behavior

By

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To my parents, Anne and Ben,

for their consistent support and encouragement

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CHAPTER I: Introduction

The neural control of motivated behavior

In 2015, the Nikon Small World in Motion prize was awarded to a video of a single-celled ciliate orienting toward, and then consuming, a smaller single-celled ciliate. This video illustrates a fundamental principle of life: in order to live, an organism must identify a source of energy and put itself in a position to consume it. To find a source of energy, that organism must collect and interpret information from the environment and use that information to guide its actions. This fundamental feature of life is not restricted to animals, as the bacteria *E. coli* will also move towards an energy source or salt, a response that can be disrupted by deleting the receptors that sense these nutrients (Adler, 1978; Qi and Adler, 1989). Thus, the ancient roots of motivated behavior are observable from the beginning of life on this planet.

All forms of life, including bacteria, must process information from the environment in order to efficiently find sustenance. While in a single-celled organism it is sufficient to simply orient toward an area of higher nutrient concentration, an animal with a more complex body in a more complex environment must balance competing needs with the many different potential strategies to fulfill them. This problem led to the evolution of the neural circuits that control motivated behavior. In what is arguably the most complete attempt to date at describing a wiring diagram for the neural control of motivated behavior, Swanson proposed that the behaviors necessary for the survival of a species are controlled by "behavioral control columns" (Swanson, 2000). These columns, located in the hypothalamus and upper brainstem, mediate the production of survival behaviors including ingestion, defense, reproduction, and exploration. The indirect

connections of these regions to the muscles controlling movement are critical to the full expression of these behaviors. Animals with lesions below the control column lose the ability to perform the complex behavior, and instead express fragments (Kelley, 2004a; Swanson, 2000). Animals with lesions above the control column retain the ability to perform these behaviors, but perform them inappropriately in response to stimuli (Grill and Norgren, 1978). These results depict the crucial importance of the communication of environmental information to brain circuits for the proper expression of motivated behavior.

In addition to external information, an animal with a complex body must also be attuned to their internal environment, their internal state. Each motivated behavior begins with a need, deriving from an imbalance in homeostasis that invigorates a drive within the animal. This drive is then broadcast across the brain, to sensory processing areas, emotional and motivational centers, and motor controlling regions. The ability of behavioral circuits to sense internal state is not a recent addition but rather a fundamental principle of all nervous systems. Moreover, the chemical, genetic, and molecular components that communicate internal state are highly conserved through evolution (monoamines and neuropeptides, as well as their g protein-coupled receptors and effector pathways). Although the components are similar, through the course of evolution the systems exerting and processing the influence of state have become more complex. The construction of increasingly detailed anatomical and molecular intricacy on top of the hypothalamic/brainstem behavioral control column has allowed for the remarkable adaptive behavior seen in animals. Along the vertical axis of this system, state is integrated repeatedly, into both cortical and subcortical regions projecting to the control column. In this way, our current needs shape our perception and interactions with the world around us.

The goal of the work presented here is to increase our understanding of how neuropeptides shape the control of motivated behavior. To understand the context of this work, I will first introduce the brain region examined in these studies. The locus of these studies is a subregion of a forebrain structure perched atop the behavioral control column, the nucleus accumbens. Next, I will introduce the current understanding of how whole-body energy state impacts neuropeptidergic control of behavior, with a focus on the arcuate nucleus. Through the work presented here, I detail how multiple neuropeptide modulatory systems shape neurotransmission within the nucleus accumbens and how this impacts motivated behavior. In Chapter II, I describe how hunger promotes changes to food-seeking strategy by engaging neuropeptide signaling within the nucleus accumbens. In Chapter III, I describe how neuropeptide signaling modulates glutamatergic neurotransmission in the nucleus accumbens and promotes social interaction. Finally, I discuss implications and future directions of this research.

The nucleus accumbens

Early anatomical studies of the nucleus accumbens led to the hypothesis that this region integrated affective and cognitive processing to direct the motor associated brain regions to control behavior (Graybiel, 1976; Mogenson et al., 1980). Identification of inputs from cortical and allocortical structures like the amygdala, hippocampus, prefrontal cortex, and thalamus made it clear that the nucleus accumbens was a crossroads where a wide variety of motivationally relevant information was integrated. Outputs to the pallidum and ventral midbrain suggested a role for nucleus accumbens projections in motor control. This prediction, that the nucleus accumbens is

the interface between “motivation and action” has been examined over four decades of experimentation and has proven to be an apt definition of nucleus accumbens function.

Nucleus accumbens structural organization

Examining the nucleus accumbens in greater detail identifies a great deal of heterogeneity. The nucleus accumbens can be divided into two main territories: the core directly surrounding the anterior commissure, and the shell extending out from the core medially, laterally, and ventrally. These two regions display subtle differences in the source of their glutamatergic input. The core receives projections from the prelimbic area of the prefrontal cortex, while the shell receives input from the infralimbic portion (Brog et al., 1993). The shell region receives a particularly dense input from the ventral subiculum, while the dorsal subiculum targets the core (Brog et al., 1993). Similar differences in amygdalar input also exist (Wright et al., 1996). The two subregions further differ in their outputs (Fig. 1). The core sends projections to more classical basal ganglia targets like the substantia nigra in the midbrain and the dorsal ventral pallidum, while the shell provides output to the ventral tegmental area, ventromedial ventral pallidum, and additionally targets the lateral hypothalamus (Groenewegen et al., 1999).

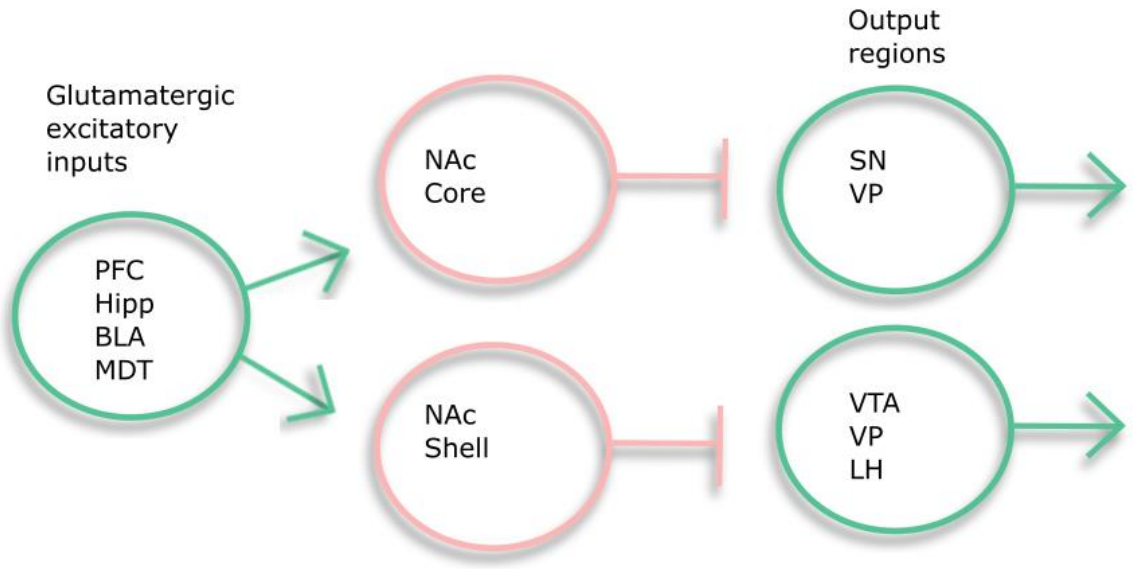


Figure 1 | The nucleus accumbens core and shell receive similar glutamatergic input but project to distinct brain regions

Schematic depicting glutamatergic input to the nucleus accumbens (NAc) core and shell and their distinct outputs. Both the core and the shell of the nucleus accumbens receive input from the prefrontal cortex (PFC), the hippocampus (Hipp), the basolateral amygdala (BLA), and the mediodorsal thalamus (MDT). The nucleus accumbens shell uniquely projects to the lateral hypothalamus, and sends input to a different region of the midbrain compared to the nucleus accumbens core. The nucleus accumbens shell projects to the more medial ventral tegmental area (VTA), while the nucleus accumbens core projects to the more lateral substantia nigra (SN). Both the core and the shell project to the ventral pallidum (VP).

These differences in midbrain projection targets lead to a medial to lateral gradient of striatal input to the midbrain. Medial sections of the shell send projections to the medial midbrain dopamine nuclei, while more ventral and lateral parts innervate lateral dopamine cell groups (Groenewegen et al., 1999). The nucleus accumbens core sends projections further laterally, reaching into the substantia nigra (Groenewegen et al., 1999). Similar gradients exist in the dopaminergic input to the nucleus accumbens, where more medial dopaminergic cell groups project to more medial aspects of the nucleus accumbens. This organization leads to a spiral like relationship, where nucleus accumbens input influences the dopaminergic cell groups that project to "higher" striatal regions (Fig. 2) (Haber et al., 2000; Voorn et al., 2004). Moving up the spiral also coincides with greater and greater dopaminergic input to the specific striatal compartment. Thus, computations in lower regions of the striatum influence processing in higher regions, an anatomical realization that has interesting implications when one considers the functional role of different striatal regions.

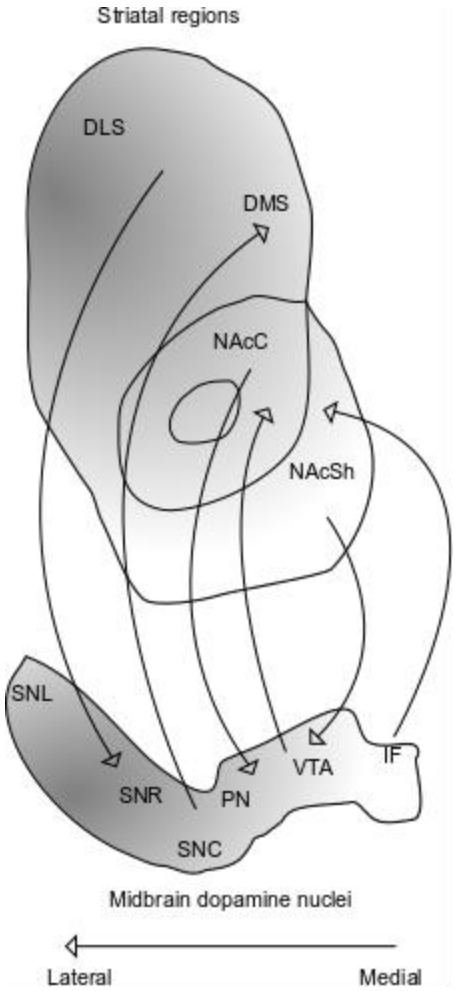


Figure 2 | Reciprocal ascending spiral connecting midbrain dopamine nuclei with striatal subregions

Diagram depicting the medial to lateral gradient of midbrain dopamine nuclei that corresponds to a ventral to dorsal gradient of striatal nuclei (Haber et al., 2000). The most dorsal region of the medial nucleus accumbens shell (NAcSh) receives dopaminergic input from the intrafasicular nucleus (IF), but does not itself project to midbrain dopamine nuclei (Thompson and Swanson, 2010). Moving ventrally in the accumbens shell, projections to the ventral tegmental area (VTA) begin to appear (Soden et al., 2020). The VTA sends projections to both the nucleus accumbens core (NAcC) and the NAcSh. The NAcC in turn projects to paranigral and substantia nigra regions. These areas innervate the dorsal striatum, which projects back to the substantia nigra pars reticulata, a basal ganglia output nuclei. At each step of this spiral, terminal fields overlap such that there are midbrain dopamine nuclei both receiving input and not receiving input from the "previous" striatal subregion.

Nucleus accumbens cell types

Medium spiny neurons

Approximately 95% of the neurons within the nucleus accumbens are γ-aminobutyric acid (GABA) producing projection neurons, first described by Ramón y Cajal as cells with medium sized somas (~15 μM) and a high density of dendritic spines (Fig. 3) (Cajal RS, 1911; Meredith et al., 2008). Based on these properties, these neurons came to be known as medium spiny neurons (MSNs). The synaptic organization of these spines speaks to the function of the nucleus accumbens as a whole, and the importance of excitatory transmission to that function. MSN spines commonly exhibit an asymmetric, glutamatergic, input on the head, with a dopaminergic input at its base (Freund et al., 1984; Kemp and Powell, 1971). This organization allows the modulatory processing of information before it is channeled to the soma of the MSN. It should be noted however that this synaptic arrangement is not true of every spine, and this coincident glutamatergic and dopaminergic input only occurs in about half of spines in the nucleus accumbens core and even fewer in the nucleus accumbens shell (Zahm, 1992). Regardless of dopaminergic innervation, these spines are important sites of synaptic plasticity (Grueter et al., 2012). Further indicating the importance of excitatory transmission in MSN activity is their low baseline firing rate. This phenotype is due to the expression of inwardly rectifying potassium channels (Mermelstein et al., 1998; Wilson and Kawaguchi, 1996). These potassium channels maintain the hyperpolarized resting membrane potential of MSNs (~ -90 mV), a level that is close to the equilibrium potential for potassium(Mermelstein et al., 1998). This hyperpolarized membrane state is referred to as the "down" state, and coincident or repeated excitatory transmission is required to transition MSNs to an "up" depolarized state where they are more likely to fire action potentials (O'Donnell and

Grace, 1995). Thus MSNs integrate excitatory transmission with dopaminergic modulation to selectively pass information through the nucleus accumbens.

Though similar in morphology as a whole, MSNs can be divided into subtypes based on their neurochemical and receptor expression profiles. One class of MSNs expresses the dopamine D1 receptor and the neuropeptides dynorphin and substance P (D1+ MSNs). Another class expresses the dopamine D2 receptor, the A2a receptor, and the neuropeptide enkephalin (D2+ MSNs). These expression profiles are also associated with specific projection targets, with D1+ MSNs making up a "direct" pathway to the midbrain and D2 MSNs making up an "indirect" pathway to the pallidum. However, these pathways are less clear cut in the nucleus accumbens than in the dorsal striatum, where this nomenclature originated (Gerfen and Surmeier, 2011). In both the nucleus accumbens core and shell, D1+ MSNs project to both the pallidum and the midbrain (Baimel et al., 2019; Kupchik et al., 2015). Additionally, in the NAc shell D1+ MSNs send a unique projection to the lateral hypothalamus, which in turn projects to the ventral tegmental area in the midbrain (Nieh et al., 2016, 2015; O'Connor et al., 2015; Thoeni et al., 2020). It is intriguing to consider that this pathway may also be "indirect" in nature. However, this region of the lateral hypothalamus also projects directly to hypothalamic motor controlling regions, allowing shell D1+ MSNs unique indirect access to regions controlling motor patterns (Thompson and Swanson, 2010). Regardless, the D1+ and D2+ classes are distinct not only along the genetic dimension, but also in their electrophysiological properties and synaptic plasticity (Gertler et al., 2008; Grueter et al., 2013, 2010). These features have led to widespread adoption of the categorization of MSNs based on their dopamine receptor expression.

Single cell RNA sequencing of nucleus accumbens cell types has identified much greater diversity in MSNs beyond dopamine receptor expression. Using expression patterns to distinguish

groups of MSNs yielded 30 D1+ subtypes and 27 D2+ subtypes (Chen et al., 2021). Important for the work outlined in this thesis, the medial nucleus accumbens shell exhibits multiple unique D1+ cell types including those identified by *Serpineb2* expression or those identified by *Tac2* expression. Subsequent fluorescent *in situ* hybridization experiments confirmed that these genes mark distinct, spatially restricted, D1+ cell types within the medial nucleus accumbens shell. These results hint at the complexity yet to be uncovered in the diversity of MSN heterogeneity, and the implications this has for nucleus accumbens function.

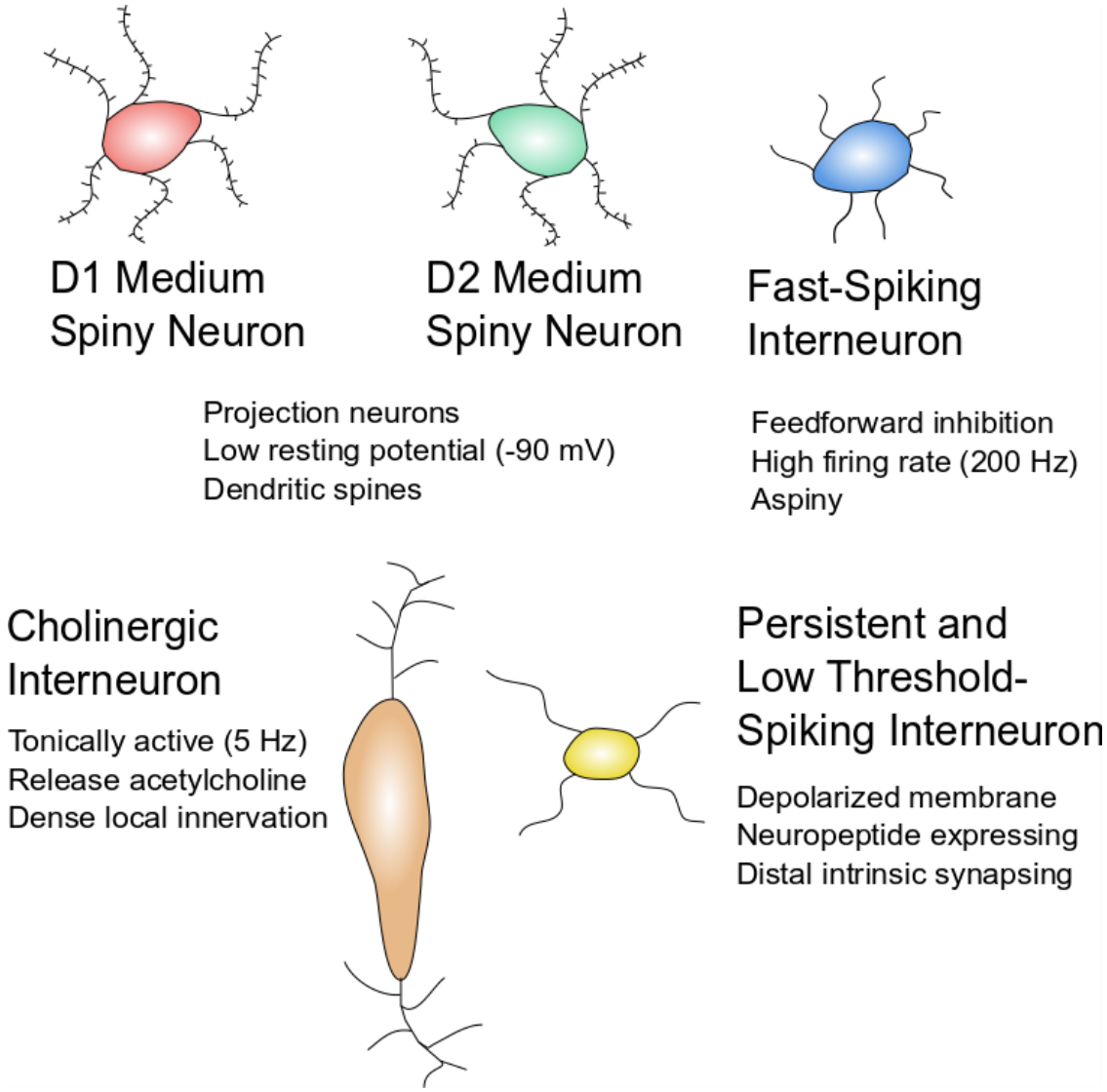


Figure 3 | Neuronal cell types of the nucleus accumbens

The nucleus accumbens contains five neuronal cell types. The medium spiny neurons are the only projection neurons and can be classified by their dopamine receptor expression. Fast-spiking interneurons receive similar excitatory input as medium spiny neurons, but are much quicker to fire and provide feedforward inhibition to projection neurons. Cholinergic interneurons are tonically firing and release acetylcholine from their dense processes within the nucleus accumbens. Persistent and low threshold-spiking interneurons receive weak excitatory input and form synapses at long ranges onto the distal processes of medium spiny neurons. However, they express neuropeptides including somatostatin and neuropeptide Y which may be critical to their function within the nucleus accumbens.

Cholinergic interneurons

Cholinergic interneurons (CINs) are sparse, tonically firing cells with a relatively large diameter soma (~ 20-30 μM) (Fig. 3) (Bolam et al., 1984; Y. Ma et al., 2014). Interestingly, because of the poor axonal staining using the Golgi method, Cajal originally identified cholinergic interneurons as the projection neuron of the striatum based on their size (Zhou et al., 2002). These neurons are the only tonically firing cell type within the nucleus accumbens and fire at approximately 5 Hz (Zhou et al., 2002). Released acetylcholine is rapidly degraded by acetylcholinesterase to avoid the desensitization of nicotinic acetylcholine receptors (Exley and Cragg, 2008; Jongen-Rêlo et al., 1994). Cholinergic interneurons extend dense axonal arbors across the nucleus accumbens and are considered to be the main source of striatal acetylcholine, although cholinergic innervation from the brainstem has been identified (Dautan et al., 2014). Using an antibody for the acetylcholine synthesis enzyme choline acetyltransferase (ChAT), dense axonal arborizations extending from cholinergic interneurons are visible (Ligorio et al., 2009). This observation, coupled with the near ubiquitous expression of acetylcholine receptors and acetylcholinesterase, has led to the hypothesis that acetylcholine acts in the nucleus accumbens via volume transmission (Descarries et al., 1997). However, there are significant arguments against this hypothesis, mainly based on the timescale of some acetylcholine dependent phenotypes (Sarter et al., 2009). Regardless, the debate speaks to the broad reach of the acetylcholine neuromodulation within the nucleus accumbens.

While cholinergic interneurons fire tonically, the rate of firing is variable and is responsive to environmental information. High levels of acetylcholinesterase, an efficient enzyme with fast kinetics, enables quick clearance of released acetylcholine (Quinn, 1987; Zhou et al., 2001). This high rate of clearance means that transient changes in acetylcholine release can meaningfully

change the extracellular levels of the transmitter and cause a physiological response. Thus, both bursting and pausing phenotypes of cholinergic interneurons firing have been thoroughly investigated in their role in controlling striatal output and the resulting behavior. Following a salient event, cholinergic interneurons exhibit a brief pause in firing (Aosaki et al., 1994). This pause can be elicited experimentally by stimulating both GABAergic and dopaminergic input from the ventral tegmental area, implicating the cue sensitivity of this region in controlling cholinergic interneuron dynamics (Brown et al., 2012; Chuhma et al., 2014; Gallo et al., 2021). Interestingly, glutamatergic innervation from the ventral tegmental area also has the ability to modulate cholinergic interneuron firing, although this influence varies by striatal subregion and is highest in the medial nucleus accumbens shell (Chuhma et al., 2014). This results in a complex burst-silence pattern of cholinergic interneuron firing in response to stimulation of ventral tegmental area input (Chuhma et al., 2014). Regulation of cholinergic interneurons by inputs from the ventral tegmental area is not unidirectional, as cholinergic signaling powerfully regulates dopamine release in the nucleus accumbens (Cachope et al., 2012; Threlfell et al., 2012). Thus cholinergic interneurons exhibit complex firing patterns dependent on cue information from the environment, and this shapes neurotransmission within the nucleus accumbens.

If the activity of cholinergic interneurons is dependent on salient environmental cues, it would follow that manipulations of cholinergic interneurons would alter the response of the animal to these cues. Indeed, disruptions of cholinergic interneurons have been found to alter behavior in response to cues, specifically by reducing behavioral flexibility. During reversal learning, extracellular acetylcholine is increased in the dorsal medial striatum (Ragozzino et al., 2009). These changes in acetylcholine likely depend on cholinergic interneurons, as lesions of cholinergic interneurons in the dorsal medial striatum impair the ability to shift a reward-seeking strategy and

attend to a previously irrelevant cue (Aoki et al., 2015). Additionally, lesions of cholinergic interneurons in the nucleus accumbens results in an increase in perseverative errors when animals are required to alter their strategy in response to a novel cue (Aoki et al., 2015). Disruption of excitatory input to dorsal striatum cholinergic interneurons reduces the animals sensitivity to changes in the action-outcome contingency, indicating a shift to habitual control of behavior (Bradfield et al., 2013). Beyond behavioral flexibility, the activity of cholinergic interneurons has also been implicated in the control of reward-related behaviors, as inhibition of cholinergic interneurons reduces cocaine conditioning (Witten et al., 2010). Together, these results suggest that cholinergic interneurons are important for the ability to use cue information to guide behavior, and that this is especially critical for changing behavior strategy.

Fast-spiking interneurons

The fast-spiking interneurons (FSIs) of the nucleus accumbens are sparse GABAergic cells, similar in soma size to MSNs (~15 μM), but without the extensive expression of dendritic spines (Fig. 3) (Kawaguchi, 1993). FSIs are primarily identified by their ability to undergo extended periods of high-frequency action potential firing (Manz et al., 2020; Tepper et al., 2010). This fast-spiking phenotype for which this class of interneurons is named is due to the expression of a unique complement of sodium and potassium channels (Kv3.1), resulting in shorter spike width and faster recovery of the afterhyperpolarization (Golomb et al., 2007; Kawaguchi, 1993). FSIs provide powerful inhibitory input to MSNs, and a single FSI can contact many neighboring MSNs, synapsing directly onto the soma and proximal region (Schall et al., 2021). FSIs receive similar excitatory input as MSNs, including input from the hippocampus, basolateral amygdala, the prefrontal cortex, and the thalamus (Coleman et al., 2021; Manz et al., 2021; Trouche et al., 2019; Yu et al., 2017). However, potentially due in part to the lack of dendritic spines in FSIs,

these excitatory inputs are much stronger on FSIs than on MSNs (Wright et al., 2017). This, coupled with the aforementioned firing properties of FSIs, leads to a feed-forward inhibition circuit motif, where excitatory input onto PVs is able to quickly promote inhibition of downstream MSNs. This creates an interesting level of complexity in nucleus accumbens microcircuitry, where FSI mediated feedforward inhibition has the ability to select MSNs ensembles by quickly inhibiting certain MSNs while they are still integrating excitatory input. This is compounded by the electrical coupling of FSIs, which express gap junction proteins and form electrical synapses with each other (Kita et al., 1990). However, it is important to note that when MSNs are in the downstate (near -90 mV) and below the reversal potential for chloride, activation of GABA A would result in chloride efflux and membrane depolarization. It is possible then to imagine that FSIs could also prime ensembles in specific cases, rather than inhibit them. The extent to which this occurs, if at all, *in vivo* is unclear. Regardless, these properties position FSIs as critical determinants of nucleus accumbens output, and the resulting behavioral phenotypes.

Persistent and low threshold spiking interneurons

The persistent and low threshold spiking interneurons (PLTS) are also commonly characterized by their expression of somatostatin, neuropeptide Y, and nitric oxide synthase (Fig. 3) (Castro and Bruchas, 2019; Tepper et al., 2010). Like FSIs, these interneurons are also GABAergic and similarly sized. Additionally, PLTS interneurons receive input from canonical nucleus accumbens glutamatergic inputs, similar to FSIs and MSNs, however this input is much weaker compared to FSIs (Gittis et al., 2010; Ribeiro et al., 2019). PLTS interneurons are readily distinguishable from FSIs based on their electrophysiological properties, including a lower maximal firing rate, a higher resting membrane potential and a higher input resistance (Gittis et al., 2010). They also form synapses onto MSNs in a unique manner compared to FSIs. Using rabies

tracing and precise optical circuit mapping, dorsal striatal PLTS interneurons were found to synapse onto distal dendrites of MSNs with an average distance of 570 µm between the two cells (Straub et al., 2016). These synapses are much weaker than FSI to MSN synapses in terms of evoked current measured in the MSN soma (Gittis et al., 2010). Thus, it is possible that PLTS interneurons primarily exert their effects on nucleus accumbens function via their neuropeptide transmitters, including somatostatin and neuropeptide Y. However, the function of these neuropeptides are understudied in the nucleus accumbens, and most studies use exogenous neuropeptides, making connections to PLTS interneurons difficult. Global manipulation of PLTS interneurons impacts the rewarding properties of cocaine and the expression of anxiety-like behavior, indicating their importance in NAc behavioral phenotypes (Ribeiro et al., 2018; Yamada et al., 2020). Further study is needed to understand how these neurons contribute to NAc microcircuitry, and what proportion of their influence is driven by their neuropeptides.

Nucleus accumbens circuitry

The regions controlling voluntary behavior in the hypothalamus and brainstem, grouped by Swanson as the behavioral control column (Swanson, 2000), receive input from cortical regions, the hippocampus, the amygdala, the striatum and the pallidum. These inputs send direct projections to the behavioral control column, but also communicate amongst themselves, processing information before it is passed to behavioral control regions. In these indirect circuits, differing drives are pitted against one another, aspects of the environment are imbued with salience, and emotion is breathed into our experience. The striatum receives a massive amount of afferent input communicating varied information from the hippocampus, the amygdala, the thalamus, and the prefrontal cortex. This information is communicated to the principal output

neurons of the striatum while concurrent neuromodulatory input provides the context with which to interpret it. Striatal output reaches motor regions that ultimately determine our behavior. Thus, the striatum serves as a critical interface between the complex information of the animal's internal state and environment, synthesizing this information to promote advantageous behavior.

Nucleus accumbens glutamatergic neurotransmission

Overview of glutamatergic neurotransmission

Glutamate is able to signal though α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kinate receptors. Most excitatory transmission within the brain occurs via AMPA receptors (Henley and Wilkinson, 2016). AMPA receptors are ligand-gated ion channels that flux sodium and potassium, formed from four subunits (GluR1-4). Intracellular loops of AMPA receptor subunits contain multiple sites for palmitoylations, phosphorylations, and ubiquitinations (Lu and Roche, 2012). These post-translational modifications can alter the biophysical properties or trafficking of AMPA receptors by regulating single channel properties or association with other synaptic accessory proteins (Lu and Roche, 2012). AMPA receptors at the synapse are highly dynamic, diffusing laterally across the membrane until "trapped" via interactions with anchoring proteins (Lu and Roche, 2012). NMDA receptors, which are calcium-permeable, regulate function of AMPA receptors through the activation of protein kinases like CaMKII (Herring and Nicoll, 2016). NMDA receptors are also tetrameric (made up of GluN1, GluN2, and GluN3 subunits) glutamate-gated ion channels, but they exhibit a voltage-dependent magnesium block that limits their conductance in the absence of membrane

depolarization (Paoletti et al., 2013). Thus, coincidence of postsynaptic depolarization and presynaptic glutamate release triggers an influx of calcium, activating postsynaptic signaling pathways that can regulate AMPA receptor signaling at that synapse (Herring and Nicoll, 2016). This is the prototypical mechanism of long-term potentiation, a form of synaptic plasticity that underlies learning and memory.

Hippocampus

The hippocampus is an allocortical structure in the medial temporal lobe containing pyramidal projection neurons that is involved in episodic memory and spatial navigation (Moser et al., 2017; Strange et al., 2014). In order to perform efficient motivated behavior, an animal must be able to remember important environments where they have found resources. The hippocampus provides critical spatial information that guides the proper expression of contextual stress and reward (Moser et al., 2017). The projection from the hippocampus to the nucleus accumbens terminates in the medial aspect of the nucleus accumbens, the nucleus accumbens shell (Kelley and Domesick, 1982). This projection is most dense in the dorsal portion of the shell, adjacent to the septum (Britt et al., 2012; Kelley and Domesick, 1982). This input is critical to the proper production of memory and spatially guided behaviors. Disruption of the connection between the hippocampus and the nucleus accumbens results in an inability of the animal to form a preference for an environment that previously contained a reward (Ito et al., 2008). Upregulation of the connection between the hippocampus and nucleus accumbens medium spiny neurons, via induction of a long-term potentiation, is able to induce a place preference (LeGates et al., 2018). Interestingly, the hippocampus also synapses onto Parvalbumin-expressing FSIs in the nucleus accumbens, and this connection is required for the retrieval of a conditioned place preference

(Trouche et al., 2019). Beyond spatial conditioning, activation of the hippocampal input to the nucleus accumbens has been found to enhance behavioral phenotypes associated with food palatability, indicating a broader role in reward processing (Yang et al., 2020). The connection between the hippocampus to MSNs is relatively strong compared to other glutamatergic inputs, and it can initiate transitions from a hyperpolarized state to an active, "up", state (O'Donnell and Grace, 1995). Perhaps exogenous activation of this input could take advantage of the power this input exerts over MSNs membrane state in order to enhance the rewarding properties of an experience. Indeed, this has been proposed as a future therapeutic approach for anhedonia (LeGates et al., 2018). These results identify the hippocampus as a source of environmental

Amygdala

The amygdala, another temporal lobe structure, is subdivided into basolateral and central nuclei. The amygdala receives input from the thalamus and sensory cortices which is passed to glutamatergic projection neurons in the basolateral amygdala that project to the nucleus accumbens (Janak and Tye, 2015). The amygdala is essential to learned behaviors that rely on emotional significance, of both positive and negative valance (Tye, 2018). In both cases, the experienced value of an event is critical to guiding the behavior of the animal when that event arises in again in the future. Disconnections of the basolateral amygdala and the nucleus accumbens results in impaired ability of the animal to use changes in outcome value to drive instrumental behavior (Shiflett and Balleine, 2010). However, the amygdala to nucleus accumbens projection is perhaps best recognized for its role in promoting behavior in response to neutral cues. Disruption of the basolateral amygdala input to the nucleus accumbens damages the ability of a conditioned stimulus to promote instrumental responding (Di Ciano and Everitt, 2004; Setlow et al., 2002; Shiflett and Balleine, 2010). Optogenetic inhibition of this projection is also able to reduce cue-induced

reinstatement of instrumental behavior (Stefanik and Kalivas, 2013). The neural response to cues in the nucleus accumbens depends on input from the amygdala, as inhibition of basolateral amygdala input to the nucleus accumbens reduces cue evoked accumbens excitation (Ambroggi et al., 2008). Thus, communication between the amygdala and the nucleus accumbens allows outcome valence and cues to inform behavior.

The valence of transmission between the amygdala and the nucleus accumbens is complex and likely dependent on multiple differing cell groups. Optogenetic stimulation of basolateral amygdala inputs has been found to be rewarding, and supports self-stimulation (Stuber et al., 2011). However, activation of a subpopulation of amygdala to nucleus accumbens projection neurons, those expressing cholecystokinin, has been found to be aversive (Shen et al., 2019). Further, optogenetic activation of this pathway decreases social interaction, a phenotype that is ameliorated by augmentation of inhibitory endocannabinoid receptors (Folkes et al., 2020). These results suggest that the basolateral amygdala is able to influence behavior both positively and negatively depending on the specific parameters of the communication. Further studies are needed to uncover what parameters determine the valence of this input.

Prefrontal cortex

The prefrontal cortex is a neocortical region with vast connections to a vast range of sensory and motor systems which allow it to exert top-down control to guide thoughts and actions (Shen et al., 2019). Complex environments often present multiple possible strategies in order to obtain one or several different rewards. Animals must choose the most appropriate strategy for their current goal and follow through with the actions required to obtain that goal. These complex actions require learning, memory, inhibitory control and behavioral flexibility. Together, these processes are commonly referred to as executive function, or the cognitive control of behavior,

and they rely on a number of subregions within the prefrontal cortex (Ragozzino, 2007; Rushworth et al., 2011). These complex cognitive functions are supported by equally complex microcircuitry embedded with a number of different neuromodulatory systems (Shen et al., 2019). The prefrontal projection to the nucleus accumbens is subregion specific, with the nucleus accumbens core receiving input from the prelimbic prefrontal cortex, and the nucleus accumbens shell receiving input from the infralimbic prefrontal cortex (Sesack et al., 1989). The connection between the prefrontal cortex and the nucleus accumbens has been studied extensively in the context of cocaine seeking. Lesion of the infralimbic projection to nucleus accumbens shell after extinction training increases cocaine seeking(Peters et al., 2008). Lesion before extinction training had no effect. This indicates that extinction learning results in recruitment of the prefrontal cortical input to the nucleus accumbens. Conversely, artificial activation of the prefrontal to nucleus accumbens shell projection reduces reinstatement of cocaine seeking (Augur et al., 2016). This control of drug seeking is paralleled by synaptic changes in the prefrontal to nucleus accumbens circuit. Withdrawal from cocaine self-administration results in the formation of silent synapses at inputs from both the prelimbic and infralimbic cortices, followed by the recruitment of calcium-permeable AMPARs (Y. Y. Ma et al., 2014). *In vivo* imaging of infralimbic neurons that project to the nucleus accumbens has revealed activity patterns that are tied to the salience of the environment, this has been shown in the context of cocaine seeking (Cameron et al., 2019). Engaging in a drug seeking behavior reduces the activity of nucleus accumbens projecting infralimbic neurons, and optogenetically activating these neurons reduces drug seeking (Cameron et al., 2019). Together, these results suggest that the prefrontal input is shaped by extinction learning, and that the plastic changes in this circuit underlie the “stop” signal for drug seeking brought on by extinction.

Thalamus

The nucleus accumbens receives input from the paraventricular nucleus of the thalamus (PVT), a highly heterogeneous region that receives significant modulatory input communicating internal state (McGinty and Otis, 2020). Generally, the PVT is appreciated to underlie decision making during motivational conflict, such as a mixed reward/danger stimuli triggering an approach/avoidance behavioral conflict (Kelley et al., 2005; Kirouac, 2015; McGinty and Otis, 2020). In order to assess such conflict, the PVT must take in a diverse range of information, both appetitive and aversive. Within the neural computations of the PVT, these environmental stimuli are considered in the context of the energy state of the animal via projections from both the lateral and arcuate hypothalamus, as well as the circadian state via the input from the suprachiasmatic nucleus (Horio and Liberles, 2021; Kelley et al., 2005). The diversity of this region is reflected in the experimental outcomes of PVT manipulations, which can seemingly produce opposing results depending on the cell type and region of the PVT targeted. Stimulating the anterior PVT (generally 0.1 to 1 mm posterior from bregma) projections to the nucleus accumbens, typically inhibits sucrose seeking (Do-Monte et al., 2017; Kessler et al., 2021). However, activation of GluT2 expressing cells that project to the nucleus accumbens in the anterior PVT promotes sucrose seeking (Labouèbe et al., 2016). GluT2 expressing neurons are activated by hypoglycemia, and promote food intake, while Glucokinase expressing cells, which are also found in the anterior PVT, are excited by hyperglycemia and suppress food intake (Kessler et al., 2021; Labouèbe et al., 2016). The posterior PVT (more than 1 mm posterior from bregma) projection to the nucleus accumbens also inhibits food-seeking behavior during periods of reward omission (Lafferty et al., 2020). Additionally, the posterior projection to the NAc mediates aspects of opiate withdrawal, and starvation induced arousal (Do-Monte et al., 2017; Hua et al., 2018). Specifically, Calretinin

expressing neurons in the PVT are required for the expression of starvation induced arousal (Hua et al., 2018). Together, these results indicate that the PVT can exert complex control over reward seeking behavior based on a number of different internal states, and that this influence is exerted by differing cell types depending on anatomical and genetic factors.

An integrated view of excitatory transmission

It should be clear from the preceding sections that the nucleus accumbens receives diverse excitatory input from several brain regions, and the local microcircuitry and cytoarchitecture is well equipped to parse this information in a meaningful way. When we consider specific excitatory inputs, it becomes increasingly clear that they contribute distinct information. What is less clear, is how this information differentially contributes to propagation through the nucleus accumbens. Experimental evidence indicates not all excitatory input is treated the same by nucleus accumbens circuitry. For example, seminal experiments from O'Donnell and Grace identified a unique role for the hippocampus in transitioning MSNs to the depolarized upstate, an ability not granted to prefrontal inputs (O'Donnell and Grace, 1995) (however, it is important to note that *repeated* stimulation of the prefrontal input is able to transition MSNs to the upstate, indicating that temporal properties are also important (Gruber and O'Donnell, 2009)). If we consider this ability in the context of the information communicated by these inputs, it is attractive to speculate that spatial information from the hippocampus elevates specific neuronal ensembles within the nucleus accumbens to have the potential to fire, and concurrent executive control from the prefrontal cortex selects from these ensembles. Said another way, hippocampal input elevates the behavioral options for a specific environment, and prefrontal input decides on which option to pursue. Combining this view with our knowledge of input specific plasticity, it is clear how synaptic modulation of the hippocampal input would differ in behavioral outcomes when compared to synaptic

modulation of prefrontal input. Hippocampal input plasticity might result in an "across the board" change to the level of network and thus behavioral activation, whereas prefrontal input plasticity may exert more targeted effects on specific ensembles and behaviors.

Nucleus accumbens GABAergic neurotransmission

The inhibitory neurotransmitter GABA acts through both ionotropic and metabotropic receptors. The $GABA_A$ receptor is a ligand-gated chloride channel made up of five subunits (Chua and Chebib, 2017). The $GABA_B$ receptor is a g protein-coupled receptor that couples to $G\alpha_{i/o}$ g proteins and commonly is found to inhibit calcium channels and activate potassium channels (Chalifoux and Carter, 2011). Through these receptors, GABAergic signaling dampens presynaptic vesicular release, hyperpolarizes membranes, and shunts depolarization (Tritsch et al., 2016).

In addition to feedforward inhibitory transmission, which was described above in the context of FSIs, there is additional intrinsic GABAergic transmission within the striatum between MSNs (Lalchandani et al., 2013; Tunstall et al., 2002). This collateral transmission, termed lateral inhibition, allows MSNs to provide $GABA_A$ receptor mediated inhibition to a subset of neighboring MSNs (Czubayko and Plenz, 2002; Taverna et al., 2008). This lateral inhibition microcircuit motif has been described as important to cocaine-induced hyperlocomotion (Dobbs et al., 2016).

While there is significant intrinsic GABAergic transmission within the nucleus accumbens due to feed-forward and lateral inhibition, the nucleus accumbens also receives extrinsic GABAergic input. This input arises from multiple areas including the ventral tegmental area and the lateral hypothalamus (Hurley and Johnson, 2014; Van Bockstaele and Pickel, 1995). The

nucleus accumbens also receives inhibitory input from the ventral pallidum. In the nucleus accumbens shell, this inhibitory input drives consumption, consistent with findings from GABA agonist infusions (Stratford and Kelley, 1997; Vachez et al., 2021). Interestingly, this pallidial input also targets interneurons, including CINs, raising the implication this input may alter the cue processing of CINs (Vachez et al., 2021).

Nucleus accumbens neuromodulators

Dopamine

Dopamine in the nucleus accumbens is a well-studied but incredibly complex neuromodulator which has been elegantly reviewed elsewhere (Berke, 2018; Berridge, 2007; Sulzer et al., 2016). The nucleus accumbens receives dopaminergic input from the ventral tegmental area (VTA), a nucleus in the midbrain that responds to rewards and the cues that predict them (Morales and Margolis, 2017; Schultz, 1998). Once released, dopamine is able to signal through five different g protein-coupled receptor subtypes that are divided into two classes. The D1 and D5 subtypes of dopamine receptors belong to the D1-like class and act through $G\alpha_s$ (Beaulieu and Gainetdinov, 2011). The D2, D3 and D4 subtypes of dopamine receptors belong to the D2-like class and act though $G\alpha_{i/o}$ (Beaulieu and Gainetdinov, 2011). In the striatum, D1 and D2 dopamine receptors are expressed on different classes of MSNs, and have been used as a genetic marker to identify these MSN cell types (Grueter et al., 2010).

Learning about the environmental cues that predict reward results in enhanced excitatory synaptic strength onto VTA dopamine neurons, providing a potential mechanism for changes in firing rate based on learning (Stuber et al., 2008). Cue-evoked activity in dopamine neurons and nucleus accumbens dopamine levels both track with the size and delay of the predicted reward

(Day et al., 2010; Roesch et al., 2007). These results suggest that dopamine neuron activation, and subsequent release of dopamine in downstream regions like the nucleus accumbens, is critical to learning about how to pursue rewards in the environment.

As dopamine is critical to the pursuit of rewards, it follows logically that it is regulated by the importance of those rewards based on the drive of the animal (Hsu et al., 2018). Food deprivation results in increases in extracellular dopamine in response to both the anticipatory and consummatory phases of feeding (Wilson et al., 1995). Food deprivation alters multiple components of the dopamine system, by increasing the firing rate of dopamine neurons and by reducing expression and function of the dopamine transporter (Patterson et al., 1998; White et al., 2008). These results indicate that the dopamine system is influenced by energy state and that this state is included in calculations of cue/reward value.

If the dopamine system is modulated by energy state, it must be receiving energy state information via circuit or neuromodulatory mechanisms. Indeed, the mesolimbic dopamine nuclei express a wide variety of receptors for energy state-related hormones and transmitters, including insulin, leptin, and amylin (Marks et al., 1990; Paxinos et al., 2004; Scott et al., 2009). The impact of these hormones on nucleus accumbens dopamine has been reviewed elsewhere (Hsu et al., 2018). As I reported in a recent review, one satiety hormone, glucagon-like peptide 1 (GLP-1), significantly regulates the dopamine system by suppressing dopamine release and enhancing its reuptake (Smith et al., 2019). Conversely, ghrelin, a food-intake promoting hormone, acts in the VTA to promote dopamine neuron firing (Abizaid et al., 2006). Additionally, less well-studied systems also have the potential to impact dopamine transmission. Work that I contributed to shows that circulating bile acids act within the nucleus accumbens to dampen cocaine-mediated elevations in dopamine, and this mechanism can be engaged by bariatric surgery procedures that

elevate circulating bile acids (Reddy et al., 2018). Thus, energy state is an important signal in determine dopamine signaling within the nucleus accumbens.

Acetylcholine

Acetylcholine exerts diverse effects within the nucleus accumbens due to the widespread expression of nicotinic and muscarinic acetylcholine receptors. Notably, despite acting as a direct excitatory neurotransmitter in the periphery, in the central nervous system acetylcholine is better described as a neuromodulator (Picciotto et al., 2012). Within the nucleus accumbens, cholinergic interneurons (described above) are thought to be the main source of acetylcholine, although input from a brainstem acetylcholine producing nucleus has been reported (Dautan et al., 2014). Nicotinic acetylcholine receptors (nAChRs) are pentameric, ligand-gated, and non-selective ion channels consisting of either homomeric or heteromeric combinations of α (α2-α7) or β (β2-β4) subunits (Picciotto et al., 2000). nAChRs are expressed extensively across the brain, especially presynaptically, where they regulate the release of nearly every major neurotransmitter (Exley and Cragg, 2008). Subunit composition is determined by expression and can vary depending on loci within the same nucleus. In the dorsal striatum, α7 and α4β2 receptors are both expressed, and both modulate glutamate release. However, α7 nAChRs enhance glutamate release, while α4β2 inhibit it via a dopaminergic mechanism (Howe et al., 2016). This diversity of nAChR subtypes can even occur within the same neuron (Gotti et al., 2006). Thus, nAChRs allow acetylcholine to acutely influence vesicular release in a variable manner depending on subunit expression.

Nicotinic signaling exerts significant control over striatal dopamine release. nAChRs are expressed presynaptically on dopamine terminals in the striatum (Exley et al., 2008; Jones et al., 2001). These presynaptic nAChRs regulate the release of dopamine following activation of cholinergic interneurons (Cachope et al., 2012; Exley et al., 2008; Threlfell et al., 2012). nAChRs

in the nucleus accumbens shell are critical to the expression of nicotine self-administration, as antagonism of α6β2 nAChRs reduces progressive ratio breakpoint for nicotine (Brunzell et al., 2010). Additionally, nicotine also acts upstream in the VTA. Within the VTA, α7 nAChRs are expressed on excitatory inputs and potentiate glutamate release, while α4β2 nAChRs are expressed on inhibitory inputs and potentiate GABA release (Mansvelder et al., 2002). *In vivo* these nAChR populations likely contribute to downstream dopamine release in the nucleus accumbens.

nAChRs are also recognized to stimulate glutamate release in the nucleus accumbens. Early studies found that peripherally administered nicotine enhanced glutamate release in the nucleus accumbens (Reid et al., 2000). This ability remained intact in animals with dopaminergic lesions, indicating that it was distinct from nicotine's effect on dopamine signaling. *Ex vivo* studies revealed that direct application of nicotinic agonists enhanced AMPAR currents, and that this ability persisted in the presence of tetrodotoxin and altered the paired pulse ratio, suggesting it was due to a presynaptic nAChR (Zhang and Warren, 2002). α7 containing nAChRs are expressed on excitatory terminals within the nucleus accumbens and may mediate the effect of acetylcholine on glutamatergic transmission (Zappettini et al., 2014). However, experiments using an α7 agonist fail to produce increases in extracellular glutamate in the nucleus accumbens (Huang et al., 2014). Interestingly, in the prefrontal cortex, α7 nAChRs activates signaling pathways that promote release of calcium from intracellular stores to promote presynaptic release of glutamate, rather than through direct activation of voltage-gated calcium channels (a mechanism used by β2 containing receptors) (Dickinson et al., 2008). It is possible that a similar signaling pathway exists in the nucleus accumbens, which may contribute to a more long term effect of nicotinic signaling.

Acetylcholine also signals through muscarinic receptors, which are g-protein coupled receptors. The M1 muscarinic receptor is present on MSNs, where it acts through the $G\alpha_q$ g protein

to stimulate endocannabinoid release (Wang et al., 2006). M1 has also been found to positively regulate MSN excitability (Zucca et al., 2018). M2 is found on glutamatergic terminals, where it inhibits glutamate release (Hersch et al., 1994). The M5 muscarinic receptor inhibits dopamine release in the striatum by acting presynaptically on dopamine terminals (Foster et al., 2014). Conversely, M2/M4 muscarinic receptors on cholinergic terminals inhibit dopamine release by reducing further nAChR signaling (Shin et al., 2015). Thus, muscarinic receptors open diverse pathways for acetylcholine to influence neuronal firing and neurotransmission within the nucleus accumbens.

Neuropeptide Y

Neuropeptide Y (NPY) is one of the most abundant neuropeptides within the brain and has been implicated in the control of feeding and anxiety-induced behaviors (Reichmann and Holzer, 2016). NPY is a 36 amino acid peptide which belongs to a family of peptides including peptide YY (PYY) and pancreatic polypeptide (PP) (Kask et al., 2002). NPY is expressed broadly across the brain but is found in relatively high levels in the hypothalamus, septum, locus coeruleus, periaqueductal gray, and the nucleus accumbens (Kask et al., 2002). Mammals express five NPY receptors, Y1r, Y2r, Y4r, Y5r and y6r, although y6r is not found in rats and in humans the protein is truncated and produces a non-functional receptor (Michel et al., 1998; Rose et al., 1997). Y4r has been determined to be a receptor for PP, and exhibits reduced affinity for NPY and PYY (Lundell et al., 1995). NPY and PYY both exhibit essentially equivalent, nanomolar, affinities for Y1r, Y2r, and Y5r (Michel et al., 1998). They are both degraded by dipeptidyl peptidase IV (DPP-IV), to produce NPY (3-36) and PYY (3-36) respectively (Grandt et al., 1996). Interestingly, NPY (3-36) and PYY (3-36) both exhibit similar affinities for Y2r and Y5r as their intact precursor, but

significantly lose affinity for Y1r (Gerald et al., 1996; Grandt et al., 1996). Therefore, degradation by DPP-IV reduces activation of Y1r, while retaining efficacy at Y2r and Y5r.

Y1r, Y2r, and Y5r are all expressed in the nucleus accumbens. Y1r is found in both the shell and the core (Pickel et al., 1998). Studies using electron microscopy have localized Y1r immunoreactivity to soma, dendrites, and axons (Pickel et al., 1998). Y1r immunoreactivity is found on asymmetric junctions, likely corresponding to excitatory synapses. Additionally, Y1r immunoreactivity is also found on astrocytic processes. Y2r mRNA is found in the nucleus accumbens, and immunoreactivity is found in both processes and cell bodies (Stanić et al., 2006). Y5 immunoreactivity is also found in the nucleus accumbens, and is visible on cell bodies (Wolak et al., 2003). Thus, multiple NPY receptor subtypes likely contribute to the role of NPY within the nucleus accumbens.

Exogenous infusion of NPY into the nucleus accumbens is rewarding. Infusion of NPY results in a conditioned place preference that is blocked by the mixed D1/D2 dopamine receptor antagonist *cis*-flupenthixol (Brown et al., 2000; Josselyn and Beninger, 1993). In both published cases these infusions were localized medially within the nucleus accumbens, although they were not restricted to the shell. Further implicating dopamine in the rewarding effect of NPY, ICV infusion of NPY causes increases in tissue content and extracellular dopamine in the striatum (Goff et al., 1992; Heilig et al., 1990). Direct infusion of NPY into the nucleus accumbens shell results in a large increase in extracellular dopamine, suggesting that NPY is able to modulate local release of dopamine (Sørensen et al., 2009). This interaction between NPY and dopamine may be mediated in part by the Y5r, as global Y5 antagonism or knockout reduces the behavioral effects of cocaine (Sørensen et al., 2012). However, the specific mechanism of NPY's effect on dopamine and the contribution this has to its rewarding effect is not fully clear.

In many brain circuits NPY acts to promote food intake (Andermann and Lowell, 2017; Smith and Grueter, 2021). Intra-nucleus accumbens infusion of NPY promotes consumption of high-fat diet, a phenotype that is dependent on the Y1r (Van Den Heuvel et al., 2015). There are mixed results on whether intra-nucleus accumbens NPY promotes sucrose intake, with some studies reporting an increase and others no change (Pandit et al., 2014; Van Den Heuvel et al., 2015). However, intra-nucleus accumbens NPY infusion does not increase chow intake (Brown et al., 2000; Van Den Heuvel et al., 2015). Together, these results suggest that NPY in the nucleus accumbens promotes food consumption by enhancing the rewarding properties of the food, rather than motivating animals simply for calories. This may be linked to nucleus accumbens systems regulating palatability (Peciña and Berridge, 2005; Vachez et al., 2021; Yang et al., 2020). NPY infusion into the nucleus accumbens does increase operant responding on a progressive ratio schedule for sucrose, indicating NPY treated animals are more motivated to obtain a palatable food reward (Pandit et al., 2014). It is attractive to link this behavioral phenotype to the recognized interaction between NPY and dopamine, however this specific hypothesis has not been tested.

Despite the relatively long history of studies examining NPY within the nucleus accumbens, many questions still remain. While it is clear that there are multiple NPY receptors expressed in the nucleus accumbens across different loci, and dopamine release is likely altered by NPY, it is unclear what receptors underlie this neurochemical phenotype. Additionally, how this effect on dopamine interacts with NPY's intra-nucleus accumbens effect on food intake and reward is unclear. Beyond these specific questions, the source for NPY release in the nucleus accumbens is unknown. The most likely source appears to be local NPY-expressing interneurons (PLTS interneurons), however there are no published reports of NPY release from these neurons (Tepper et al., 2010; Yamada et al., 2020). NPY containing projections from the arcuate nucleus

have also been reported (Van Den Heuvel et al., 2015). However, this finding has not been widely supported (Betley et al., 2013). Regardless of the source of NPY in the nucleus accumbens, the impetus for its release is similarly unknown. NPY release from arcuate nucleus neurons is stimulated by hunger (Alhadeff et al., 2018; Atasoy et al., 2012). It seems unlikely that a similar mechanism mediates NPY release in the nucleus accumbens, although it would be consistent with an increase in food reward. Further work in this area is required to more completely understand the role of NPY in modulating nucleus accumbens circuits. *In vivo* recordings indicate that NPY generally depresses firing of neurons in the nucleus accumbens, but how this is achieved is unclear (Van Den Heuvel et al., 2015). Additionally, how NPY alters firing of specific neurons during certain behavioral patterns is unexplored. Future work should address these questions, considering the reach and power of the NPY system in other neural circuits.

Nucleus accumbens control of behavior

The nucleus accumbens is most commonly associated with reward, and in most media references is referred to, incorrectly, as a "pleasure center" in the brain. This usually arises from the association of the nucleus accumbens with dopamine, regardless of the fact that dopamine is also not encoding pleasure (Berridge, 2007). In academic texts, the nucleus accumbens is commonly described as critical to the expression of motivated behavior. However, the nucleus accumbens is not required for the expression of consummatory behavior, and feeding is enhanced by inhibition of the nucleus accumbens (Reynolds and Berridge, 2002; Stratford and Kelley, 1997). The nucleus accumbens is also not necessary for the development or expression of simple instrumental behavior (Cardinal and Cheung, 2005; Corbit and Balleine, 2011). Even the acquisition and expression of first-order drug-seeking instrumental behavior is not dependent on

an intact nucleus accumbens (Ito et al., 2004). Together, these findings suggest a deeper complexity of the contribution of the nucleus accumbens to motivated behavior.

The behavioral tasks described above lack a critical aspect of most real world motivated behavior, the competition of differing available courses of action. How does the nucleus accumbens contribute to behavioral decisions when there are multiple different strategies available to obtain a reward? When lidocaine injections are used to disrupt the connection between the nucleus accumbens and the hippocampus, animals lose the ability to efficiently search for food in an 8-arm maze (Floresco et al., 1997). A similar disconnection strategy for the prefrontal cortex and the nucleus accumbens results in deficits in the ability of the animal to shift rule sets for obtaining a reward (Block et al., 2007). Similarly, nucleus accumbens lesions result in increased perseverative responding in the 5-choice serial reaction time task, meaning following a correct response lesioned animals were more likely to simply repeat the same response regardless of cue (Christakou et al., 2004). Thus while the nucleus accumbens is not necessary to perform a reward-seeking action, it seems to be necessary for the animal to efficiently choose between reward-seeking options.

As previously mentioned, the nucleus accumbens consists of two subregions with distinct anatomical connectivity. This difference in connectivity is mirrored by differences in function. Put simply, the nucleus accumbens core is more important for directing approach to salient stimuli, while the shell is more important in inhibiting the development of behaviors that will interfere with effective reward seeking (Fig. 4) (Floresco, 2015).

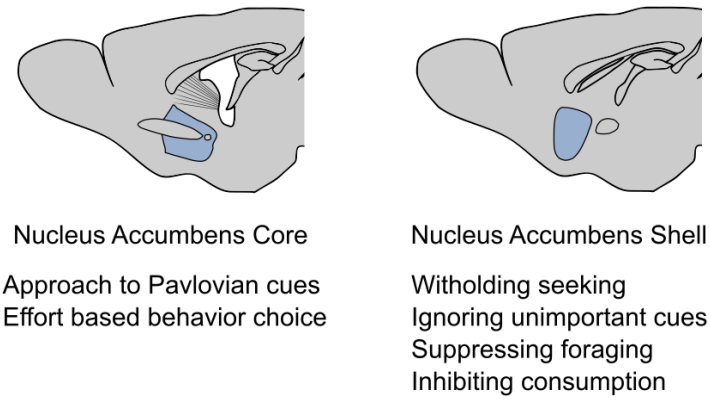


Figure 4 | Behavioral roles of nucleus accumbens subregions

The nucleus accumbens core and shell serve different behavioral functions. The core generally promotes behavior, by mediating the invigorating aspects of cues. The shell generally promotes behavioral inhibition, inhibiting learned behavior strategies and mediating the ability to ignore irrelevant cues.

Nucleus accumbens core

Experimental evidence suggests that the nucleus accumbens core is necessary for allowing salient cues to invigorate behavior. This has been extensively demonstrated using tasks dependent on Pavlovian conditioned approach, where presentation of a cue triggers approach. Lesions of either excitatory or dopaminergic input to the nucleus accumbens core results in a deficit in the ability to use learned Pavlovian cues to stimulate approach (Dalley et al., 2002; Hall et al., 2001; Parkinson et al., 2000). Disconnecting the BLA and nucleus accumbens core while interfering with dopamine signaling disrupts the ability of the animal to properly respond to reward-predictive cues (Ambroggi et al., 2008). Neurons in the BLA responds to salient cues, and this response precedes and is required for the neuronal response in the nucleus accumbens core (Ambroggi et al., 2008). This cue evoked firing in the nucleus accumbens core correlates with the vigor of the resulting approach toward the reward, as measured by the movement initiation latency and the animal's speed (McGinty et al., 2013). Together, these results suggest a model where cues activate BLA inputs to the nucleus accumbens, which excite nucleus accumbens MSNs in a manner modified by coincident dopaminergic input based on the salience of the cue. The resulting MSN firing then determines the vigor of the resulting approach behavior. Thus, the computations in the nucleus accumbens core allow animals to use cues in the environment to direct efficient motivated behavior.

Additionally, the nucleus accumbens core is necessary for making effort-based behavioral decisions. GABA agonist induced inactivation of the core reduces choice of a high-effort high-reward behavior option (Ghods-Sharifi and Floresco, 2010). Core inactivation did not disrupt choice of the greater reward option when the effort cost was the same.

Nucleus accumbens shell

In contrast to the nucleus accumbens core, the shell region of the nucleus accumbens is required for the inhibition of inappropriate or incorrect reward seeking strategies. Pharmacological inactivation of the nucleus accumbens shell increases incorrect win-stay behavior in a reversal learning paradigm (Dalton et al., 2014). These data can be interpreted to mean that once an animal has been reinforced in a specific reward seeking strategy, the nucleus accumbens shell is required to inhibit that action pattern when a shift in strategy is required. This interpretation appears consistent with another study which inactivated excitatory input to the nucleus accumbens shell. This inactivation of excitatory input resulted in inappropriate operant responding during period of reward availability, again indicating an inability to suppress a behavior pattern (Lafferty et al., 2020). Similarly, inactivation of the nucleus accumbens shell disrupts the ability of the animal to avoid a foot shock by withholding operant responding (Piantadosi et al., 2018). Together these results describe the nucleus accumbens shell as a critical component of the neural systems mediating the behavioral suppression of learned action patterns.

The nucleus accumbens shell also important for suppressing attention to cues previously learned to be irrelevant. This is best demonstrated in experiments studying latent inhibition. In latent inhibition paradigms, an animal is exposed to a cue that is not linked to a reward repeatedly. Later, when the cue is paired with a reward, animals with previous exposure to the cue take longer to form a cue-reward association. Lesions of the nucleus accumbens shell abolish the latent inhibition phenotype (Weiner et al., 1999). Further experiments have shown that the nucleus accumbens shell is critical to the expression, rather than the acquisition, of latent inhibition (Gal et al., 2005). Again, these results implicate the nucleus accumbens shell in an aspect of behavioral suppression, in this case suppressing attention to stimuli learned to be irrelevant.

The nucleus accumbens shell is also contributes to foraging behavior in complex environments. When searching for a food reward in a radial arm maze, animals will usually avoid arms they have already determined to be devoid of food. However, animals with nucleus accumbens shell inactivation will continually visit non-baited arms (Seamans and Phillips, 1994). This spatial memory based process has been linked to the dopaminergic modulation of hippocampal input to the nucleus accumbens (Floresco and Phillips, 1999). Specifically, inhibition of D1 receptor activation significantly increased inefficient maze arm re-entries. These results indicate that when an animal learns that certain spatial regions do not contain reward, a dopamine modulated hippocampus to nucleus accumbens shell circuit mediates the suppression of the foraging action pattern for that location. Additionally, these results communicate a critical distinction for the role of dopamine in the nucleus accumbens shell. Typically thought of as a motivating force, in this case dopamine is critical to the proper *suppression* of action. This highlights the subregion specificity of dopamine's role, as well as informing more generally on nucleus accumbens shell function.

The above examples paint a picture of the nucleus accumbens shell as a region dedicated to the suppression of less than optimal behavior strategies. The ability to pursue high value goals is obviously critical to efficient reward-seeking, but so is the ability to stay on task and avoid distraction by less relevant strategies. However, from an animal's perspective it can be difficult to assess what is the most optimal strategy. Further, the most optimal strategy can shift depending on the needs of the animal. Should a hungry animal be more willing to try a new strategy to find food? Should they pay attention to a previously irrelevant cue? Should they check an environment that previously hasn't had food, just in case? It is clear that the definition of productive depends on the

risk vs. reward calculation each animal must make. This idea is central to the work described in the body of this thesis

Nucleus accumbens control of feeding

In addition to controlling the higher-order motivated behavior required to seek out food, the nucleus accumbens shell also exerts inhibitory control over the consummatory act. Early microinjection experiments from Ann Kelley's lab identified a significant role for amino acid transmitters, glutamate and GABA, in controlling feeding behavior in the nucleus accumbens (Maldonado-lrizarry et al., 1995; Stratford and Kelley, 1997). Inhibiting glutamatergic signaling in the nucleus accumbens shell resulted in a significant feeding response, while inhibition in the nucleus accumbens core did not (Maldonado-lrizarry et al., 1995). Activation of $GABA_A$ receptors also promoted feeding behavior (Stratford and Kelley, 1997). This feeding phenotype caused by manipulations in the nucleus accumbens was linked to activity in the lateral hypothalamus (Maldonado-lrizarry et al., 1995; Stratford and Kelley, 1999). Inhibition of cells in the nucleus accumbens shell resulted in a significant increase in expression of the immediate early gene product Fos in the lateral hypothalamus (Stratford and Kelley, 1999). These experiments led to the idea that this circuit allows the ability to send a stop signal to areas controlling the feeding motor program (Kelley, 2004b). Kelley pioneered the idea of the nucleus accumbens shell as a sensory sentinel, collecting environmental information communicating potential danger and allowing that information to interrupt ongoing feeding (Kelley, 2004b). This circuit conceptualization evokes the direct connection between the striatum and the behavioral control column in Swanson's neural organization, which envisioned the striatum as exerting an inhibitory influence over the behavioral control column. Indeed, this circuit has been used as an anatomical model for that idea (Swanson, 2000; Thompson and Swanson, 2010).

Following the study of amino acid neurotransmitters came similarly designed experiments to assess the role of neuromodulators. Microinfusions of pharmacological reagents targeting opiate receptors identified the nucleus accumbens as a key center for the effects of opioids on feeding behavior. Infusion of morphine or Mu receptor agonists into the nucleus accumbens shell results in hyperphagia (Bakshi and Kelley, 1993; Zhang et al., 1998). Conversely, infusion of opioid antagonists reduces food intake, suggesting there is ongoing opioid signaling during normal feeding (Kelley et al., 1996). Studying conserved orofacial reactions to taste, Mu opiate receptor signaling in the dorsal medial nucleus accumbens shell was identified as a significant regulator of palatability (Peciña and Berridge, 2005, 2000). This specific striatal subregion became known as the "hedonic hot spot" (Peciña and Berridge, 2005). These results were replicated with both Delta and Kappa opiate receptor agonists, indicating that each system is able to modulate the hedonic response to food (Castro and Berridge, 2014). Additionally, this same region of the nucleus accumbens shell produced similar hedonic enhancement in response to exogenous administration of anandamide, an agonist for cannabinoid and TRPV1 receptors (Mahler et al., 2007). While rostral injections of opioid and amino acid pharmacology regulate feeding behavior, caudal injections produce fear-related behaviors, indicating the anatomical specificity of the behavior phenotypes engaged by different regions of the nucleus accumbens (Reynolds and Berridge, 2003, 2001).

As the hedonic hotspot has generated significant interest, Thompson and Swanson undertook a detailed anatomical analysis of the neural circuitry surrounding this rostrodorsal region of the medial nucleus accumbens shell (Thompson and Swanson, 2010). Generally similar to other areas of the nucleus accumbens, the inputs to this region included infralimbic cortex, the ventral subiculum, and the amygdalar basomedial nucleus. Interestingly, despite the typical

architecture of the ventral striatum, this region of the nucleus accumbens sends projections exclusively to the lateral hypothalamus and ventral pallidum. Only injections more ventral to the hedonic hotspot region yielded a labeled terminal field in the ventral tegmental area. Further anatomical mapping focusing on the area of the lateral hypothalamus targeted by this region of the nucleus accumbens yielded a projection to the anterodorsal preoptic nucleus, a region involved in visceromotor pattern generating. This circuit (nucleus accumbens -> lateral hypothalamus -> preoptic nucleus) likely mediates the ability of the nucleus accumbens to acutely interrupt ongoing feeding behavior.

Following pharmacological studies, an unanswered question was how the endogenous activity of nucleus accumbens neurons changed during food consumption. *In vivo* recordings revealed that a significant population of nucleus accumbens neurons experience inhibition during food consumption (Nicola et al., 2004). These consumption-inhibited neurons have been found across the nucleus accumbens, however they are much more numerous within the nucleus accumbens shell (Krause et al., 2010). This result tracked with anatomical bias in results from exogenous application of classically inhibitory compounds, which increased food intake (Stratford and Kelley, 1997; Zhang et al., 1998). A smaller subset of nucleus accumbens neurons were found to respond with excitations relative to the palatability of the food reward (Taha and Fields, 2005). Further work indicated that the character of a tastant impacts the firing patterns of nucleus accumbens neurons, as intraoral infusions of sucrose or quinine inhibited or excited the majority of recorded neurons, respectively (Roitman et al., 2005). This inhibition during consumption was found to extend throughout the duration of a goal-directed sequence of behavior, suggesting that these neurons gate behavioral patterns, and that inhibition permits the expression of that specific behavioral pattern (Taha and Fields, 2006). Indeed, electrical stimulation of the nucleus accumbens

shell during a consummatory bout interrupted feeding (Krause et al., 2010). The areas of the nucleus accumbens that were most effective in interrupting feeding were mostly found in the anterior portion (Krause et al., 2010). However, the authors note that areas that were effective and ineffective in interrupting feeding were often tightly grouped, with neighboring electrodes in an array producing different results (Krause et al., 2010). This suggests that even within nucleus accumbens subregions, there are likely different ensembles mediating different facets of behavior. These studies, while they paint a consistent role for the nucleus accumbens shell in inhibiting feeding, also hint at the complexity and heterogeneity of the region.

With significant evidence supporting the idea that activity in the nucleus accumbens regulates food intake, researchers began to question whether experience resulted in changes to the nucleus accumbens that promoted further consumption. This was suggested as a neural mechanism for overeating, similar to plasticity discovered in the nucleus accumbens following addictive drugs (Thomas et al., 2001). This idea was also informed by imaging experiments in human participants with obesity, who exhibited lower D2 receptor availability in the striatum (Wang et al., 2001). Indeed, placing animals on a palatable feeding regimen resulted in increased food-intake in response to intra-nucleus accumbens infusions of a $GABA_A$ agonist (Newman et al., 2013). This sensitized response was interpreted as a result of changes within the nucleus accumbens that promote endogenous inhibition. Further studies indicated that intermittent access to a sucrose solution, a protocol that results in binge-feeding behavior, altered the expression of dopamine, opioid, and endocannabinoid receptors in the nucleus accumbens (Soto et al., 2015). However, these receptors do not appear to be necessary for binge-consumption, as antagonism of opioid or dopamine receptors does not reduce binge feeding that results from an intermittent access schedule (Lardeux et al., 2015). Converse to access to high-energy diets, food-restriction was found to result

in a sensitized response to dopamine agonists in both the nucleus accumbens core and shell (Carr et al., 2003). This change was accompanied by changes to glutamatergic synaptic properties, similar to those seen in drug addiction (Anderson et al., 2008; Carr et al., 2010). Other drug addiction-like synaptic adaptations have been observed following a "junk-food" diet. Short access to the junk-food diet resulted in increased expression of calcium permeable AMPARs in the nucleus accumbens core (Oginsky et al., 2016). Further, antagonism experiments suggest that this increase in calcium permeable AMPAR expression drives increased incentive motivation (Derman and Ferrario, 2018). A similar protocol using a "cafeteria diet" resulted in downregulation of D2 dopamine receptors in the dorsal striatum, similar to those seen in human patients with obesity (Johnson and Kenny, 2010). Together, these results indicated that not only does the nucleus accumbens control feeding, but experience shapes neurotransmitter elements within the nucleus accumbens in ways that alter feeding.

Using optogenetic tools, a team in the Lüscher lab followed up on Anne Kelley's work investigating the connection between the nucleus accumbens and the lateral hypothalamus and put the sensory sentinel hypothesis to the test (O'Connor et al., 2015). Using transgenic reporter mice, they first identified the projection to the lateral hypothalamus as overwhelmingly made up of dopamine D1 receptor expressing MSNs. Utilizing genetically-driven optogenetics to "tag" D1 MSNs *in vivo*, they observed a pause in firing during food consumption, suggesting that D1 MSNs make up some of the population previously identified by unidentified recordings (Krause et al., 2010). Inhibiting D1 MSNs during food intake prolonged consumption, and activating them interrupted it. Interestingly, this inhibition of D1 MSNs reduced the ability of a distractor stimuli to interrupt feeding. These data supported Anne Kelley's original hypothesis of the nucleus accumbens to lateral hypothalamus circuit as a sensory sentinel, allowing food consumption to be

interrupted by salient events. Further confirming previous findings, they also determined that nucleus accumbens neurons did not synapse onto orexin or melanin concentrating hormone-expressing cells in the lateral hypothalamus, two cell types associated with increased food intake (Sano and Yokoi, 2007). Rather, they report that projections from the nucleus accumbens synapse onto VGaT-expressing neurons of the lateral hypothalamus. Follow up work from the Lüscher lab identified that food deprivation results in a potentiation of this nucleus accumbens to lateral hypothalamus synapse, further implicating this circuit in the control of feeding behavior (Thoeni et al., 2020). These results identify the nucleus accumbens to lateral hypothalamus circuit as a powerful brake on feeding behavior, one that is activated by environmental stimuli to shift behavior.

Building on the power of genetically-encoded biosensors and opsins, multiple studies have investigated the role of specific inputs into the nucleus accumbens in controlling feeding behavior. Similar to reduced neuronal activity phenotype described above in the nucleus accumbens, excitatory inputs to the nucleus accumbens also exhibit a decrease in activity during food consumption (Krause et al., 2010; Reed et al., 2018). Inhibition of these inputs, specifically the basolateral amygdala, the ventral hippocampus, and the midline thalamus, increases food consumption similar to inhibitions of nucleus accumbens neurons (Reed et al., 2018). These results highlight the importance of excitatory input in driving changes in MSN firing. Conversely, activation of an inhibitory input, specifically from the ventral pallidum, enhances food consumption and palatability (Vachez et al., 2021). These results added greater detail to previous conceptualizations of how excitation and inhibition of the nucleus accumbens shell modulates food intake.

Excitatory input to this region is not simply a brake on feeding behavior. Perhaps related to the different ensembles of nucleus accumbens neurons identified during food intake, low frequency optogenetic activation of the input from the ventral hippocampus enhances the apparent palatability of a food reward (Yang et al., 2020). Additionally, while optogenetic activation of the input from the prefrontal cortex inhibited high-fat diet intake, activation of the input from the anterior paraventricular nucleus of the thalamus increased it (Christoffel et al., 2021). Thus the role of excitatory transmission in the nucleus accumbens shell is varied, depending on region and likely intra-NAc connectivity. An additional caveat is the stimulation protocol used to assess the roles of these inputs. It is possible that stimulating the same input at different frequencies could result in different behavioral outcomes. It may be useful to attempt to mimic endogenous patterns of activity recorded during behavior, or to specifically inhibit inputs to identify their contribution to behavior.

As mentioned previously, intra-nucleus accumbens infusions of Mu opioid receptor agonists into the nucleus accumbens has long been appreciated to promote food intake (Zhang et al., 1998). For decades, the endogenous opioid circuit that took advantage of this signaling system was unknown. Using targeted knockout of the Mu opioid receptor, Castro et al. identified presynaptic Mu opioid receptors expressed on a dorsal raphe input to the nucleus accumbens as necessary for mediating the consummatory response to endogenous opioids (Castro et al., 2021). This inhibition was driven by endogenous enkephalin release from nucleus accumbens MSNs during food deprivation, thus allowing food deprivation to promote food consumption. How food deprivation drives enkephalin release within the nucleus accumbens remains unclear.

Nucleus accumbens summary

The above section details the structure and function of the nucleus accumbens. The nucleus accumbens is critical to the production of efficient motivated behavior. In concert with various microcircuit elements, medium spiny neurons in the nucleus accumbens process motivationally relevant information arriving in the form of glutamatergic neurotransmission. This transmission is shaped by various neuromodulators, which selectively tune inputs to guide neural computations and thus behavior. The functioning within the nucleus accumbens allows for efficient motivated behavior, promoting the proper investment of effort and allowing the flexibility to abandon strategies that are no longer useful. Additionally, the nucleus accumbens exerts significant control over food consumption. While we have identified many important neuromodulatory mechanisms within the nucleus accumbens, there are likely still many more that remain unknown. Despite the role of the nucleus accumbens in controlling feeding and reward-seeking behavior, it is unknown whether hunger acutely regulates nucleus accumbens neurotransmission to regulate these behaviors. This is an idea that will be explored in Chapter II. Further, despite the evidence detailing the presence of the neuropeptide Y system within the nucleus accumbens, it is unknown whether neuropeptide Y modulates excitatory glutamatergic transmission within the nucleus accumbens or nucleus accumbens-driven behaviors. This will be explored in Chapter III.

Hunger-driven adaptive prioritization of behavior

Neuropeptides are commonly messengers of the animals internal state, modulating neural circuits in order to promote restoration of homeostasis (van den Pol, 2012). Hunger, or the drive for food initiated by a low energy state, exerts a powerful control over behavior. In this portion of the introduction, I detail how energy state is communicated to the brain by exploring one of the best studied central integrators of energy state, the arcuate nucleus. Understanding how the arcuate nucleus collects information about energy state and propagates that information across the brain provides us with principles that may generalize to other integrators. Thus, understanding this model system may inform on computations within the nucleus accumbens, the subject of this dissertation.

In order to survive, an animal must adapt its behavioral priorities to accommodate changing internal and external conditions. Hunger, a universally recognized interoceptive signal, promotes food intake though increasingly well understood neural circuits. Within the brain, agouti-related peptide (AGRP) neurons in the arcuate nucleus of the hypothalamus have been found to powerfully stimulate feeding in addition to mediating other hunger-driven behavioral phenotypes. The study of these neurons has greatly expanded our understanding of how an internal drive is communicated to the brain.

Hunger biases behavior toward feeding

Animals engage in a wide range of behaviors to fulfill goals critical to life and reproduction. In order to thrive, an animal must appropriately prioritize the behaviors that address their most pertinent needs. The need for food represents one of nature's most fundamental obligations. Hunger, the interoceptive reporter of energy deficit, is a universally recognized signal that triggers a pervasive shift in behavior (Fig. 5). The hungry animal must engage in a significantly altered

behavioral complement relative to the fed animal to fulfill this need. Disruption in this complex interaction between energy level and behavior, leading to an abnormal behavioral response to energy deficit or surplus, is a hallmark of eating disorders. However, the mechanisms that underlie this adaptive prioritization of behavior remain largely unknown.

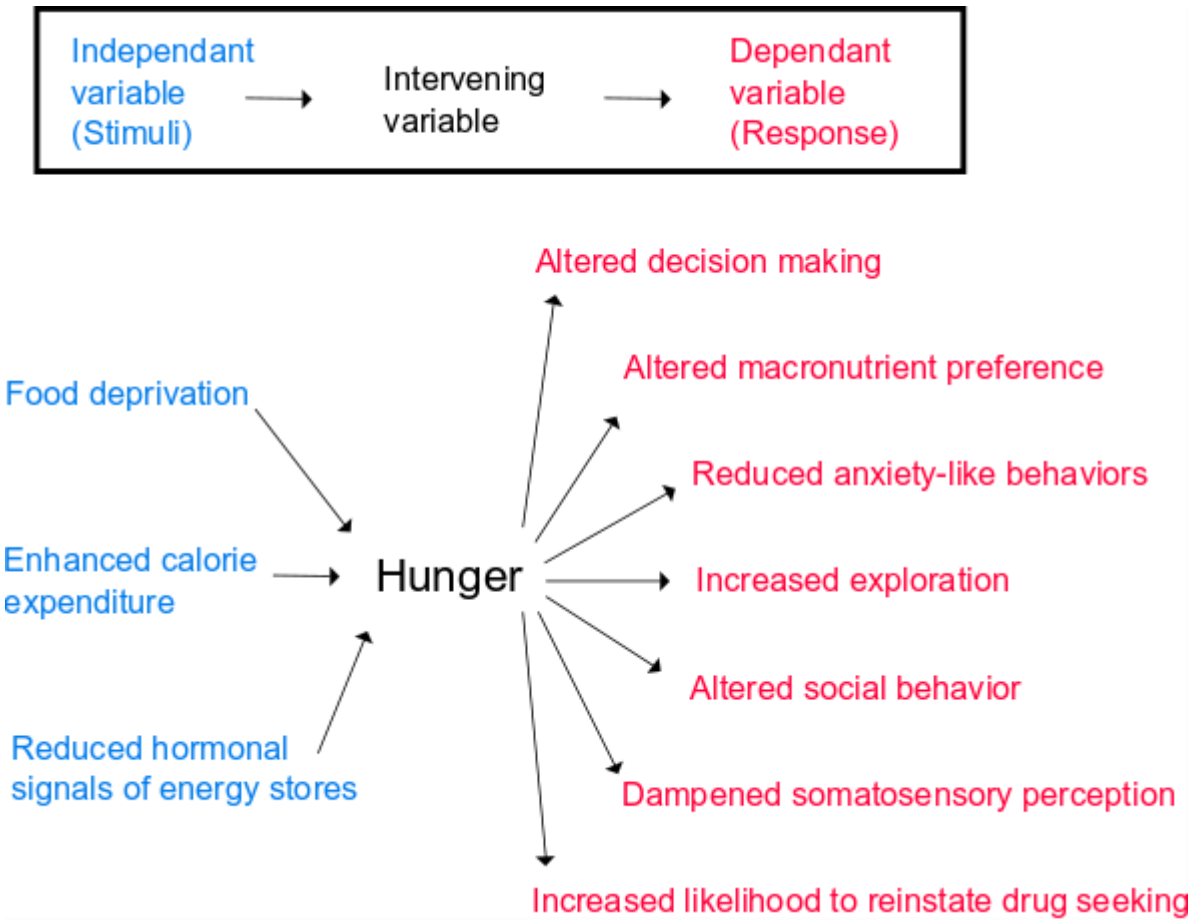


Figure 5 | Intervening variables allow consolidated central representation of drive

Hunger as an intervening variable connecting stimuli that alter energy state and the behavioral shifts that support feeding (Andermann and Lowell, 2017; Berridge, 2004). Many stimuli can trigger the need to consume food, and there are similarly many behavioral responses that support the finding and consumption of food. A neural and conceptual organization that allows an intervening variable to collect and communicate these stimuli allows the coordination of a range of behavioral responses to a number of disparate stimuli.

Hunger, while familiar to every animal, can be difficult to operationally define. How do you measure hunger? In humans, you can simply ask them. But what are they assessing internally when they introspect in search of an answer? The consequences of hunger can be measured by appetite, or how much food an animal will consume. While difficult to pin down, hunger as a concept serves an important purpose in the study of motivated behavior. From a behavioral perspective, a drive such as hunger can act as an "intervening variable" between a great number of stimuli and responses (Andermann and Lowell, 2017; Berridge, 2004; Richter, 1947) (Fig. 5). For example, a number of stimuli (food deprivation, increased calorie expenditure, hormonal imbalance) can lead to a great number of behavioral responses (food seeking, food consumption, suppression of other behaviors). Assuming a strict one to one stimulus response organization, these stimulus-response relationships would quickly become cumbersome, both theoretically and biologically. However, an intervening variable between stimulus and response allows a range of stimuli to contribute to the ultimate expression of the appropriate behavioral response. In studying homeostatic behaviors, this intervening variable is most commonly known as a drive. Hunger can be conceptualized as the aggregate neural representation of the many stimuli that can result in increased appetite, food reward, and the behavioral shifts that support feeding.

Arcuate nucleus, a central integrator of energy state

While drives such as hunger or thirst represent nebulous motivational states that have no single locus, certain brain regions like the arcuate nucleus of the hypothalamus play an outsized role in communicating these states to the nervous system. The arcuate nucleus is a region in the mediobasal hypothalamus that contains multiple neuronal subtypes responsible for controlling distinct aspects of feeding and hunger-related behaviors. Two arcuate cell types, cells that express Agouti-related peptide (AGRP) and cells that express Proopiomelanocortin (POMC), act in

opposition to promote and reduce energy intake, respectively. By integrating signals from circulating hormones, ascending circuits from the gut and brainstem, and incoming sensory information, these neurons coordinate neural systems to ensure maintenance of energy homeostasis (Andermann and Lowell, 2017; Sternson and Eiselt, 2017). Multiple experimental approaches have shown AGRP neuron activation invigorates a behavioral program to find and consume food. Both optogenetic (Aponte et al., 2011) and chemogentic (Krashes et al., 2011) excitation dramatically enhances food intake in fed animals. Conversely, chemogenetic silencing of AGRP neurons is able to rapidly attenuate feeding in hungry mice (Krashes et al., 2011). Additionally, AGRP neurons prepare the animal for feeding by altering behavioral strategy (Burnett et al., 2016) (Padilla et al., 2016) while also coordinating peripheral metabolism (Engström Ruud et al., 2020; Steculorum et al., 2016). Ablation of AGRP neurons in adult mice results in a dramatic reduction in food intake, potentially leading to death (Gropp et al., 2005; Luquet et al., 2005). However, if AGRP neurons are ablated in neonates, a yet unknown compensatory mechanism results in normal feeding behavior and survival (Luquet et al., 2005). The necessity of these neurons in adult animals indicates their central role in connecting energy state with an appropriate behavioral response. AGRP neurons have provided immense utility in allowing researchers to produce an “artificial hunger” state, however, there are more neuron populations and hormones that contribute to the central representation of energy deficit (Andermann and Lowell, 2017). Here we focus on AGRP neurons mainly because they have been most extensively studied using the genetically encoded tools (Cre-dependent opto- and chemogenetics) that have allowed the dissection of a wide range of hunger-driven behavioral phenotypes (Lowell, 2019). As the focus of this section is hunger-driven adaptations, we will focus on the AGRP neuron component of the arcuate nucleus. The arcuate nucleus as a whole, as well

as the concept of satiety and associated neural correlates have been comprehensively reviewed elsewhere (Andermann and Lowell, 2017; Sternson and Eiselt, 2017).

In response to an energy deficit, AGRP neurons engage several synaptic plasticity mechanisms resulting in significantly upregulated action potential firing frequency (Takahashi and Cone, 2005). Excitatory synaptic input onto AGRP neurons is likely upregulated via multiple mechanisms. Different groups have identified presynaptic enhancements in glutamate vesicle release probability and postsynaptic dendritic spinogenesis. Presynaptic glutamate release probability is enhanced by ghrelin signaling, which engages a 5' adenosine monophosphate-activated protein kinase (AMPK)-dependent positive feedback loop (Yang et al., 2011). This presynaptic enhancement is negatively regulated by leptin, which is able to inhibit presynaptic release in response to increasing energy stores following food intake. Postsynaptically, activation of NMDA receptors leads to spinogenesis which also acts to enhance excitatory transmission onto AGRP neurons (Liu et al., 2012). This spinogenesis is downstream of postsynaptic AMPK mediated activation of p21-activated kinase (PAK), and activation of AMPK in AGRP neurons is sufficient to drive feeding and weight gain (Kong et al., 2016). Ultimately, these changes result in a significantly increased firing rate of AGRP neurons, presumably leading to increased release of neurotransmitters, GABA and neuropeptides, in downstream regions. However, the significance of these mechanisms in promoting the behavioral phenotypes of AGRP neurons has not been studied.

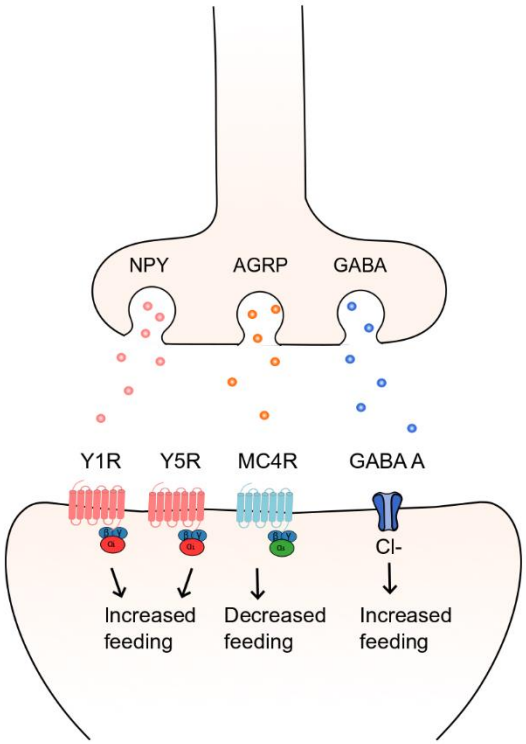


Figure 6 | AGRP neuronal transmission occurs via NPY, AGRP, and GABA

AGRP neurons release NPY, AGRP, and GABA to communicate low energy state. NPY acts through Y family GPCRs, of which Y1 and Y5 have been described as the primary effectors of NPY's effect on feeding behavior. AGRP acts as an inverse agonist at melanocortin GPCRs, including MC4R and MC3R. GABA acts at the GABA A receptor, a ligand gated ion channel, to allow immediate inhibition of postsynaptic targets. AGRP neuron neurotransmission has mainly been studied in the context of feeding behavior, and many AGRP neuron phenotypes independent from feeding are not associated with a specific transmitter or receptor system.

The production of multiple neurotransmitters by AGRP neurons leads to diverse signaling capabilities. They utilize the inhibitory neurotransmitter GABA, as well as multiple neuropeptides, Including Agouti-related peptide (AGRP) and Neuropeptide Y (NPY) (Fig. 6). AGRP produces its behavioral effect by acting as an inverse agonist at melanocortin G-protein-coupled receptors (GPCRs) MC3R and MC4R (Andermann and Lowell, 2017). These receptors are activated by the POMC product α-MSH and are typically coupled to Gαs G proteins. Thus, AGRP acts to enhance appetite by reducing endogenous, satiety promoting, MC3R and MC4R signaling. As mentioned in the previous section, NPY acts through a family of GPCR Y receptors known as Y1, Y2, Y4 and Y5 in humans (Zhang et al., 2019). All Y receptors are typically coupled to Gαi G proteins and exert an inhibitory influence. Postsynaptic Y1 and Y5 receptors are most commonly associated with the food intake promoting action of central NPY (Zhang et al., 2019), however the presynaptically localized Y2 receptor has been found to mediate anxiolytic and anti-depressant properties of NPY (Reichmann and Holzer, 2016). Further work will need to examine these signaling systems in greater detail in downstream regions in order to more clearly define the generation of different behavioral phenotypes.

Studies utilizing different genetic manipulations to perturb specific neurotransmitters released by AGRP neurons has identified distinct roles for these signaling systems. Similar to AGRP neuron ablation, constitutive knockout of *Agrp* or *Npy* results in no long-term feeding or body weight phenotype (Qian et al., 2002)(Erickson et al., 1996). Conversely, ablating GABA transmission from AGRP neurons utilizing an *Agrp-Ires-Cre, Vgat*$^{flox/flox}$ cross results in a reduction in both body weight and ghrelin stimulated food intake (Tong et al., 2008). In light of the underwhelming phenotype of the single knockout strains, a triple knockout mouse was generated lacking *Mc4r,* and with *Agrp-ires-Cre* driving AGRP specific knockout of *Npy* and *Vgat*

(Krashes et al., 2013). Chemogenetic stimulation of AGRP neurons in this triple knockout mouse failed to evoke food intake, indicating the combined necessity of these three signaling systems to allow AGRP neuron stimulation induced feeding behavior (Krashes et al., 2011). Further double knockout crosses identified GABA and NPY signaling as sufficient to drive the acute increase in food intake characteristic of AGRP neuron stimulation on a time scale of minutes (Krashes et al., 2013). Indeed, analysis of single *NPY* knockout mice finds that NPY is necessary for AGRP neuron stimulation to promote short-term increases in food intake (Engström Ruud et al., 2020). AGRP signaling through MC4r on the other hand was found to drive a more chronic increase in food intake on a time scale of hours (Krashes et al., 2013). While many studies of AGRP neuron modulation of feeding behavior utilize concurrent stimulation paradigms, endogenous AGRP neuron activity seems to be dramatically reduced upon food intake. In paradigms mimicking this activity pattern by utilizing optogenetic pre-stimulation prior to food introduction, NPY signaling is necessary for enhanced food intake (Chen et al., 2019). Importantly, the existence of GABA, NPY, and AGRP in the same cell type, all contributing uniquely to feeding behavior, illustrates that a single cell type can contain multiple transmitters each participating in unique interactions toward a shared phenotype. As the role of individual neurotransmitters has only been assessed for the food intake phenotype of AGRP neuron activation, it is possible that a variety of neurotransmitters may be key to initiating other behavioral phenotypes downstream of AGRP neurons.

In addition to these three well studied neurotransmitters, there appear to be other neuropeptides also produced in AGRP neurons, including the products of the propeptide ProSAAS (Wardman et al., 2011). ProSAAS is likely involved in the regulation of feeding behavior, as knockout of ProSAAS results in reduced body weight (Morgan et al., 2010). Conversely,

overexpression of ProSAAS results in obesity (Wei et al., 2004). In the paraventricular nucleus of the hypothalamus, one of the brain regions targeted by AGRP neurons, the ProSAAS product BigLEN acts to suppress excitatory glutamatergic transmission (Wardman et al., 2011). BigLEN has been found to act through a Gαi/o coupled receptor, GPR171 (Gomes et al., 2013). Genetic suppression of GPR171 or antibody-mediated inhibition of BigLEN signaling reduces food consumption, linking this receptor system to the control of feeding (Gomes et al., 2013; Wardman et al., 2011). Radiolabeled BigLEN accumulates in multiple regions across the brain, including the striatum (Gomes et al., 2013). Further, food deprived rats exhibit increased concentrations of BigLEN within the nucleus accumbens (Ye et al., 2017). Together, these results suggest that BigLEN and GPR171 may modulate the nucleus accumbens, in addition to their role in the hypothalamus, although this has not been assessed prior to the work presented here.

AGRP neurons send projections to brain regions both within and outside the hypothalamus, but only some of these terminal fields are able to stimulate food intake (Fig. 7). Within the hypothalamus, AGRP neurons project to the paraventricular nucleus of the hypothalamus (PVH) as well as the lateral hypothalamus (LH) (Betley et al., 2013). Beyond the hypothalamus, AGRP terminals reach the bed nucleus of the stria terminalis (BNST), the paraventricular nucleus of the thalamus (PVT), the amygdala (Amyg), and the periaqueductal gray (PAG) (Betley et al., 2013). Additionally, a projection from AGRP neurons to the nucleus accumbens (NAc) has been reported (Van Den Heuvel et al., 2015). A number of these projections are able to enhance food intake (Amyg, BNST, PVH, PVT, LH) (Betley et al., 2013; Padilla et al., 2016). Other projection fields appear to modulate different behaviors, allowing enhanced food seeking and feeding through the inhibition of competing behavioral drives, such as projections to the PBN which mediate the suppression of pain, projections to the Amyg that mediate territorial aggression, and projections to

the BNST that mediate aspects of glucose homeostasis (Alhadeff et al., 2018; Padilla et al., 2016; Steculorum et al., 2016). Additionally, AGRP neurons have been found to be able to trigger cascades of circuit changes leading to phenotypes in regions several synapses away, such as biasing food cue responses in the insular cortex via a circuit containing the PVT and Amyg (Livneh et al., 2017). The wide reach of these hunger neurons suggests a critical role of energy state in neural computations made across the brain. Through AGRP neuron modulation of downstream circuits, animals are able to consider their current hunger level when deciding to engage, or avoid, specific behaviors.

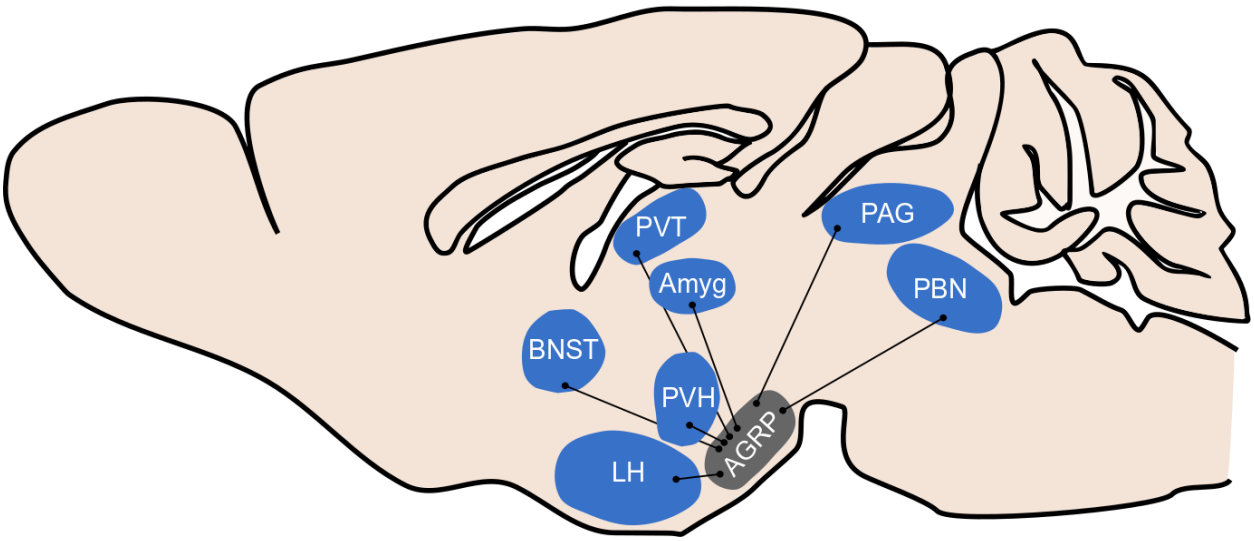


Figure 7 | AGRP neurons project widely to regions controlling feeding behavior

First order projection targets of AGRP neurons in the arcuate nucleus. AGRP neurons exhibit a broad range of projection targets, contacting regions involved in a diverse set of behaviors. Interestingly, there appear to be different neuronal populations of AGRP neurons for each projection target. These projection targets support AGRP neuron modulation of a wide range of behavioral phenotypes, including influencing food consumption through terminal fields in the PVH, LH, BNST, and PVT. Terminal fields in the Amyg and PBN have been found to influence territorial behavior and pain associated behaviors, respectively. Mesolimbic regions like the VTA and NAc are thought to play a role in food reward and food-seeking behavior, but exact mechanisms downstream of AGRP neurons are unknown.

In addition to aforementioned regulation by long-term energy state, AGRP neurons are also regulated on distinct time scales by both external sensory information as well as internal information communicating nutrient intake in the gut. Upon food presentation, AGRP neurons exhibit rapidly reduced intracellular calcium as well as reduced action potential firing frequency (Betley et al., 2015; Chen et al., 2015; Mandelblat-Cerf et al., 2015). This immediate response, before food is ingested, is thought to rely on sensory information transmitted to AGRP neurons and has been found to be transient in nature. A similar, slower effect can be produced by intragastric nutrient infusion or gastric distension (Bai et al., 2019; Beutler et al., 2017; Su et al., 2017). Further, administration of satiety hormones like CCK and PYY is able to reduce AGRP neuron calcium response (Beutler et al., 2017; Su et al., 2017). Finally, leptin is able to durably inhibit AGRP neuron calcium on a timescale of hours (Beutler et al., 2017). Together, these results suggest that when a hungry animal encounters food, AGRP neurons are suppressed in distinct temporal phases by differing mechanisms that exhibit individualized staying power. Interestingly, these mechanisms seem to be disrupted following exposure to a high fat diet, suggesting a mechanism by which calorically rich foods produce long term changes in the neural response to nutrient detection and intake (Beutler et al., 2020; Mazzone et al., 2020). These results indicate that we still have much to learn about how current and future whole-body energy state is assessed on a second by second basis, and how that communication is altered in disease states.

Energy-state dependent regulation of synaptic plasticity

The preceding section details how central neuronal populations exhibit altered activity during changes in energy state. These changes result from and subsequently cause alterations in

neurotransmission. In this section, I will describe known mechanisms of energy-state dependent regulation of synaptic plasticity in order to introduce the possible mechanisms that may be found in other contexts. This section will also serve as the introduction for general mechanisms of synaptic plasticity.

Food-deprivation results in significant changes in neural circuit activity and behavior. Many of these changes are downstream of hormones that act on central regions to promote changes in circuit function (Xu et al., 2018). In addition to changing cell-autonomous determinants of neuronal activity, these hormones regulate synaptic function, leading to differential regulation of behaviorally relevant cell types by incoming information. Here I describe some classic examples of energy-state dependent synaptic plasticity.

In dopamine neurons of the ventral tegmental area, application of the hormone ghrelin results in a glutamate receptor dependent increase in spontaneous action potential firing (Abizaid et al., 2006). In addition to a change in action potential generation, ghrelin increases the number of asymmetric synapses and decreases the number of symmetric synapses onto dopamine neurons, changes that are reflected in postsynaptic excitatory and inhibitory currents (Abizaid et al., 2006). Administration of a ghrelin receptor antagonist to the ventral tegmental area blunts refeeding in response to food-deprivation, suggesting that this plasticity is important for energy-state dependent regulation of food intake (Abizaid et al., 2006). These results suggest that, via ghrelin, hunger reorganizes synapses in the ventral tegmental area to promote feeding behavior.

AGRP neurons of the arcuate nucleus also exhibit synaptic plasticity in response to energy-state. Food-deprivation results in increased firing rate and increased expression of AGRP, NPY, and c-Fos in AGRP neurons (Liu et al., 2012). These changes co-occur with increases in action potential firing rate and AMPA receptor-mediated synaptic transmission (Liu et al., 2012). These

increases are abolished by deletion of NMDA receptors in AGRP neurons, indicating a critical role for these receptors in the induction of this plasticity (Liu et al., 2012). Energy state regulates the expression of NMDA receptor subunits in both AGRP neurons and POMC neurons, a cell type thought to regulate satiety that acts in opposition to AGRP neurons (Qi and Yang, 2015). These changes in NMDA receptor subunit expression result in different forms of synaptic plasticity in response to tetanic stimulation. The same protocol that causes an LTP at AGRP neuron excitatory synapses in fed animals causes an LTD in food deprived animals (Qi and Yang, 2015). Both of these changes depend on NMDA receptors, as inhibition of different NMDA receptor subunits is able to block either LTP or LTD (Qi and Yang, 2015). These results suggest that hunger initiates a change in expression of different NMDA receptor subunit populations in AGRP neurons, and this results in postsynaptic changes that increase synaptic drive, enhancing AGRP neuron output.

In addition to postsynaptic changes to AGRP neuron synapses, they also exhibit presynaptic plasticity. Food deprivation and ghrelin results in increases in influx of calcium presynaptically, activating AMPK, which in turn activates a positive feedback loop by stimulating intracellular calcium stores (Yang et al., 2011). This positive feedback loop can be terminated by Mu opioid receptor signaling activated by β-endorphin, a peptide released from POMC neurons (Yang et al., 2011). Thus, hunger is able to initiate both pre- and postsynaptic changes at excitatory synapses onto AGRP neurons, forming the synaptic basis of the many phenotypes downstream of AGRP neurons.

AGRP neurons receive excitatory input from multiple cell types in the paraventricular nucleus of the hypothalamus, and these inputs are sufficient to activate AGRP neurons and drive food intake (Krashes et al., 2014). The paraventricular nucleus is regulated by a number of energy-state communicating hormones, which initiate changes in various cell types to promote or

discourage feeding. Glucagon like peptide 1 (GLP-1) is a post-prandial hormone that acts through the commonly $G\alpha_s$ coupled GLP-1r to promote satiety (Smith et al., 2019). In the paraventricular nucleus of the hypothalamus, activation of GLP-1r stimulates protein kinase A-dependent phosphorylation of serine 845 on the GluA1 subunit of AMPA receptors (Liu et al., 2017). This results in increased insertion of GluA1 containing AMPA receptors, and increased amplitude of excitatory postsynaptic currents (Liu et al., 2017). This plasticity is important in the regulation of body weight, as knockdown of GluA1 in these cells is sufficient to induce obesity (Liu et al., 2017). These results identify GLP-1 as an important regulator of the paraventricular nucleus of the hypothalamus though AMPA receptor-dependent plasticity.

These examples show the diverse synaptic plasticity found across brain regions in response to acute changes in energy state. The sensitivity of hypothalamus synapses specifically to energy state is consistent with its prominent role in the regulation of feeding and metabolism. As described previously, the nucleus accumbens is a pivotal region in the control of food seeking and consumption. However, comparatively little plasticity following acute changes in energy state have been identified in the nucleus accumbens. In the development of the work presented here, I reasoned that the nucleus accumbens is likely regulated by energy state, in order to ensure the proper adaptive prioritization of behavior in response to need. In particular, the nucleus accumbens to lateral hypothalamus circuit, which exerts negative control over both food consumption and reward seeking, seemed to be a reasonable target for energy state-dependent plasticity (Gibson et al., 2018; O'Connor et al., 2015). This idea was the genesis of the work presented in Chapter II.

Studying neuropeptidergic modulation of the nucleus accumbens

The goal of the introduction for this dissertation is to familiarize the reader with the background that led to the work described here in subsequent chapters. The introduction of the nucleus accumbens was designed to communicate the contribution of different cell types and neurotransmitter species to the behaviors that depend on the nucleus accumbens. The introduction of hunger and its effect on neuronal populations was intended to communicate how the internal state of the animal can mobilize neuropeptide signaling to shift behavior. Putting these two pieces together, in the remainder of this dissertation I will describe two neuropeptidergic mechanisms within the nucleus accumbens that modulate neurotransmission and alter motivated behavior.

CHAPTER II

Hunger dampens a nucleus accumbens circuit to drive persistent food seeking

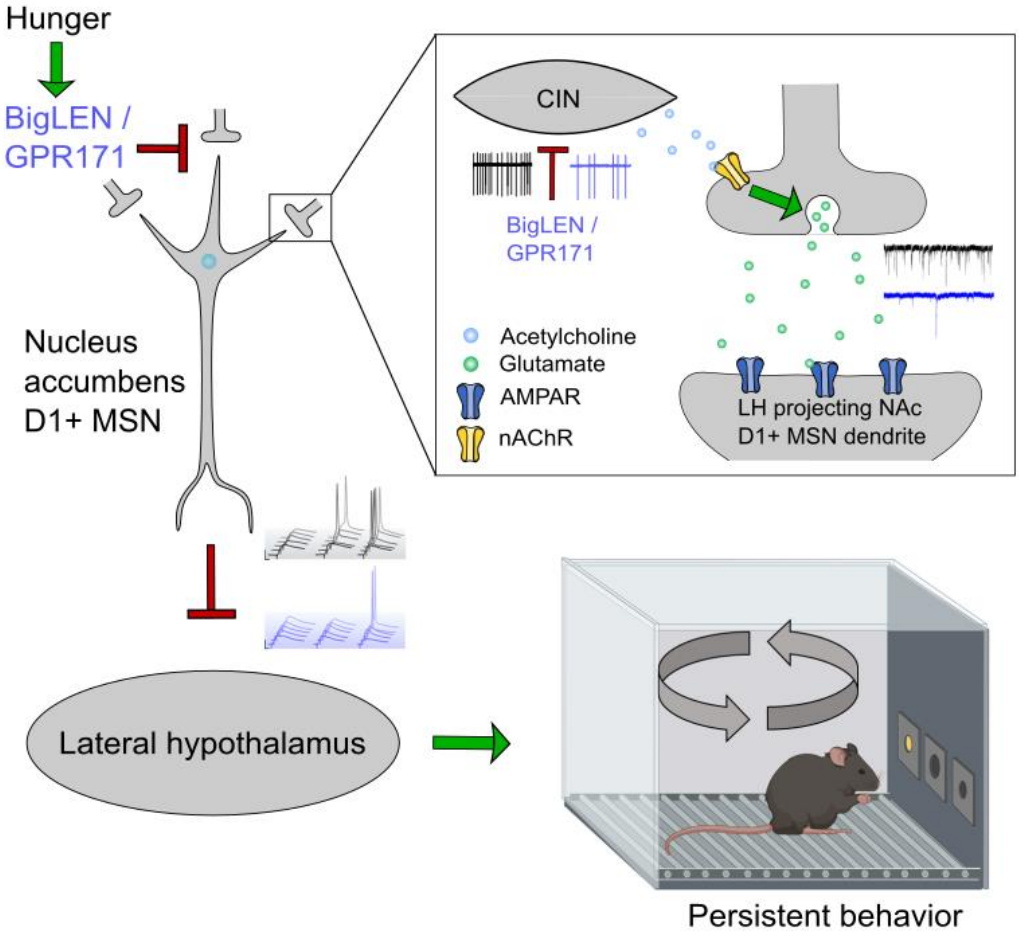


Figure 8 | Graphical abstract for hunger dampens a nucleus accumbens circuit to drive persistent food seeking

To find food efficiently, a hungry animal engages in goal-directed behaviors that rely on nucleus accumbens (NAc) circuits. Synaptic alterations within these circuits underlie shifts in behavior across motivational states. Here we show that hunger dampens a NAc to lateral hypothalamus (LH) circuit to promote persistent food seeking (Fig. 8). BigLEN, a hunger-driven neuropeptide, acts through its receptor GPR171 to inhibit glutamate transmission onto NAc shell *Drd1*+ LH-projecting medium spiny neurons by suppressing cholinergic signaling. Antagonism of GPR171 in food-deprived animals reduces persistent unrewarded food-seeking behavior but does not alter effortful food seeking or overall food intake. Chemogenetic upregulation of the NAc to LH circuit reduces this persistent unrewarded responding in hungry animals. These results describe how hunger-driven neuromodulation targets a distinct dimension of motivated behavior by shaping information flow through anatomically defined circuit elements.

Introduction

Food seeking is heavily dependent upon experience, as food-seeking behaviors that have proved fruitful in the past are more likely to be repeated. Often, these food-seeking behaviors become difficult to change and persist without respect to the current physiological need, a behavioral phenotype associated with obesity (Brunner et al., 2021; Craigie et al., 2011) that is also exacerbated in eating disorders (Tchanturia et al., 2013). Eating disorders are defined by a disruption in the ability to enact flexible behavioral strategies, resulting in ridged feeding patterns that inadequately adapt to internal need states, like hunger (Tchanturia et al., 2012; Voon et al., 2015; Zastrow et al., 2009). Despite significant advances in understanding how hunger alters food consumption (Atasoy et al., 2012) and satiety (Campos et al., 2016), we have little understanding of how hunger changes food-seeking behavior beyond increasing the effort an animal will expend to obtain a food reward.

Since the inception of behavioral science, It has been recognized that internal drives, such as hunger, are able to potentiate food-seeking behavior (Jones and Skinner, 1939). Heightened motivation is characterized by a willingness to work harder and expend additional effort in order to obtain a reward (Bock et al., 2013; Brown et al., 2017; Deroche-Gamonet et al., 2004). In addition, motivated animals exhibit a tendency to engage in continued seeking behavior during periods of reward unavailability, a phenotype we will refer to as persistence (Bock et al., 2013; Brown et al., 2017; Deroche-Gamonet et al., 2004). Both aspects of heightened motivation, increased effort expenditure and persistence, are observed in animals following experience with addictive drugs or highly palatable foods (Bock et al., 2013; Brown et al., 2017; Deroche-Gamonet et al., 2004). Hungry animals are similarly willing to expend more effort in order to obtain a food reward and are thus considered more motivated in their food-seeking behavior (Hodos, 1961). Additionally, hungry animals engage in persistent food seeking even when this seeking behavior is no longer reinforced under conditions of extinction (Perin, 1942). However, the mechanisms underlying these hunger-driven changes in persistence remain poorly understood.

Changes in motivated behaviors are associated with synaptic adaptations within the nucleus accumbens (NAc) (Bock et al., 2013; Brown et al., 2017; Christoffel et al., 2021; Derman and Ferrario, 2018; Kasanetz et al., 2010). Alterations in glutamatergic transmission to the NAc underlie drastic shifts in motivated behavior; from the pathological reward-seeking seen in addiction to the diminished interest in reward seen in anhedonia (Grueter et al., 2012; Lim et al., 2012; Pascoli et al., 2014; Turner et al., 2018; Zinsmaier et al., 2021). The NAc is particularly important in mediating behavioral flexibility and guiding behavior during periods of uncertainty

(Floresco, 2015). A subregion of the NAc, the NAc shell (NAcSh), is integral to the suppression of reward-seeking behavior when it is inappropriate, such as during periods of reward unavailability (Ambroggi et al., 2011; Blaiss and Janak, 2009; Feja et al., 2014; Lafferty et al., 2020; Reading et al., 1991). The NAcSh also exerts a powerful inhibitory control over food consumption via its projection to the lateral hypothalamus (LH) (O'Connor et al., 2015; Stratford and Kelley, 1999). Concordantly, excitatory input into this region opposes food consumption and constrains learned food-seeking behaviors (Lafferty et al., 2020; Reed et al., 2018). However, whether and how hunger gates NAc function to promote food-seeking behavior is unknown.

Internal state-dependent changes in neuropeptide signaling regulate neural circuits to prioritize behavior towards fulfillment of the animal's current need (Smith and Grueter, 2021; Sutton and Krashes, 2020). Within the NAc, food deprivation results in elevated concentrations of the neuropeptide BigLEN (Ye et al., 2017). BigLEN is produced from the precursor ProSAAS, and genetic manipulation of ProSAAS has been found to bidirectionally modulate body weight (Morgan et al., 2010; Wei et al., 2004). In the hypothalamus, BigLEN is expressed in agouti-related peptide expressing neurons, and suppresses excitatory transmission onto parvocellular neurons (Wardman et al., 2011). BigLEN activates the deorphanized g protein-coupled receptor (GPCR) GPR171, a $G\alpha_{i/o}$ coupled receptor that promotes feeding behavior and regulates anxiety (Bobeck et al., 2017; Wardman et al., 2016). BigLEN and GPR171 likely act within the NAc, as ProSAAS is expressed in multiple regions that project to the NAc, BigLEN is found in the NAc and its infusion there promotes food intake, and accumulation of radiolabeled BigLEN in the striatum suggests the presence of a receptor (Feng et al., 2001; Gomes et al., 2013; Wardman et al., 2011; Zhang et al., 2008). However, whether BigLEN and GPR171 regulate NAc circuitry and how this

could impact food-seeking behavior is unknown. Here, using complementary behavioral, molecular, and electrophysiological assays, we describe hunger-driven GPR171-dependant modulation of a NAcSh to LH circuit as a mechanism underlying enhanced food-seeking persistence.

Methods

Animals

Animal care and experimental protocols were approved and conducted in accordance with the Vanderbilt University Institutional Animal Care and Use Committee. Mice used for electrophysiological experiments were 8-12 weeks of age, mice used for operant behavior started training at 8-10 weeks of age. Mice were housed in groups of 3-5 and were maintained on a 12:12 light dark cycle. Behavioral experiments were conducted using male C57BL/6J mice ordered from Jackson Labs. Electrophysiological experiments were conducted using male C57BL/6J mice bred to carry a bacterial artificial chromosome directing the expression of the tdTomato fluorophore under the control of the Drd1a promoter (Tg(Drd1a-tdTomato)6Calak, Stock #: 016204 Jackson Labs).

Operant food seeking

Operant food-seeking experiments were performed at the Vanderbilt University Mouse Neurobehavioral Core. Animals were placed in a Med Associates Operant Chamber in a sound attenuating cubicle under a lit house light and presented with two nose poke holes, one illuminated, active hole, and another dark inactive hole. House light signaled trial start, where nose poking in the illuminated hole would be counted as one response and trigger dipper extension for a 5 second presentation of 0.1 mL of 50% Ensure (Abbott). Trials were followed by a 10 second timeout (TO) period where house lights were turned off and additional nosepokes were recorded but produced

no effect. Animals began training on a fixed ratio one (FR1) schedule, where one nosepoke on the active hole leads to dipper extension. Progression from FR1 to FR3 required three consecutive days of obtaining at least 80% of the 50 maximum possible rewards within the one hour trial. Progression from FR3 to FR5 required three consecutive days >80%. Progression from FR5 to testing required four consecutive days >80%. For unrewarded responding testing, animals were returned from the operant chamber on the final day of FR5 to a clean cage without food (if food deprived), or a clean cage with food (if fed) and single housed overnight. For testing in the unrewarded responding assay, animals were placed into the operant box with cues signaling reward availability (house light on, nosepoke hole illuminated) present. In this assay, nosepoke triggered the TO period and house light off without delivery of reward. Initial nosepokes and nosepokes during the TO period were counted. Animals were allowed to respond an unlimited number of times in the one hour test period. Following testing on unrewarded responding, animals were returned to their home cage, fed ad-lib and maintained on a daily FR1 schedule with reward for 5 days, FR3 for one day, and then food deprived again for PR testing using established protocols84.

For systemic administration experiments, the GPR171 antagonist (MS21570, 3.5 mg/kg I.P., 6% DMSO in saline vehicle39) was given shortly before the onset of the dark cycle (within an hour), and again 15 minutes prior to behavioral testing. For intra-NAc infusions, 20 nmol MS21570 in 0.5 uL vehicle (10% tween-80, 10% DMSO in saline39) was infused over two minutes shortly before the onset of the dark cycle, and again 15 minutes prior to behavioral testing.

For chemogenetic experiments, all animals received an injection of a retrograde virus encoding Cre (pENN.AAV.hSyn.HI.eGFP-Cre.WPRE.SV40, AAVrg serotype, Addgene) into the lateral hypothalamus. Experimental animals received a Cre-dependent Gq DREADD (pAAV-hSyn-DIO-

hM3D(Gq)-mCherry, AAV5 serotype, Addgene), and control animals received a Cre-dependent fluorophore (pAAV-hSyn-DIO-mCherry, AAV5 serotype, Addgene) delivered into the NAcSh. All animals were given CNO (1 mg/kg, Tocris Bioscience) 15 minutes prior to behavioral testing.

Slice preparation

Animals were deeply anesthetized with isoflurane and rapidly decapitated. Following brain dissection, the brain was washed in ice cold, oxygenated (95% O2 / 5% CO2), N-methyl-D-glucamine (NMDG) containing recovery solution (in mM: 2.5 KCl, 20 HEPES, 1.2 NaH2PO4, 25 Glucose, 93 NMDG, 30 NaHCO3, 5.0 Sodium ascorbate, 3.0 sodium pyruvate, 10 MgCl2, and 0.5 CaCl-2H20) and blocked. Parasagittal slices were obtained in ice cold, oxygenated, recovery solution using a Leica VT 1200S vibratome. Slices were then transferred to a holding chamber containing oxygenated recovery solution at 32° C for 10 minutes before being transferred to a holding chamber containing room temperature oxygenated artificial cerebrospinal fluid (ACSF) (in mM: 119 NaCl, 2.5 KCl, 1.3 MgCl2-6H2O, 2.5 CaCl2-2H2O, 1.00= NaH2PO4-H2O, 26.2 NaHCO3, and 11 glucose; 287-295 mOsm) and allowed to recover for an additional hour before recording. For recording, slices were transferred to a recording chamber perfused with oxygenated ACSF at a rate of 2 mL/min (Gilson Minipuls 3 Peristaltic Pump) at 30° C using an inline heater (Warner Instruments SH-27B).

Electrophysiology

Electrophysiology experiments were performed on a Scientifica Slicescope Pro System and have been described in detail previously85. The NAc shell was identified using established anatomical markers including the anterior/posterior position of the anterior commissure and the absence of the

dorsal striatum. NAc shell recordings took place in the rostral, medial portion of the shell. NAc core was identified by the elongation of the anterior commissure. NAc core recordings took place rostrally, dorsal to the commissure. Neurons were visualized using an upright microscope (Scientifica) allowing both infrared-differential interference contrast and fluorescence optics. Neurons were patched with 3-6 MΩ recording pipettes prepared using a pipette puller (P-1000, Sutter Instruments). D1+ MSNs were identified by expression of the tdTomato fluorophore visualized using 550 nM LED light (CoolLED pE-100). Recordings were made using a Multiclamp 700B amplifier, data was filtered at 2 kHz and digitized at 10 kHz.

For voltage-clamp recordings, neurons were patched with recording pipettes filled with a CsMeSO3 based internal solution (in mM: 120 CsMeSO3, 15 CsCl, 8 NaCl, 10 HEPEs, 0.2 EGTA, 10 TEA-Cl, 4 Mg2-ATP, 0.3 Na2-GTP, 0.1 spermine, 5 QX-314 bromide; pH 7.4, 290 mOsm). Membrane potential was clamped at -70 mV and EPSCs were isolated by the inclusion of picrotoxin (50 μM) in the bath. All cells were allowed 5 minutes to equilibrate following establishment of the whole-cell configuration. Membrane resistance and series resistance were continuously monitored throughout the experiment and a change greater than 20% resulted in omission of the experiment. sEPSCs were collected for five consecutive minutes for each cell. sEPSC traces were analyzed using a stringent best fit template (Clampfit 10.4) generated from manual analysis and training on a control D1+ MSN. Electrically evoked EPSCs were obtained by placing a bipolar stimulating electrode at the corticoaccumbens barrier and stimulating at 0.1 Hz with a 0.1 ms stimulus duration. PPR was obtained by delivering two 0.1 ms pulses at differing interstimulus intervals (in ms: 20, 50, 100, 200, 400) and calculating the ratio of the second EPSC to the first (PPR = EPSC2/EPSC1). For current clamp recordings, neurons were patched with recording pipettes filled with a potassium-gluconate based internal solution (in mM: 135 K-

gluconate, 5 NaCl, 2 MgCl2, 10 HEPES, 0.2 EGTA, 2.5 Mg2-ATP, 0.2 Na2-GTP). For current injection experiments, cells were allowed to equilibrate following establishment of the whole-cell configuration for 3 minutes. Progressively increasing current injections were applied for 0.8 seconds. For synaptically evoked action potential experiments, cells were injected with current to reach a membrane potential of -50 mV, then subjected to electrical stimulation at a magnitude found to produce a postsynaptic current between 100-200 pA in average amplitude. For current injection and synaptically evoked action potential experiments, each cell went through three technical replicates, and the average of those three trials was taken as the response of that cell. For cell-attached CIN recordings, CINs were identified by their characteristic size, morphology, and basal firing rate. Spontaneous action potentials were recorded beginning approximately one minute after forming a seal onto the cell in the voltage clamp configuration. Cells that were firing irregularly or at a very low frequency (<1 Hz) were excluded.

Stereotaxic surgery

Animals were deeply anesthetized with a ketamine (75 mg/kg I.P.) and dexdomitidor (0.5 mg/kg I.P., Zoetis) cocktail and provided with preoperative analgesia with ketoprofen (5 mg/kg I.P., Zoetis). The skull was shaved and cleaned with alcohol and iodine. A small incision was made to expose the skull and animals were placed in a stereotaxic apparatus. For viral/tracer injection studies, small holes were drilled bilaterally into the skull above the injection site and a 32g, 1 μL Neuros Hamilton syringe was lowered into the brain for viral delivery. Virus solutions were infused at a rate of 100 nL/ minute and solutions were allowed to disperse for 10 minutes before retracting the syringe. Injections into the LH were made at (AP: -0.94, ML: ±1.15, DV: -5.00). Injections into the VTA were made at a 10° lateral angle relative to the midline and targeted to

(AP: -3.08, ML: ±0.35, DV: -4.42). Injections into the NAcSh were made at a 10° lateral angle relative to the midline and targeted to (AP: 1.20, ML: ±0.50 , DV: -4.40). For cannula microinfusion studies, two, 25 gauge 4.3 mm cut, unilateral stainless-steel guide cannula (C315GS-5SP, Plastics One) were installed at a 15° lateral angle relative to the midline above the medial NAcSh (AP: 1.20, ML: ±0.50 , DV: -4.40). Cannula were secured with Metabond (Parkell) and dental cement (A-M Systems). Dummy cannula (Plastics One) were inserted to prevent cannula from becoming occluded. Following surgery, anesthesia was reversed with Antiseden (0.5 mg/kg I.P., Zoetis) and animals were kept on a warming pad until conscious and ambulatory. Post-surgery analgesia was provided using ketoprofen (Zoetis).

Immunohistochemistry

Fresh 100 μm sections from C57BL/6J mice were taken and fixed in 4% PFA overnight at 4°. Slices were washed 4x with 1x PBS with 0.2% Triton x-100 (PBST) over 24 hours. Slices were incubated in 5% bovine serum albumin PBST for 1 hour at room temperature before incubation in primary antibody for both GPR171 (1:250 GTX108131, GeneTex Rabbit39) and Choline Acetyltransferase (1:500 AB144P, Millipore Sigma Goat) in 5% bovine serum albumin PBST for 48 hours at 4°. Following 4x washes in PBST over 24 hours slices were incubated in secondary antibodies in 5% BSA PBST for 24 hours at 4°. Slices were again washed 4x in PBST over 24 hours before being mounted using ProLong Gold Antifade Mountant with DAPI. Fluorescent imaging was performed at the Vanderbilt Cell Imaging Shared Resource using a Zeiss LSM710 confocal microscope.

Food consumption

For food consumption experiments, animals were single housed and food deprived for 20 hours. Animals were then given either a vehicle, or MS21570 injection 15 minutes prior to the addition of either chow (5L0D, PicoLab) or 50% Ensure (Abbott) to the cage. The amount of food

consumed was measured at 30 minutes, and again at 2 hours. Caloric intake was calculated using published caloric densities of 5L0D chow diet and Ensure.

Fasted blood glucose

To measure fasted blood glucose, animals were single housed and food deprived for 20 hours. Following deprivation, blood glucose was assessed by measuring glucose content of tail vein blood with a handheld OneTouch Ultra blood glucose meter.

Quantification and Statistical Analysis

All data presented in bar or line graphs represents group average ± S.E.M. Dots represent an individual data point, either one animal in behavioral experiments or one cell in electrophysiology experiments. Sample sizes were determined using a power calculation with preliminary data as well as analysis of similar published experiments in the literature. Each dataset was tested for normality using the Shapiro-Wilk normality test and was subjected to the appropriate parametric or nonparametric statistical test. Two-tailed t-tests, Mann Whitney tests, one-way ANOVA, Kruskal-Wallis tests, and two-way ANOVA with Sidak multiple comparisons tests were used where appropriate and completed with GraphPad Prism software (GraphPad, Inc.) after coalescing data with Microsoft Excel (Microsoft Corp.).

Results

GPR171 dampens excitatory input to the NAc

The principal neurons of the NAc, medium spiny neurons (MSNs), are quiescent, thus MSNs rely primarily on excitatory transmission to generate action potentials. This excitatory

transmission is critical in guiding reward-seeking behaviors (Lafferty et al., 2020; O'Connor et al., 2015; Reed et al., 2018). Modulation of NAc excitatory transmission underlies extreme affective states, from addiction to anhedonia, and their associated behavioral phenotypes (Kasanetz et al., 2010; Lim et al., 2012). BigLEN is upregulated in the NAc of food deprived (FD) animals (Ye et al., 2017), and thus may induce synaptic changes that contribute to FD behavioral patterns. To examine the ability of BigLEN to modulate excitatory synaptic transmission in the NAcSh, we applied BigLEN to mouse brain slices containing the NAcSh and assessed electrophysiological properties of *Drd1* expressing MSNs (D1+ MSNs) using whole-cell patch-clamp techniques. Acute application of BigLEN (100 nM for 10 minutes) produced no change in evoked excitatory transmission onto D1+ MSNs over a 20 minute period as compared to baseline (data not shown: post-BigLEN average (n=11): 92.39% of baseline, t_{20}=1.459, p=0.16). However, we found that following 2-4 hour incubation in BigLEN (100 nM), D1+ MSNs exhibited a significantly reduced average frequency of spontaneous excitatory postsynaptic currents (sEPSCs) without altered average sEPSC amplitude (Fig. 9A-9D), relative to ACSF incubated controls. Paired pulse ratio (PPR), a measure that correlates inversely with presynaptic vesicular release probability, was increased on average at D1+ MSNs in NAcSh slices incubated in BigLEN (Fig. 9E and 9F). Incubation in BigLEN had no discernable effect on resting membrane potential or the number of action potentials produced by current injection in D1+ MSNs (Fig. 9G-9I). These BigLEN induced changes in sEPSC frequency and PPR in D1+ MSNs are consistent with a reduction in presynaptic vesicular release probability. We found a similar but distinct phenotype in NAcSh D1- MSNs, which exhibited a decrease in sEPSC frequency following BigLEN incubation, but no change in sEPSC amplitude or PPR (Fig. 10A-10C). Based on the role of NAcSh D1+ MSNs in feeding behavior, we focused on that population (O'Connor et al., 2015; Thoeni et al., 2020).

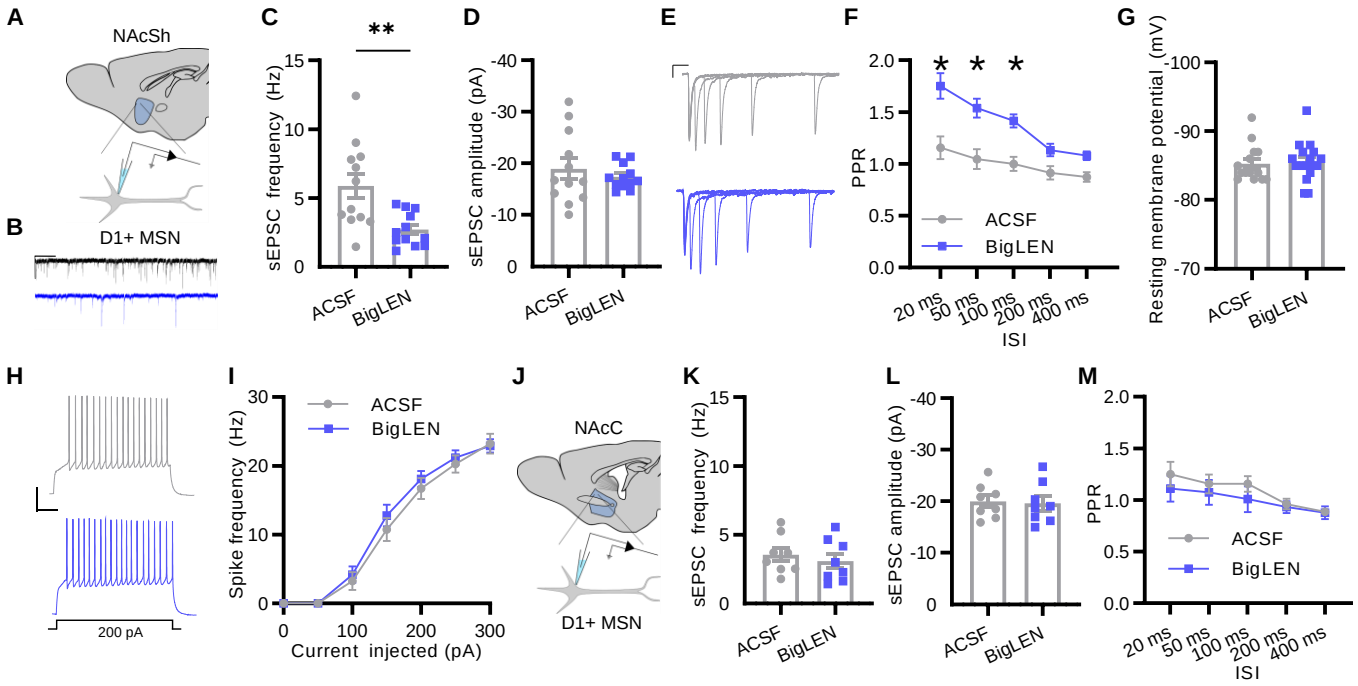


Figure 9 | BigLEN suppresses excitatory transmission onto D1+ MSNs in the NAcSh

(**A**) Schematic depicting anatomical location of NAcSh recordings. (**B**) Representative trace of sEPSCs from a NAcSh D1+ MSN in an ACSF (black) or BigLEN (blue) incubated slice. Average frequency (**C**) and amplitude (**D**) of spontaneous excitatory postsynaptic currents (sEPSCs) recorded from NAcSh D1+ MSNs treated with either ACSF (n=12) or BigLEN (n=12) from 8 mice. Representative traces (**E**) and average PPR (**F**) at five different ISIs in D1+ MSNs in the NAcSh following incubation in ACSF (gray, n=11) and BigLEN (blue, n=10) from 7 mice. (**G**) Average resting membrane potential values recorded from cells treated with either ACSF (n=14) or BigLEN (n=18) from 3 mice. (**H**)**.** Representative traces depicting action potentials in response to current injection (200 pA) in D1+ MSNs in the NAcSh following incubation in ACSF (gray) and BigLEN (blue). (**I**) Average spike frequency elicited from differing current injections from cells treated with ACSF (n=10) or BigLEN (n=10) from 3 mice. (**J**) Schematic depicting anatomical location of NAcC recordings. Average frequency (**K**) and amplitude (**L**) of sEPSCs recorded from NAcC cells treated with either ACSF (n=8) or BigLEN (n=8) from 5 mice. (**M**) Average PPR values recorded from NAcC cells treated with either ACSF (n=5) or BigLEN (n=5) from 5 mice. Data presented as mean ± S.E.M. *p<0.05, **p<0.01. Statistical tests and results can be found in table 1.

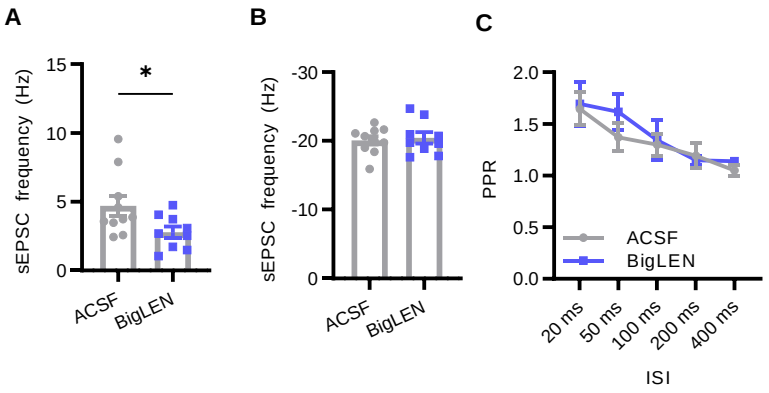


Figure 10 | BigLEN suppresses sEPSC frequency in NAcSh D1- MSNs

Average frequency (**A**) and amplitude (**B**) of spontaneous excitatory postsynaptic currents (sEPSCs) recorded from NAcSh D1- cells treated with either ACSF (n=10) or BigLEN (n=9) from 5 mice. (**C**) Average PPR at five different ISIs in D1- MSNs in the NAcSh following incubation in ACSF (gray, n=6) and BigLEN (blue, n=7) from 4 mice. Data presented as mean ± S.E.M. *p<0.05. Statistical tests and results can be found in table 1.

The NAc consists of functionally distinct subregions, and within these subregions different anatomical projection populations of neurons have been found to govern unique aspects of behavioral strategy (Gibson et al., 2018; Kelley, 2004b; Maldonado-lrizarry et al., 1995). Further, molecular and behavioral manipulations generate differential synaptic adaptations in NAcSh and NAc core (NAcC) (Grueter et al., 2013; Thomas et al., 2001). Based on these differences, we decided to explore whether modulation by GPR171 was present in an adjacent NAc subregion, the NAcC (Fig. 9J). We found no effect of BigLEN on excitatory synapses onto D1+ MSNs in the NAcC (Fig. 9K-9M).

GPR171 is required for BigLEN to suppress NAc excitatory transmission

To test directly whether BigLEN signals via GPR171, we pre-incubated slices in the GPR171 antagonist (MS21570, 1 μM (Bobeck et al., 2017)) which blocked the BigLEN induced decrease in synaptic release probability (Fig. 11A-11E). Incubation in MS21570 alone had no effect on excitatory synaptic transmission (Fig. 11F-11H).

If BigLEN is elevated in the NAc of FD animals, we would expect to observe synaptic adaptations on NAc MSNs in FD mice when compared to fed mice. We found that when compared to fed mice, FD mice exhibited no change in average sEPSC frequency or amplitude (Fig. 11I and 11J), and only an increase in 50 ms PPR (Fig. 11K). However, when slices from FD mice were incubated in BigLEN, we saw no change in the sEPSC frequency or PPR, but found decreased

sEPSC amplitude (Fig. 11L-11N), suggesting the effect of exogenous BigLEN was disrupted in FD animals.

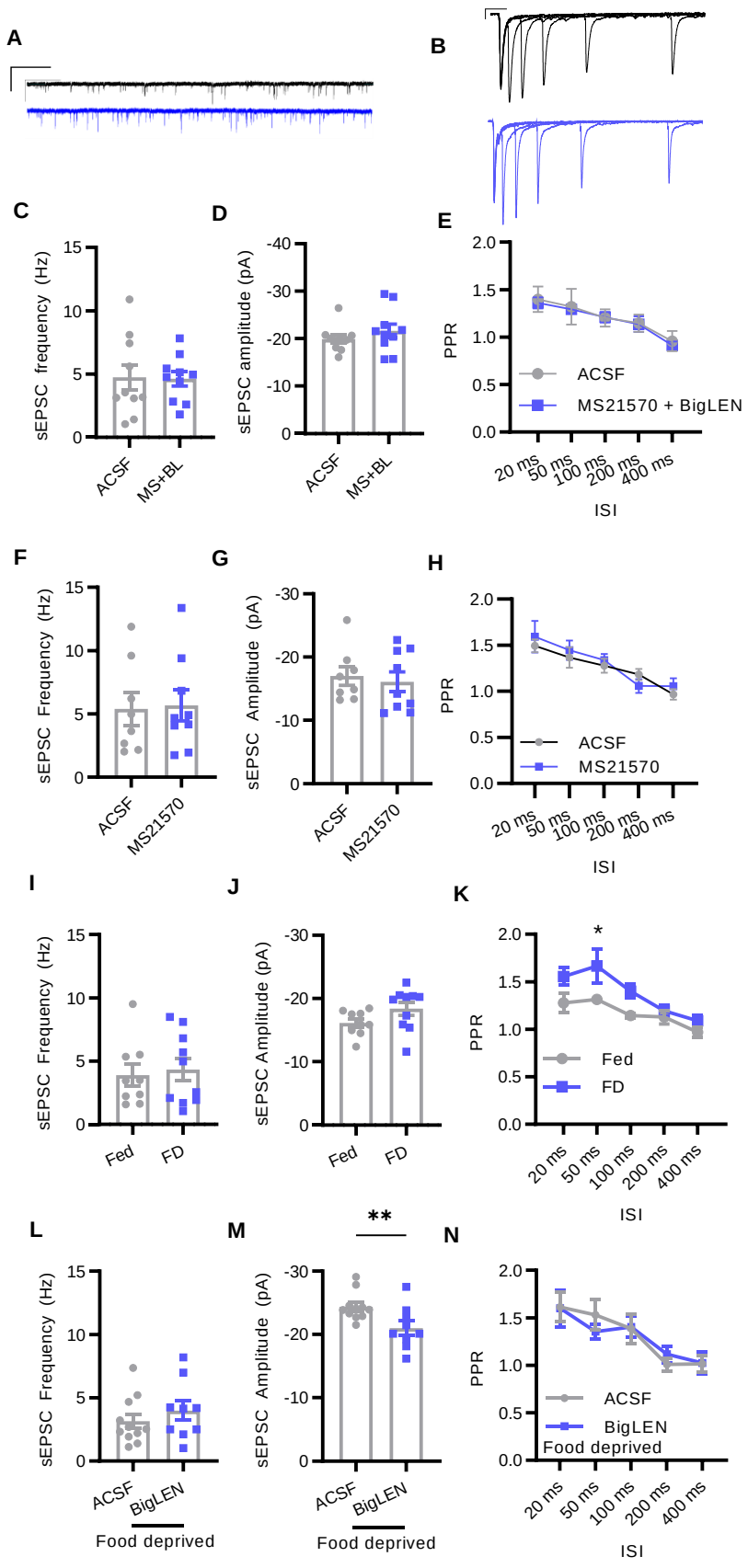

A
B
C
D
E
sEPSC frequency (Hz)
sEPSC amplitude (pA)
PPR
ACSF
MS+BL
MS21570 + BigLEN
20 ms
50 ms
100 ms
200 ms
400 ms
ISI
F
G
H
sEPSC Frequency (Hz)
sEPSC Amplitude (pA)
MS21570
I
J
K
Fed
FD
*
L
M
N
**
BigLEN
Food deprived

Figure 11| GPR171 is required for BigLEN to suppress NAc excitatory transmission, and this suppression does not occur in food deprived animals

Representative trace of sEPSCs (**A**) or PPR (**B**) from a NAcSh D1+ MSN in an ACSF (black) or MS21570 (MS, 1 μM) + BigLEN (BL, 100 nM) (blue) incubated slice. Average frequency (**C**) and amplitude (**D**) of spontaneous excitatory postsynaptic currents (sEPSCs) recorded from NAcSh cells treated with either ACSF (gray, n=10) or MS21570 + BigLEN (MS+BL) (blue, n=10) from 5 mice. Average PPR (**E**) at five different ISIs in D1+ MSNs in the NAcSh following incubation in ACSF (gray, n=9) and MS21570 + BigLEN (blue, n=13) from 6 mice. Average frequency (**F**) and amplitude (**G**) of sEPSCs recorded from NAcSh cells treated with either ACSF (gray, n=8) or MS21570 (blue, n=9) from 4 mice. (**H**) Average PPR values recorded from NAcSh cells treated with either ACSF (gray, n=7) or MS21570 (blue, n=8) from 5 mice. sEPSC frequency (**I**) and amplitude (**J**) from D1+ NAcSh MSNs in slices prepared from either fed (n=10 from 4 mice) or FD (n=10 from 4 mice) mice. (**K**) Average PPR of D1+ NAcSh MSNs in slices prepared from either fed (n=10 from 4 mice) or FD (n=9 from 4 mice) mice. sEPSC frequency (**L**) and amplitude (**M**) from D1+ NAcSh MSNs in slices prepared from FD mice and incubated in either ACSF (n=11) or BigLEN (n=9) from 3 mice. (**N**) Average PPR of D1+ NAcSh MSNs in slices prepared from FD mice and incubated in either ACSF (n=8) or BigLEN (n=8) from 3 mice. Data presented as mean ± S.E.M. *$p<0.05$. Scale bar for (**A**) is 50 pA by 1 s. Scale bar for (**B**) is 25 pA by 50 ms. Statistical tests and results can be found in table 1.

Suppression of glutamatergic transmission by BigLEN requires cholinergic transmission

Next, we considered how GPR171 was suppressing excitatory transmission on NAcSh D1+ MSNs. GPR171 has been found to couple to $G\alpha_{i/o}$ in hypothalamic neurons (Gomes et al., 2013). Presynaptic $G\alpha_{i/o}$ GPCRs are able to inhibit vesicular release via inhibition of voltage-dependent calcium channels or direct inhibition of exocytotic machinery (Betke et al., 2012), processes that occur within the presynaptic cell and do not depend on action potentials. Following incubation in BigLEN, we used tetrodotoxin (TTX, 500 nM) to block spontaneous action potentials. We found that mini excitatory postsynaptic current (mEPSC) frequency was depressed following incubation in BigLEN, with no change in mEPSC amplitude, indicating that once BigLEN has already signaled through GPR171, the expression of this effect does not require action potentials (Fig. 13A-13C). However, we found that following pre-incubation in TTX, subsequent BigLEN incubation caused no change in mEPSC frequency or amplitude compared to ACSF incubated controls (Fig. 12A-12C). This indicates that the induction of the BigLEN effect requires spontaneous action potential generation. Within the NAc, cholinergic interneurons (CINs) tonically fire action potentials and are thought to maintain a basal acetylcholine tone (Zhou et al., 2002). We found that pre-incubating slices in a nicotinic acetylcholine receptor (nAChR) antagonist (mecamylamine, 10 μM) blocked the ability of BigLEN to suppress vesicular release probability (Fig. 12D-12F). nAChRs are found presynaptically in the striatum and elsewhere (Jones et al., 2001), where they act to promote vesicular release (Gray et al., 1996; Sharma and Vijayaraghavan, 2003; Zhang and Warren, 2002). Application of the prototypical nAChR agonist, nicotine (100 nM), resulted in, on average, a pronounced and long-lasting elevation in mEPSC frequency (Fig. 12G and 12H), indicating that nAChRs are able to enhance presynaptic vesicular release at these synapses. Additionally, we found that nicotine application resulted in a slight

decrease in average mEPSC amplitude (Fig. 13D and 13E). Both outcomes of nicotine were blocked by the nAChR antagonist mecamylamine (Fig. 13F-13I). We performed immunohistochemistry to identify whether GPR171 expression patterns in the NAc supported a cholinergic mechanism. Notably, we find that while GPR171 immunoreactivity is widespread across the NAc, it is also found in choline acetyltransferase (CHAT) expressing CINs (Fig. 14A-14E), suggesting BigLEN and GPR171 could be inhibiting a CIN mediated nicotinic potentiation. Indeed, we found that while recording from NAc CINs in the cell-attached configuration, application of BigLEN significantly decreased action potential frequency relative to vehicle application (Fig. 12I-12K). Together, these results suggest a model where BigLEN inhibits NAc CINs, resulting in decreased activation of presynaptic nicotinic receptors and lowered vesicle release probability at glutamatergic terminals onto D1+ MSNs.

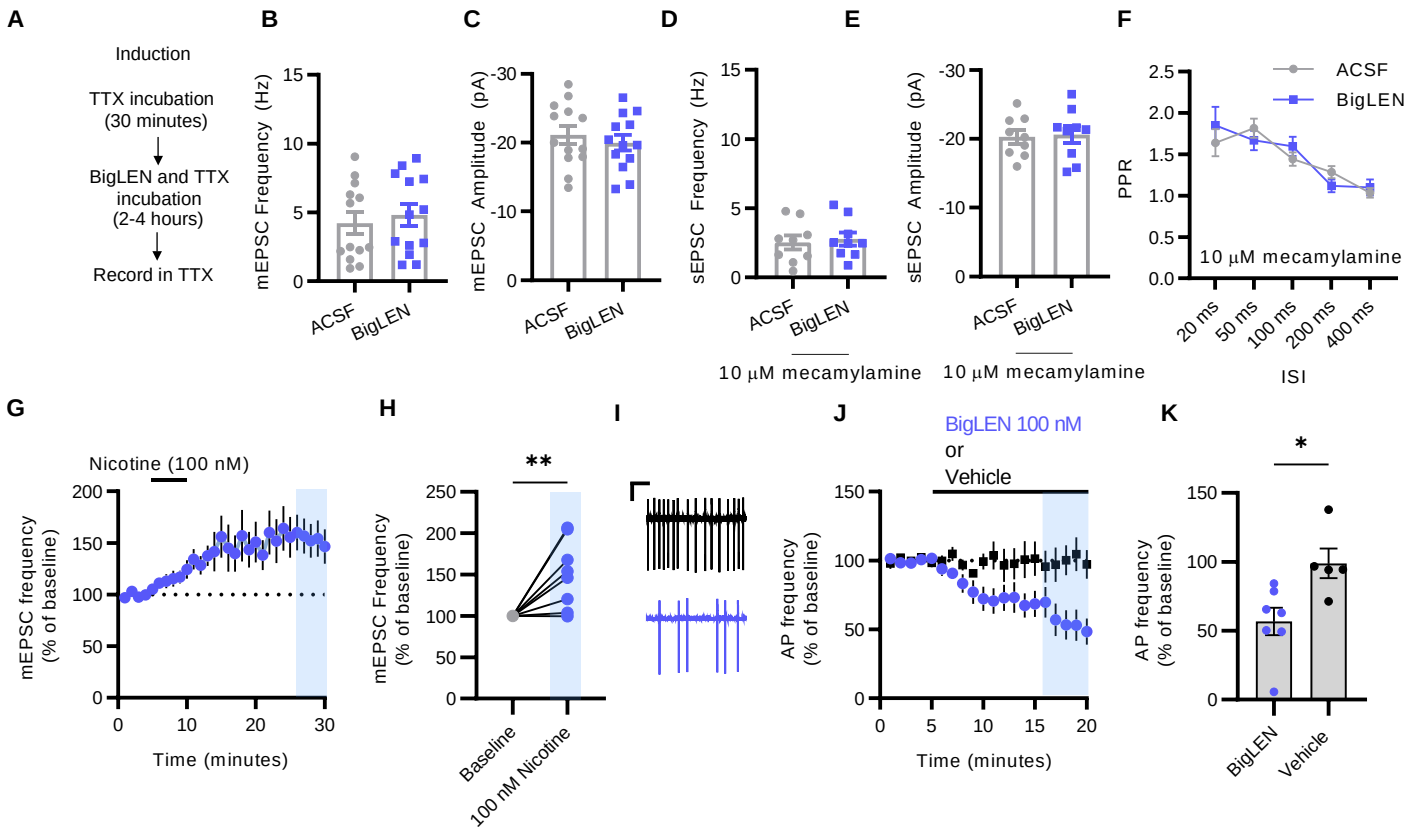


Figure 12 | Suppression of excitatory transmission by BigLEN requires nicotinic acetylcholine receptors, and BigLEN suppresses cholinergic interneuron firing

(**A**) Schematic depicting incubation in TTX followed by incubation in BigLEN or ACSF. mEPSC frequency (**B**) and amplitude (**C**) of NAcSh D1+ MSNs after pre-incubation in TTX and incubation in either ACSF (n=13) or BigLEN (n=13) from 4 mice. sEPSC frequency (**D**) and amplitude (**E**) of NAcSh D1+ MSNs after pre-incubation in mecamylamine (10 μM) followed by incubation in either ACSF (n=9) or BigLEN (n=9) from 3 mice. Average PPR (**F**) of NAcSh D1+ MSNs after pre-incubation in mecamylamine (10 μM) followed by incubation in either ACSF (n=7) or BigLEN (n=7) from 3 mice. (**G**) Time course of mEPSC frequency following 5 minute application of 100 nM nicotine normalized to a percent of baseline average. (**H**) Quantification of mEPSC frequency following application of nicotine (n=8) from 4 mice. (**I**) Representative traces of cell-attached CIN action potential recordings before (black) and after BigLEN wash (blue). (**J**) Time course of CIN action potential frequency following either vehicle (black) or BigLEN (blue) wash. (**K**) Quantification of CIN action potential frequency as a % of baseline following either BigLEN (blue, n=7 from 5 mice) or vehicle (black, n=5 from 5 mice) wash. Data presented as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001. Scale bar for (**I**) is 20 pA / 1 s. Statistical tests and results can be found in table 1.

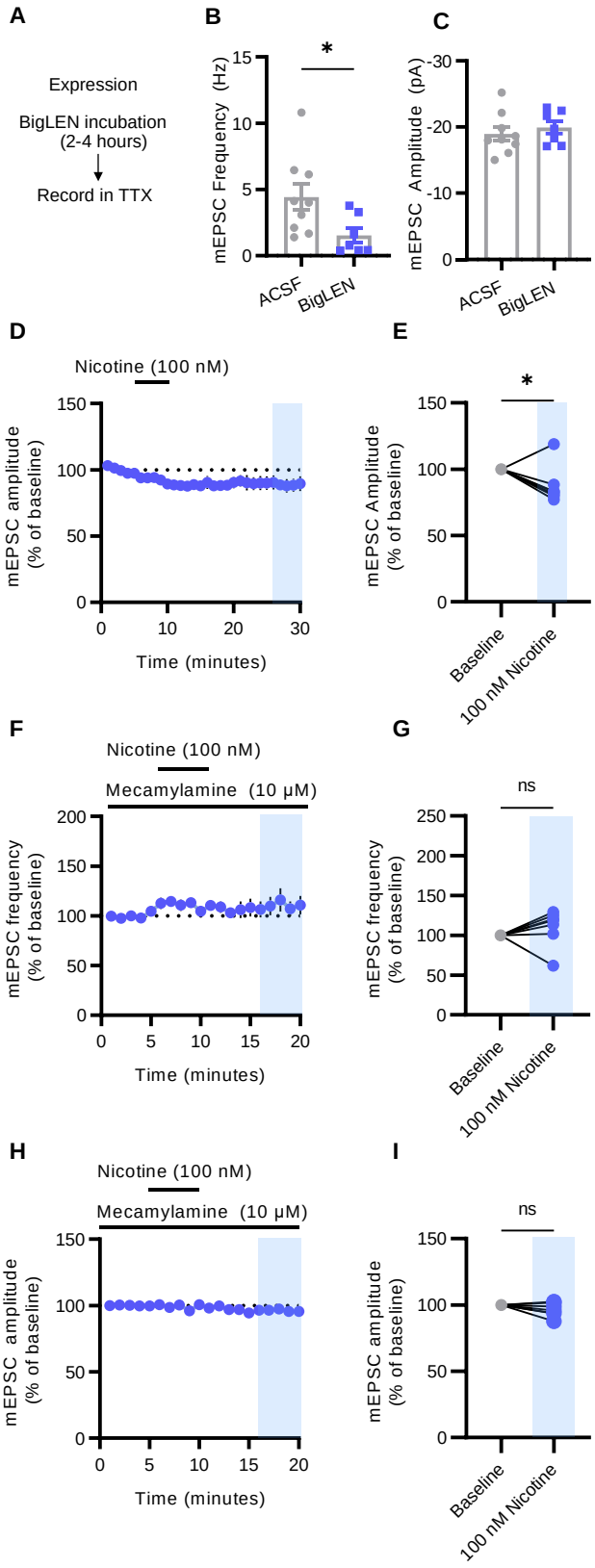


Figure 13 | Nicotine decreases mEPSC amplitude onto NAcSh D1+ MSNs, and nicotine's effect on D1+ MSN excitatory transmission is blocked by mecamylamine

(**A**) Schematic depicting incubation of slices followed by recording in 500 nM TTX. mEPSC frequency (**B**) and amplitude (**C**) of NAcSh D1+ MSNs while recording in TTX following incubation in ACSF (n=9) or BigLEN (n=6) from 3 mice. (**D**) Time course and (**E**) quantification depicting average mEPSC amplitude following application of 100 nM nicotine, normalized to percent of baseline average (n=8) from 4 mice. (**F**) Time course and (**G**) quantification of average mEPSC frequency following application of 100 nM nicotine in the presence of the nAChR antagonist mecamylamine (10 µM) (n=7) from 5 mice. (**H**) Time course and (**I**) quantification of average mEPSC amplitude following application of 100 nM nicotine in the presence of the nAChR antagonist mecamylamine (10 µM) (n=7) from 5 mice. Data presented as mean ± S.E.M. *p<0.05, **p<0.05. ns = not significant. Statistical tests and results can be found in table 1.

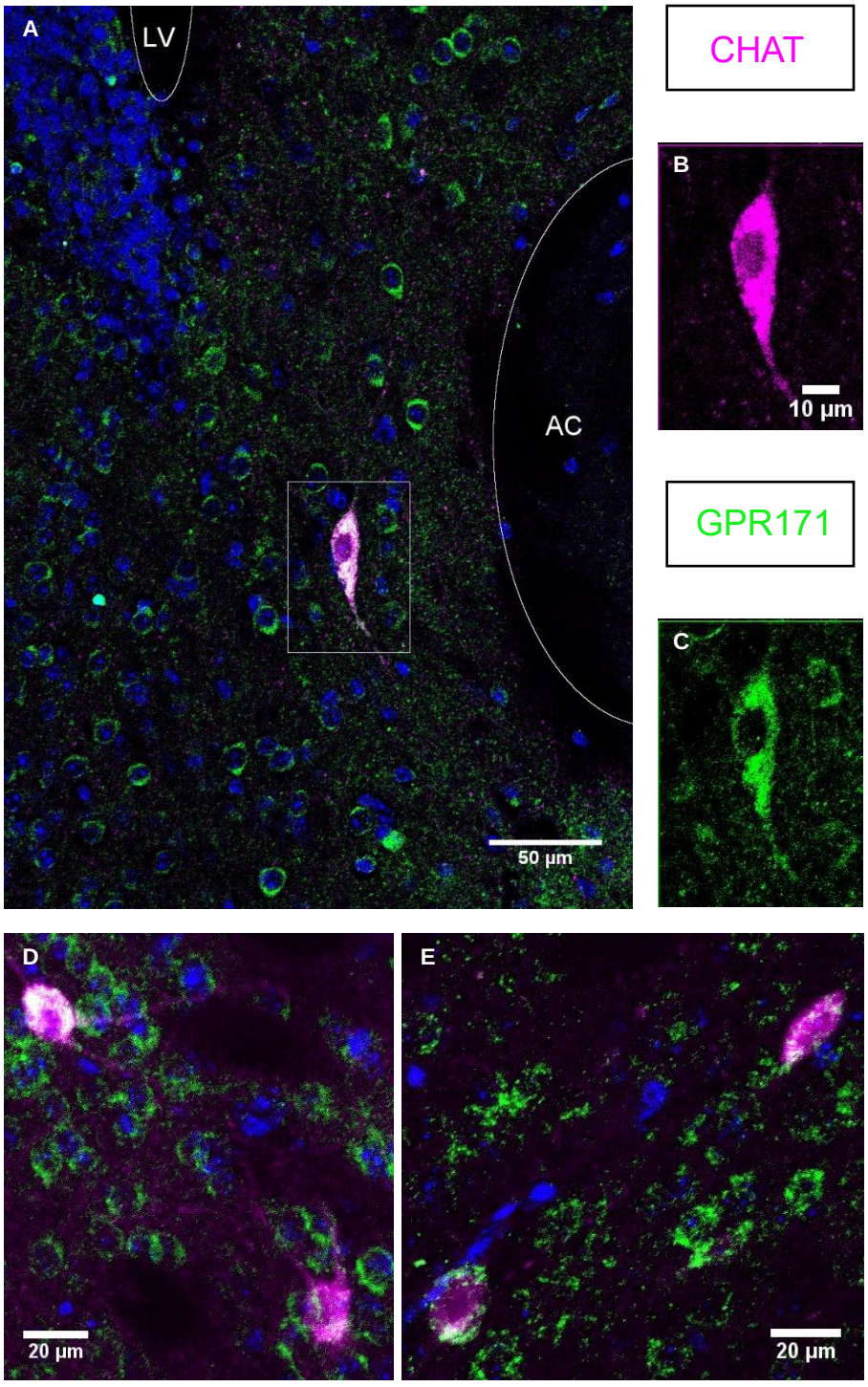


Figure 14 | GPR171 is expressed in CHAT positive cells

(**A**) 20x magnification image showing GPR171 (green) and CHAT (magenta) immunoreactivity and DAPI (blue) in the NAc. Overlap shown in white. Inset (white box) highlighting dual stained CIN with single channel images in (**B**) (CHAT) and (**C**) (GPR171). (**D**) and (**E**) Overlap of GPR171 and CHAT immunoreactivity in the NAc.

BigLEN suppresses excitatory transmission onto a NAc → LH circuit

While D1+ MSNs in the NAcSh send projections to both the LH and the ventral tegmental area (VTA), the NAcSh is the only striatal region that projects to the hypothalamus (Gibson et al., 2018; O'Connor et al., 2015). Due to the lack of effect of GPR171 stimulation on excitatory transmission outside of the NAcSh and the well described role of the NAc → LH circuit in controlling feeding behavior (Kelley et al., 2005; O'Connor et al., 2015), we hypothesized that this NAcSh to LH circuit is modulated by GPR171. To isolate D1+ MSNs in the NAcSh that project to the LH, we injected a retrograde cholera toxin subunit B – Alexa Fluor 488 conjugate (CT-B 488) into the lateral hypothalamus of *Drd1a-tdTomato* mice (Fig. 15A). The genetic identity of the isolated LH projecting population was primarily D1+ (93% of 46 cells co-labeled with CT-B 488 and tdTomato, Figure 15B), confirming previous reports in the literature (Thoeni et al., 2020). After incubating these slices in BigLEN, we found a significant reduction in the frequency of sEPSCs, with no change in the average amplitude (Fig. 15C-15E) in co-labeled cells. Similarly, we found an enhanced PPR at 20 and 50 ms interstimulus intervals (Fig. 15F and 15G). To determine if this synaptic modulation was present in other D1+ cell types, we repeated these experiments in D1+ MSNs projecting to the VTA (Fig. 15H). Consistent with prior reports, we found that the majority of VTA projecting cells in the NAcSh are D1+ (78% of 49 cells co-labeled with CT-B 488 and tdTomato, Fig. 15I) (Bocklisch et al., 2013). However, In the VTA projecting population, we found no change in sEPSC frequency or amplitude following BigLEN incubation (Fig. 15J-15L). Further, we found no change in PPR following BigLEN incubation in VTA projecting D1+ MSNs (Figures 15M and 15N). Thus, the synaptic modulation engaged by BigLEN and GPR171 dampens excitatory transmission onto NAcSh D1+ MSNs that project to the LH.

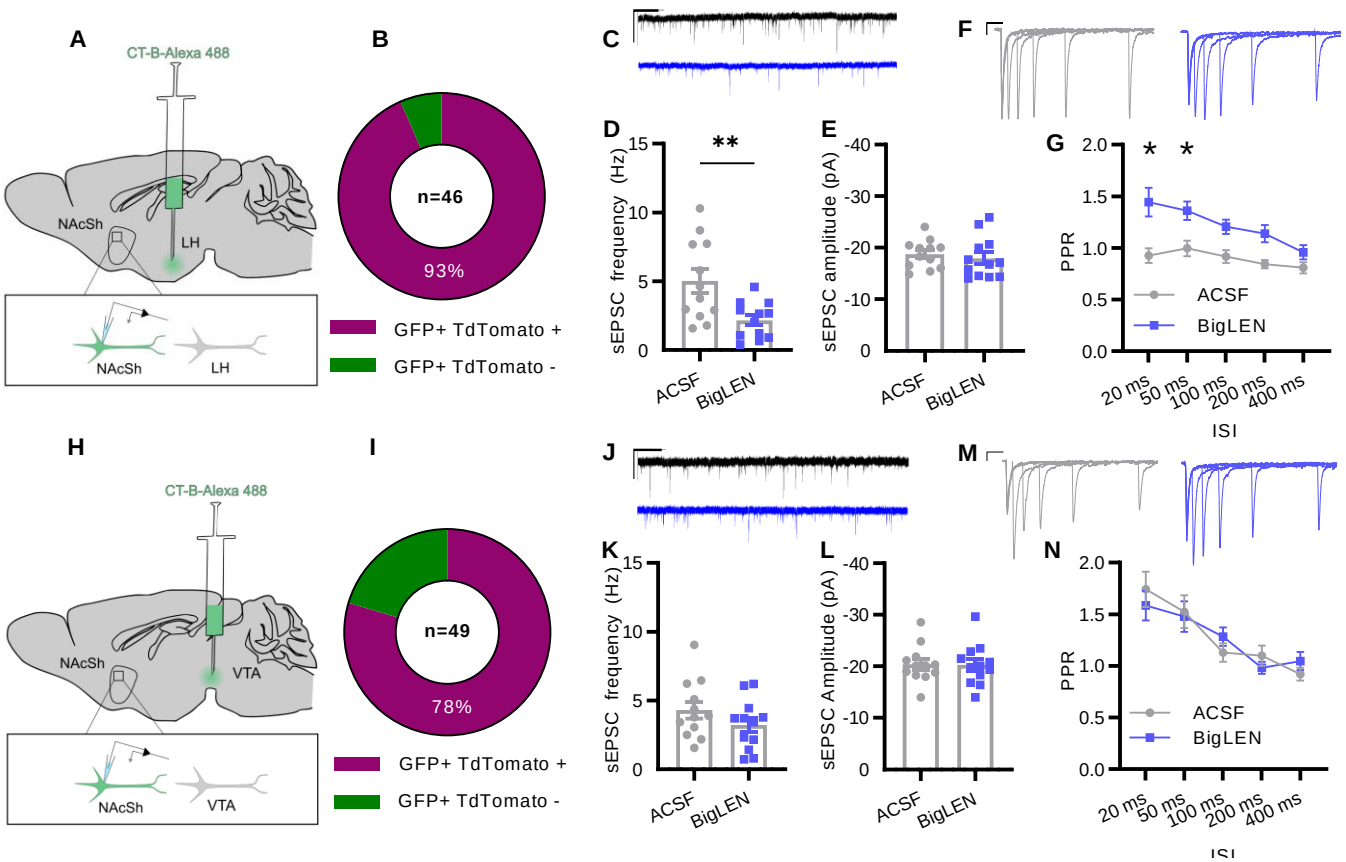


Figure 15 | BigLEN suppresses excitatory input onto NAcSh D1+ MSNs that project to the LH but not those that project to the VTA

(**A**) Schematic depicting CTB-488 injection used to label LH projecting MSNs and (**B**) proportion of cells co-expressing CTB-488 and Drd1-tdTomato following LH CTB-488 injection. (**C**) Representative trace of sEPSCs from LH projecting D1+ MSNs in an ACSF (black) or BigLEN (blue) incubated slice. Average frequency (**D**) and amplitude (**E**) of sEPSCs recorded from NAcSh, LH projecting D1+ MSNs following ACSF (n=12) or BigLEN (n=12) incubation from 7 mice. Representative traces (**F**) and average PPR (**G**) in LH projecting, D1+ MSNs in the NAcSh following ACSF (gray n=8) or BigLEN (blue, n=13) incubation from 7 mice. (**H**) Schematic depicting CTB-488 injection used to label VTA projecting MSNs, and (**I**) proportion of cells co-expressing CTB-488 and Drd1-tdTomato. (**J**) Representative trace of sEPSCs from a VTA projecting D1+ MSNs following ACSF (black) or BigLEN (blue) incubation. Average frequency (**K**) and amplitude (**L**) of sEPSCs recorded from NAcSh, VTA projecting D1+ MSNs following ACSF (n=12) or BigLEN (n=12) incubation from 6 mice. Representative traces (**M**) and average PPR (**N**) in VTA projecting, D1+ MSNs in the NAcSh following ACSF (gray, n=7) or BigLEN (blue, n=8) incubation from 5 mice. Data presented as mean ± S.E.M. *p<0.05, **p<0.01. Statistical tests and results can be found in table 1.

Hunger drives food-seeking persistence via NAc GPR171

Hunger powerfully stimulates food-seeking behavior. To investigate how hunger potentiates specific dimensions of food-seeking behavior, we examined operant responding for a food reward in FD mice. Mice trained in an operant food-seeking task, in which they perform a nose poke to earn a palatable food reward (50% ensure) (Fig. 16A-16C), were split into two groups: FD and control mice fed *ad libitum* (fed). We first tested mice under conditions of extinction (unrewarded responding), where active nosepoke no longer leads to the delivery of a food reward. After 20-hour food deprivation (average weight loss 16% of body weight, Fig. 16D), FD mice exhibited increased unrewarded operant responding in the active nosepoke hole relative to fed mice, without affecting previously unreinforced responding in the inactive hole (Fig. 16E and 16F). This overall increase in unrewarded responding by the FD mice was accompanied by an increase in nosepokes during the 10 second timeout period following the initial active hole nosepoke (Fig. 16G). FD animals also reach the food port faster following a nosepoke, and end proportionally fewer trials without accessing the food port compared to fed mice, indicating the enhanced salience of the food reward in FD animals (Fig. 17A and 17B). As expected, in the progressive ratio (PR) task, we found that food deprivation enhances effortful food seeking, evidenced by increasing the number of total responses, the breakpoint reached, and the number of rewards earned by FD mice (Fig. 16H) (Fig. 17C and 17D). Again, no change in unreinforced inactive hole responding was observed in FD mice in the PR task (Fig. 16I). Thus, FD animals are willing to expend more effort to obtain a food reward and are also more persistent in a food-seeking strategy that is no longer productive.

BigLEN suppresses excitatory input to the NAcSh to LH circuit, and NAcSh output is thought to regulate reward-seeking behavior. As BigLEN is upregulated in the NAc of FD animals (Ye et al., 2017), its signaling through GPR171 may underlie the potentiation of food-seeking behaviors seen in FD animals. We found that systemic antagonism of GPR171 (MS21570, 3.5 mg/kg I.P.) (Bobeck et al., 2017), significantly reduced unrewarded responding in food deprived animals to a level no different from fed, vehicle treated, controls (Fig. 16J). Again, we found that FD animals treated with vehicle responded significantly more during the timeout period, and that treatment with the GPR171 antagonist significantly reduced this timeout period responding (Fig. 16K). Importantly, treatment with the GPR171 antagonist in fed animals had no effect on total active responses or responses during the timeout period in the unrewarded responding assay (Fig. 17E and 17F). We reasoned that these MS21570-treated animals likely responded fewer times in the unrewarded responding assay because they were less motivated to engage in effortful food seeking. Surprisingly, we found that systemic antagonism of GPR171 had no effect on the ability of food deprivation to enhance effortful food seeking, as evidenced by the lack of change in responding on the progressive ratio task (Fig. 16L). Together, these results describe GPR171 as necessary for the increased unrewarded persistence, but not the increased effort, seen in food deprived animals.

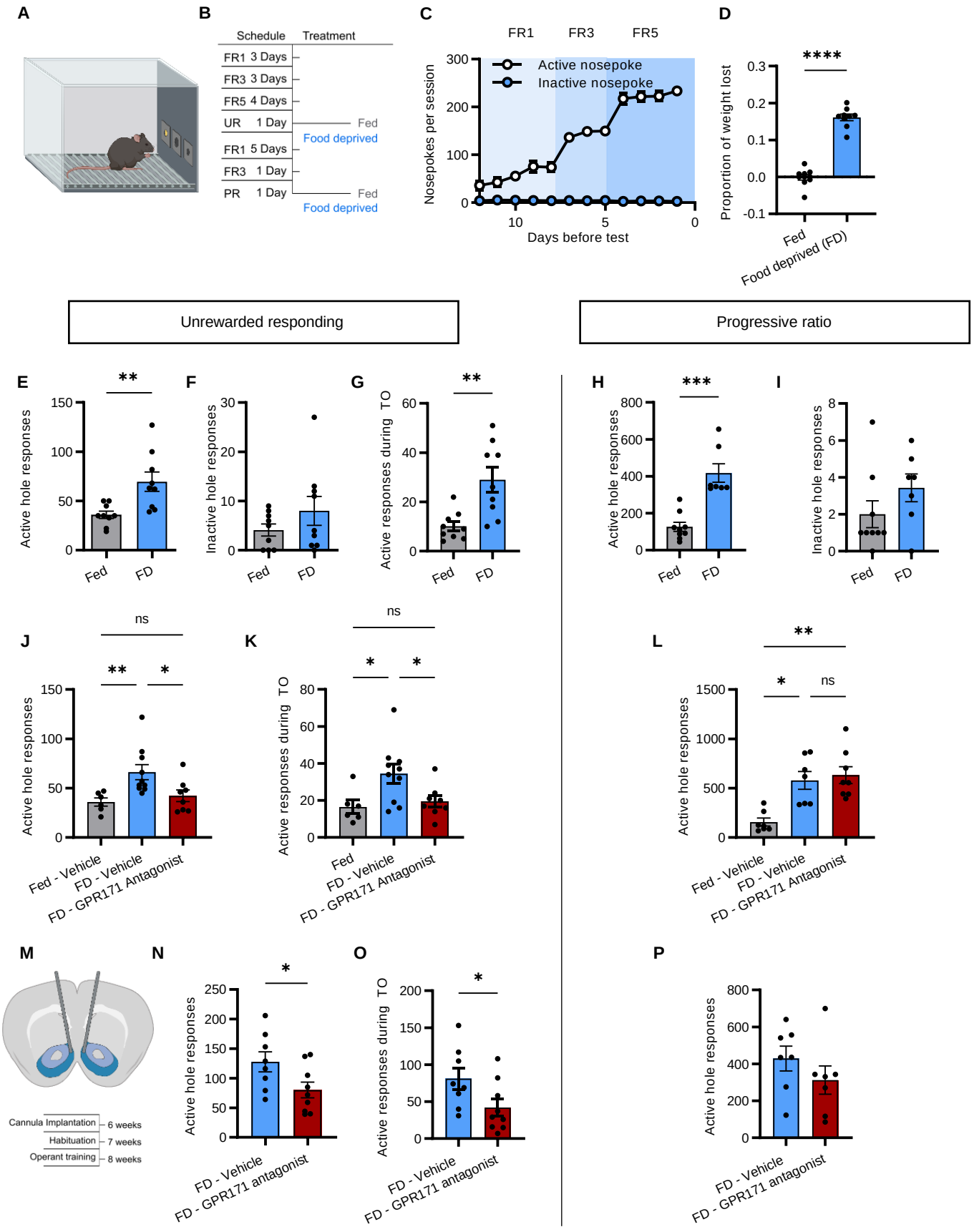

A
B
Schedule
Treatment
FR1 3 Days
FR3 3 Days
FR5 4 Days
UR 1 Day
Fed
Food deprived
FR1 5 Days
FR3 1 Day
PR 1 Day
Fed
Food deprived
C
FR1
FR3
FR5
Active nosepoke
Inactive nosepoke
Nosepokes per session
Days before test
D
****
Proportion of weight lost
Fed
Food deprived (FD)
Unrewarded responding
Progressive ratio
E
**
Active hole responses
Fed
FD
F
Inactive hole responses
G
**
Active responses during TO
H
***
I
J
ns
**
*
Fed - Vehicle
FD - Vehicle
FD - GPR171 Antagonist
K
L
M
Cannula Implantation
6 weeks
Habituation
7 weeks
Operant training
8 weeks
N
FD - Vehicle
FD - GPR171 antagonist
O
P

Figure 16 | Food deprivation requires NAc GPR171 in order to enhance unrewarded but not effortful food-seeking

(**A**) Diagram depicting operant chamber with two nosepoke holes (active and inactive), and reward port with dipper (created with BioRender.com). (**B**) Schematic of behavioral training paradigm. Animals complete increasing fixed ratio (FR) schedules prior to food deprivation and testing in unrewarded responding (UR) and progressive ratio (PR). (**C**) Nosepokes in active and inactive holes over the course of operant training on increasing fixed ratio schedules. (**D**) Proportion of weight lost during food deprivation prior to behavioral testing. Active (**E**) and inactive (**F**) hole nosepokes of fed (n=9) and food deprived (FD, n=9) mice in the unrewarded responding task. (**G**) Average responses during the 10 second time out (TO) period following initial active hole nosepoke between Fed and FD animals during the unrewarded responding task. Active (**H**) and inactive (**I**) hole nosepokes of fed (n=8) and FD (n=7) mice in the PR task. (**J**) Active hole nosepokes of fed (n=6), FD mice treated with vehicle (n=10), and FD mice treated with the GPR171 antagonist MS21570 (n=8) in the unrewarded responding task. (**K**) Average responses during the 10 second TO period in the UR task between fed, fed vehicle treated, and FD GPR171 antagonist treated animals during the unrewarded responding task. (**L**) Active hole nosepokes of fed (n=7), FD mice treated with vehicle (n=7), and FD mice treated with the GPR171 antagonist (n=8) in the PR task. (**M**) Schematic depicting dual unilateral cannulation of the NAcSh and surgery timeline based on animal age. (**N**) Active hole nosepokes of FD mice treated with either intra-NAc vehicle (n=8) or GPR171 antagonist (MS21570, 20 nmols) (n=9) in the unrewarded responding task. (**O**) Average responses during the 10 second TO period by FD animals treated with either intra-NAc vehicle or GPR171 antagonist during the unrewarded responding task. (**P**) Total active hole responses during the PR task between FD animals treated with intra-NAc GPR171 antagonist (MS21570) or vehicle. Data presented as mean ± S.E.M. *$p<0.05$, **$p<0.01$, ***$p<0.001$. ****$p<0.0001$, ns = not significant. Statistical tests and results can be found in table 1.

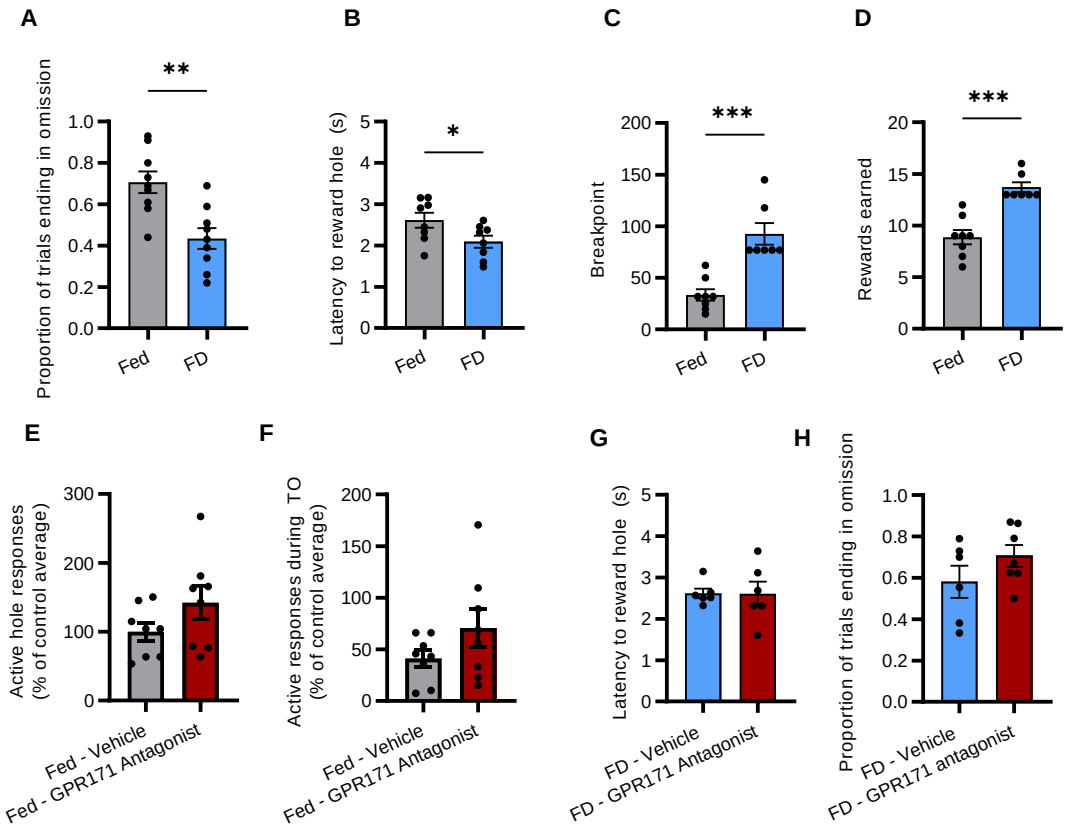


Figure 17 | Additional measures for operant behavior experiments

(**A**) Proportion of total trials ending without head entry into reward port, an omission, in the unrewarded responding task between fed and FD animals. (**B**) Average latency between active nosepoke and head entry into reward port in the unrewarded responding task between fed and FD animals. (**C**) Breakpoint reached in the PR task by fed and FD animals. (**D**) Number of rewards earned in the PR task by fed and FD animals. (**E**) Average active hole responses during the unrewarded responding task between fed animals given either vehicle or the GPR171 antagonist MS21570. (**F**) Average responses during the 10 second TO period in the unrewarded task by fed animals given either vehicle or the GPR171 antagonist MS21570. (**G**) Average latency between active nosepoke and head entry into reward port in the unrewarded responding task between FD animals treated with intra-NAc GPR171 antagonist or vehicle. (**H**) Proportion of total trials ending without head entry into reward port, an omission, in the unrewarded responding task between FD animals treated with intra-NAc GPR171 antagonist (MS21570) or vehicle. Data presented as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001. Statistical tests and results can be found in table 1.

Additionally, we found no effect of GPR171 antagonism on body weight during food deprivation and after refeeding (Fig. 18A). Further, we found no effect of GPR171 antagonism on fasted blood glucose, or food consumption of either chow or the palatable food reward during refeeding (Fig. 18B-18F). Thus, we find that GPR171 activation is not necessary for food deprivation to increase food intake.

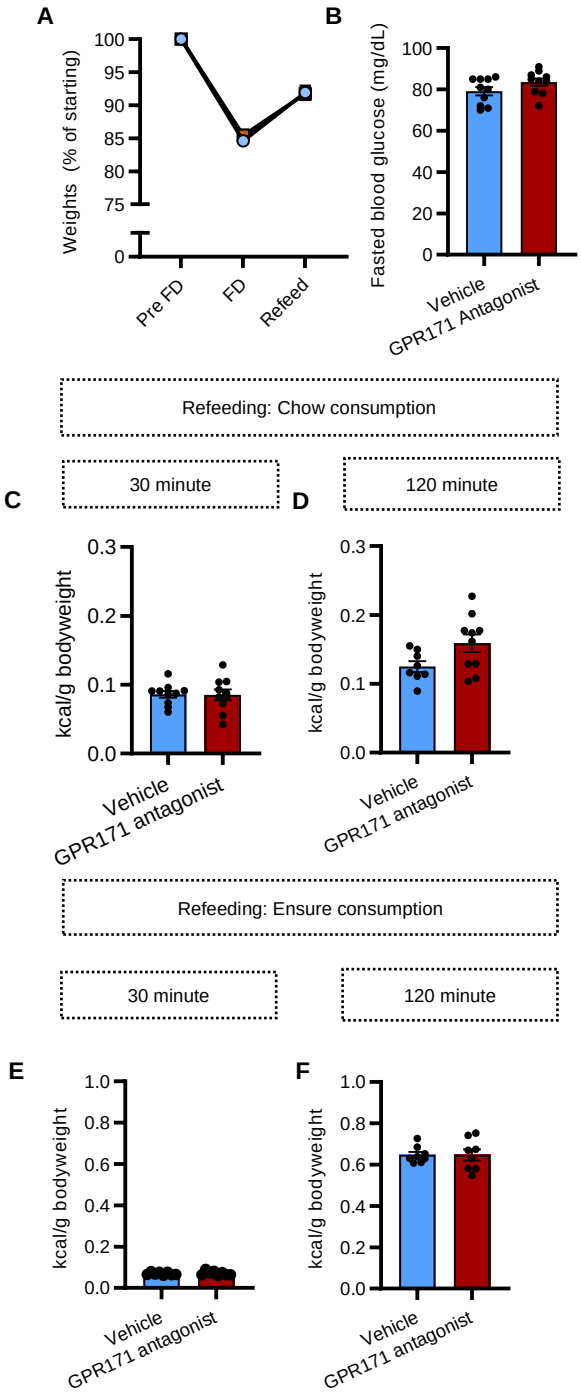

A
B
Weights (% of starting)
100
95
90
85
80
75
0
Pre FD
FD
Refeed
Fasted blood glucose (mg/dL)
100
80
60
40
20
0
Vehicle
GPR171 Antagonist
Refeeding: Chow consumption
30 minute
120 minute
C
D
kcal/g bodyweight
0.3
0.2
0.1
0.0
Vehicle
GPR171 antagonist
Refeeding: Ensure consumption
30 minute
120 minute
E
F
1.0
0.8
0.6
0.4
0.2
0.0

Figure 18 | GPR171 activation is not necessary for increased food consumption following food deprivation, and does not change weight loss or blood glucose

(**A**) Percent of starting weight lost during food deprivation, and percent regained following refeeding on chow between animals treated with GPR171 antagonist (MS21570, I.P. 3.5 mg/kg) or vehicle. (**B**) Fasted blood glucose levels between animals treated with GPR171 antagonist or vehicle. 30 minute (**C**) and 120 minute (**D**) chow consumption on refeeding following food deprivation between animals treated with GPR171 antagonist or vehicle. 30 minute (**E**) and 120 minute (**F**) consumption of 50% ensure on refeeding following food deprivation between animals treated with GPR171 antagonist or vehicle. Data presented as mean ± S.E.M. Statistical tests and results can be found in table 1.

Powerful alterations in reward seeking behavior are associated with synaptic adaptations in the NAc (Brown et al., 2017; Kasanetz et al., 2010; Pascoli et al., 2014). Additionally, NAcSh output controls food consumption and unproductive reward-seeking behavior (Lafferty et al., 2020; O'Connor et al., 2015; Stratford and Kelley, 1999). Thus, we hypothesized that GPR171 within the NAcSh is responsible for this unrewarded persistence phenotype. To test this, we surgically implanted NAcSh targeted cannula (Fig. 16M and 19). Infusion of the GPR171 antagonist (20 nmol MS21570 in 0.5 uL vehicle (10% tween-80, 10% DMSO in saline) (Bobeck et al., 2017)), into the NAc of FD animals resulted in significantly reduced unrewarded food seeking (Fig. 16N). We also found that NAcSh targeted antagonism of GPR171 significantly reduced responding during the timeout period (Fig. 16O). This manipulation did not alter the latency to access the food port, or the proportion of trials ending without accessing the food port (Fig. 17G and 17H). In agreement with systemic antagonist experiments, we found that intra-NAc infusion of the GPR171 antagonist did not change responding in the PR assay (Fig. 16P). Together, these results indicate that the NAc GPR171 population is critical for the ability of hunger to enhance food-seeking persistence.

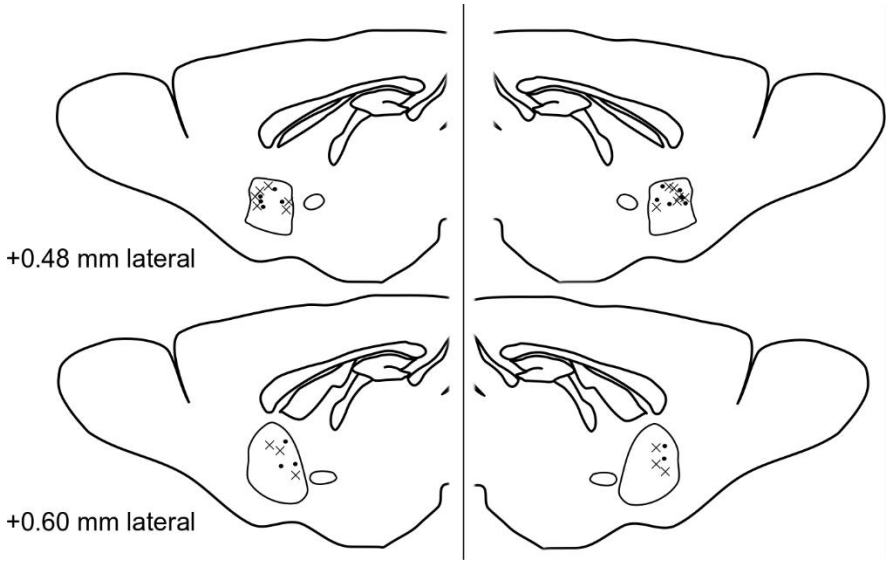


Figure 19 | Diagram depicting intra-NAcSh cannula placements

Termination point of the cannula tract in animals with NAcSh cannula. Dots represent animals infused with vehicle, Xs represent animals infused with GPR171 antagonist.

BigLEN dampens NAc to LH circuit output

NAcSh output determines feeding and reward-seeking behavioral patterns (Gibson et al., 2018; O'Connor et al., 2015). We wondered whether dampened glutamatergic transmission due to GPR171 stimulation would translate into reduced action potential generation. To test this, we clamped the membrane potential of NAcSh D1+ MSNs at -50 mV and applied electrical stimulation at an increasing range of frequencies. This resulted in the generation of action potentials with a probability that increased with stimulation frequency (Fig. 20). Importantly, we were unable to evoke action potentials using this protocol in the presence of the AMPA antagonist NBQX (10 μM), indicating the dependence of this generation of action potentials on AMPA receptor mediated currents (Fig. 20). We found that after incubating slices in BigLEN, LH projecting D1+ MSNs exhibited a significantly lower action potential probability during 10, 20 and 30 Hz stimulation (Fig. 21A and 21B). Repeating these experiments in VTA projecting D1+ MSNs, we found no effect of BigLEN on electrically evoked action potentials, consistent with the inability of BigLEN to suppress excitatory transmission onto VTA projecting D1+ MSNs (Fig. 21C and 21D).

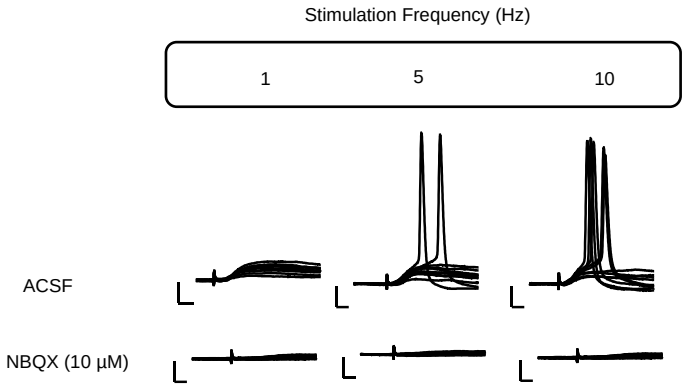
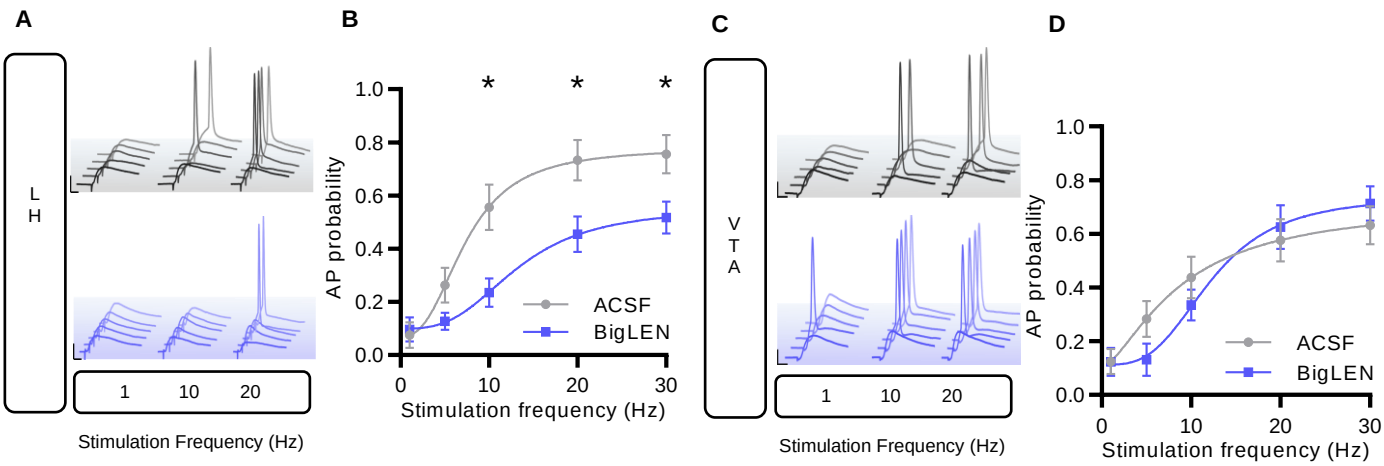


Figure 20 | Action potentials evoked by electrical stimulation require AMPA receptor currents

Representative action potential traces following increasing stimulation frequencies before and after application of the AMPA receptor antagonist NBQX (10 µM).


A
L
H
1
10
20
Stimulation Frequency (Hz)
B
AP probability
Stimulation frequency (Hz)
ACSF
BigLEN
C
V
T
A
1
10
20
Stimulation Frequency (Hz)
D
AP probability
Stimulation frequency (Hz)
ACSF
BigLEN

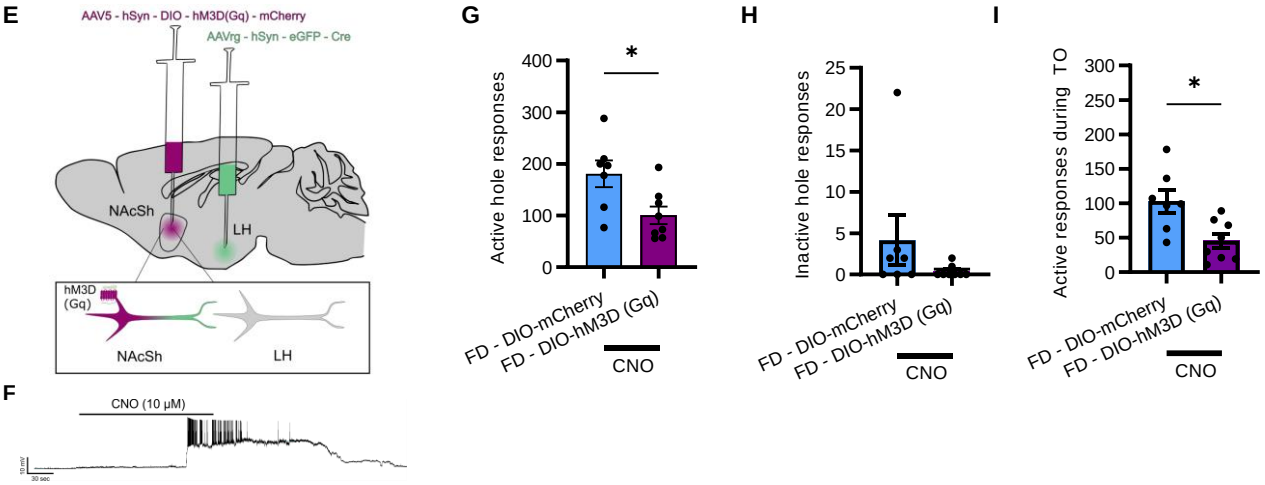

E
AAV5 - hSyn - DIO - hM3D(Gq) - mCherry
AAVrg - hSyn - eGFP - Cre
NAcSh
LH
hM3D
(Gq)
NAcSh
LH
F
CNO (10 μM)
G
Active hole responses
FD - DIO-mCherry
FD - DIO-hM3D (Gq)
CNO
H
Inactive hole responses
FD - DIO-mCherry
FD - DIO-hM3D (Gq)
CNO
I
Active responses during TO
FD - DIO-mCherry
FD - DIO-hM3D (Gq)
CNO

Figure 21 | BigLEN dampens NAc to LH circuit output and enhancing NAc to LH circuit output reduces unrewarded food-seeking in food deprived animals

Representative traces (**A**) and action potential probability (**B**) in response to electrically evoked excitatory synaptic transmission at three different stimulation frequencies in LH projecting, D1+ MSNs in the NAcSh incubated in either ACSF (gray, n=8) or BigLEN (blue, n=11) from 5 mice. Representative traces (**C**) and action potential probability (**D**) in response to electrically evoked excitatory synaptic transmission at three different stimulation frequencies in VTA projecting, D1+ MSNs in the NAcSh incubated in either ACSF (gray, n=9) or BigLEN (blue, n=8) from 5 mice. (**E**) Schematic depicting viral strategy to selectively express the hM3D (Gq) DREADD in LH projecting NAcSh MSNs. (**F**) Representative trace depicting spontaneous action potentials in response to CNO application (10 μM) in a eGFP positive, mCherry positive MSN in the NAcSh. (**G**) Active hole nosepokes completed by CNO treated mice injected with either control (DIO-mCherry, n=7) or DIO-hM3d (Gq) (n=8) virus into the NAcSh during the unrewarded responding task. (**H**) Number of inactive hole nosepokes during the UR task between FD animals expressing either control virus (DIO mCherry, n=7) or DIO-hM3D (Gq) DREADD (n=8). (**I**) Average number of active hole nosepokes during the 10 second timeout period following initial active hole nosepoke during the unrewarded responding task between FD animals expressing either control virus (DIO mCherry, n=7) or DIO-hM3D (Gq) DREADD (n=8). Data presented as mean ± S.E.M. *$p<0.05$, **$p<0.01$. Statistical tests and results can be found in table 1.

We reasoned that because GPR171 acts within the NAc to LH circuit to suppress action potential output and is necessary for hunger to enhance food-seeking persistence, then upregulating activity of LH projecting MSNs *in vivo* should result in reduced unrewarded food seeking. To test this, we paired a retrograde virus encoding Cre injected into the LH with viral mediated expression of a Cre-dependent designer excitatory chemogenetic receptor (DREADDs, DIO-hM3D(Gq)) in the NAcSh (Fig. 21E). This resulted in expression of the hM3D(Gq) DREADD in NAcSh LH projecting MSNs, which responded *ex vivo* with a high rate of spontaneous actions potentials in response to CNO application (Fig. 21F). We found that when compared to control animals expressing DIO-mCherry in NAcSh LH projecting MSNs, those expressing the DIO-hM3D(Gq) DREADD completed significantly fewer active nosepokes in the unrewarded responding task following CNO administration (Fig. 21G), with no change in inactive responses (Fig. 21H). The behavioral profile of mice expressing the DIO-hM3D (Gq) DREADD was similar to that previously seen in MS21570 treated mice, including a significant reduction in nosepokes during the timeout period (Fig. 21I). We found no change in latency to access reward port or in the proportion of trials omitted (Fig. 22A and 22B). These results show that BigLEN, a hunger-driven neuropeptide, inhibits excitatory transmission in a NAc to LH circuit, and this results in increased unrewarded food-seeking behavior in food deprived animals.

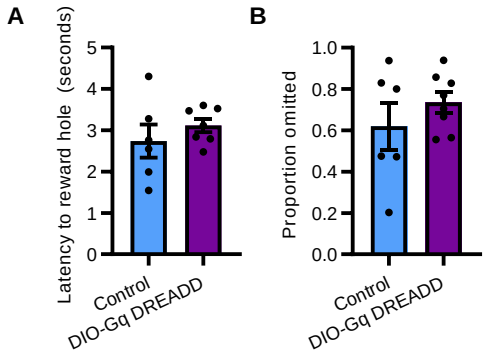


Figure 22 | CNO administration in animals expressing the Gq DREADD hM3D in LH projecting MSNs does not alter latency to reward port or proportion of trials ending in omission

(**A**) Average latency to access reward port following initial active hole nosepoke during the unrewarded responding task between FD animals expressing either control virus (DIO mCherry, n=6) or DIO-hM3D (Gq) DREADD (n=7). (**B**) Proportion of trials ending in omission during the unrewarded responding task between FD animals expressing either control virus (DIO mCherry, n=6) or DIO-hM3D (Gq) DREADD (n=7). Data presented as mean ± S.E.M. **p<0.01. Statistical tests and results can be found in table 1.

Discussion

Hunger exerts a powerful influence over behavior, promoting both food seeking and food consumption. We find that activation of GPR171 by the neuropeptide BigLEN results in the suppression of excitatory transmission onto NAcSh D1+ MSNs that project to the LH. Our results suggest this suppression of excitatory transmission occurs via inhibition of NAc CINs, resulting in reduced presynaptic facilitation of vesicular release probability by nAChRs. Food-seeking behavior is enhanced in hungry animals across multiple dimensions, including increased effortful food seeking as well as increased persistence when a food-seeking strategy is no longer rewarded. We describe GPR171 signaling as necessary for hunger enhanced unrewarded food-seeking persistence, and the receptor population within the NAc as critical to this phenotype. Conversely, antagonism of GPR171 has no effect on increased effortful food seeking or enhanced food consumption in the food-deprived state. Chemogenetic upregulation of the NAc to LH circuit, which circumvents hunger-driven changes to excitatory transmission in this circuit, reduces unrewarded persistence in hungry animals. These results define a hunger-driven neuropeptidergic modulation of NAc circuitry as a critical determinant of internal state-dependent motivated behavior.

Deficits in motivated behavior are a common feature of many psychiatric diseases, including eating disorders. While hunger is known to enhance both effortful (Hodos, 1961) and persistent unrewarded (Perin, 1942) responding, and both of these measures are related to the drive of the animal, they represent distinct aspects of behavior. We find that these different facets of motivated behavior are encouraged by separable hunger-driven modulatory mechanisms, as

GPR171 is necessary for persistent unrewarded responding but is not necessary for rewarded, effortful responding. These findings highlight the importance of assessing a diverse range of parameters when describing the impact of neuromodulation on higher-order motivated behavior. Further, they broaden our understanding of how hunger alters motivated behavior and indicate that it may be possible to specifically address distinct aspects of food-seeking behavior that are disrupted in disease states. This would allow for therapeutic options that are tailored toward specific behavioral deficits. For example, patients with binge eating disorder, anorexia nervosa, and bulimia nervosa exhibit reduced behavioral flexibility and are more likely to persist with a unproductive strategy when compared to healthy volunteers (Tchanturia et al., 2012; Voon et al., 2015; Zastrow et al., 2009). In these patient populations, these phenotypes have been associated with reduced volume or activation of the ventral striatum, indicating an importance of NAc circuitry in their genesis (Nagahama et al., 2005; Voon et al., 2015; Zastrow et al., 2009). Importantly, patients who have recovered from anorexia nervosa exhibit increased flexibility compared to current patients, suggesting that targeting this phenotype may prove clinically beneficial (Tchanturia et al., 2012). Interventions aimed at altering distinct aspects of motivated behavior would help combat the immense etiological diversity of disordered eating at the population level.

We find that food-deprived animals continually engage in a food-seeking behavior despite discontinued reward, revealing a deficit in behavioral inhibition. In addition to an overall increase in persistent unrewarded responding, FD animals make significantly more responses during the 10 second timeout period following the initial nosepoke. Both of these phenotypes are reduced by antagonizing NAc GPR171. This behavioral phenotype may appear habitual. However, our data

suggests this phenotype is distinct from habit in that it critically depends on internal state, and thus is linked to the current value of the reward. Habitual behaviors are resistant to reward devaluation (Dickinson et al., 1983; Everitt and Robbins, 2005), and although we do not specifically test that here, animals fed *ad libitum* likely value the food reward less than their FD counterparts, as evidenced by reduced responding in the PR assay. The phenotype presented here may be related to impulsivity or compulsivity, however there is a notable absence of any direct benefit of inhibiting responding, or any avoidance of negative consequence by withholding responding (Fineberg et al., 2010). The repetition of unrewarded responding most closely matches the definition of perseverative behavior, a phenotype that has been observed in animals with NAc lesions (Christakou et al., 2004). Similar to our results, animals with NAc lesions appear to perseverate on the responding behavior immediately after completing a failed trial that does not result in reward (Christakou et al., 2004). Together, the results presented here indicate hungry animals are more resistant to learning from changes in task outcomes, and this lack of adaptation manifests as a perseveration on the previously learned behavior.

The NAc, and specifically the NAcSh, has long been understood to enable efficient reward-seeking in part by suppressing reward-seeking behavior when it is inappropriate (Floresco, 2015). Inactivation of the NAcSh results in increased responding during periods of reward unavailability (Ambroggi et al., 2011; Blaiss and Janak, 2009; Feja et al., 2014; Lafferty et al., 2020; Reading et al., 1991), and neuronal activity in NAcSh MSNs is increased during periods where reward-seeking behavior is suppressed (Lafferty et al., 2020). Conversely, when animals engage in reward-seeking, NAcSh neurons are inhibited (Ambroggi et al., 2011). As NAc MSNs are quiescent and rely primarily on excitatory transmission to generate action potentials, it is likely the changes in

NAcSh neuronal activity during these periods are due in part to changes in excitatory input. Indeed, inhibition of excitatory input to the NAcSh results in inappropriate, unproductive, reward-seeking behavior (Lafferty et al., 2020). Additionally, during food consumption these excitatory inputs exhibit a coordinated reduction in activity that coincides with NAc neuronal inhibition (Krause et al., 2010; Reed et al., 2018). Here we describe a mechanism that allows whole-body energy state to shift the definition of productive in the NAcSh by modulating excitatory transmission. In the hungry animal, GPR171 dampens excitatory input to the NAcSh, disinhibiting food-seeking behavior to allow pursuit of avenues that in more replete times may be considered unproductive. Whether GPR171 inhibits a specific excitatory input to achieve this is unknown, however inhibition of both the input from the basaolateral amygdala or the paraventricular nucleus of the thalamus have been reported to increase unproductive reward-seeking behavior (Lafferty et al., 2020). Additionally, lesions to the ventral hippocampus, the strongest glutamatergic input to the NAcSh(Britt et al., 2012), also result in unproductive behavior (Abela et al., 2013; Maruki et al., 2001). These results highlight the influential role internal state plays in producing efficient motivated behavior, and how this state is communicated to NAc circuitry.

The NAc, and its connection to the LH, exerts inhibitory control over feeding behavior that is shaped by on excitatory transmission within the NAc (Kelley et al., 2005). Our experiments identified the ability of BigLEN and GPR171 to suppress glutamate release onto D1+ MSNs as present at synapses onto LH projecting D1+ MSNs in the NAcSh. Inhibiting glutamate receptors, or activating GABA receptors within the NAcSh results in a robust increase in food consumption (Maldonado-lrizarry et al., 1995; Reynolds and Berridge, 2003, 2001; Stratford and Kelley, 1997; Urstadt et al., 2013). This occurs alongside increases in immediate early gene expression in the

LH (Baldo et al., 2004; Stratford and Kelley, 1999), and inactivation of the LH blocks increases in food consumption triggered by NAcSh manipulations (Maldonado-lrizarry et al., 1995; Urstadt et al., 2013). During food consumption, NAc neurons, and specifically D1+ MSNs, exhibit reduced firing rates (O'Connor et al., 2015; Roitman et al., 2010; Vachez et al., 2021), a pattern that is also seen in the activity of excitatory inputs into the NAcSh (Reed et al., 2018). This excitatory input has been described as communicating novel or potentially dangerous environmental information which triggers the interruption of the consummatory motor program (Kelley, 2004b). Modulation of this glutamatergic input then, modulates endogenous "stop" signals, setting a filter for what is deemed critical enough to interrupt feeding or food seeking. This conceptualization of the role of glutamatergic signaling within the NAc to LH circuit fits with the behavioral phenotypes we observe downstream of GPR171 activation during food deprivation; it alters the ability of the animal to integrate environmental information relevant to food seeking but does not disrupt consumption in a familiar environment where there is no urgent stop signal. It would be interesting to examine whether GPR171 signaling within the NAc alters consumption when animals are presented with distracting stimuli (O'Connor et al., 2015). This role for glutamatergic transmission stands in contrast to NAcSh GABAergic transmission from the ventral pallidum, which has been found to alter consumption and palatability (Vachez et al., 2021). Modulation of this GABAergic "go" signal would likely alter consumption, however it is unclear whether it would alter food seeking during uncertainty in a manner similar to glutamatergic modulation. Our results add a critical piece to this body of literature by describing how the flow of information through this NAc to LH circuit is controlled by internal state via modulation of excitatory transmission.

The NAc consists of multiple output pathways with varied genetic identities. The majority of LH projecting MSNs are D1+ (O'Connor et al., 2015), and comprise a distinct population of MSNs from those targeting the VTA or the ventral pallidum (Thoeni et al., 2020). We find that that VTA projecting D1+ MSNs were insensitive to BigLEN incubation, suggesting that excitatory inputs onto these two distinct MSN populations in the NAcSh are differentially regulated. Indeed, it is understood that these different MSN populations are able to encourage separable behavioral phenotypes. Studies of alcohol-seeking behavior have described the VTA projection population as promoting relapse, while the LH projection population promotes extinction (Gibson et al., 2018). If the outputs of these cells mediate different behavioral outcomes, it follows that they could then receive different input. Indeed, the NAcSh has been described as containing multiple parallel output pathways with unique inputs and plasticity (Baimel et al., 2019). It is yet unclear whether this difference in input modulation arises from distinct regions, cells within regions, differential modulation of inputs, or even compartmentalized modulatory influences within the same cell. Further studies are needed in order to better understand whether and how differential plasticity based on projection target contributes to motivated behavior.

We find that the ability of BigLEN and GPR171 to suppress glutamate release depends on action potential generation and nAChR activation, and that application of the nAChR agonist nicotine results in enhanced mEPSC frequency. Further, we found that application of BigLEN reduces CIN firing rate, and that GPR171 immunoreactivity is present in CHAT expressing CINs. Within the NAc, tonically-firing CINs are considered to be the primary source of ACh (Bolam et al., 1984) (Descarries et al., 1997; Descarries and Mechawar, 2000), although projections from other ACh containing brain regions have been found (Dautan et al., 2014). We find that treatment

with TTX is able to block the induction of the BigLEN induced suppression of glutamate release, indicating that a tonically firing ACh source is responsible for this phenotype. Together, these results suggest a model where BigLEN via GPR171 inhibits spontaneously firing CINs, reducing the acetylcholine available to activate nAChRs. This model, and the behavioral implication that nAChR activation opposes food-seeking behavior, is consistent with previous literature where cholinergic signaling within the NAc has been described as communicating a satiety signal (Aitta-Aho et al., 2017; Helm et al., 2003; Mark et al., 1992; Stouffer et al., 2015). Food intake increases extracellular ACh within the NAc (Mark et al., 1992), and postprandial hormones like cholecystokinin and insulin both promote ACh release within the NAc (Helm et al., 2003; Stouffer et al., 2015). This increase in extracellular ACh likely impacts glutamatergic transmission, as ACh signaling through nAChRs has been found to enhance extracellular glutamate in the NAc (Reid et al., 2000). This is in line with studies from other brain regions, where nAChRs have been found to act presynaptically to promote glutamate release (Garduño et al., 2012; Gray et al., 1996; Sharma and Vijayaraghavan, 2003). Importantly, despite the well-studied role of nAChRs in promoting striatal dopamine release, nAChR-mediated enhancement of extracellular glutamate within the NAc is independent of dopamine signaling (Reid et al., 2000). These results increase our understanding of how neuropeptides influence NAc microcircuits to communicate state via modulation of afferent excitatory transmission.

The development of tools that enable the precise manipulation of neural circuits coupled with extensive anatomical mapping has provided a wealth of information describing how behaviors are generated by discrete circuits. What is still unclear, is how internal state is communicated to these circuits in order to produce adaptive behaviors based on pertinent needs.

We find that hunger engages GPR171 signaling to inhibit synaptic transmission onto a NAcSh to LH circuit, ultimately suppressing its output. It is possible that this suppression targets a specific input into the NAc, and thus this neuromodulation could be selectively dampening information encoding a unique reason to terminate the food-seeking behavior (Christoffel et al., 2021; Lafferty et al., 2020). This may prove to be a repeating principle across the brain, that state-dependent neuromodulation can not only select, but also suppress certain circuit elements in order to bias behavior towards a "desired" outcome. Beyond assembling the roadmap of circuits that drive behavior, an important future problem will be identifying the neuromodulatory elements that direct the traffic, and further understanding the rules which they implement to guide the flow of information through the brain.

Table 1. Statistics for all Chapter II figures

Fig	Panel	Test	Group	Test Statistic	*P*-value	*n*
9	C	Unpaired t-test	ACSF vs. BigLEN	t_{22}=3.264	0.0036	12,12
9	D	Unpaired t-test	ACSF vs. BigLEN	t_{22}=0.7436	0.465	12,12
9	F	Two-way RM ANOVA	ACSF vs. BigLEN	Interaction $F_{(4,76)}$=4.260	0.0037	11,10
		Holm - Sidak's multiple comparisons	20 ms	t_{95}=5.221	<0.0001	
			50 ms	t_{95}=4.306	0.0002	
			100 ms	t_{95}=3.632	0.0014	
9	G	Mann Whitney test	ACSF vs. BigLEN	U=104.5	0.42	14,18
9	I	Mixed effects model	ACSF vs. BigLEN	Interaction $F_{(9, 158)}$= 0.5724	0.8183	11,10
9	K	Unpaired t-test	ACSF vs. BigLEN	t_{14}=0.6277	0.5403	8,8
9	L	Unpaired t-test	ACSF vs. BigLEN	t_{14}=0.2167	0.8316	8,8
9	M	Two-way RM ANOVA	ACSF vs. BigLEN	Interaction $F_{(4, 56)}$= 0.6685	P=0.6165	9,7
11	C	Unpaired t-test	ACSF vs. MS+BL	t_{18}=0.08788	0.9309	10,10
11	D	Unpaired t-test	ACSF vs. MS+BL	t_{18}=1.016	0.3233	10,10
11	E	Two-way RM ANOVA	ACSF vs. MS+BL	$F_{(4, 80)}$= 0.06419	0.9923	9,13
11	F	Unpaired t-test	ACSF vs. MS21570	t_{15}=0.1565	0.8778	8,9
11	G	Unpaired t-test	ACSF vs. MS21570	t_{15}=0.4293	0.6738	8,9
11	H	Two-way RM ANOVA	ACSF vs. MS21570	Interaction $F_{(4, 52)}$= 1.040	0.3958	7,8
11	I	Unpaired t-test	Fed vs. FD	t_{17}=0.3549	0.7271	9,10
11	J	Unpaired t-test	Fed vs. FD	t_{17}=1.775	0.0938	9,10
11	K	Two-way RM ANOVA	Fed vs. FD	ISI $F_{(4, 16)}$= 8.348	0.0107	9,9
		Holm – Sidak's multiple comparisons	50 ms	t_{80}=2.891	0.0245	9,9
11	L	Unpaired t-test	ACSF vs. BigLEN	t_{18}=0.8942	0.383	11,9

11	M	Mann Whitney test	ACSF vs. BigLEN	U=14	0.0057	11,9
11	N	Two-way RM ANOVA	ACSF vs. BigLEN	Interaction $F_{(4, 56)}$= 0.6898	0.6021	8,8
12	B	Unpaired t-test	ACSF vs. BigLEN	t_{24}=0.5277	0.6025	13,13
12	C	Unpaired t-test	ACSF vs. BigLEN	t_{24}=0.6600	0.5155	13,13
12	D	Unpaired t-test	ACSF vs. BigLEN	t_{16}=0.3552	0.7271	9,9
12	E	Unpaired t-test	ACSF vs. BigLEN	t_{16}=0.1980	0.8455	9,9
12	F	Two-way RM ANOVA	ACSF vs. BigLEN	Interaction $F_{(4, 48)}$= 1.681	0.1697	7,7
12	H	Unpaired t-test	Baseline vs. 100 nM Nicotine	t_{14}=3.423	0.0041	8,8
12	K	Unpaired t-tst	BigLEN vs. Vehicle	t_{10}=2.849	0.0173	5,7
15	D	Unpaired t-test	ACSF vs. BigLEN	t_{22}=3.066	0.0057	12,12
15	E	Unpaired t-test	ACSF vs. BigLEN	t_{22}=0.5557	0.584	12,12
15	G	Two-way RM ANOVA	ACSF vs. BigLEN	Interaction $F_{(4, 76)}$= 3.104	0.0202	8,13
		Holm - Sidak's multiple comparisons	20 ms	t_{95}=4.009	0.0006	8,13
			50 ms	t_{95}=2.833	0.0223	8,13
15	K	Unpaired t-test	ACSF vs. BigLEN	t_{22}=1.322	0.1998	12,12
15	L	Unpaired t-test	ACSF vs. BigLEN	t_{22}=0.1049	0.9174	12,12
15	N	Two-way RM ANOVA	ACSF vs. BigLEN	Interaction $F_{(4, 52)}$= 2.159	0.0866	7,8
16	D	Unpaired t-test	Fed vs FD	t_{17}=14.14	<0.0001	10,9
16	E	Unpaired t-test	Fed vs. FD	t_{16}=3.212	0.0054	9,9
16	F	Unpaired t-test	Fed vs. FD	t_{16}=1.229	0.2368	9,9
16	G	Unpaired t-test	Fed vs. FD	t_{16}=3.541	0.0027	9,9
16	H	Mann Whitney test	Fed vs. FD	U=0	0.0003	8,7
16	I	Mann Whitney test	Fed vs. FD	U=17	0.2089	8,7

16	J	Kruskal-Wallis test	Fed-vehicle vs. FD-vehicle vs. FD-MS21570	KW=11.14	0.0038	6,10,8
		Dunn's multiple comparisons	Fed-vehicle vs. FD-vehicle	Z=3.052	0.0068	6,10
			Fed-vehicle vs. FD-MS21570	Z=0.7427	>0.9999	6,8
			FD-vehicle vs. FD-MS21570	Z=2.477	0.0397	10,8
16	K	One-way ANOVA	Fed-vehicle vs. FD-vehicle vs. FD-MS21570	$F_{(2,21)}$=5.003	0.0167	6,10,8
		Holm-Sidak's multiple comparisons	Fed-vehicle vs. FD-vehicle	$t_{21)}$=2.777	0.0335	6,10
			Fed-vehicle vs. FD-MS21570	$t_{21)=}$0.445	0.6609	6,8
			FD-vehicle vs. FD-MS21570	$t_{21)=}$2.517	0.0397	10,8
16	L	Kruskal-Wallis test	Fed-vehicle vs. FD-vehicle vs. FD-MS21570	KW=12.45	0.0004	7,8,8
		Dunn's multiple comparisons	Fed-vehicle vs. FD-vehicle	Z=2.716	0.0198	7,7
			Fed-vehicle vs. FD-MS21570	Z=3.332	0.0026	7,8
			FD-vehicle vs. FD-MS21570	Z=0.526	>0.9999	7,8
16	N	Unpaired t-test	FD-vehicle vs. FD-MS21570	$t_{(14)}$=2.701	0.0172	7,9
16	O	Unpaired t-test	FD-vehicle vs. FD-MS21570	$t_{(15)}$=2.153	0.048	8,9
16	P	Unpaired t-test	FD-vehicle vs. FD-MS21570	$t_{(12)}$=1.147	0.2739	7,7
21	D	Two-way RM ANOVA	ACSF vs. BigLEN	Interaction $F_{(4, 68)}$= 3.456	0.0125	8,11
		Holm - Sidak's	10 Hz	$t_{(85)}$=3.712	0.0018	8,11
			20 Hz	$t_{(85)}$=3.22	0.0072	8,11
			30 Hz	$t_{(85)}$=2.767	0.0207	8,11

		multiple comparisons				
21	F	Two-way RM ANOVA	ACSF vs. BigLEN	Interaction F(4, 60)= 2.085	0.0939	9,8
21	I	Unpaired t-test	FD-DIO-mCherry vs. FD-DIO-hM3D (Gq)	$t_{(13)}$=2.658	0.0197	7,8
21	J	Mann Whitney test	FD-DIO-mCherry vs. FD-DIO-hM3D (Gq)	U=16	0.145	7,8
21	K	Unpaired t-test	FD-DIO-mCherry vs. FD-DIO-hM3D (Gq)	$t_{(13)}$=3.397	0.0048	7,8
10	A	Unpaired t-test	ACSF vs. BigLEN	$t_{(17)}$=2.206	0.0414	10,9
10	B	Unpaired t-test	ACSF vs. BigLEN	$t_{(17)}$=0.3647	0.7198	10,9
10	C	Two-way RM ANOVA	ACSF vs. BigLEN	Interaction $F_{(4,44)}$= 0.5235	0.7189	6,7
13	B	Mann Whitney test	ACSF vs. BigLEN	U=9	0.0164	9,7
13	C	Unpaired t-test	ACSF vs. BigLEN	$t_{(14)}$=0.6838	0.5052	9,7
13	E	Unpaired t-test	Baseline vs. 100 nM Nicotine	$t_{(14)}$=2.825	0.0135	8,8
13	G	Unpaired t-test	Baseline vs. 100 nM Nicotine	$t_{(12)}$=1.153	0.2714	7,7
13	I	Unpaired t-test	Baseline vs. 100 nM Nicotine	$t_{(12)}$=1.871	0.0859	7,7
17	A	Unpaired t-test	Fed vs. FD	$t_{(16)}$=3.731	0.0018	9,9
17	B	Unpaired t-test	Fed vs. FD	$t_{(14)}$=2.221	0.0433	8,8
17	C	Mann Whitney test	Fed vs. FD	U=0	0.0003	8,7
17	D	Mann Whitney test	Fed vs. FD	U=0	0.0003	8,7
17	E	Unpaired t-test	Fed-vehicle vs. fed-MS21570	$t_{(14)}$=1.541	0.1456	8,8
17	F	Unpaired t-test	Fed-vehicle vs. fed-MS21570	$t_{(14)}$=1.481	0.1606	8,8
17	G	Unpaired t-test	FD-vehicle vs. FD-MS21570	$t_{(10)}$=0.02296	0.9821	6,6

17	H	Unpaired t-test	FD-vehicle vs. FD-MS21570	$t_{(11)}$=1.359	0.2014	6,7
18	A	Two-way RM ANOVA	Vehicle vs. MS21570	Interaction $F_{(2, 36)}$= 0.4417	0.6464	10,10
18	B	Unpaired t-test	Vehicle vs. MS21570	$t_{(18)}$=1.630	0.1204	10,10
18	C	Unpaired t-test	Vehicle vs. MS21570	$t_{(18)}$=0.08694	0.9317	10,10
18	D	Unpaired t-test	Vehicle vs. MS21570	$t_{(16)}$=2.104	0.0515	8,10
18	E	Unpaired t-test	Vehicle vs. MS21570	$t_{(14)}$=0.3564	0.7269	8,8
18	F	Unpaired t-test	Vehicle vs. MS21570	$t_{(14)}$=0.01486	0.9884	8,8
22	A	Unpaired t-test	Control vs. DIO-Gq-DREADD	$t_{(11)}$=0.9327	0.371	6,7
22	B	Unpaired t-test	Control vs. DIO-Gq-DREADD	$t_{(12)}$=1.025	0.3257	6,8

CHAPTER III

Neuropeptide Y modulates excitatory transmission and promotes social behavior in the nucleus accumbens

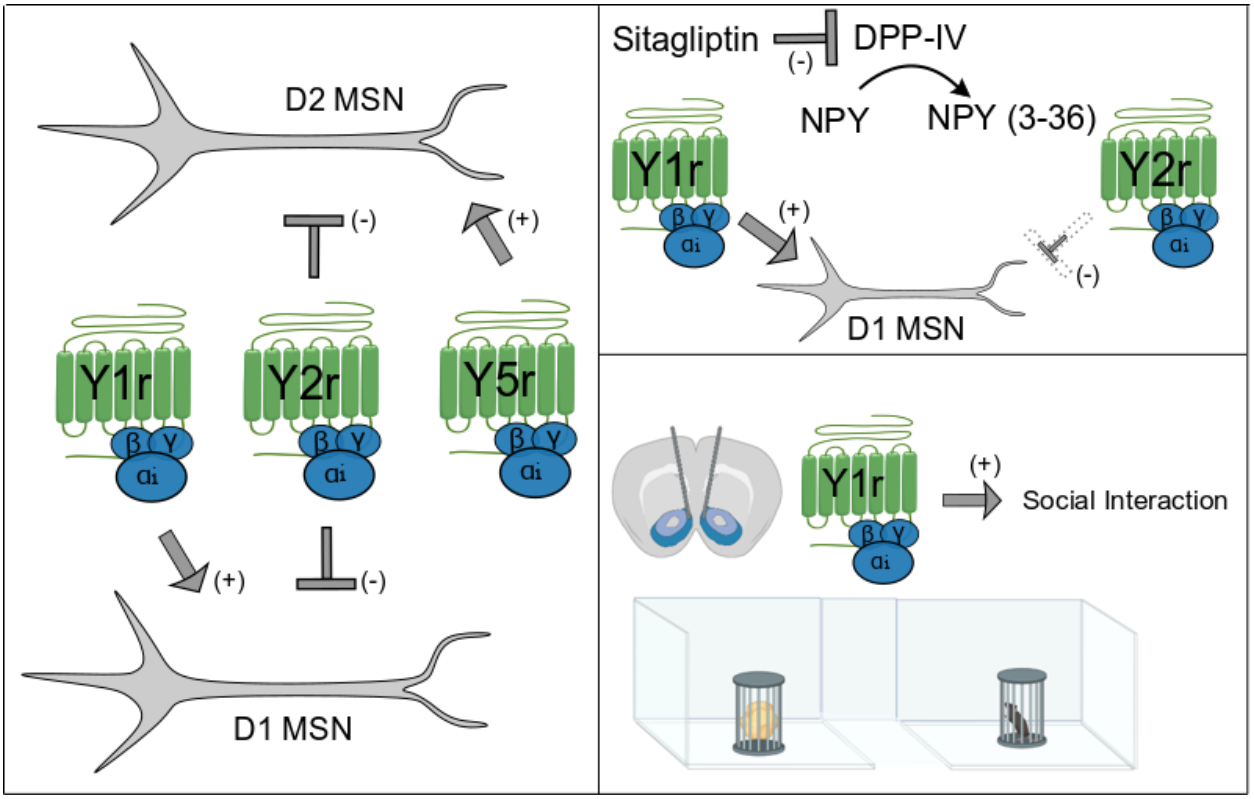


Figure 23 | Graphical abstract for Neuropeptide Y modulates excitatory transmission and promotes social behavior in the nucleus accumbens

Social interactions define the human experience, but these integral behaviors are disrupted in most common psychiatric disorders creating a need for novel therapeutic interventions. Social behaviors have evolved over millennia, and neuromodulatory systems that promote social behavior in invertebrates are still present in human brains. One such neuromodulator, neuropeptide Y (NPY), acts through several receptors including the Y1r, Y2r, and Y5r. These receptors are present in brain regions that control social behavior, including the nucleus accumbens (NAc), a critical node in the reward pathway. However, how NPY modulates NAc neurotransmission is unknown. Using whole-cell patch-clamp electrophysiology of NAc neurons, we find that multiple NPY receptors regulate excitatory synaptic transmission in a cell-type specific manner. At excitatory synapses onto D1+ MSNs, Y1r activity enhances while Y2r suppresses transmission. At excitatory synapses onto D1- MSNs, Y5r activity enhances while Y2r suppresses transmission. Behaviorally, Infusing NPY or the Y1r agonist [Leu31, Pro34]-NPY into the NAc significantly increases social interaction with an unfamiliar conspecific. Inhibition of an enzyme that breaks down NPY, dipeptidyl peptidase IV (DPP-IV) with sitagliptin shifts the effect of NPY on excitatory transmission onto D1+ MSNs to a Y1r dominated phenotype. Together, these results increase our understanding of how NPY regulates neurotransmission in the nucleus accumbens, and identify a novel mechanism underlying the control of social behavior (Figure 23). Further, they reveal a potential strategy to shift NPY signaling for therapeutic gain.

Introduction

Social relationships are beneficial for a great number of species, decreasing the likelihood of predation, promoting foraging efficiency and enhancing child rearing (Matthews and Tye, 2019).

This has led to the evolution of neuromodulatory systems that shape circuit function to promote social behavior, with robust conservation, including Neuropeptide Y (NPY). The NPY system and its homologues encourages prosocial behaviors across the animal kingdom from nematodes to mammals (De Bono and Bargmann, 1998; Desai et al., 2014; Shiozaki et al., 2020; Wu et al., 2003). For instance, higher levels of striatal NPY are found in primates that exhibit complex social behaviors (Raghanti et al., 2018). Behaviorally, NPY promotes social interaction by acting in multiple brain regions, a phenomenon that has been attributed to a reduction in anxiety (Kask et al., 2001; Sajdyk et al., 1999; Villarroel et al., 2018). However, due to the ubiquitous central expression of NPY and its receptors, this is likely not the only mechanism by which NPY promotes social behavior.

The mesolimbic dopamine circuit, including the ventral tegmental area and nucleus accumbens (NAc), are critical to the expression of social behaviors (Golden et al., 2019; Gunaydin et al., 2014; Hung et al., 2017; Van Erp and Miczek, 2000). Social interaction with an unfamiliar conspecific triggers dopamine release in the medial NAc shell (NAcSh) (Gunaydin et al., 2014). Dopamine projections to this region of the NAc are also recruited by the prosocial hormone oxytocin to increase social interaction (Hung et al., 2017). To exert its effects on motivated behavior, dopamine modulates medium spiny neurons (MSNs), the principal neurons of the NAc. MSNs are classically characterized by their expression of dopamine receptors, with different cell groups expressing either the dopamine D1 receptor (D1+ MSN) or the dopamine D2 receptor (D2+ MSN) (Castro and Bruchas, 2019). NAc MSNs are hyperpolarized, and their activity is mostly derived from glutamatergic excitatory input arriving from the prefrontal cortex, hippocampus, thalamus and basolateral amygdala (O'Donnell and Grace, 1995; Turner et al., 2018). Alterations in this excitatory input to the NAc corresponds with changes in motivational state (Grueter et al., 2012;

Lim et al., 2012; Pascoli et al., 2014; Turner et al., 2018). Previous work has shown that excitatory transmission within the NAc is disrupted in an animal model of autism spectrum disorder, where social interaction is devalued (Folkes et al., 2020). Thus, this excitatory transmission is likely integral to NAc control of social behavior.

In the human brain, the NAc contains some of the highest levels of NPY, likely due to expression within NPY containing interneurons (Adrian et al., 1983; Castro and Bruchas, 2019; Chronwall et al., 1985). The NAc also contains the NPY receptors Y1r (Kishi et al., 2005; Kopp et al., 2002; Pickel et al., 1998), Y2r (Caberlotto et al., 1998; Chen et al., 2021; Gustafson et al., 1997; Stanić et al., 2006), and Y5r (Quarta et al., 2011; Wolak et al., 2003). NPY within the NAc has been found to be rewarding, and supports a conditioned place preference (Brown et al., 2000; Josselyn and Beninger, 1993). Within the NAc, NPY receptors are found at a variety of loci, including MSN cell bodies, as well as excitatory and monoaminergic terminals (Massari et al., 1988). Whether NPY regulates excitatory neurotransmission within the NAc, and whether NPY within the NAc promotes social interaction is unknown.

NPY, one of the most abundant peptides in the brain, is a 36 amino acid peptide that belongs to a family of peptides including Peptide YY (PYY) and Pancreatic Polypeptide (PP) (Sajdyk et al., 2004). In mammals, NPY acts through five different receptors (Y1, Y2, Y4, Y5 and y6). Y1, Y2 and Y5 are the primary NPY receptors within the brain, while the endogenous ligand for Y4 is PP, and y6 is non-functional in humans (Reichmann and Holzer, 2016). NPY receptors are g protein-coupled receptors (GPCRs) that are $G_{i/o}$ g coupled (Lin et al., 2004). Generally, NPY modulates neuronal activity by activating GIRK channels, and inhibiting calcium channels both pre- and postsynaptically (Acuna-Goycolea et al., 2005; Acuna-Goycolea and Van Den Pol, 2005; Fu et al., 2004; McQuiston et al., 1996; Roseberry et al., 2004; Sun et al., 2001b, 2001a; Van Den Pol et al.,

2004; West and Roseberry, 2017). Additionally, NPY has been found to act presynaptically to inhibit vesicular release of both glutamate and GABA (Acuna-Goycolea et al., 2005; Acuna-Goycolea and Van Den Pol, 2005; Chen and Van Den Pol, 1996; Cowley et al., 1999; Fu et al., 2004; Gilpin et al., 2011; Kash and Winder, 2006; Molosh et al., 2013; Sun et al., 2001a; Van Den Pol et al., 2004; West and Roseberry, 2017). However, it is currently unclear whether NPY engages any of these mechanisms in the NAc to regulate synaptic transmission.

Methods

Reagents

Neuropeptide Y (human, rat), [Leu31,Pro34]-NPY (human, rat), peptide YY (3-36), [cPP1-7,NPY19-23,Ala31,Aib32,Gln34] - hPancreatic Polypeptide, BIBO 3304 trifluoroacetate, BIIE 0246 hydrochloride, L-152,804, and Sitagliptin were all acquired from Tocris.

Animals

Animal care and experimental protocols were approved by and conducted in accordance with the Vanderbilt University Institutional Animal Care and Use Committee. Mice used for electrophysiology experiments ranged from 8-12 weeks of age. Mice used for social interaction experiments ranged from 9-10 weeks of age. Mice were maintained on a 12:12 light dark cycle. For electrophysiology experiments, mice were housed in groups of 3-5.For social interaction experiments, mice were housed in pairs following cannulation surgery. Social interaction experiments were performed using male C57BL/6J mice from Jackson Labs. Electrophysiological experiments were performed using male and female C57BL/6J mice bred to carry a bacterial artificial chromosome directing the expression of the tdTomato fluorophore under the control of the Drd1a promoter (Tg(Drd1a-tdTomato)6Calak, Stock #: 016204 Jackson Labs).

Slice preparation

Animals were deeply anesthetized using isoflurane and were rapidly decapitated. Brains were dissected and washed in cold, oxygenated (95% O2 / 5% CO2) N-methyl-D-glucamine (NMDG) containing recovery solution (in mM: 2.5 KCl, 20 HEPES, 1.2 NaH2PO4, 25 Glucose, 93 NMDG, 30 NaHCO3, 5.0 Sodium ascorbate, 3.0 sodium pyruvate, 10 MgCl2, and 0.5 CaCl-2H2O). Parasagittal slices were taken in ice cold, oxygenated NMDG solution with a Leica VT 1200S vibratome. Slices were transferred to 32° oxygenated NMDG solution in a recovery chamber for 10 minutes before being stored at room temperature oxygenated artificial cerebrospinal fluid (ACSF) (in mM: 119 NaCl, 2.5 KCl, 1.3 MgCl2-6H2O, 2.5 CaCl2-2H2O, 1.00 NaH2PO4-H2O, 26.2 NaHCO3, and 11 glucose; 287-295 mOsm). Slices were allowed to rest for one hour prior to recording. For electrophysiological recording, slices were moved to a recording stage perfused with oxygenated ACSF at a rate of 2 mL/min (Gilson Minipuls 3 Peristaltic Pump) at 30° C using an inline heater (Warner Instruments SH-27B).

Electrophysiology

Electrophysiological experiments were performed on a Scientifica Slicescope Pro system described in detail (Manz et al., 2021). All recordings were performed in the NAc shell, which was identified in sagittal slices by the anterior/posterior position and shape of the anterior commissure and the absence of the dorsal striatum. Neurons were selected from the rostral, dorsal portion of the NAc shell. Neurons were visualized using an upright microscope (Scientifica) allowing both infrared differential interference contrast and fluorescence optics. Neurons were patched with 3-6 MΩ recording pipettes using a potassium gluconate based internal solution (in mM: 135 K-gluconate, 5 NaCl, 2 MgCl2, 10 HEPES, 0.2 EGTA, 2.5 Mg2-ATP, 0.2 Na2-GTP). All recordings took place with the membrane clamped at -70 mV and in the presence of picrotoxin (50 μM) to isolate excitatory post synaptic currents (EPSCs). Following break-in, neurons were allowed to

dialyze for five minutes prior to the beginning of baseline. Throughout the experiment, membrane and series resistance were monitored and a deviation of greater than 20% of the base value resulted in an omission of the experiment from analysis. EPSCs were evoked by placing a bipolar stimulating electrode at the corticoaccumbens barrier and stimulating at 0.1 Hz with a 0.1 ms stimulus duration. 50 ms paired pulse ratio was collected with each sweep, and was calculated by taking the ratio of the second EPSC to the first EPSC (PPR = EPSC2/EPSC1).

Stereotaxic surgery

For cannula implantation, animals were deeply anesthetized with a ketamine (75 mg/kg I.P.) and dexdomitidor (0.5 mg/kg I.P., Zoetis) cocktail and given preoperative analgesia with ketoprofen (5 mg/kg I.P., Zoetis). Once anesthetized, the scalp was shaved and cleaned with iodine and alcohol pads. A small incision was made in the scalp to expose the skull and animals were placed into a stereotaxic apparatus. Small holes were drilled above the implantation site and two, 25 gauge 4.3 mm cut unilateral stainless-steel guide cannula (C315GS-5SP, Plastics One) were installed at a 15° lateral angle relative to the midline targeted to the NAcSh (AP: 1.20, ML: ±0.50 , DV: -4.40). Cannula were first secured with Metabond (Parkell) followed by dental cement (A-M Systems). Dummy cannula (Plastics One) were inserted. Following cannula implantation, anesthesia was reversed with Antisedan (0.5 mg/kg I.P., Zoetis). Animals were kept on a warming pad post-surgery until fully ambulatory. Post-surgery analgesia was provided using ketoprofen.

Social interaction assay

The social interaction assay was performed in a three chamber apparatus with two 8 inch x 8 inch chambers and one 5 inch x 6.5 inch connecting chamber. First, animals were habituated to the empty assay chamber for 10 minutes with no stimuli. Following habituation, animals were infused with either vehicle (ACSF) or drug (NPY or [Leu31,Pro34]-NPY (94 pmol/ 200nL per side)). Animals were then returned to the assay chamber and allowed to recover and interact with two

empty wire cups for 10 minutes. Wire cups were topped with an Erlenmeyer flask to prevent climbing. Locomotion was scored during this period. Following this 10 minute period, test subjects were returned briefly to the home cage while a non-social object and a younger, unfamiliar male conspecific (approximately 6 weeks old) were added to the wire cups. Test subjects were returned to the middle chamber of the social interaction apparatus and allowed to interact for 10 minutes. Unfamiliar conspecifics used as a social stimulus were used for a maximum of two assays per day to prevent social fatigue. Each test subject was run in both drug and vehicle conditions in a randomized order on different testing days. Social interaction was manually scored by two blinded experimenters and the average of the two scores was taken as the final value for that animal. Social interaction was defined as sniffing or rearing onto the wire cup regardless of the position or participation of the target mouse. Cannula placements were histologically confirmed post hoc.

Statistics and experimental design

All data presented in timecourse or bar graphs represent group average ± S.E.M. For electrophysiology experiments, a single dot represents one experiment from one cell. For social interaction experiments, one dot represents data from one animal. For social interaction experiments animals were run in both drug and vehicle conditions on different days. Sample sizes for all experiments were determined using a power calculation with preliminary data as well as analysis of similar published experiments in the literature. Statistical tests were completed using GraphPad Prism software (GraphPad, Inc.) after coalescing data in Microsoft Excel (Microsoft Corp.).

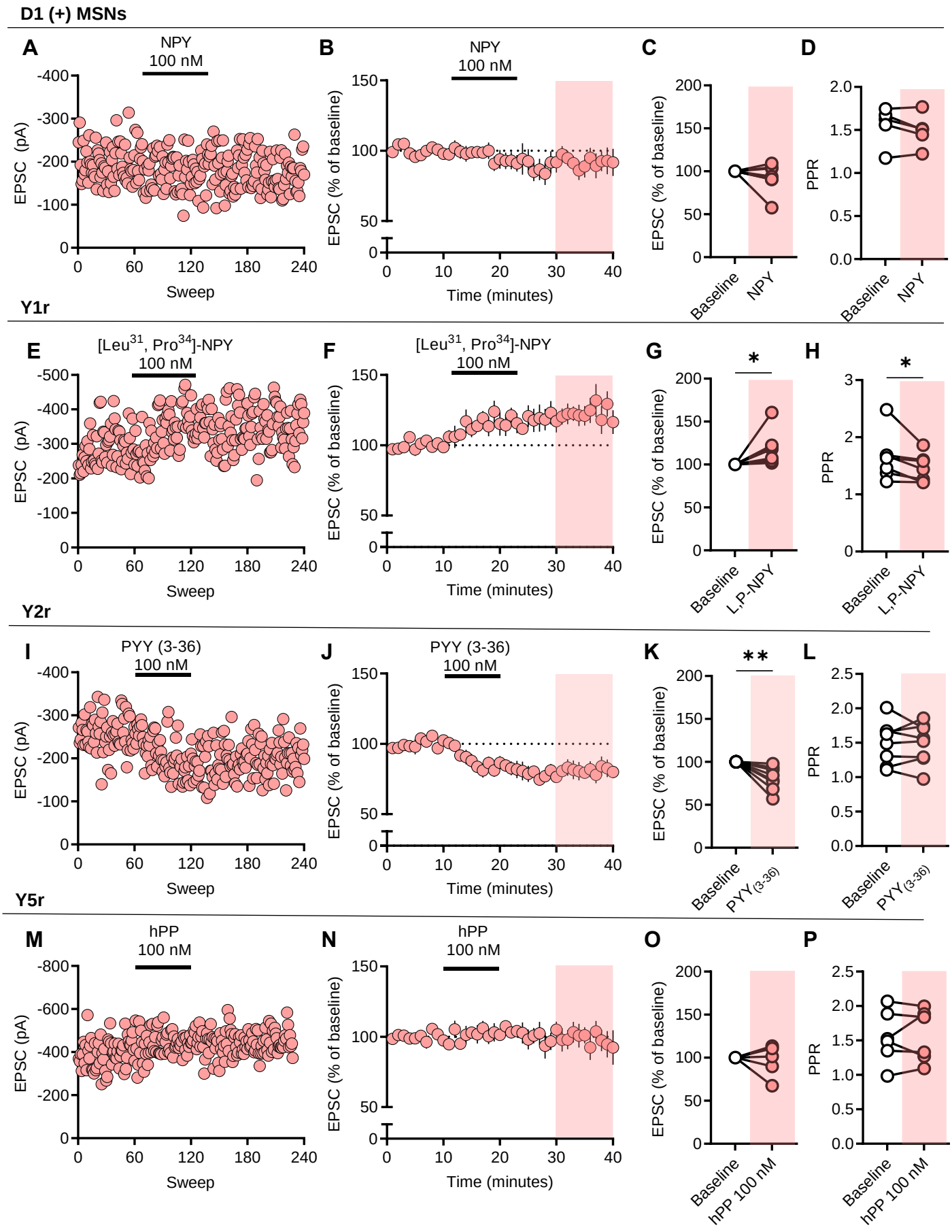
Results

Y1r and Y2r modulate excitatory transmission onto NAcSh D1+ MSNs

To investigate whether NPY modulates excitatory transmission within the NAcSh, we prepared brain slices from *Drd1 tdTomato* reporter mice and performed whole-cell patch clamp electrophysiology. Acute bath application of NPY (Fig. 24A-D) to brain slices containing the NAcSh resulted in no change in average excitatory post synaptic current (EPSC) amplitude (92.9±6.5% of baseline, n=7 cells, paired t-test, $t_{(6)}$=1.104, p=0.312) or 50 ms paired pulse ratio (mean difference of -0.06±0.04, n=5 cells, paired t-test, $t_{(4)}$=1.525, p=0.202) (PPR) in D1+ NAcSh MSNs. However, the NAc contains multiple NPY receptors, including the Y1r (Kopp et al., 2002; Van Den Heuvel et al., 2015; Wolak et al., 2003), Y2r (Stanić et al., 2006), and Y5r (Wolak et al., 2003), creating the possibility of multiple opposing mechanisms. Indeed, we found that acute application of the Y1r selective agonist [Leu^{31},Pro^{34}]-NPY (Fig. 24E-H) resulted in an increase in electrically evoked EPSC amplitude on D1+ MSNs (119.4±7.5% of baseline, n=7 cells, paired t-test, $t_{(6)}$=2.572, p=0.042), and a decrease in paired pulse ratio (PPR) (mean difference of -0.197±0.08, n=7 cells, paired t-test, $t_{(6)}$=2.518, p=0.045). In contrast, application of the Y2r selective agonist PYY(3-36) (Fig. 24I-L) resulted in a decrease in average EPSC amplitude on D1+ MSNs (80.9±4.8% of baseline, n=8 cells, paired t-test, $t_{(7)}$=3.944, p=0.006), also without any change in PPR (mean difference of -0.01±0.06, n=8 cells, paired t-test, $t_{(7)}$=0.139, p=0.893). Application of the Y5r selective agonist [cPP$^{1-7}$,NPY$^{19-23}$,Ala^{31},Aib^{32},Gln^{34}] – hPancreatic Polypeptide (hPP) (Fig. 24M-1P) caused no change in EPSC amplitude (99.1±7.3% of baseline, n=6 cells, paired t-test, $t_{(5)}$=0.122, p=0.907) or PPR (mean difference of 0.02±0.08, n=6 cells, paired t-test, $t_{(5)}$=0.224, p=0.831).

We found the effect of [Leu^{31}, Pro^{34}]-NPY was due to activation of the Y1r, as application of the Y1r antagonist BIBO 3304 blocked the increase in EPSC amplitude caused by [Leu^{31}, Pro^{34}]-NPY (Fig. 25A-C). In the presence of BIBO 3304, application of [Leu^{31}, Pro^{34}]-NPY resulted in a

decrease in EPSC amplitude (85.1±3.7% of baseline, n=5 cells, paired t-test, $t_{(4)}$=3.987, p=0.016) and no change in PPR (mean difference of -0.07±0.07, n=5 cells, paired t-test, $t_{(4)}$=1.02, p=0.364). This Y1r antagonist insensitive decrease in EPSC amplitude suggests an off target effect of [Leu^{31}, Pro^{34}]-NPY. Similarly, application of the Y2r antagonist BIIE 0246 (Fig. 25D-E) blocked the decrease in EPSC amplitude caused by PYY (3-36) (98.2±4.6% of baseline, n=5 cells, paired t-test, $t_{(4)}$=0.395, p=0.713). Together, these results suggest that in D1+ MSNs, excitatory transmission is enhanced presynaptically by Y1r, and depressed by Y2r.


D1 (+) MSNs
A
NPY
100 nM
EPSC (pA)
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B
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Time (minutes)
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hPP 100 nM
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Baseline
hPP 100 nM

Figure 24 | Excitatory transmission onto D1+ MSNs is enhanced by the Y1r agonist [Leu31, Pro34]-NPY and depressed by the Y2r agonist PYY (3-36)

(A) Representative time course of EPSC amplitude recorded from a D1+ MSN following NPY application. **(B)** Average EPSC amplitude in D1+ MSNs following NPY application. **(C)** NPY application does not change average EPSC amplitude (shaded area) in D1+ MSNs compared to baseline. **(D)** NPY application does not change average PPR (shaded area) in D1+ MSNs compared to baseline. **(E)** Representative time course of EPSC amplitude recorded from a D1+ MSN following [Leu^{31}, Pro^{34}]-NPY application. **(F)** Average EPSC amplitude in D1+ MSNs following [Leu^{31}, Pro^{34}]-NPY application. **(G)** [Leu^{31}, Pro^{34}]-NPY application increases average EPSC amplitude (shaded area) in D1+ MSNs compared to baseline. **(H)** [Leu^{31}, Pro^{34}]-NPY application decreases average PPR (shaded area) in D1+ MSNs compared to baseline. **(I)** Representative time course of EPSC amplitude recorded from a D1+ MSN following PYY (3-36). **(J)** Average EPSC amplitude in D1+ MSNs following PYY (3-36) application. **(K)** PYY (3-36) application decreases average EPSC amplitude (shaded area) in D1+ MSNs compared to baseline. **(L)** PYY (3-36) application does not change average PPR (shaded area) in D1+ MSNs compared to baseline. **(M)** Representative time course of EPSC amplitude recorded from a D1+ MSN following hPP application. **(N)** Average EPSC amplitude in D1+ MSNs following hPP application. **(O)** hPP application does not change average EPSC amplitude (shaded area) in D1+ MSNs compared to baseline. **(P)** hPP application does not change average PPR (shaded area) in D1+ MSNs compared to baseline.

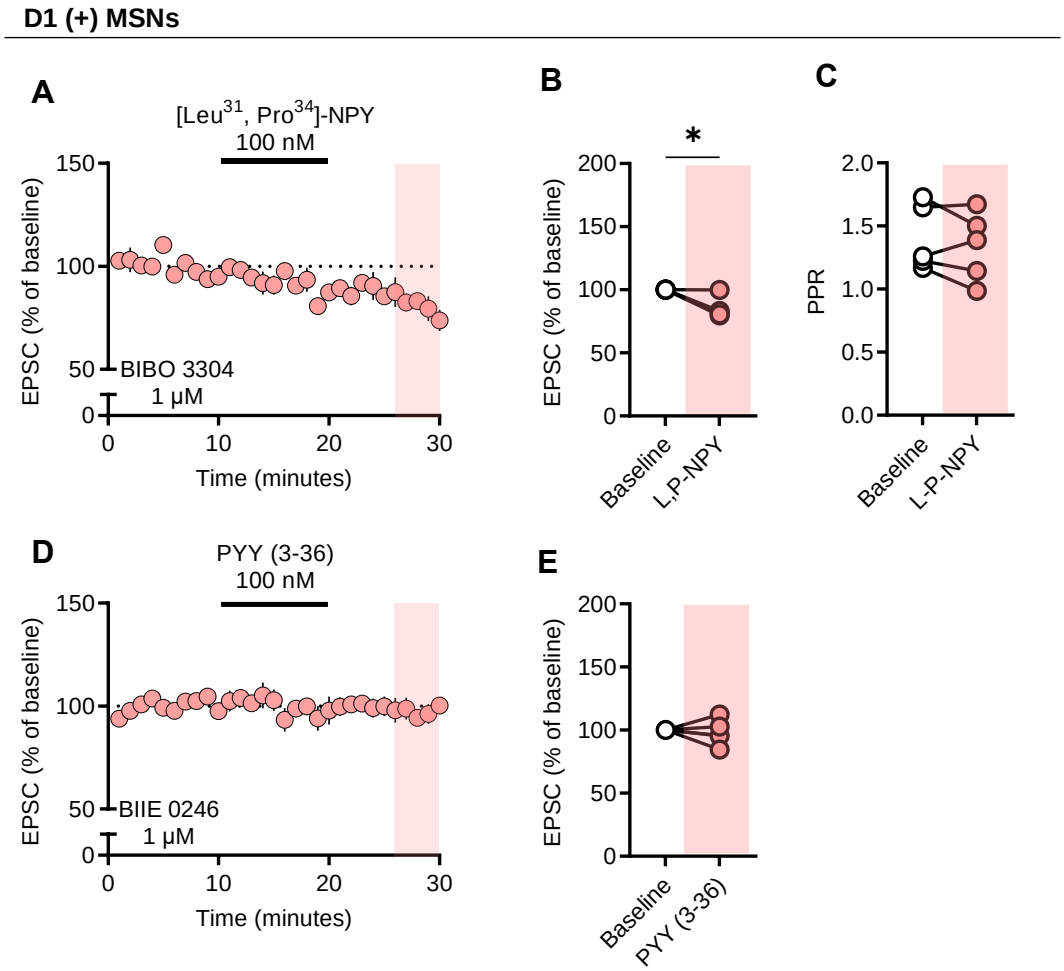


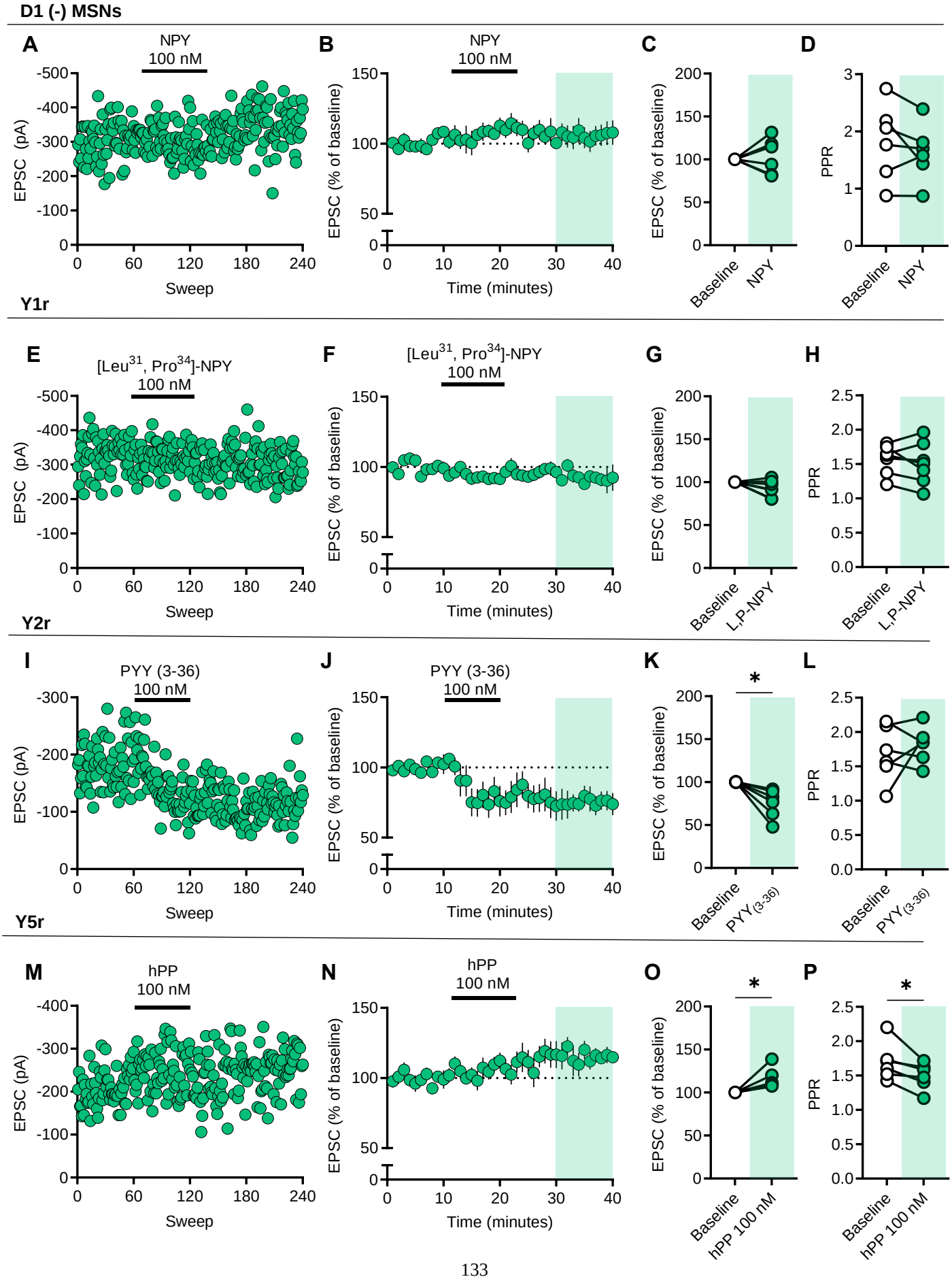
Figure 25 | [Leu31, Pro34]-NPY mediated enhancement of excitatory transmission onto D1+ MSNs requires Y1r, and PYY (3-36) mediated depression of excitatory transmission onto D1+ MSNs requires Y2r

(A) Average EPSC amplitude in D1+ MSNs following [Leu^{31},Pro^{34}]-NPY application in the presence of BIBO 3304.**(B)** [Leu^{31},Pro^{34}]-NPY application does not change average EPSC amplitude (shaded area) in D1+ MSNs compared to baseline in the presence of BIBO 3304.**(C)** [Leu^{31},Pro^{34}]-NPY application does not change average PPR (shaded area) in D1+ MSNs compared to baseline in the presence of BIBO 3304.**(D)** Average EPSC amplitude in D1+ MSNs following PYY (3-36) application in the presence of BIIE 0246.**(E)** PYY (3-36) application does not change average EPSC amplitude (shaded area) in D1+ MSNs compared to baseline in the presence of BIIE 0246.**(F)** PYY (3-36) application does not change average PPR (shaded area) in D1+ MSNs compared to baseline in the presence of BIIE 0246.

Y2r and Y5r modulate excitatory transmission onto NAcSh D1– MSNs

In order to assess the effect of NPY on D2+ MSNs, we recorded from MSNs that did not exhibit expression of Drd1 tdTomato (D1- MSNs), which correspond to the D2+ MSN population. Similar to D1+ MSNs, we found that acute application of NPY (Fig. 26A-D) resulted in no change to average EPSC amplitude (105.6±7.5% of baseline, n=7 cells, paired t-test, $t_{(6)}$=0.743, p=0.485) or PPR (mean difference of -0.19±0.14, n=6 cells, paired t-test, $t_{(5)}$=1.346, p=0.236) in D1- MSNs. In D1- MSNs, [Leu^{31}, Pro^{34}]-NPY (Fig. 26E-H) also had no effect on average EPSC amplitude (92.5±3.6% of baseline, n=7 cells, paired t-test, $t_{(6)}$=2.081, p=0.083) or PPR (mean difference of -0.06±0.07% of baseline, n=7 cells, paired t-test, $t_{(6)}$=0.935, p=0.386). PYY (3-36) application (Fig. 26I-L) resulted in a decrease in EPSC amplitude (75.2±6.9% of baseline, n=6 cells, paired t-test, $t_{(5)}$=3.597, p=0.016) and no change in PPR (mean difference of 0.13±0.17, n=6 cells, paired t-test, $t_{(5)}$=0.732, p=0.496) in D1- MSNs. In D1- MSNs, application of hPP (Fig. 26M-P) caused an increase in EPSC amplitude (115.7±5.0% of baseline, n=6 cells, paired t-test, $t_{(5)}$=3.126, p=0.026), and a decrease in PPR (mean difference of -0.203±0.64, n=6 cells, paired t-test, $t_{(5)}$=3.179, p=0.025).

Similar to D1+ MSNs, the effect of PYY (3-36) in D1- MSNs was blocked by applying the Y2r antagonist BIIE 0246. Application of PYY (3-36) in the presence of BIIE 0246 (Fig. 27A-B) had no effect on EPSC amplitude in D1- MSNs (109.1±4.3% of baseline, n=5 cells, paired t-test, $t_{(4)}$=2.13, p=0.10). Application of the Y5 antagonist L-152,804 blocked the effect of hPP (Fig. 27 C-E) on both EPSC amplitude (97.9±2.5% of baseline, n=5 cells, paired t-test, $t_{(4)}$=0.83, p=0.45) and PPR (mean difference of -0.06±0.04, n=5 cells, paired t-test, $t_{(4)}$=1.41, p=0.23) in D1- MSNs. Thus, in D1- MSNs excitatory transmission is enhanced presynaptically by Y5r, and depressed by Y2r.


D1 (-) MSNs
A
NPY
100 nM
EPSC (pA)
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NPY
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EPSC (% of baseline)
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EPSC (% of baseline)
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NPY
D
PPR
Baseline
NPY
Y1r
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[Leu31, Pro34]-NPY
100 nM
EPSC (pA)
Sweep
F
[Leu31, Pro34]-NPY
100 nM
EPSC (% of baseline)
Time (minutes)
G
EPSC (% of baseline)
Baseline
L,P-NPY
H
PPR
Baseline
L,P-NPY
Y2r
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100 nM
EPSC (pA)
Sweep
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PYY (3-36)
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EPSC (% of baseline)
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PYY(3-36)
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PPR
Baseline
PYY(3-36)
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hPP
100 nM
EPSC (pA)
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N
hPP
100 nM
EPSC (% of baseline)
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O
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hPP 100 nM
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hPP 100 nM

Figure 26 | Excitatory transmission onto D1- MSNs is depressed by the Y2r agonist PYY (3-36) and enhanced by Y5r agonist hPP

(A) Representative time course of EPSC amplitude recorded from a D1- MSN following NPY application. **(B)** Average EPSC amplitude in D1- MSNs following NPY application. **(C)** NPY application does not change average EPSC amplitude (shaded area) in D1- MSNs compared to baseline. **(D)** NPY application does not change average PPR (shaded area) in D1- MSNs compared to baseline. **(E)** Representative time course of EPSC amplitude recorded from a D1- MSN following [Leu^{31}, Pro34]-NPY application. **(F)** Average EPSC amplitude in D1- MSNs following [Leu^{31}, Pro34]-NPY application. **(G)** [Leu^{31}, Pro34]-NPY application does not change average EPSC amplitude (shaded area) in D1- MSNs compared to baseline. **(H)** [Leu^{31}, Pro34]-NPY application does not change average PPR (shaded area) in D1- MSNs compared to baseline. (I) Representative time course of EPSC amplitude recorded from a D1- MSN following PYY (3-36) application. **(J)** Average EPSC amplitude in D1- MSNs following PYY (3-36) application. (K) PYY (3-36) application decreases average EPSC amplitude (shaded area) in D1- MSNs compared to baseline. (L) PYY (3-36) application does not change average PPR (shaded area) in D1- MSNs compared to baseline. (M) Representative time course of EPSC amplitude recorded from a D1- MSN following hPP application. (N) Average EPSC amplitude in D1- MSNs following hPP application. (O) hPP application increases average EPSC amplitude (shaded area) in D1- MSNs compared to baseline. (P) hPP application decreases average PPR (shaded area) in D1- MSNs compared to baseline.

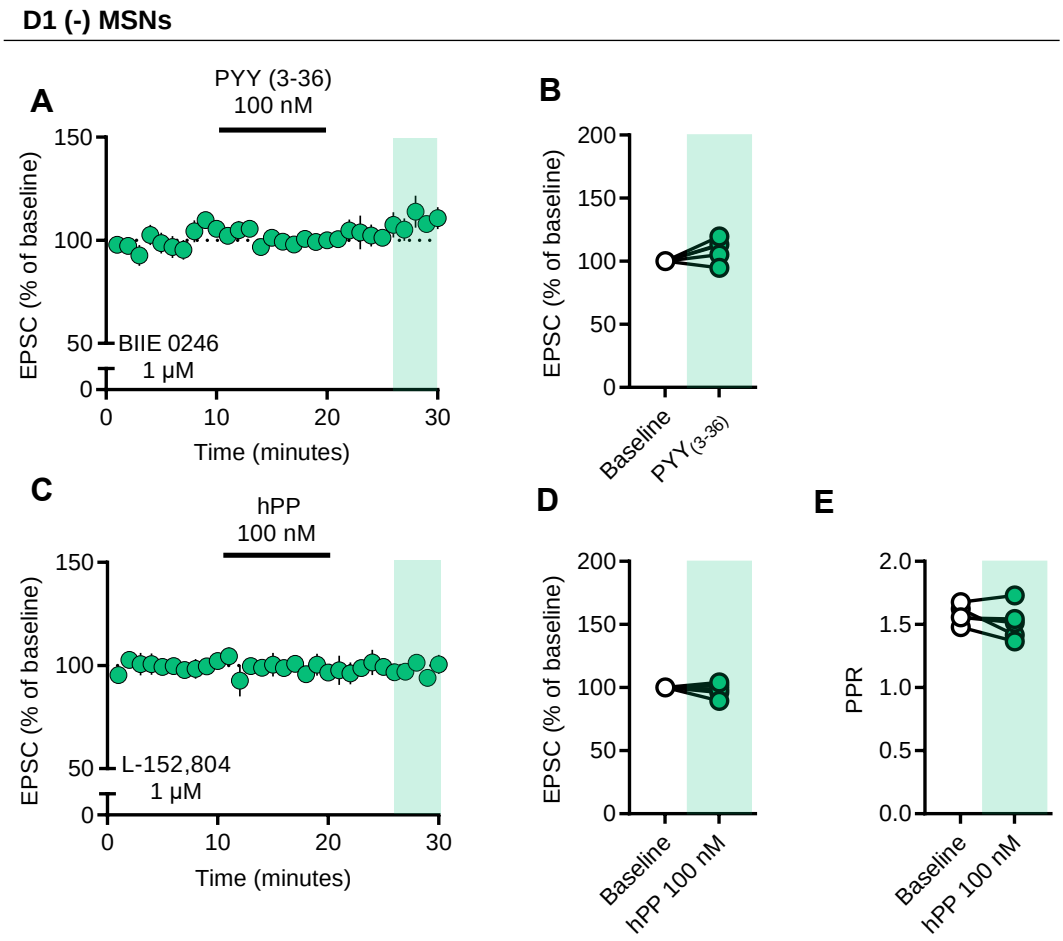

Figure 27 | PYY (3-36) mediated decrease in EPSC amplitude onto D1 – MSNs requires Y2r and hPP mediated enhancement in EPSC amplitude onto D1- MSNs requires Y5r

(A) Average EPSC amplitude in D1- MSNs following PYY (3-36) application in the presence of BIIE 0246. **(B)** PYY (3-36) application does not change average EPSC amplitude (shaded area) compared to baseline in the presence of BIIE 0246. (C) Average EPSC amplitude in D1- MSNs following hPP application in the presence of L-152,804. (D) hPP application does not change average EPSC amplitude (shaded area) compared to baseline in the presence of L-152,804. (E) hPP application does not change average PPR (shaded area) compared to baseline in the presence of L-152,804.

Intra-NAcSh NPY and [Leu^{31}, Pro^{34}]-NPY stimulates social interaction

Based on the effect of NPY on NAc excitatory transmission, we questioned whether administration of NPY to the NAc would alter social interaction. We surgically implanted NAc shell targeted cannula (Fig. 28A) and infused NPY (94 pmol/ 200nL per side) into the NAc of male mice 10 minutes prior to interaction with a novel conspecific in a three chamber apparatus (Fig. 28B). Treatment with NPY significantly increased locomotion in the social interaction apparatus during the pretest period (Fig. 28C) (mean difference of 906.2±264.9 cm, n=7 animals, paired t-test, $t_{(6)}$=3.42, p=0.01). NPY treatment resulted in a significant change in interaction time (Fig. 28D, n=7 animals, 2-way RM ANOVA, Drug: $F_{(1,12)}$=15.30, p=0.002). We found that animals treated with NPY engaged in significantly more social interaction compared to vehicle treated animals (mean difference of 109.9 seconds, Sidak's multiple comparisons, $t_{(24)}$=3.733, p=0.002) without altering interaction with a non-social stimulus (mean difference of 21.92 seconds, Sidak's multiple comparisons, $t_{(24)}$=0.745, p=0.713). Additionally, we found that NPY treatment significantly changed the average interaction bout size (Fig. 28E, n=7 animals, 2-way RM ANOVA, Drug: $F_{(1,12)}$=6.493, p=0.026). This change was driven by a significant increase in the average social interaction bout size (mean difference of 2.91 seconds, Sidak's multiple comparisons, $t_{(24)}$=2.923, p=0.015) without any change in non-social interaction bout size (mean difference of 0.39 seconds, Sidak's multiple comparisons, $t_{(24)}$=0.390, p=0.910). Thus, NPY infusion to the NAc enhances locomotion and increases social interaction.

Y1r enhances excitatory transmission onto D1+ MSNs (Fig. 24E-G), and activity in D1+ MSNs increases social interaction (Gunaydin et al., 2014), we hypothesized that activating NAc Y1r would enhance social interaction. Infusion of the Y1r agonist, [Leu^{31}, Pro^{34}]-NPY (94 pmol/ 200nL per side), into the NAcSh did not change locomotion in the social interaction apparatus (Fig. 28F)

(mean difference of -170.5±280.3 cm, n=7 animals, paired t-test, $t_{(6)}$=0.61, p=0.57). Treatment with [Leu^{31}, Pro^{34}]-NPY significantly changed interaction time with a novel conspecific (Fig. 28G, n=7 animals, 2-way RM ANOVA, Drug: $F_{(1\text{-}12)}$=11.42, p=0.006). [Leu^{31}, Pro^{34}]-NPY treatment significantly increased social interaction with a novel conspecific (mean difference 110.4 seconds, Sidak's multiple comparisons, $t_{(24)}$=4.256, p=0.001) without any change in interaction with the non-social stimulus (mean difference 0.829 seconds, Sidak's multiple comparisons, $t_{(24)}$=0.032, p=0.999). Again, we found this increase is accompanied by a change in the average interaction bout size (Fig. 28H, n=7 animals, 2-way RM ANOVA, Drug: $F_{(1,12)}$=8.51, p=0.013). Treatment with [Leu^{31}, Pro^{34}]-NPY significantly increased the size of an average interaction bout with the social stimulus (mean difference of 8.344 seconds, Sidak's multiple comparisons, $t_{(24)}$=4.458, p=0.0003) without changing the size of an average interaction bout with the non-social stimulus (mean difference of 0.174 seconds, Sidak's multiple comparisons, $t_{(24)}$=0.093, p=0.995).

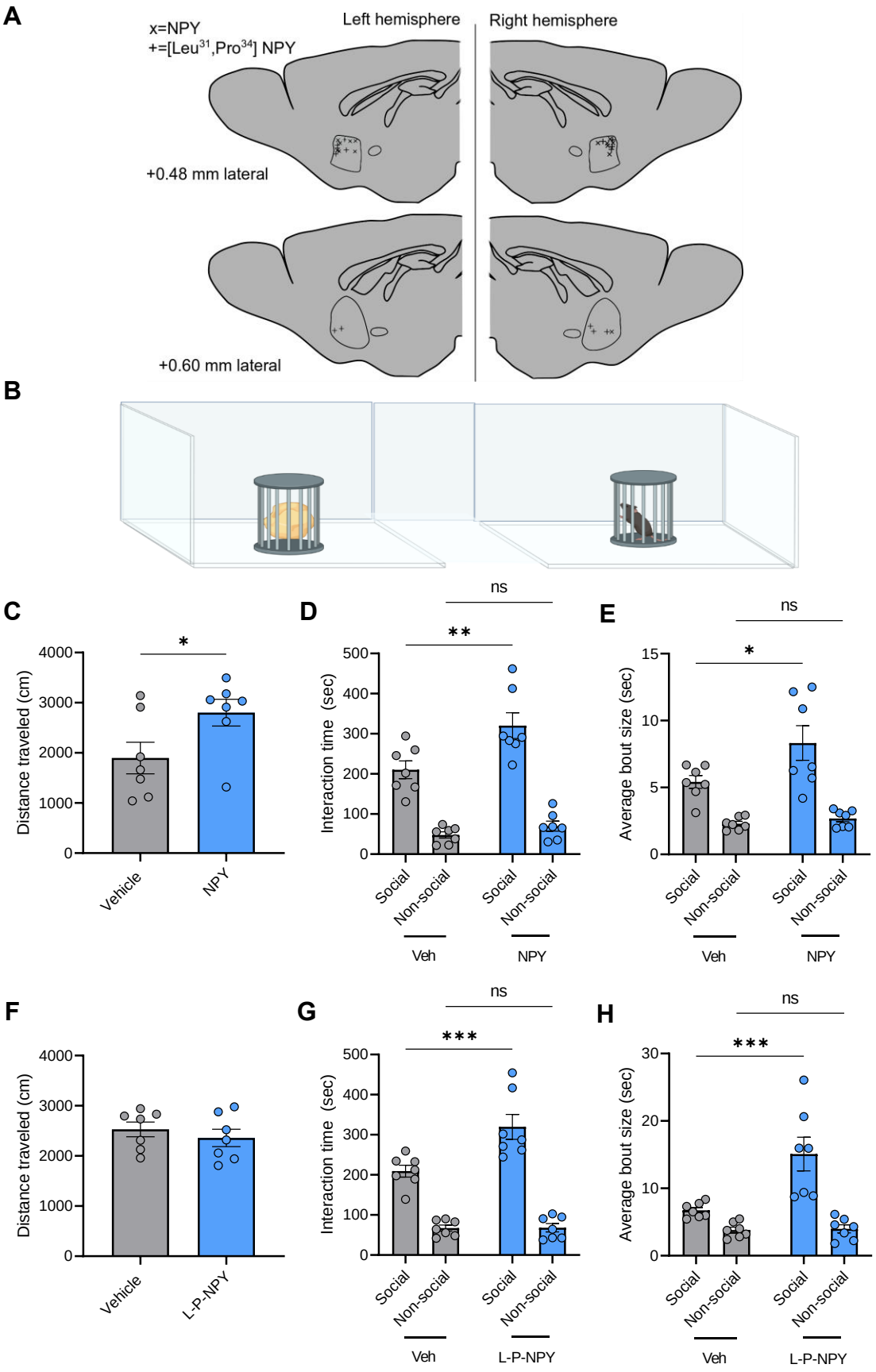
A
x=NPY
+=[Leu31,Pro34] NPY
Left hemisphere
Right hemisphere
+0.48 mm lateral
+0.60 mm lateral
B
C
Distance traveled (cm)
Vehicle
NPY
D
Interaction time (sec)
ns
**
Social
Non-social
Veh
NPY
E
Average bout size (sec)
ns
*
F
Distance traveled (cm)
Vehicle
L-P-NPY
G
Interaction time (sec)
ns
***
Veh
L-P-NPY
H
Average bout size (sec)
ns
***

Figure 28 | Intra-NAc NPY and [Leu31, Pro34]-NPY enhance social interaction time with an unfamiliar conspecific

(A) Diagram of identified cannula termination sites. **(B)** Diagram of three chamber social interaction assay, created with BioRender. **(C)** Intra-NAc NPY increases distance traveled in the pretest period. **(D)** Intra-NAc NPY increases interaction time with the social stimulus relative to vehicle treatment but does not increase interaction time with the non-social stimulus. **(E)** Intra-NAc NPY increases the average size of an interaction bout with the social stimulus relative to vehicle treatment but does not increase the average size of an interaction bout with the non-social stimulus. **(F)** Intra-NAc [Leu^{31}, Pro34]-NPY does not alter the distance traveled during the pretest period. **(G)** Intra-NAc [Leu^{31}, Pro34]-NPY increases interaction time with the social stimulus relative to vehicle treatment but does not increase interaction time with the non-social stimulus. **(H)** Intra-NAc [Leu^{31}, Pro34]-NPY increases the average size of an interaction bout with the social stimulus relative to vehicle treatment but does not increase the average size of an interaction bout with the non-social stimulus.

DPP-IV inhibition shifts the balance between NPY receptors

Because NPY caused no effect on excitatory transmission alone, but specific receptor agonists caused opposing changes in excitatory transmission, we wondered whether we could shift the balance of NPY receptor activation to encourage a specific phenotype. Once released into the extracellular space, neuropeptide transmission is terminated through breakdown of the peptide by peptidases. Degradation of neuropeptides has been shown to acutely regulate their modulatory effects on synaptic neurotransmission (Saleh et al., 1996). NPY is degraded by dipeptidyl peptidase-IV (DPP-IV), an enzyme which has been targeted therapeutically because it also breaks down incretin hormones like glucagon-like peptide 1 (GLP-1). Interestingly, cleavage of NPY by DPP-IV results in a truncated peptide NPY (3-36), which exhibits a significantly different pharmacological profile than native NPY (Fig. 29A). Specifically, NPY (3-36) exhibits a higher specificity for Y2r and Y5r, and a lowered affinity for Y1r. A single brain region can contain multiple NPY receptors, expressed in the same or different cells, sometimes mediating opposing effects (Fu et al., 2004; Gilpin, 2012; Kash and Winder, 2006; Pleil et al., 2015). Inhibition of DPP-IV should reduce production of the Y2r and Y5r selective agonist NPY (3-36), potentially reducing the activation of these receptors. However, whether the degradation of NPY by DPP-IV contributes to the balance between these receptors in the brain is unknown.

Due to the opposing regulation of excitatory transmission onto D1+ MSNs by Y1r and Y2r, we tested whether inhibition of DPP-IV could shift this balance to favor the Y1r effect. We applied the DPP-IV inhibitor sitagliptin (Drucker et al., 2007) alone while recording from D1+ MSNs (Fig. 29B-C), and found no change in EPSC amplitude on average (102.0±6.0% of baseline, n=7 cells, paired t-test, $t_{(6)}$=0.33, p=0.75). However, following application of sitagliptin, NPY (Fig. 29D-F) caused an increase in average EPSC amplitude (127.5±10.2% of baseline, n=10 cells, paired t-test,

$t_{(9)}$=2.70, p=0.02) and a decrease in average PPR (mean difference of -0.17±0.05, n=10 cells, paired t-test, $t_{(9)}$=3.211, p=0.011) in D1+ MSNs. Notably, this electrophysiological phenotype mimics that seen in D1+ MSNs following Y1r agonism. Indeed, we found that in the presence of the Y1r antagonist BIBO 3304 and sitagliptin (Fig. 29G-I), NPY no longer caused an increase in EPSC amplitude (90.9±5.5% of baseline, n=6 cells, paired t-test, $t_{(5)}$=1.65, p=0.16) and did not change PPR (mean difference of -0.06±0.04, n=6 cells, paired t-test, $t_{(5)}$=1.145, p=0.21).

D1 (+) MSNs

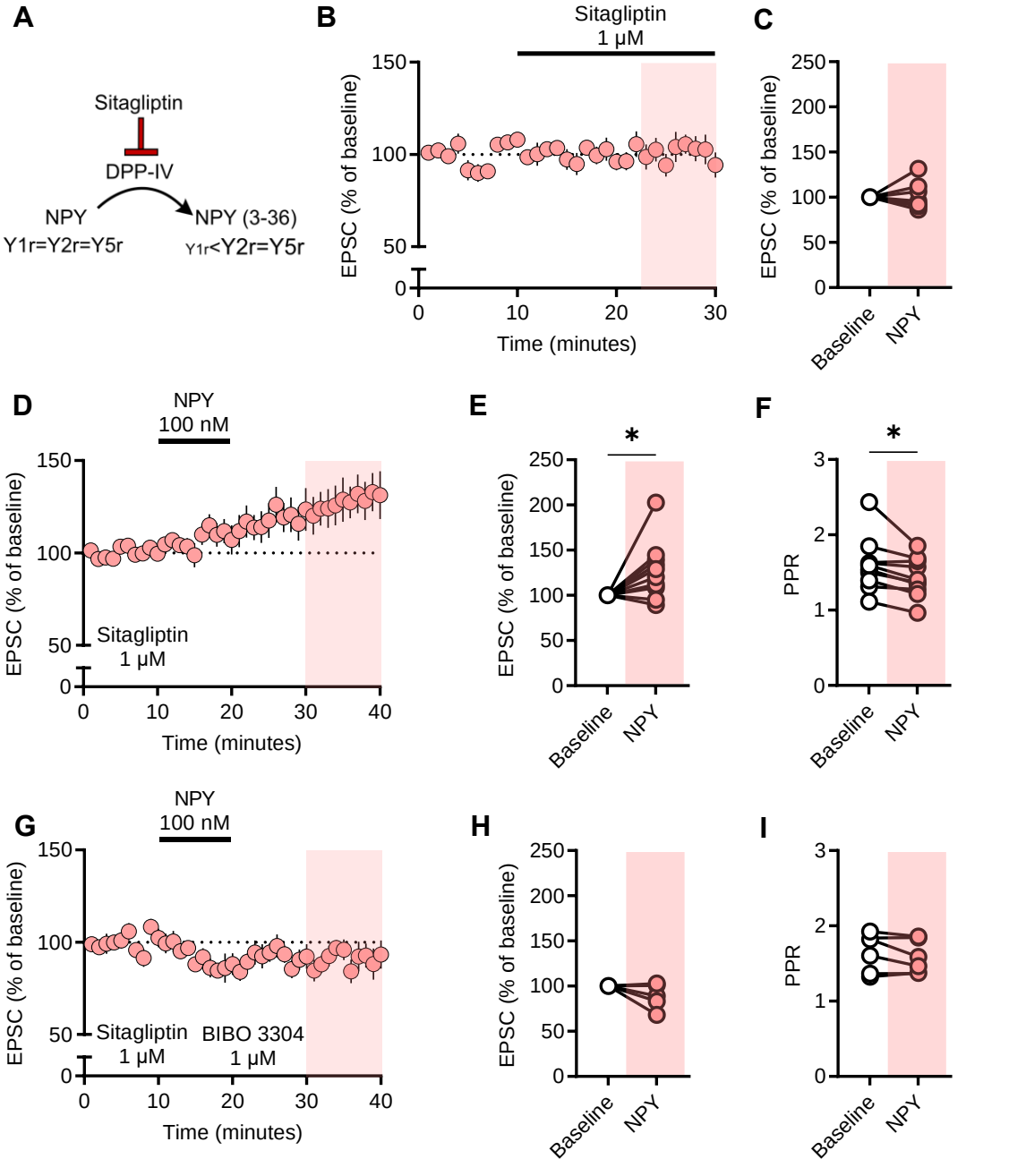
A
Sitagliptin
DPP-IV
NPY
Y1r=Y2r=Y5r
NPY (3-36)
Y1r<Y2r=Y5r
B
Sitagliptin
1 μM
EPSC (% of baseline)
Time (minutes)
C
EPSC (% of baseline)
Baseline
NPY
D
NPY
100 nM
Sitagliptin
1 μM
EPSC (% of baseline)
Time (minutes)
E
EPSC (% of baseline)
Baseline
NPY
F
PPR
Baseline
NPY
G
NPY
100 nM
Sitagliptin
1 μM
BIBO 3304
1 μM
EPSC (% of baseline)
Time (minutes)
H
EPSC (% of baseline)
Baseline
NPY
I
PPR
Baseline
NPY

Figure 29 | Inhibition of DPP-IV with sitagliptin results in a Y1r mediated enhancement of EPSC amplitude in D1+ MSNs following NPY application

(A) Diagram depicting DPP-IV mediated breakdown of NPY, producing NPY (3-36) which exhibits higher affinity for Y2r and Y5r over Y1r. DPP-IV is inhibited by the FDA approved inhibitor sitagliptin. **(B)** Average EPSC amplitude in D1+ MSNs following sitagliptin application. **(C)** Sitagliptin application does not change average EPSC amplitude (shaded area) in D1+ MSNs compared to baseline. **(D)** NPY application increases average EPSC amplitude (shaded area) in D1+ MSNs compared to baseline in the presence of sitagliptin. **(E)** NPY application decreases average PPR (shaded area) in D1+ MSNs compared to baseline in the presence of sitagliptin. **(F)** Average EPSC amplitude in D1+ MSNs following NPY application in the presence of sitagliptin and BIBO 3304. **(G)** NPY application does not change average EPSC amplitude (shaded area) in D1+ MSNs in the presence of both sitagliptin and BIBO 3304. **(H)** NPY application does not change average PPR (shaded area) in D1+ MSNs in the presence of both sitagliptin and BIBO 3304.

Discussion

We describe a multifactorial role of NPY in regulating excitatory synaptic transmission within the NAc. At synapses on D1+ MSNs, we find Y1r mediates a presynaptic potentiation, while Y2r mediates a depression, and Y5r has no discernable effect. Conversely, at D1- MSNs, while our results indicate Y2r also mediates a depression in excitatory transmission, Y1r has no discernable effect and instead Y5r mediates a presynaptic potentiation. We find that these opposing actions of NPY receptors can be manipulated by inhibiting DPP-IV, a protease that breaks down NPY to produce the Y2r and Y5r selective ligand NPY (3-36). Additionally, both NPY and [Leu^{31}, Pro^{34}]-NPY within the NAc are able to enhance social interaction time by increasing the average size of an interaction bout. These results elucidate the multiple receptors by which NPY modulates excitatory transmission across MSN cell types. Further, they identify DPP-IV inhibition as a pharmacological strategy to bias the balance of these receptors towards a therapeutic goal.

The role of NPY in promoting social behavior is highly conserved across the animal kingdom, and in mammals multiple brain regions have been implicated in contributing to this phenotype (Kask et al., 2001; Sajdyk et al., 1999; Villarroel et al., 2018). Adding to this literature, we find that intra-NAc infusion of NPY or a Y1r agonist increase social interaction. Previous research has described the prosocial effects of NPY as stemming from its anxiolytic properties (Desai et al., 2014; Reichmann and Holzer, 2016; Sajdyk et al., 1999). NAc NPY may act in a similar manner, as activation of NAc NPY-expressing neurons results in an anxiolytic phenotype (Yamada et al., 2020). Beyond anxiety, it is also possible that the increase in social interaction seen following intra-NAc NPY infusion results from an increase in the rewarding properties of the encounter. Infusion of NPY into the NAc alone is rewarding and is able to support a conditioned place preference (Brown et al., 2000). Further, intra-NAc infusion of NPY increases consumption of

highly rewarding foods, an effect that is blocked by Y1r antagonism (Van Den Heuvel et al., 2015). We find that Y1r agonism increases excitatory transmission onto D1+ MSNs, and artificial stimulation of these NAc excitatory inputs is rewarding and reinforces instrumental behavior (Britt et al., 2012). In addition to regulating excitatory synaptic transmission, ICV and intra-NAc NPY are both able to increase dopamine within the NAc (Quarta et al., 2011; Sørensen et al., 2009). Y1r immunoreactivity has been found in the NAc at non-synaptic terminals containing small clear vesicles, suggesting a direct role for Y1r in regulating dopamine release (Aoki and Pickel, 1988; Quarta et al., 2011; Sørensen et al., 2009). Thus, NAc NPY is likely able to influence reward via multiple mechanisms. Future studies should investigate how changes to reward and anxiety downstream of NAc NPY contribute to its positive modulation of social behavior.

DPP-IV inhibition is used to manipulate GLP-1 degradation in the treatment of type 2 diabetes, and the drug sitagliptin is FDA approved for this purpose (Drucker et al., 2007). However, DPP-IV catalyzes the transformation of a number of peptides, including the endogenous NPY receptor ligands NPY and PYY (Mentlein, 1999). The removal of the amino terminal dipeptide in both cases yields a pharmacologically distinct compound, NPY 3-36 and PYY 3-36 respectively, that retain their efficacy at Y2r while losing efficacy at Y1r (Grandt et al., 1996). NPY (3-36) is abundant in the brain, where it is reported to account for 35% of all NPY immunoreactivity (Grandt et al., 1996). In the present study, we find that DPP-IV regulates the response to exogenous NPY, potentially by modulating the production of NPY 3-36. Inhibition of this degradation likely results in a reduction in the activation of Y2r, shifting the balance of NPY's effect on excitatory transmission to a more Y1r dominated phenotype. It is intriguing to speculate that DPP-IV also modulates endogenous NPY neurotransmission, and thus that regulation of DPP-IV expression and function would then alter the character of NPY signaling in the NAc.

The NPY system is classically considered to exert inhibitory influence based on the consistent coupling of NPY receptors to the $G\alpha_i$ g protein (Lin et al., 2004). Postsynaptically, NPY most commonly activates GIRK channels to hyperpolarize neurons, a function that is frequently attributed to Y1r (Acuna-Goycolea et al., 2005; Fu et al., 2004; Roseberry et al., 2004; Sun et al., 2001a, 2001b; Van Den Pol et al., 2004; West and Roseberry, 2017). However, Y1r also acts postsynaptically to enhance GABA transmission in both the basolateral amygdala and the bed nucleus of the stria terminalis (Molosh et al., 2013; Pleil et al., 2015). We similarly report enhanced neurotransmission, albeit glutamatergic transmission, in D1+ MSNs following Y1r activation and in D1- MSNs following Y5r activation. NPY receptors have been found in many brain regions to modulate presynaptic release of neurotransmitters, however this is typically an inhibitory interaction (Acuna-Goycolea et al., 2005; Chen and Van Den Pol, 1996; Cowley et al., 1999; Fu et al., 2004; Gilpin et al., 2011; Kash and Winder, 2006; Sun et al., 2001a; Van Den Pol et al., 2004; West and Roseberry, 2017). Thus, our results report a relatively unique presynaptic enhancement caused by NPY. The signaling mechanism underlying this phenotype is yet unclear. It is possible this results from a direct action of a presynaptic receptor, or through the regulation of another neuromodulator. Regardless, these mechanisms may underlie reports of NPY increasing extracellular glutamate, as measured by microdialysis (Meurs et al., 2012). These results indicate an excitatory neuromodulatory role for NAc NPY.

Expression of Y1r, Y2r, and Y5r has been reported in the NAc. Y1r immunoreactivity has been identified presynaptically within the NAc at asymmetric, presumably glutamatergic, synapses (Pickel et al., 1998). However, Y1r mRNA has also been detected within the NAcSh, and has been found to be present in both dynorphin-expressing and enkephalin-expressing neurons (D1+ and D2+ MSNs, respectively) (Kishi et al., 2005; Van Den Heuvel et al., 2015). This indicates the

possibility for additional postsynaptic mechanisms not identified here, including changes to gene expression (Van Den Heuvel et al., 2015). Y2r immunoreactivity has been reported on cell bodies and fibers within the NAc (Stanić et al., 2006). We find that Y2r exerts an inhibitory effect on excitatory transmission onto both D1+ and D1- MSNs, with a lack of effect on PPR. While our results do not indicate a presynaptic role for Y2r, they do not preclude it either. Y5r immunoreactivity is found in the NAc, and binding of a radiolabeled Y5r antagonist is found to a moderate degree within the NAc (Quarta et al., 2011; Wolak et al., 2003). In both the cerebellum and the lateral hypothalamus, Y5r appears to modulate synaptic transmission via a presynaptic effect (Dubois et al., 2012; Fu et al., 2004). Thus, it is possible that like Y1r, Y5r is also expressed on presynaptic terminals in the NAc.

It is important to note that this study focused on the effect of NPY on excitatory transmission in the NAc shell. NPY likely modulates diverse neurotransmitter systems within the NAc beyond excitatory transmission. Further, while this study has focused on the role of NPY in the NAc shell, NPY interneurons and NPY receptors are also found in the NAc core (Chen et al., 2021). Future studies should address the contributions of NPY to the neural computations and behavioral outcomes of that accumbens subregion, and further investigate how NPY modulate other modes of neurotransmission.

In conclusion, our results depict the diverse ability of the NPY neuromodulatory signal to shape excitatory transmission across MSN cell types in the NAc shell. We find that intra-NAc NPY promotes social interaction with an unfamiliar conspecific. In addition to contributing to our understanding of NPY as a neuromodulator, we repurpose an existing therapeutic to shift the physiological response to NPY, identifying a novel strategy for shaping NPY signaling. We

anticipate that this strategy can be used to better understand NPY's role in the brain in future studies, as well as the regulation of neuropeptide signaling as a whole.

Chapter IV

Conclusions and future directions

Neuropeptides are complex regulators of neural function, commonly acting through multiple receptors to modulate different nodes across a nucleus (van den Pol, 2012). Here, I detail findings identifying novel electrophysiological phenotypes caused by two neuropeptides, BigLEN and neuropeptide Y (NPY). Additionally, I present results of behavioral experiments linking the actions of these peptides within the nucleus accumbens to food-seeking behavior and social interaction, respectively. Beyond the specific results of these studies, I anticipate that the methodology used here informs on future studies of neuropeptides. In the study of BigLEN, we found that a long-term incubation was required to observe an electrophysiological phenotype, similar to previous work with the melanocortin system (Lim et al., 2012). In the study of NPY, we found that the individual analysis of receptor subtypes was required to break down the components of the response to exogenous NPY. Further, we found that the character of this electrophysiological phenotype depended on the breakdown of NPY by dipeptidyl peptidase IV, which produces a pharmacologically distinct neuropeptide, neuropeptide Y (3-36). Peptidergic signaling in the brain is a diverse pool of untapped potential. The two neuropeptide systems studied here exemplify diverse mechanisms and behaviors within the NAc and inform future studies of novel peptidergic signaling.

Hunger dampens a nucleus accumbens circuit to drive persistent food seeking

In this study, we identified BigLEN, a hunger-driven neuropeptide, as able to suppress excitatory transmission onto D1+ medium spiny neurons (MSNs) in the nucleus accumbens shell. We find that this suppression requires GPR171 and occurs presynaptically, but depends on

cholinergic signaling through nAChRs. This likely occurs through a BigLEN mediated inhibition of cholinergic interneurons, which we find express GPR171. Further, this mechanism is present at synapses onto MSNs that project to the lateral hypothalamus, but is not present at synapses onto MSNs that project to the ventral tegmental area. Based on the role of the nucleus accumbens shell in behavioral inhibition and the control of feeding, we hypothesized that BigLEN and GPR171 regulate food-seeking behavior. As BigLEN concentrations are elevated in the nucleus accumbens of food-deprived rodents, we examined the ability of food-deprived animals to withhold operant responding when rewards are unexpectedly unavailable (Ye et al., 2017). Antagonizing GPR171 systemically or within the nucleus accumbens resulted in a decrease in the unrewarded responding of food-deprived mice but not fed mice. Conversely, these manipulations had no effect on effortful food seeking in food-deprived mice, measured using the progressive ratio assay. Because BigLEN caused depression of excitatory synaptic transmission in the nucleus accumbens to lateral hypothalamus circuit, we wondered whether upregulation of this circuit would reduce unrewarded responding in food-deprived animals. We first validated that, *ex vivo,* BigLEN does reduce the ability of excitatory synaptic transmission to evoked action potentials in lateral hypothalamus projecting MSNs. Next, we used circuit specific expression of a hM3D(Gq) DREADD to increase action potential generation in the nucleus accumbens to lateral hypothalamus circuit. Administration of CNO to food-deprived animals prior to operant testing resulted in a decrease in unrewarded responding. Together, these results suggest a model where BigLEN acts through GPR171 to inhibit a cholinergic interneuron-mediated potentiation of excitatory synaptic transmission onto a nucleus accumbens to lateral hypothalamus circuit, and this inhibition results in a shift to a more persistent seeking strategy in food-deprived animals faced with a suddenly ineffective food-seeking strategy.

Comparison of behavioral phenotype to studies of drug addiction

The finding that GPR171 antagonism regulated unrewarded food seeking but not effortful food seeking was surprising and highlights the importance of studying multiple aspects of reward-seeking behavior. Models of substance use disorders commonly assess different aspects of drug-seeking behavior in animals (Bock et al., 2013; Deroche-Gamonet et al., 2004). These studies sought to model similarities between features of human addiction to rodent behaviors following experience with addictive drugs. Human patients who exhibit substance dependence have: 1) difficulty stopping drug use or limiting intake, 2) high motivation to obtain and take the drug, and 3) they continue its use despite harmful consequences (Deroche-Gamonet et al., 2004). Translating these phenotypes into operant behaviors, the first phenotype can be mimicked in part by assessing the animal's operant responding during periods of drug unavailability. The second phenotype can be measured by observing operant responses and breakpoint in the progressive ratio assay. The third phenotype is more difficult to model in animals, as many of the most salient negative consequences for people suffering from substance abuse are social and not immediate. However, this phenotype has been modeled using periodic substitution of drug reward with foot shock, requiring animals to endure negative reinforcement in order to obtain drugs. Rodents with experience self-administering cocaine exhibit these three phenotypes (Bock et al., 2013; Deroche-Gamonet et al., 2004). Additionally, animals with diet-induced obesity exhibit similar behavioral phenotypes (Brown et al., 2017). While these phenotypes appear to track together in drug addiction and obesity, they represent distinct aspects of behavior that contribute a unique knowledge to our understanding of these conditions.

Operant phenotypes in the study of feeding behavior

We find that food-deprived animals also display both the 1) difficulty stopping and 2) high motivation phenotypes mentioned above in the context of food seeking, although we do not study 3) compulsive seeking during negative reinforcement. However, other studies of the neural mechanisms of hunger do not typically assess a range of operant parameters, and typically only assess effortful food seeking with the progressive ratio task. This is true for the study of agouti-related peptide neurons, a cell population considered critical for the propagation of hunger signals through the brain (Betley et al., 2015; Krashes et al., 2011). This is also true for studies of ghrelin, a hunger triggered hormone (Bake et al., 2019; Finger et al., 2012; Skibicka et al., 2011). Thus our results are unique not only because they assess an additional dimension of food seeking, unrewarded responding, but also because GPR171 is required for unrewarded responding and not effortful responding. This result indicates that there are separable hunger-driven neural mechanisms contributing to these phenotypes. To my knowledge, this is the first report of a hunger-driven mechanism that modulates behavioral strategy rather than overall consumption or food seeking as a whole. It would be interesting to apply a similar approach to the study of other hunger-driven neural modulators to see if this specificity is common.

If different neural components of hunger encourage separable aspects of motivated behavior, it would likely be possible to create therapeutics that address specific deficits in individual behavior, allowing for more personalized treatment plans. This could lead to a paradigm shift in the study and treatment of disordered eating. The etiology of obesity and eating disorders is highly complex at the population level, and different patients respond differently to available treatments. If we can identify symptoms that indicate deficits in specific aspects of food-seeking or general behavioral strategy, we could use these biomarkers to drive treatment decisions with

precision medicine designed to target those specific symptoms. The key to developing these treatments is increasing our understanding of the endogenous regulators of feeding and food-seeking in the brain. The GPR171 studies reported here show that energy state related modulators do not necessarily exert a global dampening of all food-seeking behavior. I anticipate that this result, and others like it, will change the way we think about and treat disordered eating.

Relevance to eating disorders

While manipulating motivation for food may appear to be the most critical issue when combating disordered eating, patients with these diseases also exhibit deficits in behavioral flexibility. Similar to hungry animals in our task, patients with anorexia nervosa, bulimia nervosa, and binge eating disorder all have difficulty in switching behavioral strategy when given feedback that their current strategy is no longer working (Tchanturia et al., 2012; Zastrow et al., 2009). Importantly, this phenotype is reduced in patients who have recovered from anorexia (Tchanturia et al., 2012). This suggests that either a lower expression of this phenotype at baseline predicts recovery, or that improving behavioral flexibility promotes recovery. If this is true, developing therapeutics that improve behavioral flexibility could be beneficial. Further, it is clear that in eating disorders both food seeking and consumption have become uncoupled from hunger, indicating the mechanisms linking this drive to behavior may be disrupted (Treasure et al., 2020). These ideas point to a possible therapeutic strategy targeting hunger-driven neuromodulatory mechanisms that regulate behavioral flexibility, one that could potentially be targeted via GPR171.

GPR171 expression

We found that GPR171 was widely expressed across the nucleus accumbens, as determined by immunohistochemistry. This expression was found not only in the nucleus accumbens core and

shell, but also in the dorsal striatum and septum (unpublished observations). The frequency at which GPR171 immunoreactivity was observed in nucleus accumbens cell bodies means it is almost certainly expressed in MSNs, as MSNs make up approximately 95% of the cells in the nucleus accumbens. Despite this, we found no evidence of a postsynaptic effect in D1+ MSNs. However, we did find a reduction in sEPSC frequency in D1- MSNs that did not coincide with an increase in PPR. This, along with the likely expression of GPR171 in MSNs, suggests that there may be a postsynaptic effect of GPR171 in D1- MSNs on excitatory synaptic transmission. Additionally, while we did not measure membrane properties in D1- MSNs, we found no change in D1+ MSN membrane properties following BigLEN incubation. Alternatively, GPR171 in MSNs may not only be regulating neural transmission directly, and may exert its influence through another avenue, like regulating gene expression. Regardless, the impressive reach and penetrance of GPR171 expression in striatal regions suggests that it plays an important role in regulating striatal function.

Source of BigLEN

We originally pursued BigLEN and GPR171 in part due to a report in the literature that BigLEN concentrations were elevated in the nucleus accumbens during food deprivation (Ye et al., 2017). This, coupled with the expression of BigLEN in AGRP neurons, and a reported AGRP neuron projection to the nucleus accumbens, led to a model where hunger-driven elevations in AGRP neuron firing resulted in increased BigLEN release in the nucleus accumbens (Gomes et al., 2013; Van Den Heuvel et al., 2015). However, based on my conversations with others who had performed analysis of AGRP terminal fields, AGRP neurons do not appear to synapse in the nucleus accumbens. This is interesting, as other arcuate nucleus cell populations do project to the nucleus accumbens (Lim et al., 2012).

If not AGRP neurons, what is the endogenous source of BigLEN in the nucleus accumbens? BigLEN is widely expressed across the brain, including in multiple regions that provide input to the nucleus accumbens: the hippocampus, amygdala, thalamus, and the hypothalamus (Feng et al., 2001). More puzzling, is how these inputs would be regulated by energy state to provide BigLEN to a greater degree during food deprivation. Among these candidates, the most likely is a thalamic source. The paraventricular nucleus of the thalamus, as mentioned previously in this document, is significantly regulated by energy state (Kirouac, 2015). This input to the nucleus accumbens would even have the ability to be directly regulated by glucose, as multiple cell types within the paraventricular nucleus of the thalamus have been described as glucose sensing (Kessler et al., 2021; Labouèbe et al., 2016). This would be a reasonable place to begin looking for the endogenous source of BigLEN in the nucleus accumbens. However, functional GPR171 has been described in the basolateral amygdala, which also projects to the nucleus accumbens (Bobeck et al., 2017). This raises the possibility of a source of BigLEN within the basolateral amygdala. Any number of these regions could be providing BigLEN to the nucleus accumbens, and due to the widespread expression of BigLEN, it may not be transmitted by a specific single neuronal population (Feng et al., 2001).

Ultimately, identifying the endogenous source would prove quite difficult, without the ability to knockout BigLEN specifically. Currently, the only available reagent for this purpose is a knockout mouse for the precursor protein, ProSAAS (DJ et al., 2010). Knocking out the precursor protein would impact not only the functions of BigLEN and the precursor, which serves as a chaperone for prohormone convertase 1/3, but it would also disrupt other peptides produced from ProSAAS (Fricker et al., 2000). One of these peptides, PEN, acts through GPR83, which is expressed in cholinergic interneurons within the nucleus accumbens and modulates morphine

reward (Fakira et al., 2019). One way to alleviate this issue would be to knockout ProSAAS, and reintroduce a ProSAAS transgene that lacks a functional BigLEN segment. This would allow analysis of the specific deletion of BigLEN from the nucleus accumbens and its input regions.

BigLEN and food deprivation

We describe the effect of systemic GPR171 antagonism on unrewarded food seeking as dependent on energy state, as animals fed *ad-libitum* showed no response to MS21570 administration during operant behavior. We were unable to identify significant synaptic changes in food-deprived animals at nucleus accumbens synapses, despite finding those synapses to be regulated by exogenous application of BigLEN. However, when we applied BigLEN to slices from food-deprived animals, we found no changes in synaptic properties. One way to interpret these results is that in food-deprived animals, GPR171 is already activated, and further application of BigLEN causes no difference. But if that is true, why do the basal excitatory synaptic properties of MSNs from food-deprived animals not match those from slices treated with BigLEN? We cannot answer this question with the data provided here. It is possible that there are multiple mechanisms tuning different aspects of nucleus accumbens physiology in the food-deprived state. This may manifest in input specific differences that proceed in opposite directions, such that analysis of excitatory transmission indiscriminant of input appears devoid of any difference. Indeed, this idea evokes the experiments presented here studying the effect of NPY on nucleus accumbens excitatory transmission. In that case, and this one, I suggest that opposing changes in nucleus accumbens excitatory transmission may be downstream of the same global manipulation. The effect of BigLEN on specific excitatory inputs is an avenue that I have pursued experimentally, and those results are presented later in this chapter. If we consider different excitatory inputs to be

communicating different information, we could conceptualize these opposing actions as a bidirectional rebalancing of the relative strength of distinct information. This complexity is not represented in our model, where excitatory input and MSN output both oppose food seeking. There are likely more energy state-dependent mechanisms that modulate excitatory transmission within the nucleus accumbens.

BigLEN modulation of a cholinergic microcircuit

We found that if slices were pre-incubated in tetrodotoxin to block action potential generation, subsequent incubation in BigLEN caused no change in vesicular release probability. This was a surprising result, as the electrophysiological phenotype caused by BigLEN had seemed like that of a classical presynaptic receptor, which would have been insensitive to tetrodotoxin. However, this finding was foreshadowed by work in the hypothalamus, where the effect of BigLEN on presynaptic properties was blocked by inhibiting postsynaptic g protein signaling (Wardman et al., 2011). Thus it may be possible that BigLEN and GPR171 are typically integrated into local modulatory networks, and initiate change via circuit mechanisms, rather than direct actions.

The indication that BigLEN in the nucleus accumbens relied on action potential generation led to the investigation of a cholinergic mechanism. We reasoned that because cholinergic interneurons are the only tonically firing cell type within the nucleus accumbens, it was possible that silencing them with tetrodotoxin was what made BigLEN incubation ineffective (Tepper et al., 2010). Indeed, we found that nicotinic signaling in the nucleus accumbens shell mediates a potentiation of excitatory transmission, and blocking nAChRs blocks the effect of BigLEN. Further, we found that GPR171 is expressed in cholinergic interneurons, and that application of

BigLEN reduces their action potential firing rate. These results suggest that BigLEN inhibits cholinergic interneurons via GPR171, and this reduces nAChR-mediated presynaptic potentiation.

We focused our analysis on the interaction of GPR171 and nAChRs, however there is still much unexplored regarding nAChR signaling within the nucleus accumbens. While we report that nicotine enhances mEPSC frequency suggesting increased vesicular release probability, as has been reported previously, the extent this potentiation is present at "rest" is unclear (Zhang and Warren, 2002). The basal tone of nAChR activation is likely highly regulated due to the sensitivity of nAChRs to desensitization. In the VTA, concentrations of nicotine achieved by smoking desensitize β2-containing nAChRs, but leave α7 nAChR expression intact(Exley and Cragg, 2008). This ultimately results in reduced β2 phenotypes (direct dopamine release and enhanced GABAergic transmission onto VTA dopamine neurons) but intact α7 phenotypes (increased glutamatergic input to VTA dopamine neurons). Thus, the net effect of nicotine-induced desensitization in this system is an *increase* in nAChR-stimulated dopamine neuron firing (Mansvelder et al., 2002). This example illustrates that the ultimate effect of changes in nicotinic signaling are complex and can depend on multiple receptor subtypes. These dynamics are not as well understood in the nucleus accumbens. The nAChR receptor subtype underlying enhanced glutamate release is unknown, and although α7 containing nAChRs are found on nucleus accumbens glutamatergic terminals there is some evidence that activation of these receptors is not sufficient to enhance extracellular glutamate (Huang et al., 2014; Zappettini et al., 2014). Further studies using receptor subtype specific pharmacology are needed to determine the method of this modulation within the nucleus accumbens.

Using cell-attached recordings, we identified a depression in spontaneous cholinergic interneuron firing rate following BigLEN application, as compared to vehicle application. Further,

we found expression of GPR171 in cells expressing choline acetyltransferase, the enzyme that synthesizes acetylcholine. Notably, this localization of GPR171 in cholinergic interneurons is similar to the expression pattern of GPR83, a de-orphanized receptor for PEN, a peptide produced from the same precursor as BigLEN (Fakira et al., 2019). The results presented here suggest that GPR171, a receptor that has been found to couple to $G\alpha_{i/o}$ in other contexts, inhibits tonic firing of cholinergic interneurons (Gomes et al., 2013; Wardman et al., 2016, 2011). How BigLEN achieves this suppression of cholinergic interneurons is not clear from the data presented here. While the simplest explanation is a direct inhibitory effect of GPR171 on the membrane potential of cholinergic interneurons, similar to the effect found downstream of GPR171 on pyramidal neurons in the BLA, we cannot exclude indirect effects (Bobeck et al., 2017). Further, we have not performed antagonism or cell-type specific knockout experiments to ensure this BigLEN induced phenotype is caused by GPR171. A cell-type specific knockout would be an excellent method to test the contribution of this cholinergic mechanism to the behavioral phenotype of GPR171 described here.

The idea that increased unrewarded operant responding would result from inhibition from striatal cholinergic interneurons is consistent with reports in the literature. The connection between dorsal striatal cholinergic interneurons and behavioral flexibility is well studied (Prado et al., 2017). Disrupting cholinergic function in the dorsal medial striatum reduces the ability of the animal to incorporate changes in action-outcome contingencies, leading to inflexible habitual behavior (Bradfield et al., 2013). Interestingly, the neural components underlying this system in the dorsal medial striatum appear to fail with age, resulting in reduced adaptive goal-directed behavior (Matamales et al., 2016). The contribution of cholinergic interneurons in the nucleus accumbens shell to behavioral flexibility is less well studied. However, lesion of cholinergic

interneurons in the ventral striatum as a whole resulted in increased perseverative errors when animals had to use a novel cue to guide responding on a previously trained task (Aoki et al., 2015). Thus, it is possible that cholinergic interneurons in the nucleus accumbens shell mediate the suppression of inappropriate responding when contingencies change, a role that has been described for the shell generally (Floresco, 2015; Lafferty et al., 2020). However, more targeted manipulations of cholinergic interneurons during different behavioral tasks are needed to fully parse out their role in the nucleus accumbens shell.

Sex differences in BigLEN and related peptides

There are sex differences in phenotypes reported following manipulations to the BigLEN propeptide ProSAAS and other ProSAAS product peptides. Male ProSAAS knockout mice exhibit a 10-15% reduction in body weight compared to wild type controls, while female ProSAAS knockout mice do not (Morgan et al., 2010). Additionally, genetic disruption of GPR83, the deorphanized receptor for the ProSAAS product PEN, results in sex specific changes in morphine reward (Fakira et al., 2019). These results suggest that the ProSAAS system as a whole is likely to differ across sexes. This is further compounded by the idea that cholinergic regulation of the nucleus accumbens independently exhibits sex differences, a concept that effects the ProSAAS system as both GPR171 and GPR83 appear to function via modulation of cholinergic interneurons (Fakira et al., 2019). With this in mind, it is intriguing to speculate that female mice are not simply insensitive to BigLEN, but rather integrate this signal differently, and so testing for the male phenotype is not sufficient to uncover an effect. Turning to behavior, this raises the question of whether female mice exhibit a similar persistence phenotype as is found in male food-deprived mice. Are female mice more likely to persist when food-deprived? It would not be far-fetched to

suggest that food-seeking behavior would differ between the sexes. Of course, the prevalence of eating disorders in the human population is also highly sexually dimorphic, but it would be foolish to discount the cultural drivers behind this divergence. Ultimately, the question of why the phenotype in response to BigLEN application differ between sexes is likely better answered by focusing on more fundamental differences in nucleus accumbens function between sexes. Studies of sex differences in cholinergic interneuron regulation of glutamate and dopamine will be informative of how the regulation of those neurons by peptides like BigLEN changes behavior.

Analysis of BigLEN's effect on specific excitatory inputs

As discussed in the introduction, different inputs to the nucleus accumbens communicate distinct information regarding both internal state and external environment. Consequently, these inputs regulate different behavioral outcomes. In deciding which input to pursue first, it would be quite difficult to narrow down choices using the behavioral phenotype of GPR171. Viewing the behavior as related to extinction, the input from the prefrontal cortex seems like a good candidate based on its role in extinction and behavioral flexibility (Peters et al., 2008; Ragozzino, 2007). The input from the paraventricular nucleus of the thalamus also seems like a possible option based on its importance in communicating energy balance information to the nucleus accumbens, as well as its general role in resolving behavioral decisions during conflict (Kelley et al., 2005; Kirouac, 2015). The input from the basolateral amygdala seems less likely, due to my speculation that animals in this particular behavioral task do not rely heavily on the cues available. However, both the basolateral amygdala and the paraventricular nucleus of the thalamus have been found to oppose reward-seeking during periods of reward unavailability (Lafferty et al., 2020). Thus,

inhibition of these inputs results in unproductive reward seeking, similar to what we find in food-deprived animals. It is also possible that the input from the ventral hippocampus is targeted, something that would have a large impact on the activity of the region based on the power and reach of the input in the medial nucleus accumbens shell (Britt et al., 2012; LeGates et al., 2018; Trouche et al., 2019; Yang et al., 2020). It is also possible that a smaller, less well-studied input could be specifically targeted. Modulation of these smaller inputs can still support large scale behavioral phenotypes, a concept that was well documented in a study describing the role of opiate-mediated regulation of the dorsal raphe input to the nucleus accumbens shell in producing food-deprivation induced increases in consumption (Castro et al., 2021). Finally, input specificity is not a necessity, and GPR171 modulation may exert more of a broadcast change in excitatory input. This hypothesis is supported by the cholinergic microcircuit mechanism described here, which may serve as a mechanism to amplify the GPR171 signal across a number of inputs.

Projection class differences in GPR171's modulation of excitatory transmission

We report that GPR171 depresses excitatory synapses onto LH projecting D1+ MSNs, but has no effect on excitatory synapses onto VTA projecting D1+ MSNs. Considering the behavioral role of GPR171, this observation fits well with the conceptualization of the nucleus accumbens to LH circuit as an important regulator of feeding behavior (Kelley et al., 2005). However, the projection to the VTA also regulates feeding behavior (Bond et al., 2020). Why then should the GPR171 signal be present in the LH projecting population of MSNs and not the VTA projecting population?

While both of these projection populations are able to interrupt ongoing feeding behavior, they serve distinct behavioral functions in the context of reward-seeking. Studying the extinction of alcohol-seeking behavior, it has been shown that projections to the LH mediate extinction of

seeking in a specific context, while VTA projectors mediate reinstatement of seeking (Gibson et al., 2018). It is intriguing that two subpopulations of D1+ MSNs could be mediating opposing behavior phenotypes, an idea that adds complexity to decades old dichotomies focused on D1+ and D2+ MSNs. However it seems plausible that just as different brain regions mediate different aspects of behavior, so do projections to those different brain regions. Further work will need to identify the roles of these two projection populations in controlling known nucleus accumbens-mediated behaviors.

Another unanswered question is: how is this difference in GPR171 sensitivity expressed at the molecular level? We are still beginning to understand the transcriptional complexity of MSNs within the nucleus accumbens (Chen et al., 2021). It is likely that there are differences in gene expression between projection classes which could underlie differences in response to neuromodulators. However, the effect of GPR171 is expressed presynaptically on excitatory synapses onto MSNs. We describe this effect as dependent on nAChR activation, suggesting that GPR171 inhibits nAChR-mediated potentiation. Does that mean that the nAChR-mediated potentiation is differentially expressed at synapses onto different projection classes? This certainly seems possible, considering projections classes in the nucleus accumbens have been described to exhibit distinct plasticity How would tonic acetylcholine signaling target synapses onto one projection class and not another? This could be achieved through expression of differentially selective nAChRs on inputs to different projection classes. Differential expression of nAChR subtypes on different inputs has been shown in the VTA (Mansvelder et al., 2002; Mansvelder and McGehee, 2000). These subtypes can exhibit differential desensitization dynamics, leading to differential functional expression of nAChRs at the same concentration of acetylcholine. This is one potential molecular mechanism, however there are numerous that could underlie this

phenotype. A detailed study of the role of nAChRs in modulation nucleus accumbens glutamate transmission is needed to more fully understand how this occurs and the functional implications

Neuropeptide Y modulates excitatory transmission and promotes social behavior in the nucleus accumbens

We find that NPY differentially regulates excitatory synaptic transmission via multiple receptors in a cell type specific manner. Y1r and Y2r potentiate and depress excitatory synaptic transmission onto D1+ MSNs in the nucleus accumbens shell, respectively. Y5r and Y2r potentiate and depress excitatory synaptic transmission onto D1- MSNs in the nucleus accumbens shell, respectively. While Y1r and Y5r both decrease PPR, suggesting a presynaptic locus of action, Y2r does not alter PPR in either cell type. In D1+ MSNs, we find that inhibition of dipeptidyl peptidase IV, an enzyme that degrades released NPY, results in a Y1r dominated effect on excitatory synaptic transmission. Further, we find that infusing NPY or a Y1r agonist into the nucleus accumbens results in increased social interaction relative to vehicle infusion. Together, these results show that NPY regulates excitatory synaptic transmission within the nucleus accumbens via multiple receptors at different loci, and they indicate that NPY within the nucleus accumbens is able to potentiate social interaction.

NPY and social behavior

NPY promotes social behavior across the animal kingdom. The NPY homologue, neuropeptide F, promotes social behaviors in the roundworm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* (De Bono and Bargmann, 1998; Wu et al., 2003). NPY itself promotes social interaction in zebrafish (Shiozaki et al., 2020). In primates, higher levels of striatal

NPY are found in species with more complex social structures like chimpanzees and humans (Raghanti et al., 2018). This impressive functional conservation of a neuropeptide system suggests it plays a fundamental role in controlling the circuits that regulate behavior.

In mice, NPY has been found to promote social interaction by acting in the amygdala (Sajdyk et al., 1999). This action has been considered to be a result of NPY signaling reducing the aversive properties of a novel social interaction, a hypothesis that is supported by the ability of NPY to reduce social fear conditioning (Kornhuber and Zoicas, 2019). This interpretation is likely influenced by the extensive literature linking NPY to general anxiety and fear conditioning (Gutman et al., 2008; Lach and de Lima, 2013; Verma et al., 2015, 2012; Yamada et al., 2020; Zhou et al., 2008). However, NPY is expressed across the brain in different cell types and regulates many neural circuits (van den Pol, 2012). This suggests that there could be more mechanisms by which NPY regulates social behavior. Despite this, the role of NPY in classical reward regions of the brain, like the nucleus accumbens, has not been investigated prior to this work.

The finding that NPY within the nucleus accumbens can promote social interaction suggests that NPY may increase the rewarding aspect of a social encounter. Central NPY, and NPY infused into the nucleus accumbens, is rewarding itself and can support a conditioned place preference (Brown et al., 2000; Josselyn and Beninger, 1993). Social interactions are rewarding in part due to activation of dopamine neurons that project to the medial nucleus accumbens shell, where NPY was infused in our study (Gunaydin et al., 2014). NPY infusion into the ventricles or into the nucleus accumbens increase extracellular dopamine in the nucleus accumbens (Goff et al., 1992; Liu and Borgland, 2015; Quarta et al., 2011; Sørensen et al., 2009). Thus, it seems possible that NPY acts to promote social interaction by increasing the dopamine response to a social encounter. Specifically how NPY increases dopamine in the nucleus accumbens, and whether this

underlies its impact on social behavior is unknown. It is also important to note that excitatory transmission in the nucleus accumbens is important for the regulation of social behavior, and that the phenotypes presented here relating to excitatory transmission are also a potential mechanism for NPY's effect on social interaction (Folkes et al., 2020). Specific experiments testing the contributions of these different mechanisms are needed to better understand the modulatory role NPY plays in promoting social behavior.

Contribution of different NPY receptor subtypes to behavior

We find that infusion of both NPY and a Y1r agonist into the nucleus accumbens promotes social interaction. While activating Y1r is sufficient to promote social interaction, we have no tested whether it is necessary for the NPY induced effect on social interaction. This is a critical first step for identifying the contributions of different NPY receptors to social interaction. Further, we find that on D1+ MSNs, Y1r and Y2r oppose each other, and Y1r does not appear to regulate excitatory transmission on D2+ MSNs. Does this mean that Y2r opposes social interaction? This is possible, given that in the study of anxiety phenotypes, Y1r is typically found to be anxiolytic and Y2r is anxiogenic (Reichmann and Holzer, 2016). This is likely in part due to Y2r's role as an auto-receptor. However, it is important to note that while these receptors may appear to be opposing when examining broad, electrically evoked, excitatory transmission, they may be complimentary when viewed in greater detail. It seems possible that the potentiation of some inputs could be paired with the quieting of others, in order to bias behavior toward a specific outcome. In this way, the NPY system would be applying a complex, information specific, filter to input arriving in the nucleus accumbens. The effect of Y2r in this cause would not need to be specific to a certain input, indeed we find no evidence that the Y2r mediated depression at either cell type is presynaptic. A postsynaptic depression coupled with specific presynaptic potentiation would

similarly filter out non-potentiated inputs, like a high-pass filter, allowing the potentiated input to more reliably control ensembles. Another interesting layer is that this filter would be sensitive to DPP-IV activity, as the DPP-IV product NPY (3-36) is selective for Y2r over Y1r (Grandt et al., 1996). Future studies of the NPY receptor system in the nucleus accumbens should perform more detailed analysis to solidify the synaptic locus of receptor specific modulations, and use this information to inform studies of how these different receptors shape MSN activity and behavior.

We also report a presynaptic potentiation onto D2+ MSNs caused by a Y5r agonist. Similar to Y2r, we did not investigate the role of this receptor subtype in social behavior. Drd1 receptors, and the activity of D1+ MSNs, have been found to be important for and support social interaction (Gunaydin et al., 2014). D2+ MSNs exhibit synaptic changes in a mu opioid receptor knockout mouse, which exhibits altered social behaviors, suggesting this population may also contribute to the generation of social behavior (Toddes et al., 2021). In this particular study knockout mice exhibited increased inhibitory transmission onto D2+ MSNs and reduced social interaction, suggesting that activity in D2+ MSNs could promote social interaction. Thus it seems plausible that Y5r modulation of D2+ MSNs could also promote social behavior. Again, this specific effect downstream of Y5r would need to be taken in context with the effect Y2r, assuming that both receptor populations are activated by endogenous NPY release. In this case however, the relative stimulation of Y5r and Y2r would not be as sensitive to DPP-IV activity, as the DPP-IV product NPY (3-36) is able to activate both Y5r and Y2r (Gerald et al., 1996; Grandt et al., 1996). Further studies should examine the necessity and sufficiency of Y5r for the production of social behaviors, and more broadly the role of D2+ MSNs.

Closing remarks

The work presented here informs on the ability of internal state to impact the function of motivationally relevant neural circuits, shaping behavior. Additionally, they point to the importance of assessing multiple dimensions of motivated behavior beyond effort when considering the impact of hunger. These ideas are not unique to the circuits studied here. State-dependent neuromodulation likely exerts a pervasive, subtle influence across behaviorally relevant circuits. Some of these neuromodulators communicating internal state, neuropeptide Y for example, have homologues that are present stretching far back into the evolutionary timeline. The presence of the precursors of these modulators in simpler species indicates the foundational role they play in any nervous system. We have built an impressive body of information detailing the neural circuits that control behavior with the help of new neuroscience tools allowing targeted manipulations. These circuits define the roadmap for information through the brain, but the traffic is directed by neuromodulatory elements that guide the flow. Understanding these neuromodulators, and how they are disrupted in disease states, is critical to making sense of the circuit maps we produce. Advances in pharmacology, like the development of tools for the deorphanized GPR171, or the plethora of subtype specific drugs for the NPY receptor family, have provided us with new means to assess the role of neuromodulators. Combining this pharmacological specificity with circuit maps will provide a fresh understanding of motivated behavior.

The studies outlined here detail the electrophysiological and behavioral roles of two neuropeptides in the medial nucleus accumbens shell. Both stories were produced with a similar structure, beginning with an electrophysiological phenotype and then using literature about the brain region and roles of these neuromodulators to identify a likely behavioral phenotype. I

consider the behavioral relevance of these electrophysiological phenotypes to be of the highest importance, as I think that the goal of understanding the brain is to understand its product, behavior. However, the most difficult aspect of conducting these studies has been causally linking the electrophysiological findings to the behavioral phenotypes. I hope to improve upon this aspect in future work, so that the basic science produced from studies like these can be more useful in treating deficits in human behaviors.

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