CIRCUIT DYNAMICS UNDERLYING CONTROL OF DOPAMINE NEURON ACTIVITY BY THE NUCLEUS REUNIENS OF THE MIDLINE THALAMUS

by

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The circuitry mediating top-down control of dopamine (DA) neurons in the ventral tegmental area (VTA) is exceedingly complex. Characterizing these networks will be critical to our understanding of fundamental behaviors, such as motivation and reward processing, as well as several disease states. Previous work suggests that the medial prefrontal cortex (mPFC) exerts a profound influence on VTA DA neuron firing. Recently, our group reported that inhibition of the infralimbic subdivision of the medial prefrontal cortex (ilPFC) increases the proportion of VTA DA neurons that are spontaneously active (i.e., "population activity") and that this effect depends on activity in the ventral subiculum of the hippocampus (vSub). However, there is no direct projection from the mPFC to the vSub. Anatomical evidence suggests that communication between the two structures is mediated by the nucleus reuniens of the midline thalamus (RE). The work presented in this dissertation aims to determine if RE controls VTA DA neuron firing and to describe the circuits underlying ilPFC-RE communication. In Chapter 2, we present findings demonstrating that RE can drive VTA DA neuron firing by engaging vSub. In addition, we show that ilPFC opposes this effect, likely via input to RE in the form of feedforward inhibition from TRN. In Chapter 3, we present findings that characterize the diverse array of RE neuron firing patterns in an intact preparation and describe the circuit dynamics underlying projections from ilPFC to RE. These studies suggest that ilPFC exerts control of RE neuron firing pattern through direct, monosynaptic connections

and via the thalamic reticular nucleus (TRN). Overall, the studies described in this dissertation reveal a novel role for a corticothalamic circuit including the ilPFC, RE, and vSub in controlling VTA DA neuron firing and confirm that corticothalamic input from ilPFC to RE can modulate RE neuron firing pattern in nuanced and complex ways. In addition, these findings provide a plausible circuit basis for various behavioral phenomena observed in schizophrenia, and could help inform the development of novel treatments.

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LIST OF ABBREVIATIONS

BOLD: blood-oxygen-level dependent

CBV: cerebral blood volume

CNO: clozapine-n-oxide

CS-: unpaired conditioned stimulus

CT-TC: a direct, monosynaptic projection from a cortical area onto thalamic cells (not TRN)

CT-TRN: a direct, monosynaptic projection from a cortical area onto TRN cells

DA: dopamine

DAT: presynaptic dopamine transporter

dCA1: dorsal CA1

DREADDs: designer receptors exclusively activated by designer drugs

DLPFC: dorsolateral prefrontal cortex

ilPFC: infralimbic prefrontal cortex

IPSPs: inhibitory postsynaptic potentials

LGN: lateral geniculate nucleus

MAM: methylazoxymethanol acetate

MD: mediodorsal thalamic nucleus

MGN: medial geniculate nucleus

mPFC: medial prefrontal cortex

NAc: nucleus accumbens

plPFC: prelimbic prefrontal cortex

PPTg: Pedunculopontine tegmentum

PV: parvalbumin

rCBF: regional cerebral blood flow

RE: nucleus reuniens

SLM: stratum lacunosum-moleculare

TC-TRN: a direct, monosynaptic projection from a thalamic area onto TRN cells

TRN: thalamic reticular nucleus

TRN-TC: a direct, monosynaptic projection from TRN onto thalamic cells

VBM: ventrobasal thalamic nucleus

vCA1: ventral CA1

vHipp: ventral hippocampus

VP: ventral pallidum

VPL/VPM: ventral posterolateral and ventral posteromedial thalamic nuclei

vSub: ventral subiculum

VTA: ventral tegmental area

ZI: zona incerta

PREFACE

The work presented herein is based on multiple manuscripts that are published or in preparation.

Chapter 2 is a modified version of:

Zimmerman EC, Grace AA (2016) The Nucleus Reuniens of the Midline Thalamus Gates Prefrontal-Hippocampal Modulation of Ventral Tegmental Area Dopamine Neuron Activity. J Neurosci 36:8977–8984.

Chapter 3 is a modified version of:

Zimmerman EC, Grace AA (2017) Prefrontal inputs to the nucleus reuniens of the midline thalamus modulate firing pattern via parallel corticothalamic pathways. *In submission.*

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1.0 GENERAL INTRODUCTION

1.1 SYSTEMS NEUROSCIENCE AND TRANSLATIONAL PSYCHIATRY

Systems neuroscience approaches the study of the brain with the goal of describing large-scale functional interactions among broadly distributed networks. A fundamental motif of these interactions is the interplay between cortical and subcortical structures. While cortical areas are necessary for the most sophisticated functions of the mammalian brain, it is becoming clear that subcortical structures offer a critical contribution. The dopamine (DA) system of the ventral midbrain has received much attention in this regard. DA neurons send widespread, but anatomically and functionally discreet, projections throughout the brain. These neurons are incorporated into diverse networks comprised of other cortical and subcortical structures that underlie myriad complex behaviors.

The DA system is also heavily implicated in several disease states, one of the most prominent of which is schizophrenia. The efficacy of antipsychotic medications used to treat schizophrenia depends on their antagonism of DA receptors. However, the usefulness of these medications is greatly diminished by their side effect profile, relatively low effectiveness, and problems with adherence, highlighting a need for mechanistically novel treatments. To this end, a large body of work from our group and others has focused on characterizing the firing properties of DA neurons and the afferent regions controlling their output using rodents. Rodents provide a rich toolbox of circuit-based techniques that allow for measurement and manipulation of neural activity with great spatial and temporal precision. The translational value of rodent work lies in the ability to provide detailed information about circuits that might be applicable to higher species, and to “model” the possible ways in which disrupted circuit dynamics could underlie pathology in humans.

The goal of the present work was to gain a better understanding of large-scale functional interactions involving the DA system and how these may be disrupted in schizophrenia. Here we investigate the control of DA neuron activity by a cortico-thalamo-hippocampal network centered on the nucleus reuniens of the midline thalamus (RE). In addition, we explore how cortical input modulates firing properties of RE neurons. Our findings help to explain how previously identified circuit alterations in schizophrenia could lead to symptoms and may inform the development of novel treatments.

1.2 THE DOPAMINE SYSTEM: INTRINSIC PROPERTIES AND AFFERENT REGULATION

1.2.1 *Firing Pattern in Dopamine Neurons of the Rodent Ventral Midbrain.* The majority of DA neurons of the mammalian ventral midbrain are divided into the A8, A9, and A10 cell groups (Yetnikoff et al., 2014). In rodents, the more medial A10 group occupies the ventral tegmental area (VTA), while the more lateral A9 group occupies the substantia nigra pars compacta (SN). In primates, the VTA has regressed and is largely replaced by the limbic-related dorsal tier of the SN. The motor-related functions of the SN in primate are preserved in its ventral tier (Haber and Calzavara, 2009; Haber, 2014). From this point forward we will focus on the intrinsic properties and afferent connections of DA neurons in the rodent VTA (A10), many of which are preserved in the primate brain (Fabre et al., 1983; Schultz, 2007).

The firing patterns of DA neurons can be classified into one of three categories: 1) inactive, 2) tonic, and 3) burst (Fig. 1-1). In the rodent VTA under baseline conditions approximately 50%

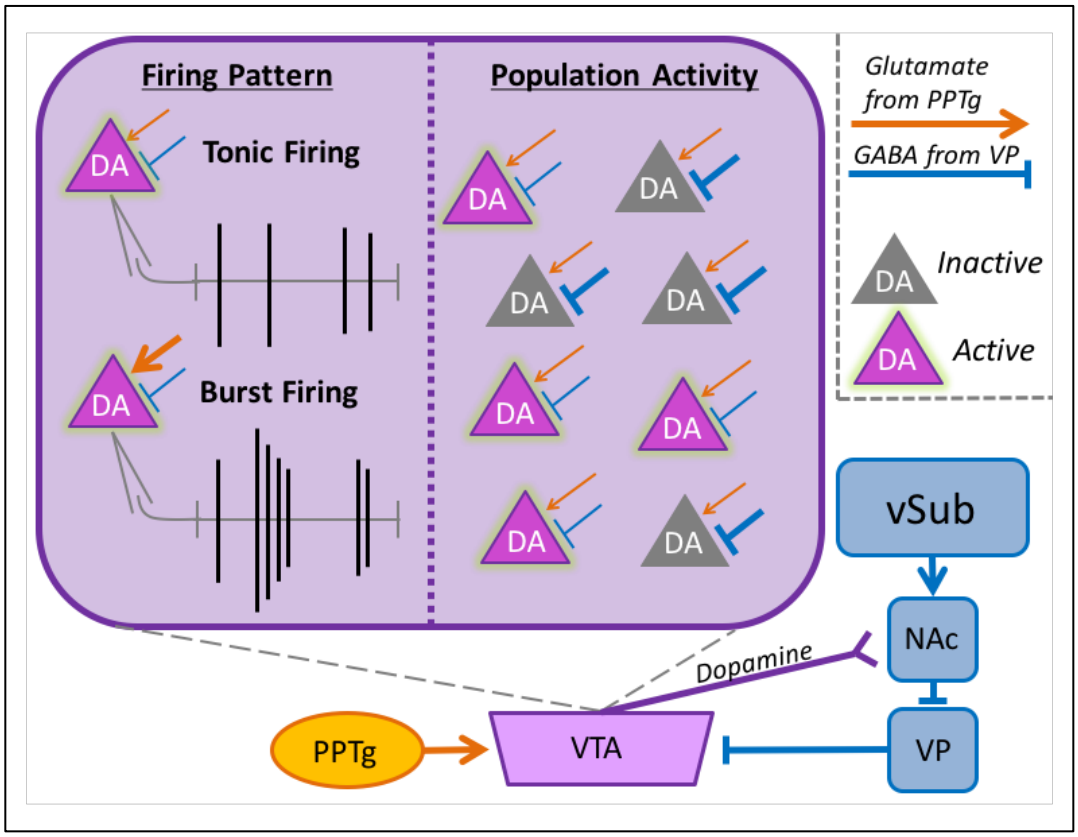

Figure 1-1: Afferent control of dopamine neuron firing.

In awake and anesthetized rats, approximately half of mesencephalic VTA DA neurons are firing spontaneously and exhibit two primary firing patterns: tonic firing and burst firing. Tonic firing is driven by intracellular pacemaker potentials. Burst firing is driven by glutamatergic input from the PPTg. It is critical to note that DA neurons that are not spontaneously active cannot fire bursts. Thus, the functional output of the DA system is primarily determined by the proportion of VTA DA neurons that are spontaneously active (i.e. population activity). Population activity is controlled by a circuit involving the vSub, NAc, and VP. Pharmacological excitation of vSub, via NAc and VP, leads to disinhibition of VTA DA neurons, increased population activity, and enhanced DA release in NAc.

of DA neurons are inactive, i.e. firing no action potentials (Freeman and Bunney, 1987). Inactive DA neurons are held in a prolonged hyperpolarized state by a constant barrage of inhibitory postsynaptic potentials (IPSPs) arising from the ventral pallidum (VP; Grace and Bunney, 1985), which fires at a consistent, high firing rate in the baseline state (Maslowski-Cobuzzi and Napier, 1994). Those DA neurons receiving relatively less VP input at any given time can fire in either tonic or burst mode. The tonic mode is defined by the presence of single spikes fired at 2-10 hz with irregular interspike intervals (Grace and Bunney, 1984a), and is driven by a pacemaker potential with contributions from a diverse complement of ion channel conductances (Grace and Onn, 1989). The burst mode is defined by the presence of a series of 2-10 action potentials fired in quick succession, typically exhibiting a 40-80 msec (i.e ~25 hz firing rate) interspike interval (Grace and Bunney, 1984b). Bursting is driven by glutamatergic afferent input and is considered to be the behaviorally-relevant output of the DA system, because DA neurons fire bursts at very short latency when an organism is presented with a salient stimulus (Schultz, 2007; Brischoux et al., 2009; Ungless et al., 2010; Grace, 2016).

The firing pattern of DA neurons determines the nature of DA release in efferent targets of the VTA. These downstream regions include the reward-related nucleus accumbens (NAc) and ventral striatum, the associative striatum, and the medial prefrontal cortex (mPFC), along with the amygdala, ventral hippocampus (vHipp), and subthalamic nucleus, among many others (Sesack and Grace, 2010; Beier et al., 2015). The magnitude of tonic firing in the VTA is determined by the proportion of spikes fired in tonic mode, as well as the proportion of DA neurons that are spontaneously active. These two parameters of tonic firing set the extrasynaptic levels of DA in target regions (~10nM range at baseline in the striatum), which changes over the course of minutes to hours and is regulated by enzymatic degradation (Floresco et al., 2003). In contrast, phasic bouts

of burst firing can raise the intrasynaptic concentration of DA to the low-mM range (Garris and Wightman, 1994), which is quickly attenuated and prevented from reaching the extrasynaptic space by the presynaptic dopamine transporter (DAT; Floresco et al., 2003). It is important to note that the level of expression of DAT varies across brain areas in mammals (Garris and Wightman, 1994; Sesack et al., 1998). Tonic and burst firing, by virtue of having dramatically different effects on the DA tone of VTA output regions, also modulate the function of these regions in different ways. Taking the striatum as an example, tonic firing is likely to affect a wider area of tissue that includes extrasynaptic targets, whereas phasic bursts might impact a more restricted population of postsynaptic neurons. DA tone can also differentially modulate the integration of inputs to the NAc (Goto and Grace, 2005). Therefore, DA neuron firing pattern is a primary determinant of the manner in which DA influences signaling in downstream structures.

1.2.2 *Afferent Control of Firing Pattern.* The firing pattern of DA neurons is heavily impacted by afferent input from a variety of brain regions (Fig. 1-1). Burst firing in VTA DA neurons is induced by depolarization: stimulation of glutamatergic inputs or direct application of glutamate in vivo both enhance bursting (Grace and Bunney, 1984b; Chergui et al., 1994; Floresco et al., 2003). The primary region that drives burst firing is the pedunculopontine tegmentum (PPTg), which sends monosynaptic glutamatergic and acetylcholinergic projections to the VTA (Lokwan et al., 1999; Floresco et al., 2003; Pan and Hyland, 2005). The PPTg receives diverse afferent input and acts as nexus for behavioral-relevant signals from the mPFC, amygdala, and sensory areas (Mori et al., 2016). In addition, the laterodorsal tegmentum (LDTg) has been identified as a region that gates burst firing, likely via projections onto intrinsic VTA GABAergic neurons or by altering cholinergic signaling within the VTA (Lodge and Grace, 2006a).

Although burst firing can be driven in DA neurons by glutamate application, it has been shown that glutamate promotes bursting by binding specifically to NMDA receptors: bursting is prevented by iontophoretic application of AP-5, but not CNQX (Overton and Clark, 1992; Chergui et al., 1993). At hyperpolarized membrane potentials ionic conductance in NMDA receptors is prevented by a membrane potential-dependent Mg2+ block (McBain and Mayer, 1994). This tethers the burst firing propensity of DA neurons to their membrane potential, allowing for modulation by inhibitory inputs. Previous studies have shown that while electrical stimulation of PTTg was sufficient to drive bursting in DA neurons, simultaneous reduction of VP activity during stimulation led to a multiplicative increase in bursting (Lodge and Grace, 2006b). Therefore, inhibitory input from the VP, through control of VTA DA neuron membrane potential, determines the amplitude of the burst firing-dependent, phasic DA signal. Said differently, DA neurons that are not spontaneously active cannot burst. Critically, this enables VP to set the gain of phasic DA system output by controlling the number of spontaneously active DA neurons.

The central role of VP in controlling DA neuron firing makes it critical to understand the afferent circuitry regulating tonic DA tone upstream of VP. To quantify the tonic output of the VTA, our group uses an electrophysiological protocol that measures the number of spontaneously active neurons encountered in a predetermined series of vertical passes through the VTA with a glass recording electrode. This method shows high inter-subject reliability and provides an estimate of the proportion of DA neurons that are spontaneously active, which we call “population activity.” Population activity serves as a quantitative readout of the gain of the DA system, based on the mechanisms described above. Several studies from our group and others have demonstrated that VTA DA neuron population activity is regulated by a circuit involving the ventral subiculum of the hippocampus (vSub), the NAc, and the VP (For review see Grace, 2016). Stimulation of

vSub drives feedforward inhibition from NAc onto VP, enhancing VTA DA neuron population activity and DA release in the NAc (Floresco et al., 2003). The control of population activity by this circuitry has been well-characterized and implicated in a wide variety of behavioral paradigms (Lodge and Grace, 2007; Chang and Grace, 2014; Belujon et al., 2016; Grace, 2016).

Recent findings suggest that additional areas are involved in the control of VTA DA neuron population activity. Previous studies have shown that manipulations of the mPFC, comprised of the infralimbic (ilPFC), prelimbic (plPFC), and anterior cingulate (ACC) subdivisions in rodents, can modulate DA tone in the striatum (Karreman and Moghaddam, 1996; Finlay and Zigmond, 1997; Jackson et al., 2001). However, the circuit mechanisms underlying this modulation have not been fully described. mPFC neurons send monosynaptic projections to the VTA, but these fibers preferentially target DA neurons and long-range GABAergic projection neurons that then project back to cortex, and do not collateralize to striatum (Loughlin and Fallon, 1984; Carr and Sesack, 2000a; 2000b). More recent findings characterizing how mPFC modulates VTA output are inconsistent and difficult to compare owing to differences in methodology (Moorman and Aston-Jones, 2010; Butts et al., 2011; Lodge, 2011; Jo et al., 2013; Ferenczi et al., 2016). However, our group has demonstrated that mPFC can control VTA DA neuron population activity (Patton et al., 2013; Moreines et al., 2017). We have shown that stimulation of the plPFC has no effect on DA neuron firing, while inhibition moderately decreases population activity. In contrast, manipulation of ilPFC exerts bidirectional effects: stimulation of ilPFC decreases population activity in a BLA-dependent manner, while inhibition of ilPFC enhances population activity in a vSub-dependent manner.

In summary, the studies reviewed here have elegantly dissected circuits controlling VTA DA neuron firing and therefore DA tone in VTA output regions. From these studies it is clear that

afferents to the VTA are critical in gating DA signaling across diverse temporal and behavioral scales. That said, the importance of many known afferents to the VTA have not been explored (Watabe-Uchida et al., 2012; Beier et al., 2015) and new regions that impact DA neuron firing are still being identified (Hikosaka, 2010; Barrot et al., 2012; Lecca et al., 2012; Moreines et al., 2017). Even more daunting is the task of describing regulation of VTA neuron firing as one component of large-scale networks (Ferenczi et al., 2016; Lohani et al., 2016; Decot et al., 2017). The present work offers a novel contribution to this effort. Based on our previous findings (Patton et al., 2013), we focus on the ilPFC specifically and describe a corticothalamic circuit that modulates VTA DA neuron population activity via the hippocampus.

1.3 THE THALAMUS: GENERAL CONCEPTS AND THE NUCLEUS REUNIENS

1.3.1 *Emerging Themes of Thalamic Function.* The thalamus has long been recognized for its role in relaying sensory information from the periphery to the neocortex (Bishop et al., 1962; Harrison and Warr, 1962; Guillery, 1967). The ascending visual pathway, comprised of retinal inputs to the lateral geniculate nucleus (LGN) and LGN inputs to V1, is a prototypical sensory circuit mediated by a thalamic relay (Sherman and Guillery, 2001; Wurtz, 2009). LGN is a prime example of a "first-order" thalamic nucleus, others of which include the medial geniculate nucleus (MGN) of the auditory system (Bartlett, 2013) and the ventral posterior nuclei (VPL/VPM) of the somatosensory system (Petersen, 2007). First-order thalamic nuclei receive the majority of their input from the periphery and engage in minimal processing of this information. In the case of the visual system, LGN cells exhibit receptive field properties that closely resemble those in the retina (Sherman, 1996), as would be expected of a relatively simple relay nucleus. First-order nuclei are well-studied and traditionally viewed as exemplars of thalamic function.

However, the thalamus is comprised of over 20 distinct subnuclei with diverse functions well beyond sensory relay from the periphery. Indeed, the participation of the thalamus in sophisticated information processing is becoming increasingly recognized (Sherman, 2016). In particular, a second category of thalamic nuclei termed "higher-order" nuclei have been shown to receive relatively little input from the periphery and instead appear to mediate information transfer between cortical areas (Mitchell et al., 2014; Saalmann, 2014). For example, inputs from the mediodorsal nucleus (MD) to the mPFC help to maintain sustained activity through the delay period of a spatial working memory task (Bolkan et al., 2017), while the pulvinar nucleus has been shown to be critical for modulating synchrony between cortical areas during attention in the delay period of a visuospatial attention task (Saalmann et al., 2012). These findings and others suggest that "transthalamic" connections between cortical areas mediated by higher-order thalamic nuclei could provide a gating function and additional pathways for information processing (Saalmann, 2014), which would make higher-order nuclei fundamentally distinct from first-order nuclei. Despite this, the properties of higher-order thalamic nuclei are much less-well characterized compared to first-order nuclei.

First-order and higher-order thalamic nuclei also differ in how their function is influenced by cortical input (Sherman, 2016). Cortical inputs to thalamus arise from layers 5/6 and consist of monosynaptic projections directly onto thalamic cells, as well as disynaptic feedforward-inhibitory projections via the thalamic reticular nucleus (TRN; see below). In first-order nuclei of sensory systems cortical input has been shown to primarily influence how information is relayed from the periphery, e.g. by shaping receptive fields (Eyding et al., 2003; Li and Ebner, 2007), enhancing sensory gain (Przybyszewski et al., 2000) and/or affecting attentional processes (Wimmer et al., 2015). In contrast, cortical inputs to higher-order nuclei likely provide not only a source of

information to be conveyed to another cortical area, but also a modulatory influence on the transfer of that information (Saalmann, 2014). Therefore, cortical input to higher-order nuclei is likely to be different from cortical input to first-order nuclei in both function and circuit physiology. However, the physiology of cortical feedback to both first-order and higher-order thalamic nuclei is not well studied.

1.3.2 *The Thalamic Reticular Nucleus and Thalamic Bursting*. Inhibitory tone in the mammalian thalamus is provided primarily by afferent inputs, the most prominent of which is the TRN (Pinault, 2004; Halassa and Acsády, 2016). This is underscored by the fact that intrinsic GABAergic interneurons are absent in the rodent thalamus (except in LGN; Sherman, 2004) and are sparse in humans (Arcelli et al., 1997). Functionally, the TRN has traditionally been viewed as a low-resolution modulator of arousal and attention based on the "searchlight hypothesis" famously advanced by Francis Crick (Crick, 1984). However, the TRN is topographically organized based on cortical afferents and thalamic projection targets (Crabtree, 1992; Pinault, 2004), and recent studies have demonstrated that it is involved in the precise modulation of myriad awake behaviors and large-scale rhythmic interactions between disparate brain regions (Blumenfeld and McCormick, 2000; Halassa et al., 2014; Wimmer et al., 2015; Clemente-Perez et al., 2017). Critically, the cortex is a primary driver of TRN and the majority of cortical input to thalamus is via feedforward inhibition from TRN (Golshani et al., 2001; Paz et al., 2011). Despite the existence of monosynaptic corticothalamic projections, the predominance of TRN input is facilitated by the higher synaptic strength of the cortico-TRN synapse and the variable conduction velocity of corticothalamic axons (Swadlow and Weyand, 1987; Paz et al., 2011).

The primary influence of TRN on thalamic nuclei is on membrane potential and firing pattern. Specifically, TRN drives burst firing in thalamic neurons (Halassa et al., 2011). This effect is mediated by the dense, ubiquitous somatic and dendritic expression of T-type calcium channels (Cav3 family, CACNA1 gene) whose physiology in the thalamus has been well-characterized (Sherman and Guillery, 2001). These channels exhibit qualitatively similar kinetics to the voltage-gated sodium channel: sufficient membrane depolarization opens the activation gate, permitting an inward calcium current (I_T) that leads to a propagating, all-or-none spike known as the "low-threshold spike." However, a key distinguishing feature of these channels is that they inactivate upon even slight (~5mV) depolarization from rest. This makes the firing pattern of thalamic neurons heavily dependent on membrane potential. If a suprathreshold depolarization arrives at a thalamic neuron that is already slightly depolarized (e.g. -59mV), T-type calcium channels will be inactivated and therefore no low-threshold spike will occur, resulting in a tonic series of single action potentials. In contrast, if the same suprathreshold depolarization arrives at a thalamic neuron that is already slightly hyperpolarized (e.g. -70mV), T-type calcium channels will be de-inactivated and the ensuing I_T current will generate a low-threshold spike, causing a burst of 2-9, high-frequency (>250 hz) action potentials. The amplitude of the low-threshold spike will also determine the number of spikes fired within any given burst (Zhan et al., 1999).

Thus, hyperpolarization in thalamic neurons enhances the propensity for burst firing, positioning the TRN as a key modulator of thalamic neuron firing pattern. However, the function of burst firing in the thalamus is not completely understood. Thalamic bursting has traditionally been associated with sleep or other unconscious states (Steriade et al., 1993) and theorized to play relatively little role in conscious brain function (Steriade, 2001). In sensory systems, bursting has been described as a "wake-up" call to cortex (Sherman, 2001a) based on the idea that in the awake

state, sensory stimuli driving burst firing in first-order thalamic nuclei likely engages cortical circuits more strongly than tonic firing. However, this conceptualization is only supported by a handful of studies (Swadlow and Gusev, 2001; Ortuño et al., 2014; Hu and Agmon, 2016). In addition, burst firing in higher-order thalamic relays is not well-studied (Ramcharan et al., 2005) and is likely to have a nuanced and multifaceted impact on cortical function. Indeed, the concept of a sensory "wake-up" call does not translate to the transfer of information between cortical areas mediated by higher-order nuclei.

1.3.3 *The Nucleus Reuniens.* Reciprocal communication between the mPFC and hippocampus has been shown to be critical for several domains of behavior (Vertes, 2006; Corcoran and Quirk, 2007; Ito et al., 2015; Spellman et al., 2015; Carreno et al., 2016; Eichenbaum, 2017). Monosynaptic projections from vSub, the primary output region of the hippocampus, terminate in the mPFC onto pyramidal neurons and interneurons (Gabbott et al., 2002; Hoover and Vertes, 2007). In contrast, reciprocal monosynaptic connections from mPFC to hippocampus are sparse (Vertes, 2003). The mPFC projects to the lateral entorhinal and perirhinal cortices, and a projection from dorsal ACC to CA1 and CA3 has recently been described (Rajasethupathy et al., 2015). However, it is likely that much of the information transfer from mPFC to hippocampus occurs through indirect, polysynaptic pathways.

One potential relay between the mPFC and hippocampus that has recently garnered increased attention is the RE (Fig. 1-2). RE is a higher-order thalamic nucleus whose dense, reciprocal connections with mPFC and hippocampus have been thoroughly described in anatomical tracing studies (Wouterlood et al., 1990; Vertes, 2002; McKenna and Vertes, 2004;

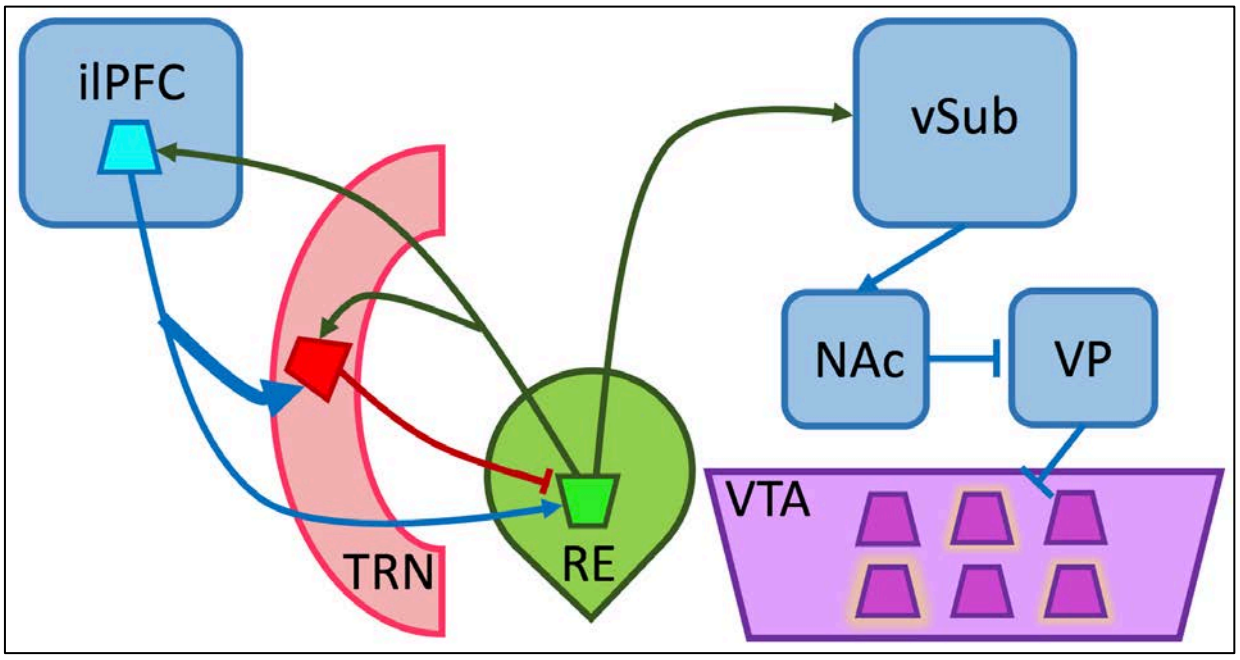

Figure 1-2: Anatomically defined pathways of cortico-thalamo-hippocampal communication in the rodent.

Anatomical tracing studies have demonstrated that RE is positioned to act as a primary relay between the mPFC, including plPFC and ilPFC, and the hippocampus. Projections from RE to the hippocampus are excitatory (triangular arrowheads) and target dorsal and ventral CA1 and subiculum, with the densest projection being to the vHipp. Corticothalamic projections to thalamus give off en passant collaterals to TRN before continuing on to their thalamic targets. These projections are reciprocated by thalamocortical efferents that are organized in the same fashion. Note the larger synaptic strength of the cortico-TRN collaterals as compared to the cortico-RE synapses (demonstrated experimentally in other corticothalamic projections but not RE). TRN inputs to the thalamus are GABAergic (flat-headed arrowheads).

Vertes et al., 2006; Çavdar et al., 2008; Hoover and Vertes, 2012; Xu and Südhof, 2013; Varela et al., 2014). RE sends a glutamatergic projection to the dorsal and ventral hippocampus that innervates the stratum lacunosum-moleculare (SLM) of CA1 and the molecular layer of vSub. This projection is the densest to the hippocampus from any thalamic nucleus and is biased towards the vHipp: ten times as many fibers from RE project to vHipp compared to dorsal hippocampus (Vertes et al., 2006). Fibers from RE also distribute throughout the mPFC, including projections to layers 1 and 5/6. In turn, RE receives reciprocal connections from vSub and mPFC, both of which also send collaterals to RE-projecting regions of TRN (Çavdar et al., 2008). Despite having relatively restricted efferent projections RE receives afferent input from several structures including the claustrum, lateral septum, preoptic area, and hypothalamus, as well as various brainstem nuclei including the VTA and dorsal raphe (McKenna and Vertes, 2004). The cortical and hippocampal connections of RE are roughly preserved in primates (See "*Technical Considerations and Limitations*").

Behavioral studies have shown that RE is involved in a variety of behaviors known to require communication between mPFC and hippocampus, including spatial navigation (Davoodi et al., 2011; Cassel et al., 2013; Jankowski et al., 2014; Ito et al., 2015), working memory (Cholvin et al., 2013; Duan et al., 2015; Jin and Maren, 2015; Layfield et al., 2015; Linley et al., 2016; Prasad et al., 2017), fear learning (Xu and Südhof, 2013; Jin et al., 2015; Sierra et al., 2017; Vetere et al., 2017), and mood regulation (Kafetzopoulos et al., 2017). Recordings performed in awake, behaving animals have shown that RE contains head-direction cells (Jankowski et al., 2014) and can encode the intended direction of movement (Ito et al., 2015). In addition, lesion or transient inhibition of RE impairs performance on spatial working memory tasks (Cholvin et al., 2013; Layfield et al., 2015), e.g. delayed alternation (Hallock et al., 2016). In fear learning paradigms,

RE lesion or transient inhibition does not impair task acquisition, but affects the consolidation and retrieval of memories (Xu and Südhof, 2013; Jin et al., 2015; Vetere et al., 2017). Taken together, these findings suggest that RE facilitates information transfer from mPFC to hippocampus during ongoing behavior and during systems-level consolidation.

In contrast to the anatomical and behavioral studies outlined above, circuit-based electrophysiological studies of RE are sparse. The effects of manipulating RE neuron firing on target structures have been examined in a small number of studies. RE stimulation leads to excitatory responses in both hippocampus and cortex, and engages both pyramidal neurons and interneurons (Dolleman-Van der Weel et al., 1997; Di Prisco and Vertes, 2006; Morales et al., 2007; Dolleman-Van der Weel et al., 2016). Consistent with the effects of stimulation, RE inactivation reduces firing rates in mPFC and dorsal CA1 (dCA1) in awake animals (Ito et al., 2015; Hallock et al., 2016). In addition, RE has been shown to mediate various aspects of oscillatory communication between mPFC and hippocampus (Ito et al., 2015; Hallock et al., 2016; Kafetzopoulos et al., 2017; Roy et al., 2017). These studies are somewhat difficult to compare owing to methodological differences, but broadly suggest that RE is necessary for maintaining coherence and synchrony between mPFC and hippocampus across multiple frequency bands at rest and during task performance.

In summary, RE is a higher-order thalamic nucleus that is anatomically positioned to function as a relay between the mPFC and hippocampus. Similar to other higher-order thalamic nuclei, RE likely provides an additional node of information processing that integrates input from afferent structures to shape communication between two or more cortical areas. However, RE is likely to be distinct from other higher-order nuclei given the unique structure and function of the hippocampus compared to other areas of cortex. Several questions remain about RE. To date, no

studies have examined the impact of manipulating afferent inputs, including TRN, on RE neuron firing. Many of the behavioral studies of RE have not assessed the causal involvement of the circuits underlying the effects they report, including the impact of TRN. In addition, those studies that have assessed the causal involvement of RE in behavior using electrophysiological recordings and/or projection-specific manipulations have focused almost entirely on RE projections to the dorsal hippocampus (Duan et al., 2015; Ito et al., 2015; Hallock et al., 2016), despite the fact that projections from RE to the vHipp are denser and likely just as critical. Therefore, the studies described in this dissertation were designed to expand what is known about the function of RE projections to vHipp and dissect the circuit dynamics of corticothalamic inputs to RE.

1.4 THE CIRCUITRY OF SCHIZOPHRENIA: INVOLVEMENT OF DOPAMINE, HIPPOCAMPUS, AND THALAMUS

Schizophrenia is a prevalent, often life-long mental illness characterized by symptoms currently categorized as positive, negative and cognitive (American Psychiatric Association, 2013). The disease is typically associated with the syndrome of psychosis, which most commonly presents with delusions and hallucinations (Kapur, 2003). However, the symptoms of schizophrenia are heterogeneous between patients, can change over the course of the illness, and are difficult to treat (Millan et al., 2016; Heilbronner et al., 2016b). Therefore, recent studies have focused on describing neurobiological phenomena associated with the diagnosis of schizophrenia, specific symptom dimensions, or specific behavioral alterations (Cuthbert and Insel, 2010; Bolkan et al., 2016). A major motivation for the studies in this dissertation was to describe circuit dynamics in rodent models that could potentially underlie neurobiological phenomena observed in patients. We

briefly review relevant clinical findings below, in order to provide a background for the design and implications of our studies.

1.4.1 *Dopamine.* Striatal hyperdopaminergia is the most widely recognized neurobiological feature of schizophrenia (Laruelle and Abi-Dargham, 1999; Howes et al., 2007; Howes and Kapur, 2009). Enhanced presynaptic DA function in the striatum, particularly in the associative striatum, is strongly associated with the psychotic symptoms of the disorder (Weinstein et al., 2017). The magnitude of DA release and severity of symptoms within a psychotic episode are correlated (Laruelle et al., 1999; Abi-Dargham et al., 2000) and drugs that induce DA release can worsen psychotic symptoms in schizophrenia patients (Janowsky et al., 1973). A hyperdopaminergic phenotype can also predict conversion to psychosis, as increased DA synthesis capacity is seen only in those ultra high-risk individuals who go on to develop the disease (Allen et al., 2012; Egerton et al., 2013). In addition, the efficacy of antipsychotic medications in treating psychotic symptoms depends on their affinity for DA receptors (Howes and Kapur, 2009). While these findings are compelling, there is little evidence for dysfunction of the DA system itself in schizophrenia (van Kammen et al., 1986; Grace, 2012a), suggesting that DA hyper-responsivity is the result of circuit dysfunction in other brain areas (Grace, 2016).

1.4.2 *The Hippocampus.* Hippocampal pathology is a well-established feature of schizophrenia (Bogerts et al., 1985; Heckers and Konradi, 2010; Konradi et al., 2011). The most consistent postmortem findings in patients are decreased GAD67 mRNA in CA2/CA3 (Benes et al., 2007) and decreased parvalbumin (PV)-positive interneuron number throughout the hippocampus (Benes and Berretta, 2001; Zhang and Reynolds, 2002; Do et al., 2015). Structural imaging studies report

decreased whole hippocampal volume with large (i.e. 0.4-0.8) effect size that is present in first-episode patients and first-degree relatives, and worsens over the course of the disease (Chakos et al., 2005; Adriano et al., 2011). Reduced hippocampal volume has in many cases been shown to be more severe in the anterior hippocampus, but this finding is not entirely consistent across studies (Heckers and Konradi, 2010).

Functional imaging studies of the hippocampus in patients with schizophrenia report enhanced regional cerebral blood flow (rCBF) in whole hippocampus that is normalized by antipsychotic treatment (Medoff et al., 2001; Malaspina et al., 2004), as well as enhanced cerebral blood volume (CBV) that is greater in anterior hippocampus (Talati et al., 2014) and specific to CA1 (Schobel et al., 2013). In addition, reduced resting-state functional connectivity between the hippocampus and several areas in the default mode network has been reported (Zhou et al., 2008; but see McHugo et al., 2015). Intriguingly, reduced functional connectivity between the anterior hippocampus and the ACC/mPFC and thalamus has been reported in a sample of psychotic patients spanning diagnostic categories (Samudra et al., 2015). Finally, task-related functional imaging studies have reported negative coupling of prefrontal regions and the hippocampus during working memory and reduced engagement of the hippocampus during memory retrieval with a concomitant increased activation of cortical regions (Meyer-Lindenberg et al., 2005; Weiss et al., 2006; Rasetti et al., 2014). Taken together, these studies show that the hippocampus in schizophrenia is hyperactive, a phenomenon that is developmental in origin and that could represent a primary etiologic driver in the disease.

1.4.3 *The Thalamus* The thalamus has long been considered as a potential node of dysfunction in schizophrenia (McGhie and Chapman, 1961), but only recently has progress been made in

describing the neurobiology that may underlie this role. Specifically, the anterior, midline, and MD nuclei of the thalamus have garnered increased attention due to their interconnections with the hippocampus and prefrontal cortex across species (see "*Technical Considerations and Limitations*"). Initial postmortem studies in patients with schizophrenia reported a decrease in both size and cell number in the whole thalamus, as well as the anterior and MD nuclei specifically (Pakkenberg, 1990; Young et al., 2000). However, results from further studies proved inconsistent (Dorph-Petersen and Lewis, 2017). More consistent are structural imaging studies of the thalamus, the majority of which report reduced whole thalamus grey matter volume and volume reductions in the anterior, midline, and MD nuclei specifically (Pergola et al., 2015). These reductions are also seen in individuals at both clinical high risk and familial high risk for schizophrenia (Harrisberger et al., 2016; Pergola et al., 2017).

Unlike the hippocampus, studies of intrinsic, resting-state activity of the thalamus in schizophrenia patients are small in number and inconsistent, especially with respect to thalamic subnuclei (Théberge et al., 2002; Kühn and Gallinat, 2013). However, task-related functional imaging studies demonstrate increased thalamic activity in patients performing a sensorimotor gating task (Tregellas et al., 2007a) and in those reporting hallucinations (Silbersweig et al., 1995), as well as decreased thalamic activation during working memory and episodic memory tasks in MD and the anterior nuclei (Andrews et al., 2006; Minzenberg et al., 2009). In addition, differences in resting-state functional connectivity between thalamus and hippocampus/prefrontal cortex have been reported: patients with schizophrenia exhibit hyperconnectivity between anterior, midline, and MD nuclei and sensory cortices, but hypoconnectivity between these nuclei and prefrontal regions (Anticevic et al., 2014; Woodward and Heckers, 2016). This finding is present in clinical

high risk populations, first-episode subjects, and predicts conversion to psychosis (Woodward et al., 2012; Anticevic et al., 2015a).

Alterations in the structure and function of the TRN have also been described in patients with schizophrenia. Postmortem studies have demonstrated reduced SST mRNA expression and enhanced excitatory amino acid transporter mRNA expression in the TRN of patients with schizophrenia compared to controls (Smith et al., 2001; Burgess et al., 2016). A reduction in sleep spindles, rhythmic events characteristic of slow-wave sleep and driven primarily by TRN, has been consistently observed in patients with schizophrenia, including first-episode and non-medicated populations (Ferrarelli and Tononi, 2011; 2017). It should be noted that functional imaging studies of the TRN are lacking due to its small size and unusual shape. However, one functional imaging study reported decreased thalamic blood-oxygen-level dependent (BOLD) response to transcranial magnetic stimulation of cortex in patients, which implies less effective cortical feedback and TRN engagement (Guller et al., 2012).

In summary, these findings demonstrate that the diagnosis of schizophrenia and its associated symptoms are correlated with structural and functional alterations in several brain areas. While a hyperdopaminergic phenotype is the most well-established finding in the disease, DA neurons are incorporated into broad networks of dozens of interconnected brain regions. This and many other previous findings suggest that hyperdopaminergia in schizophrenia is simply one discrete, measurable outcome that is indicative of a brain-wide circuit dysfunction. This idea is also supported by the fact that negative and cognitive symptoms cannot be treated adequately with DA blockade (Lieberman et al., 2005; Crossley et al., 2010). Therefore, in the current work we examine upstream regions and circuits that are potentially involved in controlling DA system output, but could also drive pathophysiology on their own.

1.5 PURPOSE OF STUDIES

In this dissertation, we describe a series of studies that dissect the dynamics of the ilPFC-RE-vSub circuit and its impact on DA neuron firing in rodents. In Chapter 2, we investigate the role of RE in gating prefrontal-hippocmpal modulation of VTA DA neuron population activity. In Chapter 3, we describe how inputs from ilPFC modulate the firing pattern of RE neurons. Taken together, our findings provide a detailed circuit model that describes how disrupted corticothalamic communication in the limbic system could lead to DA system hyperactivity and behavioral deficits in psychiatric disease.

2.0 THE NUCLEUS REUNIENS OF THE MIDLINE THALAMUS GATES PREFRONTAL-HIPPOCAMPAL MODULATION OF VENTRAL TEGMENTAL AREA DOPAMINE NEURON ACTIVITY

2.1 INTRODUCTION

The afferent circuitry regulating the activity of dopamine (DA) neurons of the ventral midbrain, including the ventral tegmental area (VTA), is exceedingly complex (Sesack and Grace, 2010; Watabe-Uchida et al., 2012; Beier et al., 2015; Lerner et al., 2015). Although a number of regions project directly to the VTA, numerous studies have shown that there are complex multi-synaptic networks that potently impact DA neuron activity states (Floresco et al., 2003; Butts et al., 2011; Patton et al., 2013; Chang and Grace, 2014; Ferenczi et al., 2016). These circuits provide multiple sites of dynamic regulation and amplification of DA neuron output. Characterizing the mechanisms by which these afferent or "upstream" networks control DA neuron firing, and therefore DA tone in VTA output regions, will be fundamental to our understanding of complex behaviors such as motivation and reward processing, as well as several disease states.

We have previously characterized a circuit comprised of the ventral subiculum (vSub), nucleus accumbens (NAc), and ventral pallidum (VP), which potently influences DA system responsivity (Floresco et al., 2001; 2003; Lodge and Grace, 2007; Valenti et al., 2011). Stimulation of vSub, via NAc and VP, leads to disinhibition of VTA DA neurons, and an increase in the proportion of VTA DA neurons that are spontaneously active, i.e. "population activity." This effect is accompanied by DA release in the NAc (Floresco et al., 2003) and an increase in amphetamine-induced hyperlocomotion (White et al., 2006), a behavioral correlate of DA system responsivity (Moore et al., 2001; Lodge and Grace, 2007; Gill et al., 2011; Valenti et al., 2011; Chang and Grace, 2013). Population activity is a key parameter of DA neuron firing, because DA neurons can only exhibit rapid, phasic, stimulus-driven burst firing (Grace and Bunney, 1984b) if they are

spontaneously active (Lodge and Grace, 2006b). Therefore, the vSub-NAc-VP circuit acts as a critical gain modulator of behaviorally-salient DA neuron outputs. In addition, this circuit controlling population activity has been shown to play essential roles in animal models of schizophrenia, depression, and drug abuse (Lodge and Grace, 2007; Chang and Grace, 2014; Belujon et al., 2016).

Hippocampal control of VTA DA neuron population activity is in turn potently modulated by the medial prefrontal cortex (mPFC). Specifically, attenuation of activity in the infralimbic subdivision of the medial prefrontal cortex (ilPFC; but not the prelimbic subdivision) increases population activity, and this effect is dependent on the vSub (Patton et al., 2013). However, no direct projection exists between ilPFC and vSub (Laroche et al., 2000; Vertes, 2003). The nucleus reuniens of the midline thalamus (RE) forms an anatomical link between the mPFC and the hippocampus, sending a potent, glutamatergic projection to hippocampus (Herkenham, 1978; Vertes et al., 2006) that densely innervates the stratum lacunosum-moleculare of dorsal and ventral CA1, as well as the molecular layer of dorsal and ventral subiculum (Bokor et al., 2002; Vertes et al., 2006; Hoover and Vertes, 2012). Electrophysiological studies show that RE excites CA1 neurons (Dolleman-Van der Weel et al., 1997). Fibers from RE also distribute throughout layers 1 and 5/6 of the mPFC, including dense projections to ilPFC (Vertes et al., 2006). In turn, RE receives reciprocal connections from vSub and ilPFC, forming a functional network (McKenna and Vertes, 2004; Varela et al., 2014).

Given these rich interconnections between ilPFC, RE, and vSub, RE could play a role in the afferent control of VTA DA neuron activity. However, this possibility has not been evaluated experimentally. In the current study, we use in vivo, circuit-based electrophysiological and

behavioral approaches in rats to explore the role of RE in the descending cortical-subcortical circuitry governing VTA DA neuron firing.

2.2 MATERIALS AND METHODS

Animals. All experiments were performed in accordance with the guidelines outlined in the USPHS Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh. All experiments were performed in adult (>PND 65) male Sprague Dawley rats (105 rats total; 300 g - 450 g).

Electrophysiology. Animals were anesthetized with an initial dose of chloral hydrate (Sigma; 400 mg/kg, i.p.) and were supplemented periodically (i.p.) to maintain suppression of the hindlimb withdrawal reflex. Rats were then placed in a stereotaxic frame (Kopf) and body temperature was maintained at 37°C with a temperature-controlled heating pad and rectal probe (Fintronics). In vivo, extracellular recordings were performed using single glass microelectrodes (WPI; impedance 6–8 MΩ) filled with a 2% Chicago Sky Blue (Sigma) solution in 2 M NaCl. This impedance ensures that we are able to clearly resolve the waveform from a single cell with a very high signal-to-noise ratio and without contamination from neighboring cells. Following a craniotomy, electrodes were lowered into the VTA in 9 sequential, vertical "tracks" at 0.2 mm intervals in a predetermined grid pattern in the x-y plane via hydraulic micropositioner (Kopf). Tracks began at the following coordinates from bregma/skull surface in mm: anteroposterior (AP) -5.3, mediolateral (ML) 0.6, dorsoventral (DV) -6.5 to -9, according to the Paxinos and Watson brain atlas (Paxinos and Watson, 2013), and sampled a block of tissue from AP: -5.3 to -5.7 and ML: 0.6 to 1.0, as previously described (Chang and Grace, 2014). Individual, putative DA neurons were recorded with open filter settings (low pass=10 Hz; high pass=16 kHz) enabling identification

using well-established criteria, including 1) slow (2-10 Hz) irregular or bursting firing pattern, 2) long duration (>2.2 ms) biphasic action potential with initial segment-somatodendritic positive phase break, and 3) temporary inhibition of firing during tail or foot pinch (Grace and Bunney, 1983; Ungless and Grace, 2012). Three properties of identified DA neurons were measured: population activity, quantified as the average number of spontaneously active DA neurons per electrode track, i.e. "cells/track" (calculated for each rat), average firing rate, and percent of spikes occurring in bursts. Burst initiation was defined as the occurrence of two spikes with an interspike interval of 80 ms, and burst termination as the occurrence of an interspike interval of 160 ms, as previously described (Grace and Bunney, 1984b). Each neuron was recorded for at least 3 min, and given the typical ~4 hz firing rate of identified DA neurons (Grace and Bunney, 1983; Ungless and Grace, 2012), this resulted in ~720 spikes being included in the analysis of firing properties for each neuron.

Intracranial Infusions in Anesthetized Rats. Local infusions were performed in anesthetized animals immediately before VTA DA neuron recordings. Using the stereotaxic frame, a guide cannula (23 gauge) was placed above the ilPFC, RE, and/or vSub at the following coordinates from bregma/skull surface in mm: ilPFC (AP: +2.7, ML: 0.5, DV: -3.5), RE (AP: -2.2, ML: 2.3, DV: -7.2, 15° angle from vertical), vSub (AP: -5.5, ML: 4.8, DV: -7.1) according to the Paxinos and Watson brain atlas (Paxinos and Watson, 2013). Subsequently, an infusion cannula (33 gauge) was inserted into the guide cannula, extending 1 mm beyond the tip of the guide cannula. Pharmacological agents dissolved in Dulbecco's phosphate-buffered saline (dPBS; Sigma) or dPBS vehicle only were administered through the infusion cannula at a rate of 0.5 µl/min. The guide cannula was left in place for 3 min following infusions to allow for adequate

diffusion of drug. Drug doses were as follows for all experiments. ilPFC: TTX (1 M in 0.5 μl); RE: NMDA (0.75 μg in 0.2 μl), TTX (1 M in 0.2 μl); vSub: TTX (1 M in 0.5 μl).

Chemical stimulation was deliberately used to enable stable, long-duration neuronal excitation without the confounds associated with current spread, activation of fibers of passage, or potential lesions during extended electrical or optical stimulation. All pharmacological agents were injected at doses reported previously to induce specific behavioral and/or neurochemical effects (Lodge and Grace, 2007; Valenti et al., 2011; Patton et al., 2013). Rats received only one injection per region and DA cell recordings were typically performed from 10 min to 2.5 hr after infusions.

Survival surgery and cannula implantation for behavioral studies. All survival surgeries were performed under general anesthesia in a sterile environment. Briefly, rats were anesthetized with isoflurane (induction: 5%, maintenance: 1-3% in oxygen) and placed in a stereotaxic apparatus using blunt, atraumatic ear bars. Bilateral cannulae (23 gauge) were implanted in RE (coordinates from bregma/skull surface in mm: AP: -2.2, ML: 2.3, DV: -7.2, 15° angle from vertical) and fixed in place with dental cement and anchor screws. Once the cement was dry, antibiotic cream was applied to the wound edge (Neosporin), and the rat was removed from the stereotaxic frame and monitored closely until conscious. Rats received postoperative analgesia (Carpofen 5 mg/kg i.p. once per day for 72 hr and Tylenol syrup in softened rat chow 5% v/w available ad libitum for 72 hr) and were allowed to recover for at least one week before behavioral experiments.

Amphetamine-induced hyperlocomotion. Following surgeries, rats were housed in a reverse light/dark cycle room (lights on 7:00PM to 7:00AM) for at least one week before behavioral experiments. Baseline locomotor activity and amphetamine-induced hyperlocomotion were measured by beam breaks in the x–y plane of an open field arena (Coulborn) and analyzed

in 5 min epochs. Following measurement of baseline locomotor activity, NMDA (0.75 µg in 0.2 µl) or dPBS vehicle was injected into RE via the previously implanted guide cannula, followed immediately by D-amphetamine sulfate (0.75 mg/kg, i.p.). Amphetamine-induced hyperlocomotion was then measured for 60 min.

Histology. After electrophysiology, the recording site was marked via electrophoretic ejection of Chicago Sky Blue dye from the tip of the recording electrode (-25 µA constant current, 20-30 min). All rats were killed, decapitated and their brains removed, fixed for at least 48 hr (8% w/v paraformaldehyde in PBS), and cryoprotected (25% w/v sucrose in PBS) until saturated. Brains were sectioned (60 µm coronal sections), mounted onto gelatin-chrom alum-coated slides, and stained with cresyl violet for histochemical verification of electrode and/or acute/chronically implanted cannula placement. All histology was performed with reference to a stereotaxic atlas (Paxinos and Watson, 1986). Only data from animals with accurate placements in all regions were included in the analysis.

Analysis. Electrophysiological analysis of DA neuron activity was performed using commercially available software (LabChart and NeuroExplorer). Locomotor behavior was recorded using TruScan software. All data are represented as the mean±SEM, unless otherwise stated. All statistics were calculated using the GraphPad Prism software program (GraphPad Software).

2.3 RESULTS

2.3.1 RE activation increases VTA DA neuron population activity

We showed previously that the vSub exerts a profound influence on VTA DA neuron activity (Floresco et al., 2003; Lodge and Grace, 2006b; 2007; Patton et al., 2013). Given the dense, excitatory projections from RE to vSub (Dolleman-Van der Weel et al., 1997; Bertram and Zhang,

1999; Vertes et al., 2006), we tested whether RE activation would also affect VTA DA neuron activity. Following infusion of vehicle into the RE (dPBS; n=8 rats and 59 neurons; Fig. 2-1A) we found an average of 1.05±0.1 spontaneously active DA neurons per electrode track in the VTA (i.e. "cells/track"), with an average firing rate of 4.57±0.3 Hz and 27.82±3.4% of action potentials fired in bursts (Fig. 2-1B–F), all of which are consistent with previous findings in untreated animals (Lodge and Grace, 2007; Chang and Grace, 2014; Gill et al., 2014). In contrast, following NMDA infusion into the RE (0.75 μg in 0.2 μl; n=8 rats and 111 neurons) we observed a nearly 63% increase in VTA DA neuron population activity over controls (1.65±0.2 cells/track; unpaired t-test, $t(14)=2.69$, $p=0.02$), with no significant change in firing rate (4.61±0.2 Hz). Infusion of NMDA into regions adjacent to RE, including the interanteromedial, ventromedial, and paraxiphoid nuclei of the thalamus had no effect on VTA DA neuron activity (data not shown). We also observed a small but significant increase in burst firing following NMDA infusion into RE (36.61±2.6%, unpaired t-test, $t(168)=2.00$, $p=0.047$). These data show that RE stimulation is sufficient to enhance VTA DA neuron population activity.

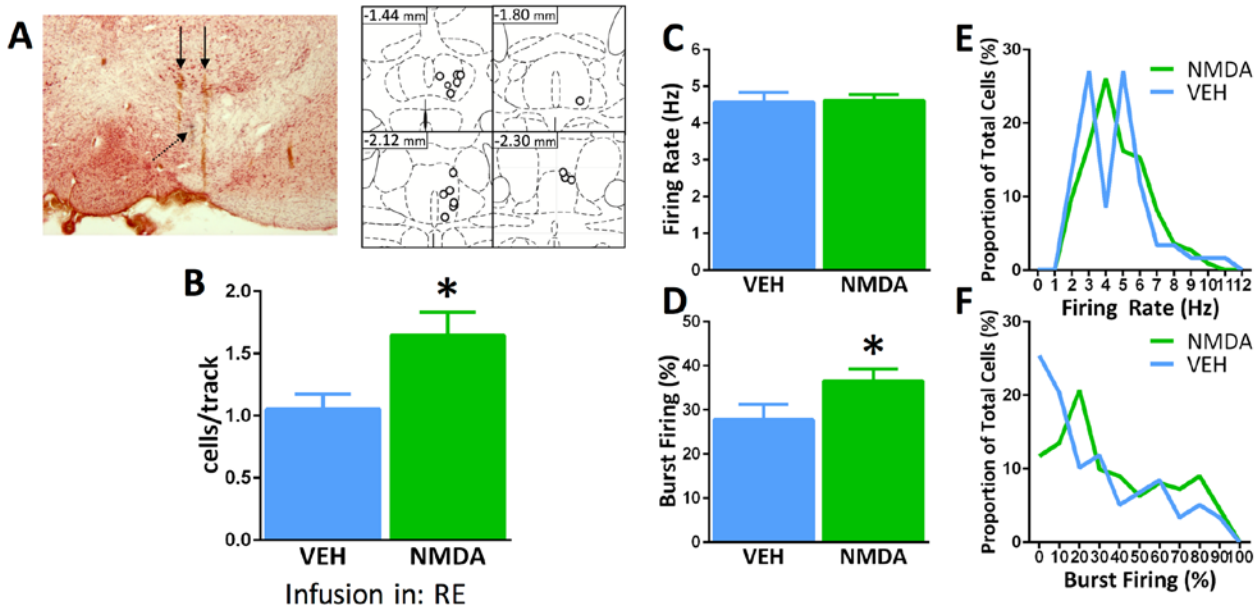

Figure 2-1: Activation of RE produced an enhancement of VTA DA neuron population activity.

A, Left, Representative image of electrode tracks (solid black arrows) and electrode tip (dashed arrow) in the VTA. *Right,* Representation of histological placements of infusion cannulae into RE (open circles). *B,* Activating the RE with NMDA enhanced the number of spontaneously active DA neurons firing in the VTA (expressed as cells/track, green bar) compared with infusion of vehicle (dPBS; blue bar). *C, D,* The average firing rate of spontaneously active dopamine cells was not affected by infusion of NMDA into RE, but the percentage of cells firing in bursts was increased. *E, F,* Distribution of firing rate and burst firing were not affected by infusion of NMDA into the RE (Kolmogorov-Smirnov test). *=$p<0.05$ (unpaired t-test). VEH n=8, NMDA n=8 rats/group; VEH n=59, NMDA=111 neurons/group. Data are represented as mean±SEM.

2.3.2 RE activation increases amphetamine-induced hyperlocomotion

Given that VTA DA neuron population activity correlates with amphetamine-induced hyperlocomotion (Moore et al., 2001; Lodge and Grace, 2007; Gill et al., 2011; Valenti et al., 2011; Chang and Grace, 2013), we measured the locomotor response to amphetamine following acute infusion of dPBS vehicle or NMDA into RE (Fig. 2-2A) in a separate group of awake, behaving rats. Rats that received an acute NMDA infusion (0.75 μg in 0.2 μl; n=8 rats) into RE displayed a significantly enhanced locomotor response to D-amphetamine sulfate injection (0.75 mg/kg, i.p.) compared to vehicle controls (n=13 rats), both when quantified as total distance traveled (Fig. 2-2B, unpaired t-test, $t(19)=2.22$, $p=0.04$) and ambulatory distance in discrete time bins (Fig. 2-2C, two-way RM ANOVA, main effect of treatment $F(1,19)=4.9$, $p=0.03$ and treatment x time interaction $F(8,152)=2.35$, $p=0.04$, Holm-Sidak post-hoc). Therefore, both VTA DA neuron population activity and the locomotor response to amphetamine are increased by RE activation.

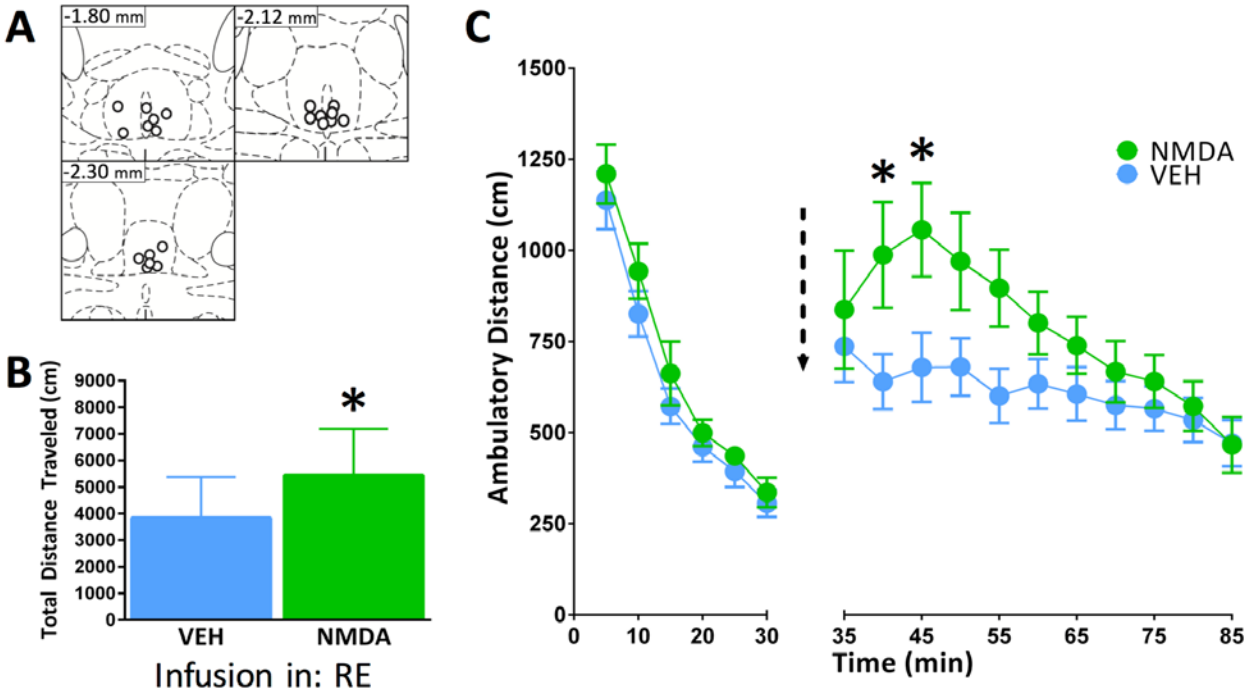

Figure 2-2: Activation of RE increased amphetamine-induced hyperlocomotion.

A, Representation of histological placements of infusion cannulae into RE (open circles). D-amphetamine sulfate (0.75 mg/kg i.p.) was delivered immediately after acute microinjection of NMDA or dPBS vehicle into RE (both represented by dashed arrow). Baseline locomotor activity (0-30min) and amphetamine-induced hyperlocomotion (35-90 min) were then measured. *B*, Pharmacological activation of RE increased total distance traveled in the 60 min post-injection period compared to controls (unpaired t-test, *=$p<0.05$). *C*, Pharmacological activation of RE increased ambulatory distance during the post-injection period (two-way RM ANOVA, Holm-Sidak post-hoc, *=$p<0.05$). VEH n=13, NMDA n=8 rats/group. Data are represented as mean±SEM.

2.3.3 The effect of RE activation on VTA DA neuron population activity requires vSub

Given evidence that the vSub potently controls VTA DA neuron activity (Floresco et al., 2003; Lodge and Grace, 2006b; 2007; Patton et al., 2013), we examined whether the enhancement of VTA DA neuron population activity following RE activation was dependent on the vSub. This was tested by pharmacologically inactivating the vSub by TTX infusion (1 μM in 0.5 μl dPBS) during simultaneous activation of the RE by NMDA infusion (0.75 μg in 0.2 μl; Fig. 2-3A). Rats receiving either vehicle infusion in both regions, or vehicle in RE and TTX in vSub, exhibited similar DA neuron firing properties (dPBS/dPBS: 0.98±0.08 cells/track, 4.40±0.3 Hz, 36.30±4.1%, n=8 rats and 40 neurons; dPBS/TTX: 0.90±0.2 cells/track, 4.46±0.3 Hz, 31.65±4.1%, n=8 rats and 54 neurons; Fig. 2-3B-F). These findings are consistent with previous data from our group in untreated animals (Lodge and Grace, 2007; Patton et al., 2013). In addition, vehicle infusion into vSub did not attenuate the enhancement of VTA DA neuron population activity observed following NMDA infusion into RE (1.56±0.2 cells/track, 4.33±0.2 Hz, 31.43±2.9%, n=10 rats and 107 neurons), which was significantly greater than in control animals (one-way ANOVA, main effect of treatment $F(3,28)=5.7$, $p=0.004$, Tukey post-hoc) and consistent with our findings above (Fig. 2-1). In contrast, infusion of TTX into the vSub completely abolished the effect of RE activation on VTA DA neuron population activity: animals in this group (n=6 rats and 41 neurons) displayed 0.98±0.08 active cells/track on average, which was not different than controls (one-way ANOVA, 95% CI of diff: -0.65-0.49). In addition, no changes in firing rate (4.55±0.2 Hz; one-way ANOVA, $F(3,238)=0.12$, $p=0.95$) or burst firing (31.32±3.6%, one-way ANOVA, $F(3,237)=0.33$, $p=0.80$) were observed in this group compared to control animals. These findings confirm that the vSub is necessary for RE stimulation to increase VTA DA neuron population activity.

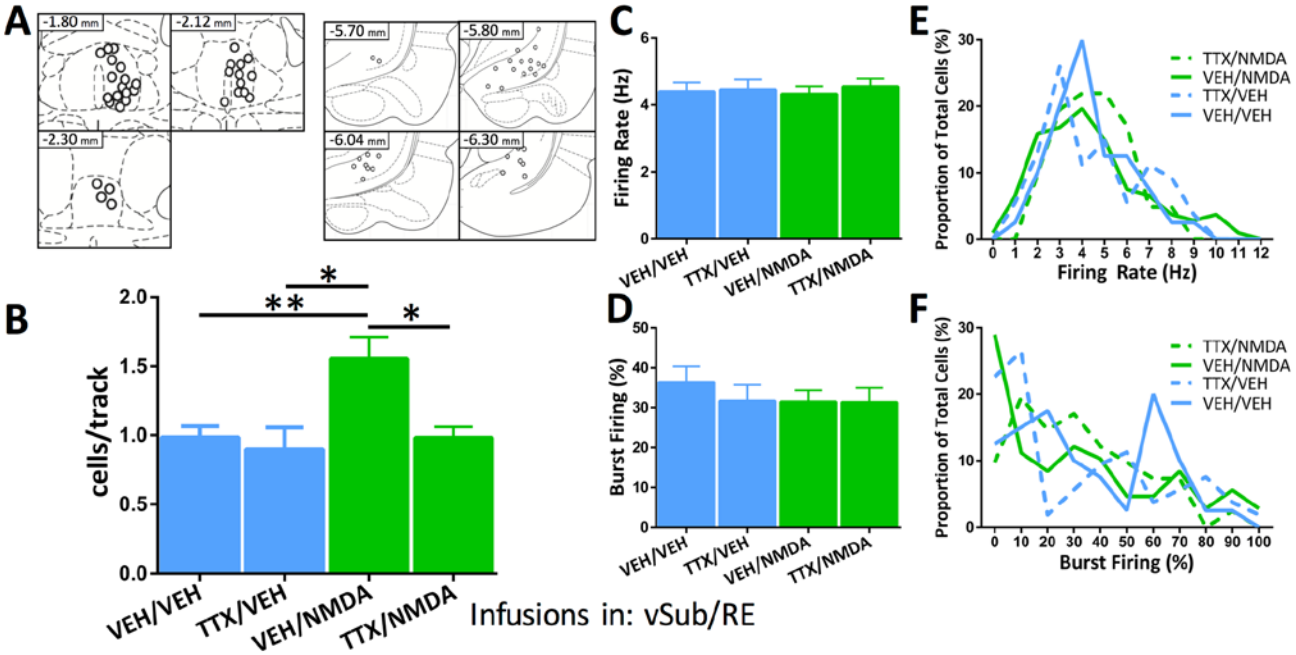

Figure 2-3: Enhanced VTA DA neuron population activity following RE activation was prevented by inhibition of vSub.

A, Representation of histological placements of infusion cannulae into RE (left) and vSub (right; open circles). TTX or dPBS vehicle was injected into vSub, followed immediately by injection of NMDA or dPBS vehicle into RE. VTA DA neuron population activity, firing rate, and burst firing were then measured. *B*, Pharmacological activation of RE increased population activity, while parallel inhibition of vSub prevented this effect. *C*, *D*, The firing rate of spontaneously active dopamine cells and the percentage of cells firing in bursts were not affected by any manipulation. *E*, *F*, Distribution of firing rate and burst firing were not affected by infusion of NMDA into the RE (Kruskal-Wallis test, firing rate: $H=1.70$, $p=0.64$; bursting: $H=2.15$, $p=0.54$). *=$p<0.05$,

**=p<0.01 (one-way ANOVA, Tukey post-hoc). X-axis in panels B-D is infusions in vSub/RE. n=6–10 rats/group; n=40–107 neurons/group. Data are represented as mean±SEM.

2.3.4 ilPFC control of VTA DA neuron population activity requires RE

We have shown previously that excitation or inhibition of ilPFC bidirectionally modulates VTA DA neuron population activity. ilPFC inactivation induces an increase in population activity that does not occur following simultaneous vSub inhibition (Patton et al., 2013). Given that there is no direct projection from ilPFC to vSub (Laroche et al., 2000; Vertes, 2003), we tested whether RE could be mediating the effect of ilPFC inhibition on VTA DA neuron population activity. This was tested by pharmacologically inactivating the RE by TTX infusion (1 μM in 0.2 μl dPBS) during simultaneous inactivation of the ilPFC by TTX infusion (1 μM in 0.5 μl dPBS; Fig. 2-4A). In rats receiving either vehicle in both regions, or vehicle in ilPFC and TTX in the RE, the number of spontaneously firing DA neurons per electrode track, firing rate, and burst firing were comparable (dPBS/dPBS: 1.0±0.1 cells/track, 4.21±0.2 Hz, 28.5±3.3%, n=10 rats and 65 neurons; dPBS/TTX: 1.0±0.2, 4.2±0.2 Hz, 30.4±3.4%, n=11 rats and 68 neurons; Fig. 2-4B-F). These findings are consistent with previous data from our group in untreated animals (Patton et al., 2013). In addition, vehicle infusion in the RE did not influence the increase in VTA DA neuron population activity observed following TTX infusion into ilPFC (1.60±0.2 cells/track, 4.67±0.2 Hz, 27.15±2.8%, n=7 rats and 83 neurons), in which we observed a significant increase over controls (one-way ANOVA, main effect of treatment $F(3,32)=6.7$, $p=0.001$, Tukey post-hoc). However, infusion of TTX into RE completely prevented the effect of ilPFC inhibition on VTA DA neuron population activity (n=8 rats and 40 neurons; 0.58±0.08 cells/track) making this group statistically indistinguishable from controls (one-way ANOVA, 95% CI of diff: -0.16-0.97). In addition, combined infusion of TTX into ilPFC and RE resulted in a small but statistically significant enhancement of burst firing (one-way ANOVA, main effect of treatment $F(3,251)=2.7$, $p=0.046$, Tukey post-hoc). These findings confirm that activity in RE is necessary for the effect of ilPFC

inhibition on increasing VTA DA neuron population activity, and suggest that RE acts as a necessary intermediary between ilPFC and vSub, playing a crucial role in cortical modulation of VTA DA neuron activity.

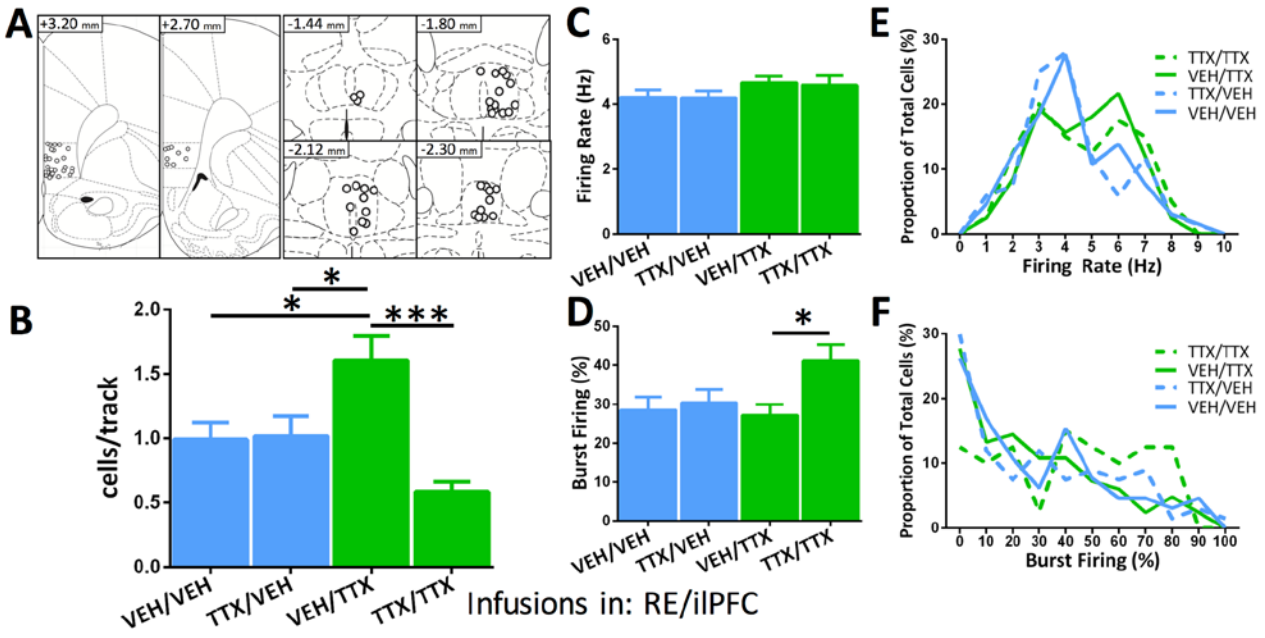

Figure 2-4: Enhanced VTA DA neuron population activity following ilPFC inactivation was prevented by inhibition of RE.

A, Representation of histological placements of infusion cannulae into ilPFC and RE (open circles). TTX or dPBS vehicle was injected into RE, followed immediately by injection of TTX or dPBS vehicle into ilPFC. VTA DA neuron population activity, firing rate, and burst firing were then measured. *B,* Pharmacological inhibition of ilPFC increased population activity, while parallel inhibition of RE prevented this effect. *C, D,* The firing rate of spontaneously active dopamine cells was not affected by any manipulation, while the percentage of cells firing in bursts was significantly enhanced in the TTX/TTX group. *E, F,* Distribution of firing rate and burst firing were not affected by infusion of NMDA into the RE (Kruskal-Wallis test, firing rate: H=0.060, p=0.99; bursting: H=0.54, p=0.91). *=p<0.05, ***=p<0.001 (one-way ANOVA, Tukey post-hoc).

X-axis in panels B-D is infusions in RE/ilPFC. n=7–11 rats/group; n=40–83 neurons/group. Data are represented as mean±SEM.

2.4 DISCUSSION

We present evidence demonstrating that RE is a novel region involved in control of VTA DA neuron population activity. Pharmacological stimulation of RE enhances DA neuron population activity, without affecting average firing rate, and mildly enhancing burst firing. The same stimulation paradigm also enhances amphetamine-induced hyperlocomotion, which strongly correlates with DA system responsivity (Moore et al., 2001; Lodge and Grace, 2007; Gill et al., 2011; Valenti et al., 2011; Chang and Grace, 2013). Furthermore, we show that the effect of RE stimulation is prevented if vSub is also inhibited, suggesting that activity in vSub is necessary for RE to drive VTA DA neuron firing. Finally, inactivation of RE prevents the increase in VTA DA neuron population activity observed following ilPFC inhibition, suggesting that the ilPFC potently regulates RE drive of VTA DA neuron activity.

We and other groups have shown previously that stimulation of the vSub increases VTA DA neuron population activity and amphetamine-induced hyperlocomotion, without affecting firing rate or burst firing (Floresco et al., 2001; 2003; Hammad and Wagner, 2006; White et al., 2006). In addition, RE has been shown to drive activity in CA1 and subiculum via asymmetric synapses onto the distal dendrites of pyramidal neurons (Wouterlood et al., 1990; Dolleman-Van der Weel et al., 1997; Bertram and Zhang, 1999). Therefore, the current findings suggest that stimulation of RE enhances population activity and amphetamine-induced hyperlocomotion via direct, as well as indirect (via CA1) excitation of vSub. Multiple previous studies have characterized the role of RE in mediating communication between the mPFC and hippocampus in behaviors requiring intact spatial and working memory (Hembrook and Mair, 2011; Hembrook et al., 2012; Cassel et al., 2013; Cholvin et al., 2013; Prasad et al., 2013; Duan et al., 2015; Ito et al., 2015; Layfield et al., 2015; Prasad et al., 2017), i.e. predominantly dorsal hippocampal functions.

However, the present work is one of the first to show that RE modulates ventral hippocampal functions, which is consistent with the fact that projections from RE to ventral hippocampus are the most dense of any RE efferents to hippocampus (Herkenham, 1978; Hoover and Vertes, 2007; Varela et al., 2014).

We have shown previously that the ilPFC bidirectionally modulates VTA DA neuron population activity: inhibition of ilPFC enhances VTA DA neuron population activity, and this effect is dependent on the vSub, whereas excitation of ilPFC attenuates VTA DA neuron population activity, and this effect depends on the basolateral amygdala (Patton et al., 2013). However, there are multiple potential pathways between ilPFC and vSub that could mediate the effect of ilPFC inhibition. The current findings provide strong evidence that RE constitutes the connection by which the ilPFC can control vSub drive of VTA DA neuron activity. This finding is consistent with several studies characterizing monosynaptic interconnections between ilPFC and RE (Vertes, 2002; McKenna and Vertes, 2004; Hoover and Vertes, 2007). ilPFC projections to RE are likely glutamatergic, making it apparently paradoxical that inhibition of ilPFC could lead to drive of vSub via RE. However, there is mounting evidence that cortical regions may influence excitability of thalamic circuits via feed-forward inhibition mediated by the reticular nucleus of the thalamus (Cornwall et al., 1990; Paz et al., 2011; Pratt and Morris, 2015; Wimmer et al., 2015). In this context, our findings would suggest that the ilPFC provides a potent down-modulation of RE drive of the hippocampus via the reticular thalamus. The finding that inhibition of RE alone did not alter VTA DA neuron population activity does not preclude this model, because RE likely exhibits low levels of activity at baseline in the anesthetized state. This model could explain the effects of ilPFC hyperactivity, which is proposed to play a role in models of depression (Belujon and Grace, 2014; Chang and Grace, 2014), in disrupting limbic emotional influences on memory

processes. It also implies that dysfunction in ilPFC would remove any feed-forward inhibition provided by the reticular thalamus, leading to RE-vSub hyperexcitability.

It should be noted that, despite anatomical evidence for direct connections, our manipulations are not projection-specific, and therefore we cannot completely rule out the involvement of intervening structures in these effects. However, we believe that the regions manipulated here form vital nodes in the circuit for several reasons. First, while the RE receives a diverse array of afferent inputs (McKenna and Vertes, 2004), its major projection targets are restricted primarily to hippocampus and mPFC (Vertes, 2002; Vertes et al., 2006). Given that our NMDA infusions targeted the portion of RE that contains primarily neurons projecting to ventral hippocampus, i.e. the rostral portion (Vertes et al., 2006), RE-mPFC projections are not likely involved in the responses observed. In addition, our data show a complete reversal of the effect of RE stimulation on VTA DA neuron population activity with vSub inhibition, supporting a direct RE-vSub action. Finally, although projections from mPFC to entorhinal cortex (Vertes, 2003) and from mPFC to VTA (Vertes, 2003) have been described anatomically, these circuits are unlikely to be involved in these findings given that RE inhibition completely reversed of the effect of ilPFC inhibition on VTA DA neuron population activity. It should also be noted that recordings were performed in anesthetized animals. Nonetheless, whereas anesthesia is likely to impact baseline activity, it should not qualitatively impact the effects of pathway activation. The fact that our behavioral studies in awake animals were consistent with our electrophysiological findings supports this contention.

Taken together, our findings support a model whereby inactivation of ilPFC leads to an increase in RE activity, driving vSub and enhancing VTA DA neuron population activity. We propose that RE is a novel, key modulator of subcortical/limbic circuits involved in the control of

VTA DA neuron firing, and as such could play a role in psychiatric illnesses involving dysfunction in these circuits. In particular, abnormally high DA tone underlies the pathophysiology of psychosis across psychiatric illnesses (Heinz and Schlagenhauf, 2010; Howes et al., 2012; Winton-Brown et al., 2014), most notably in schizophrenia (Howes et al., 2012). In addition, increased activity in the subiculum (Tregellas et al., 2007b; Allen et al., 2012; Tregellas et al., 2014) and thalamus (Silbersweig et al., 1995; Tregellas et al., 2007b) have been described in patients with schizophrenia. Our group has modeled the hippocampal hyperexcitability observed in schizophrenia using the methylazoxymethanol acetate (MAM) developmental disruption model, which exhibits enhanced VTA DA neuron population activity and aberrantly high vSub activity (Moore et al., 2006; Lodge and Grace, 2007; Modinos et al., 2015). Given that stimulation of RE was sufficient to replicate the hyperdopaminergic state observed in MAM animals, thalamic projections to hippocampus could represent a key connection that perpetuates the subcortical hyperexcitability and hyperdopaminergic state seen in patients with schizophrenia. Indeed, this idea has been proposed, but never tested experimentally (Lisman et al., 2010). In addition, these findings are particularly interesting in light of recent studies demonstrating reduced functional connectivity between the mPFC and thalamus in patients with schizophrenia (Anticevic et al., 2014), a measure that can differentiate psychotic and non-psychotic patients (Anticevic et al., 2015b). This suggests that targeting thalamic regions in humans that are interconnected with both mPFC and hippocampus could represent an effective approach for developing novel treatments for psychotic disorders, including schizophrenia.

3.0 PREFRONTAL INPUTS TO THE NUCLEUS REUNIENS OF THE MIDLINE THALAMUS MODULATE FIRING PATTERN VIA PARALELL CORTICOTHALAMIC PATHWAYS

3.1 INTRODUCTION

The thalamus has long been recognized for its role as a way station for sensory information bound for cortex, a function accomplished by its "first-order" nuclei (Sherman and Guillery, 2001; Sherman, 2016). However, the thalamus also contains subregions that facilitate information transfer between cortical areas, termed "higher-order" nuclei. This transthalamic route between cortical areas is thought to allow for enhanced modulation or gating of information not available to cortico-cortical pathways (Guillery and Sherman, 2002; Saalmann et al., 2012; Mitchell, 2015), but higher order thalamic nuclei are much less well-studied compared to first-order nuclei. In particular, it is still unclear precisely how corticothalamic input modulates thalamocortical cell firing in higher-order nuclei. Corticothalamic input to both first-order and higher-order thalamic nuclei consists of direct, monosynaptic connections, as well as feedforward inhibition from the thalamic reticular nucleus (TRN) and extrathalamic sources (Sherman and Guillery, 2001; Halassa and Acsády, 2016). This circuity is complex and corticothalamic input is functionally heterogenous across cortical areas, highlighting the importance of characterizing the circuit dynamics of these connections in different parts of the corticothalamic network (Halassa et al., 2014; Crandall et al., 2015).

The nucleus reuniens of the midline thalamus (RE) is a higher-order thalamic nucleus that represents a critical mediator of communication between the mPFC and hippocampus. Previous studies have shown that RE is involved in several different classes of behavior that share in common a dependence on the coordinated action of these two regions (Cassel et al., 2013), including spatial navigation (Jankowski et al., 2014; Ito et al., 2015), working memory (Cholvin

et al., 2013; Duan et al., 2015; Layfield et al., 2015; Hallock et al., 2016), executive function (Prasad et al., 2013; Linley et al., 2016; Prasad et al., 2017) fear learning (Davoodi et al., 2011; Xu and Südhof, 2013; Sierra et al., 2017; Vetere et al., 2017), and mood regulation (Kafetzopoulos et al., 2017). RE inputs to hippocampus selectively target apical tufted dendrites and interneurons in the stratum-lacunosum moleculare of dorsal and ventral CA1, as well as the molecular layer of the subiculum, and these connections are reciprocal (Çavdar et al., 2008; Varela et al., 2014; Vertes et al., 2015). Corticothalamic projections to RE appear to follow the basic organizing principles described for other thalamic nuclei: neurons from layers 5 and 6 of the medial prefrontal cortex (mPFC) send monosynaptic connections to RE (McKenna and Vertes, 2004; Varela et al., 2014) and layer 6 ilPFC neurons send collaterals to the antero-medial TRN (Cornwall et al., 1990), the same TRN subregion that projects to RE (Kolmac and Mitrofanis, 1997).

Despite the well-established importance of RE in a variety of behaviors and the thorough characterization of its anatomical connections, studies examining the baseline firing properties of RE neurons and their modulation by afferent structures are sparse (Walsh et al., 2017). In particular, it is unknown how mPFC controls the firing of RE neurons, and whether corticothalamic input from mPFC to RE is similar or different to that in other parts of the thalamus. Cortical input is likely to be dynamic, and critical to gating RE output in a variety of behavioral contexts. Our goal for the current study was to assess the combined and separate contributions of direct and indirect ilPFC-RE pathways to controlling RE neuron firing. We focus on inputs to RE from the infralimbic subdivision of the mPFC (ilPFC), because we have previously demonstrated that inhibition of ilPFC enhances dopamine (DA) neuron firing in the ventral tegmental area (VTA) via the ventral subiculum, likely by disinhibiting RE (Patton et al., 2013; Zimmerman and Grace, 2016). We used in vivo, extracellular, single-unit recordings in the anesthetized rat and

manipulated neural activity using targeted pharmacological manipulations, electrical stimulation, and a projection-specific implementation of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Our findings show that ilPFC can robustly modulate multiple aspects of RE neuron firing across diverse timescales.

3.2 MATERIALS AND METHODS

Animals. All experiments were performed in accordance with the guidelines outlined in the United States Public Health Service Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. All experiments were performed in adult (>65 d old) male Sprague Dawley rats (59 rats total; 300 – 500 g).

In vivo electrophysiology in anesthetized rats. Rats were anesthetized with an initial dose of chloral hydrate (400mg/kg i.p.) and supplemented periodically (i.p.) to maintain suppression of the hindlimb withdrawal reflex. Rats were placed in a stereotaxic frame and body temperature was maintained at 37°C with a temperature-controlled heating pad and rectal probe. Extracellular recordings were performed using single glass microelectrodes (impedance 6 – 8 M) filled with a 2% Chicago sky blue solution in 2 M NaCl. This impedance ensures that we are able to clearly resolve the waveform from a single neuron with a very high signal-to-noise ratio and without contamination from neighboring neurons. Following a craniotomy electrodes were lowered into the RE in vertical tracks at 0.2mm intervals in the *x-y* plane via hydraulic micropositioner. The sampling area comprised a block of tissue including the RE from bregma/dural surface (in mm) AP: -1.6 to -2.2, ML: 0.1-0.5, and DV: -5.5 to -7.5. Sampling in the AP direction was counterbalanced across animals. Every spontaneously active neuron encountered in this block of

tissue was recorded for at least 3 minutes, and given the typical 1-2 Hz firing rate of RE neurons this resulted in ~270 spikes being included in the analysis of firing properties for each neuron. Individual neurons were recorded with broad filter settings (low pass, 10 Hz; high pass, 16 kHz). Immediately following recordings, the recording site was marked via electrophoretic ejection of Chicago Sky Blue from the tip of the recording electrode. All neurons included in the analysis were confirmed to be located in the RE by referencing their position to the marked recording site.

Intracranial infusions and electrical stimulation. For intracranial infusions a guide cannula (23 gauge) was placed above the ilPFC or TRN at the following coordinates from bregma/skull surface (in mm): ilPFC AP: 2.7; ML: 0.5; DV: -3.5; TRN AP: -1.6, ML: 1.6, DV: -5.0, according to the Paxinos and Watson brain atlas (Paxinos and Watson, 2013). Subsequently an infusion cannula (33 gauge) was inserted into the guide cannula, extending 2 mm beyond the tip of the guide cannula. Pharmacological agents dissolved in Dulbecco's PBS (dPBS) or dPBS vehicle only were administered through the infusion cannula at a rate of 0.5μl/min. The guide cannula was left in place for 3 min following infusions to allow for adequate diffusion of drug. Drug doses were as follows for all experiments: TTX 1M in 0.5μl; muscimol, BODIPY® TMR-X conjugate (Thermofisher) 0.8mM in 0.2μl. All pharmacological agents were injected at doses reported previously to induce specific behavioral and/or neurochemical effects (Lodge and Grace, 2007; Allen et al., 2008; Valenti et al., 2011; Patton et al., 2013). Rats received only one injection per region and RE cell recordings were performed from 5 m to 2.5 h after infusions.

To visualize the spread of fluorescent muscimol, animals were transcardially perfused with 0.85% saline followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde overnight and cryoprotected (25% w/v sucrose in PBS) until saturated. Coronal sections (60μm thickness) were taken. During image acquisition exposure time was optimized for image quality.

The spread of the drug was then qualitatively assessed on several sections and if more than 30% of the area of fluorescence was outside the area of the TRN the animal was excluded from further analyses.

For electrical stimulation, a concentric bipolar stimulating electrode was lowered into ilPFC at the following coordinates from bregma/skull surface (in mm): ilPFC AP: 2.7; ML: 0.5; DV: -4.5. Single pulses (1mA, 0.25 msec pulse-width) were delivered at 0.5 hz. Spontaneously active neurons were recorded for 3 m before 5 pulses were delivered. If the neuron responded to these initial pulses, more pulses were delivered until the firing pattern of the neuron stabilized. Spontaneous activity was also recorded after stimulation to assess for persistent effects on spontaneous firing pattern. Multiple neurons were recorded in the same animal, but the effects of stimulation did not differ qualitatively in neurons recorded early vs. late in recordings.

Viral constructs. To achieve hM4Di DREADDs expression in ilPFC, we used an AAV vector *(rAAV2-hSyn-HA-hM4D(Gi)-IRES-mCitrine*) containing a synapsin promoter, as well as a N-terminal HA tag and mCitrine reporter. A vector lacking the DREADDs gene *(rAAV2-hSyn-EGFP)* was used for control experiments. Both vectors were obtained from the UNC Vector Core.

Survival surgeries for virus injections. All survival surgeries were performed under general anesthesia in a sterile environment. Briefly, rats were anesthetized with isoflurane (induction: 5%; maintenance: 1–3% in oxygen) and placed in a stereotaxic apparatus using blunt, atraumatic ear bars. Then 400nl of virus was injected unilaterally into the right ilPFC via a pulled glass pipette (38-42µm tip diameter) and pneumatic pressure over the course of 15min. The pipette was left in place for 15min after the infusion to allow for adequate diffusion. The wound was then closed and antibiotic cream was applied to the wound edge, and the rat was removed from the stereotaxic frame and monitored closely until conscious. Rats received analgesia (Medigel containing

carpofen, 5 mg/kg/day, p.o.) 24 h before and 24 h after surgery, and immediately following surgery (carpofen, 5mg/kg, s.c.). Rats were allowed 12 weeks to achieve sufficient construct expression in terminals in RE before experiments.

In vivo electrophysiology in DREADD-expressing animals. Animals were prepared for recording and the RE was sampled as described above. Extracellular recordings were performed using a single glass microelectrode glued to a pulled glass pipette (20μm tip diameter) filled with dPBS vehicle or clozapine-n-oxide (CNO; Tocris). The recording electrode tip and infusion pipette tip were separated by ~150μm. Spontaneously active neurons were recorded for 3 m before, during, and after vehicle or CNO (dissolved in dBPS; 100μM in 60nl) was delivered over the course of 30 to 60 s. Multiple cells were recorded in the same animal and separated by at least 200μm. Each cell received only one exposure to vehicle or CNO.

Localization of DREADDs expression. Immediately following electrophysiological recordings animals were transcardially perfused with 0.85% saline followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde overnight and cryoprotected (25% w/v sucrose in PBS) until saturated. Coronal sections (10μm thickness) were taken and mounted onto glass slides. Immunohistochemistry for HA was then performed in the following manner. Tissue was blocked in a solution of 1x PBS containing 0.3% Triton X-100 and 3% normal goat serum, then incubated in primary antibody (rabbit anti-HA, 1:500, Cell Signaling, 3724) overnight at room temperature. Slides were then washed and then incubated with a secondary fluorescent antibody (goat anti-rabbit 594, 1:500, Abcam, ab150080) for 3 h. Slices were then counterstained with DAPI and coverslipped with ProLong Gold. Viral spread of DREADDs-expressing constructs was then qualitatively assessed in ilPFC on several sections using anti-HA fluorescence and if more than 30% of the area of fluorescence was outside the ilPFC the animal

was excluded from future analyses. Terminal expression in RE was also confirmed using anti-HA fluorescence. Viral spread and terminal expression were assessed in EGFP-only animals using endogenous fluorescence. During image acquisition exposure time was optimized for image quality. All antibodies were previously validated for specificity, as described on the manufacturer's websites and the *Journal of Comparative Neurology* antibody database.

Analysis parameters for RE neuron firing properties. Bursts were defined using the following interval criteria: maximum interval to start a burst: ≤6 ms, maximum interval to end burst: 10 ms, minimum interval between burst: 200 ms, minimum duration of burst: 2 ms, minimum number of spikes within a burst: 2 (Kim et al., 2011). We used a 6 ms interspike interval (ISI) for the maximum interval to start a burst after an examination of ISI distributions in our data revealed that some putative burst spikes were occurring at intervals >4 ms, the typical but conservative cutoff used to classify thalamic bursts (Guido et al., 1992; Lu et al., 1992; Ramcharan et al., 2005). We also verified that bursts conformed to other previously established criteria for thalamic burst firing, i.e. a preceding >100 ms of no spikes and a gradual lengthening of the ISI within a burst (Guido et al., 1992). For mean spikes per burst analysis, cells in which no bursts occurred were excluded from analysis. The average number of spontaneously active RE neurons per electrode track, i.e. "cells/track" was calculated for each individual animal.

Experimental Design and Statistical Analysis Electrophysiological analysis of RE neuron activity was performed using commercially available software (LabChart and NeuroExplorer). All statistics were calculated using the GraphPad Prism software program (GraphPad Software). Experimental design for each experiment is as follows.

ilPFC Inhibition Experiment: 21 total rats were included in this experiment. 138 neurons were found to lie within RE and were included in the analysis. Multiple neurons were recorded

before and after drug injection in the same animal, as described above. Control animals received only vehicle (i.e. dPBS) infusion. Therefore, neurons were recorded under four different conditions: 1) before vehicle injection, 2) after vehicle injection, 3) before drug injection, and 4) after drug injection. Note that conditions 1 and 3 are identical. Neurons exposed to the same experimental conditions were pooled across animals for statistical analyses. Reported sample sizes indicate the number of cells recorded in total from all animals. Significance testing was performed using the Mann-Whitney U test, unpaired t-test, and Kolmogorov-Smirnov test. Regarding sample size determination, we are the first group to perform extracellular, single-unit recordings in the RE of anesthetized rats. In our studies, we found that burst firing propensity (i.e. percent spikes fired in bursts) in RE neurons, even recorded in the same animal in close spatial and temporal proximity, was highly variable and not normally distributed. Our statistical analyses of burst firing data were designed to account for this variability while also describing physiologically relevant changes across the population of recorded RE neurons. In addition, we found that the distribution of burst firing propensity under baseline conditions between experiments was broadly consistent, suggesting that our data reflect the population. In experiments #1 and #3, the mean spikes per burst also provides a less-variable indicator of burst firing propensity that has been shown to depend on the same intracellular mechanisms as burst firing (Guido et al., 1992).

ilPFC Stimulation Experiment: 3 rats were included in the experiment. 8 neurons from these rats were found to lie within RE and were included in the analysis. Each neuron was recorded before, during, and after acute electrical stimulation. All RE neurons from all animals were pooled for analyses.

TRN Inhibition Experiment: 17 rats were included in the experiment. 132 neurons from these rats were found to lie within RE and were included in the analysis. The experimental design

and justification of sample size here was identical to that described above in the "ilPFC Inhibition Experiment", but muscimol in TRN was used instead of TTX in ilPFC. Significance testing was performed using the Mann-Whitney U test, unpaired t-test, and Kolmogorov-Smirnov test.

DREADDs Experiment: 18 rats were included in the experiment. 75 neurons from these rats were found to lie within RE and were included in the analysis. Multiple neurons were recorded in the same animal. Each neuron was recorded before, during, and after vehicle or drug infusion, as described above. This resulted in cells exposed to 4 different manipulations as follows: animals expressing EGFP+vehicle injection (EGFP+VEH), animals expressing EGFP+CNO injection (EGFP+CNO), animals expressing hM4Di+vehicle injection (hM4Di+VEH), and animals expressing hM4Di+CNO injection (hM4Di+CNO). Significance testing was performed using the Wilcoxon matched pairs signed-rank test. The justification of sample size here was identical to that described above in the "ilPFC Inhibition Experiment".

3.3 RESULTS

3.3.1 Baseline firing properties of RE neurons

A growing body of evidence suggests that the RE performs a critical function as a relay between several subregions of both the mPFC and hippocampus. However, the baseline firing properties of RE neurons and the circuit dynamics governing their activity are at this point understudied. To begin to address this question, we performed extracellular recordings of spontaneously active RE neurons in anesthetized rats. Each neuron was recorded for 3min and firing rate and multiple burst firing parameters were measured. Burst firing was defined using previously established criteria (Guido et al., 1992; Kim et al., 2011) and based on our independent assessment of interspike intervals in the recorded sample. The majority of RE neurons recorded exhibited both tonic and

burst firing patterns, most often intermixed in the same neuron (Fig. 3-1 A-B). The mean firing rate of RE neurons was 2.16±0.36 Hz while the median firing rate was 1.60 Hz (Fig. 3-1C). Of the recorded neurons, >75% exhibited a firing rate at or below 3.0 Hz, while firing rates in the remaining minority of neurons ranged from 4-12 Hz. The mean percentage of spikes fired in bursts was 53.6±4.8%, while the median was 61.41% (Fig. 3-1D). Of the recorded neurons, 18% did not exhibit any burst firing, while the remaining neurons exhibited a percentage of spikes fired in bursts ranging from <5% to >95%. In addition, the mean spikes per burst in RE neurons was 2.9±0.1 spikes, while the median was 2.68 spikes (Fig. 3-1E). Finally, although observations suggested that neurons with higher firing rates tended to exhibit a lower percentage of spikes fired in bursts (Fig. 3-1F), these parameters were not correlated significantly ($F(1,46)=3.44$, $p>0.05$, 95% CI=-0.04—0.001; $R^2=0.07$).

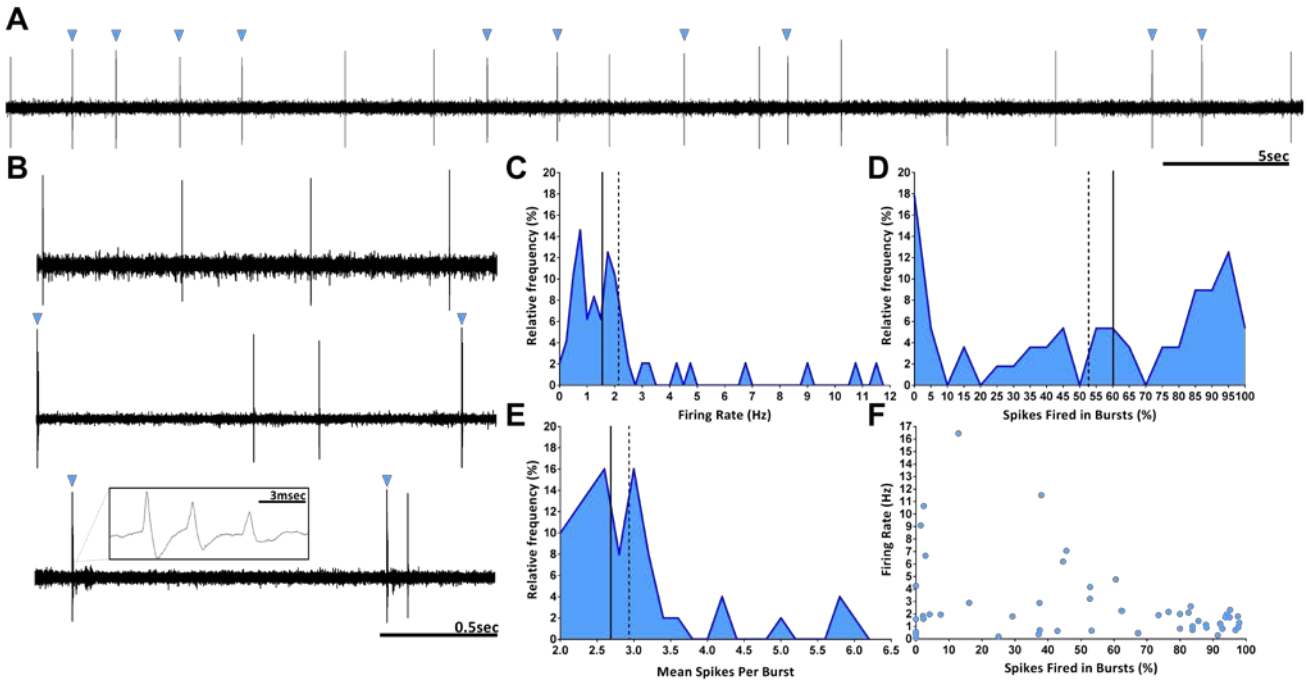

Figure 3-1: Baseline firing properties of RE neurons.

Extracellular recordings of spontaneously active RE neurons were performed in anesthetized rats under baseline conditions. ***A,*** Representative trace demonstrating the typical firing pattern of an RE neuron, with mixed tonic and burst (blue triangles) firing. ***B,*** Three representative traces demonstrating the variable amount of burst firing present in the RE neuron population, from completely absent (top), to mixed (middle), to nearly all bursting (bottom). Inset in bottom panel demonstrates typical RE burst architecture. ***C-E,*** Relative frequency histograms of firing rate, the percentage of spikes fired in bursts, and the mean spikes per burst for 48 recorded RE cells with group mean (dashed line) and median (solid line) indicated. ***F,*** Firing rate plotted against the percentage of spikes fired in bursts. These parameters were not significantly correlated in this group of recorded neurons ($F(1,46)=3.44$, $p>0.05$, 95% CI=-0.04—0.001, $R^2=0.07$). n=48 cells for panels C-F.

3.3.2 Inhibition of ilPFC reduces burst firing in RE neurons

Previous work from our group (Zimmerman and Grace, 2016) has implicated ilPFC-RE communication in controlling VTA DA neuron population activity. However, the circuit dynamics underlying ilPFC-RE communication have not been studied in detail. To address this question we recorded spontaneous activity in RE cells in anesthetized rats before and after acute infusion of TTX into ilPFC (1μmol/L in 0.5μl), in which several neurons were recorded before and after TTX infusion in the same animal (Fig. 3-2). Data from all neurons recorded in all animals were then pooled. Firing rate and burst firing parameters were assessed in each neuron recorded. To assess for effects of time under anesthesia and prior sampling in RE, RE neurons were recorded in animals in which dPBS vehicle was infused into ilPFC. Following TTX or vehicle infusion in ilPFC no differences were observed in firing rate in RE neurons recorded after infusion compared to RE neurons recorded before infusion (Fig. 3-2C; Mann-Whitney, VEH: U=622, p=0.28, 95% CI=-0.8355-0.304, TTX: U=390.5, p=0.44, 95% CI=-29.05-7.04). However, following the same manipulation there was a reduction in burst firing propensity across the population of RE neurons sampled (Fig. 3-2D), although this difference was not statistically significant (Kolmogorov-Smirnov, D=0.18, p=0.71) In addition, the average number of spikes within a burst for each neuron was measured (Fig. 3-2E); a parameter that depends on the amplitude of low-threshold calcium spikes within a neuron (Sherman, 1996). Following TTX infusion (but not vehicle), there was a large, statistically significant decrease in the average number of spikes within a burst (median before TTX=2.91, after TTX=2.29; Mann-Whitney, U=197.5, p=0.005). Finally, no change was observed in the number of spontaneously active RE neurons per track following either vehicle or TTX infusion (Fig. 3-2F, TTX: unpaired t-test, $t_{(12)}$=1.47, p=0.17, 95% CI=-1.33-0.26). These data suggest that ilPFC contributes to the maintenance of burst firing in RE neurons.

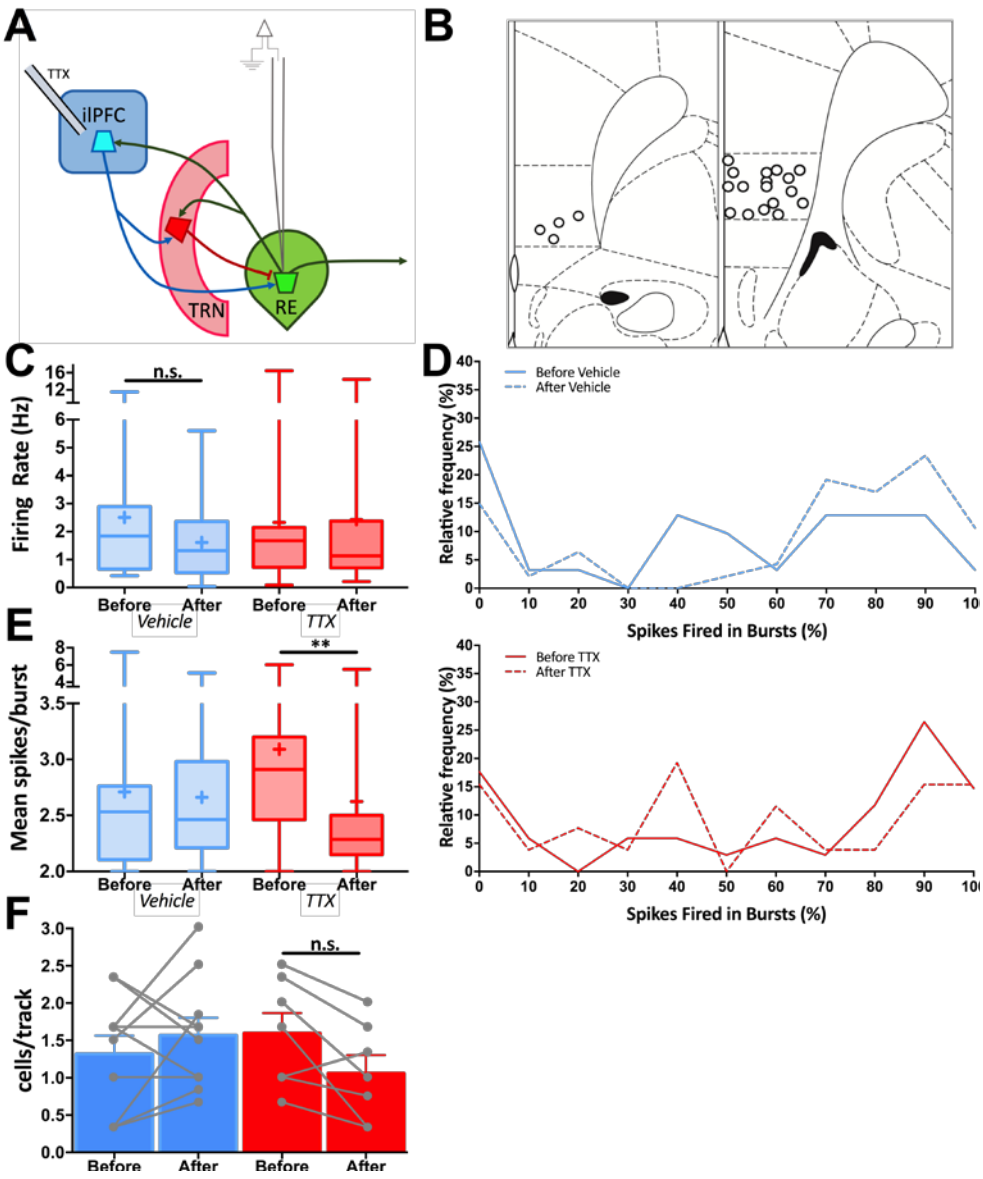

Figure 3-2: Inhibition of ilPFC reduces burst firing in RE neurons.

Spontaneous activity was recorded in multiple RE neurons in multiple animals both before and after acute infusion of TTX (1μM in 0.5μl) or dPBS vehicle into ilPFC. ***A,*** Schematic of experimental design. ***B,*** Representation of histological placements of infusion cannulae into ilPFC. ***C,*** The firing rate of RE neurons plotted as "box-and-whiskers" plots, here and in subsequent figures representing highest and lowest values (highest and lowest horizontal lines), interquartile

range (rectangle), mean ("+" symbol), and median (horizontal line in rectangle). Firing rates recorded before vehicle or TTX infusion did not differ from those recorded after infusion. ***D,*** Relative frequency distribution histograms depicting the percentage of spikes fired in bursts in neurons recorded either before or after vehicle (top) or TTX (bottom) infusion into ilPFC. No statistically significant differences were observed following vehicle or TTX infusion. ***E,*** The mean spikes per burst of RE neurons recorded after TTX infusion was decreased compared to those recorded before infusion. ***F,*** The number of spontaneously active RE neurons per electrode track did not differ following vehicle or TTX infusion. **$p<0.01$ (Mann-Whitney U test). Before Vehicle n=31 cells from 12 animals, After Vehicle n=47 cells, Before TTX n=34 cells from 9 animals, After TTX n=26 cells.

3.3.3 Electrical Stimulation of ilPFC induces burst firing and reduces tonic firing in RE neurons

Direct ilPFC projections to RE are glutamatergic, but ilPFC influence on RE likely also depends on feed-forward inhibition arising from ilPFC projections to the TRN (Cornwall et al., 1990; Çavdar et al., 2008; but see Vertes, 2003). To examine the combined contribution of these pathways in controlling RE neuron firing, spontaneous activity in single RE neurons was recorded before, during, and after electrical stimulation of ilPFC (0.5 hz, 1 mA) using a concentric bipolar electrode (Fig. 3-3). Several successive trials were performed in each neuron and multiple neurons were recorded in the same animal. In 75% of neurons recorded, individual pulses to ilPFC resulted initially in a cessation of all firing, followed ~0.6 s later by rebound spiking, predominantly consisting of bursting (Fig. 3-3C-D). In responsive neurons, several successive pulses (i.e. ~15-25) resulted in a gradual loss of tonic firing and predominance of burst firing (Fig. 3-3E), in most cases leading to a complete cessation of firing altogether. This effect of stimulation subsided within 1min following cessation of stimuli. These findings suggest that ilPFC stimulation is sufficient to entrain bursting in, and even possibly silence altogether, RE neurons. Furthermore, these effects are likely mediated by feed-forward inhibition from the ilPFC-TRN pathway (Halassa et al., 2011).

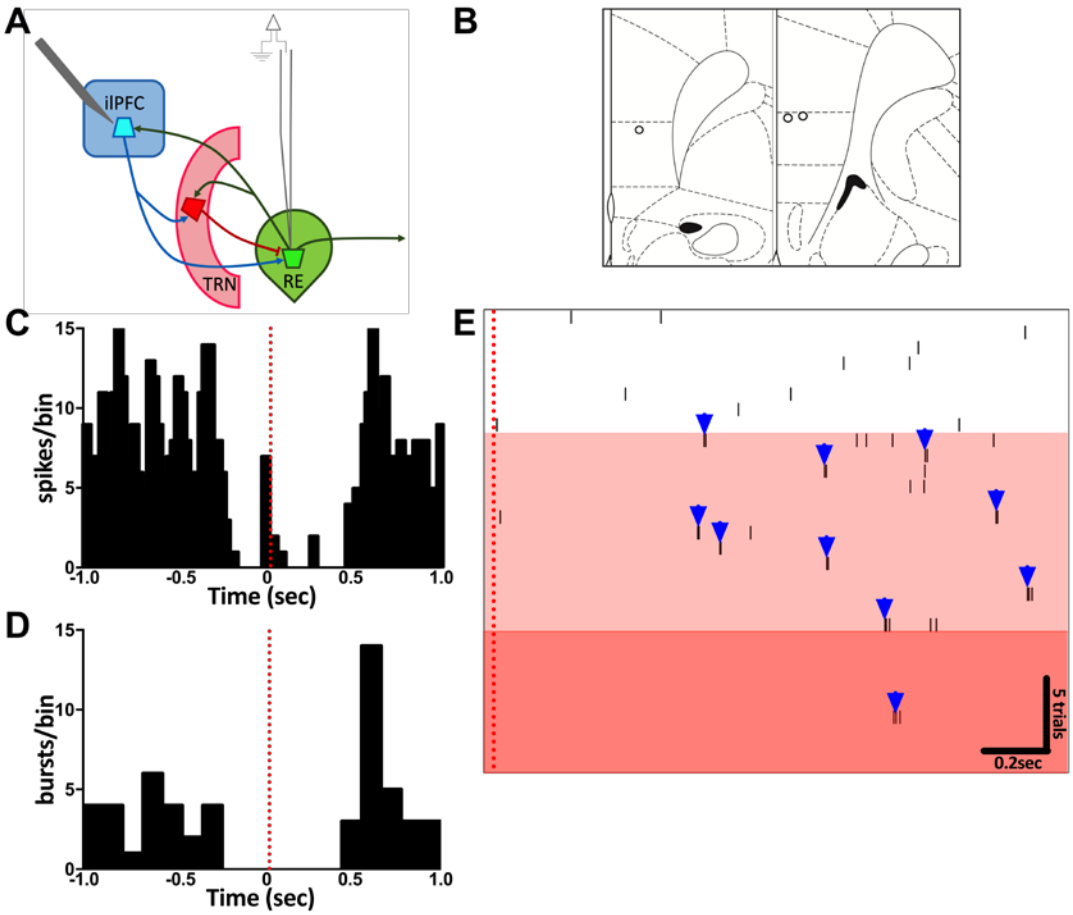

Figure 3-3: Electrical Stimulation of ilPFC induces burst firing and attenuates tonic firing in RE neurons.

Spontaneously active RE neurons were recorded both before and during electrical stimulation of ilPFC (0.5 hz, 1 mA) via a concentric bipolar stimulating electrode. Several successive trials were performed in each neuron. ***A,*** Schematic of experimental design. ***B,*** Representation of histological placements of stimulating electrodes in recorded animals. ***C,*** Cumulative peri-event histogram of firing rate for a subset of trials in all RE neurons recorded (n=10 trials/cell, n=8 cells) demonstrating robust inhibition immediately following ilPFC stimulation (red dotted line). ***D,*** Cumulative peri-event histogram of burst firing plotted from the same traces as in C demonstrating inhibition immediately following ilPFC stimulation (red dotted line), followed by rebound bursting

~0.6 sec post-stimulation. ***E,*** Perievent raster of 30 successive trials within a single RE neuron. Electrical stimulation of ilPFC (red dotted line) in successive trials gradually converted the firing pattern of this neuron from tonic to burst firing (white vs. light pink shaded areas). After 21 trials, the neuron nearly ceased firing (darker pink area). n=8 cells from 3 animals.

3.3.4 Inhibition of TRN decreases the number of spontaneously active RE neurons

Given the likely impact of feedforward inhibition from the ilPFC-TRN pathway in RE neuron firing, the effects of manipulating TRN itself was investigated (Fig. 3-4). The TRN is a large brain region that is topographically organized, sending inhibitory inputs to nearly every thalamic subnucleus. Therefore, the portion of TRN that most strongly projects to RE was targeted (Cornwall et al., 1990; Kolmac and Mitrofanis, 1997; Çavdar et al., 2008). In addition, fluorescently-conjugated muscimol (Allen et al., 2008) was used to achieve neuronal inhibition, which 1) will not affect fibers of passage and 2) permits visualization of the extent of drug spread – a critical point given the unusual shape of TRN in the coronal plane and its close proximity to RE (Paxinos and Watson, 2013). The experimental design and analyses for these experiments were identical to those in the ilPFC inhibition experiment presented above. Following muscimol infusion into TRN no changes were observed in the firing rate of RE neurons recorded after vehicle or muscimol infusion compared to those recorded before (Fig. 3-4C). Similarly, there was no change in burst firing propensity or mean spikes within a burst (Fig. 3-4D-E). However, muscimol infusion produced a substantial and statistically significant reduction in the number of spontaneously active RE neurons encountered (Fig. 3-4F; unpaired t-test, $t_{(14)}=2.29$, $p=0.04$). These data suggest that, when manipulated in isolation, TRN input to RE has a qualitatively distinct role in modulating firing pattern compared to feedforward inhibition from corticothalamic inputs.

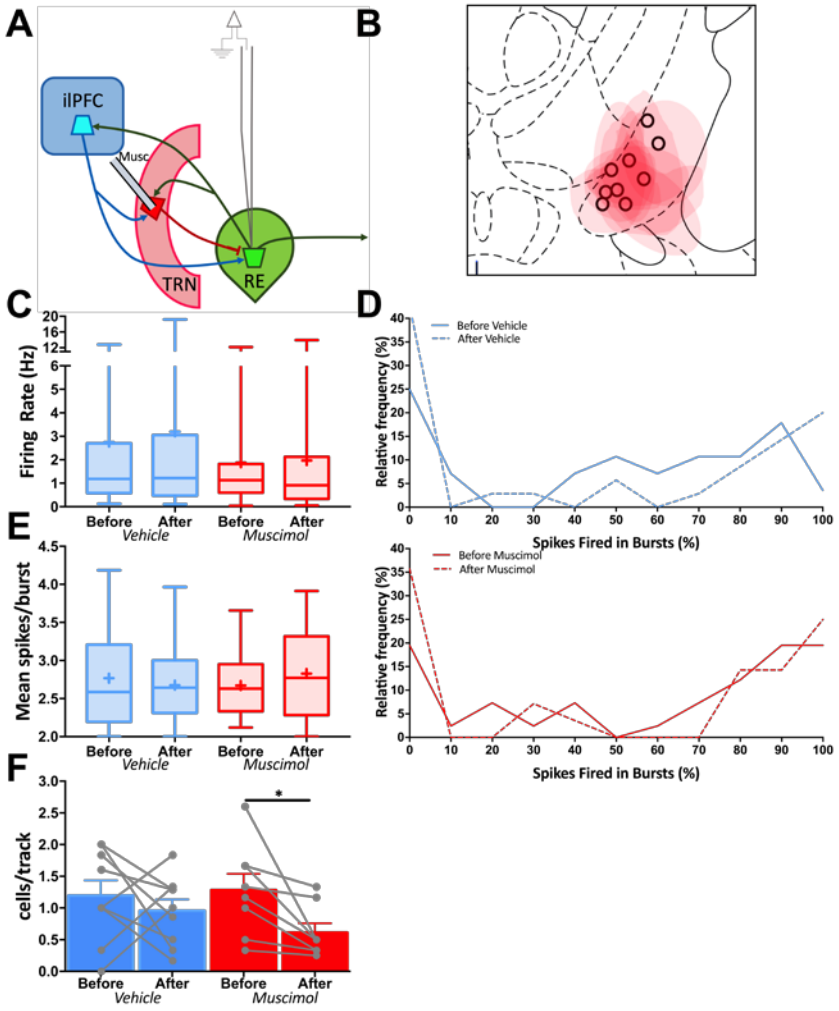

Figure 3-4: TRN inhibition decreases the number of spontaneously active RE neurons.

Spontaneous activity was recorded in multiple RE neurons in multiple animals both before and after acute infusion of fluorescently-tagged muscimol (0.8µM in 0.2µl) or dPBS vehicle into TRN ***A,*** Schematic of experimental design. ***B,*** Representation of histological placements of infusion cannulae for vehicle groups (black circles) and maximum extent of fluorescence for muscimol groups (red shading) in TRN. ***C,*** The firing rate of RE neurons recorded before vehicle or muscimol

infusion did not differ from those recorded after infusion. ***D,*** Relative frequency distribution histograms depicting the percentage of spikes fired in bursts in neurons recorded either before or after vehicle (top) or muscimol (bottom) infusion into TRN. No statistically significant differences were observed following vehicle or TTX infusion. ***E,*** The mean spikes per burst of RE neurons recorded before vehicle or muscimol infusion did not differ from those recorded after infusion. ***F,*** The number of spontaneously active RE neurons per electrode track recorded after muscimol infusion was decreased compared to those recorded before infusion. *$p<0.05$ (unpaired t-test). Before Vehicle n=28 cells from 9 animals, After Vehicle n=35 cells, Before Muscimol n=41 cells from 8 animals, After Muscimol n=28 cells.

3.3.5 Inhibition of ilPFC terminals in RE enhances burst firing in RE neurons

The findings described above are consistent with the ilPFC influencing the firing pattern of RE neurons via feedforward inhibition from the TRN. However, ilPFC also sends monosynaptic, glutamatergic projections to RE. In order to assess the role of these projections in controlling RE neuron firing, a projection-specific approach utilizing DREADDs (Mahler et al., 2014; Roth, 2016; Smith et al., 2016) was used. AAV vector constructs containing the inhibitory DREADD hM4Di fused to GFP *(rAAV2-hSyn-HA-hM4Di-IRES-mCitrine*) or EGFP only controls *(rAAV2-hSyn-EGFP*) were injected into ilPFC (Fig. 3-5). After allowing sufficient time for construct expression, individual RE neurons were recorded before and after local microinfusion of CNO (60nl of 100μM) or dPBS vehicle control (VEH) via a combined glass injection pipette-recording electrode (Fig. 3-6A). Following acute CNO or VEH application, no effect on firing rate was found in any group (Fig. 3-6B). However, following CNO application onto RE neurons in animals expressing hM4Di there was a statistically significant enhancement of the percentage of spikes fired in bursts (Fig. 3-6C-E; Wilcoxon matched pairs signed-rank test, p=0.02). This effect was observed in some individual neurons recorded in animals expressing EGFP only or following vehicle injection (Fig. 3-6D), but there were no statistically significant effects in any of these groups as a whole (Wilcoxon matched pairs signed-rank test, EGFP+VEH: p=0.38, 95% CI=-10.75-1.69; EGFP+CNO: p=0.89, 95% CI=-6.27-3.33, hM4Di+VEH: p=0.99, 95% CI=-9.86-7.30). Notably, neurons in the hM4Di+CNO group that exhibited an enhancement in bursting following CNO application did not show a consistent change in the mean number of spikes fired within a burst (Fig. 3-6F). Finally, an increase in the average number of spikes within a burst following CNO application in EGFP animals was found (Wilcoxon matched pairs signed-rank test, p=0.005) but no differences in this parameter were observed in any other group (Fig. 3-6G). Taken together,

these findings suggest that the direct ilPFC-RE projection provides an input necessary to maintain certain characteristics of burst firing in RE neurons.

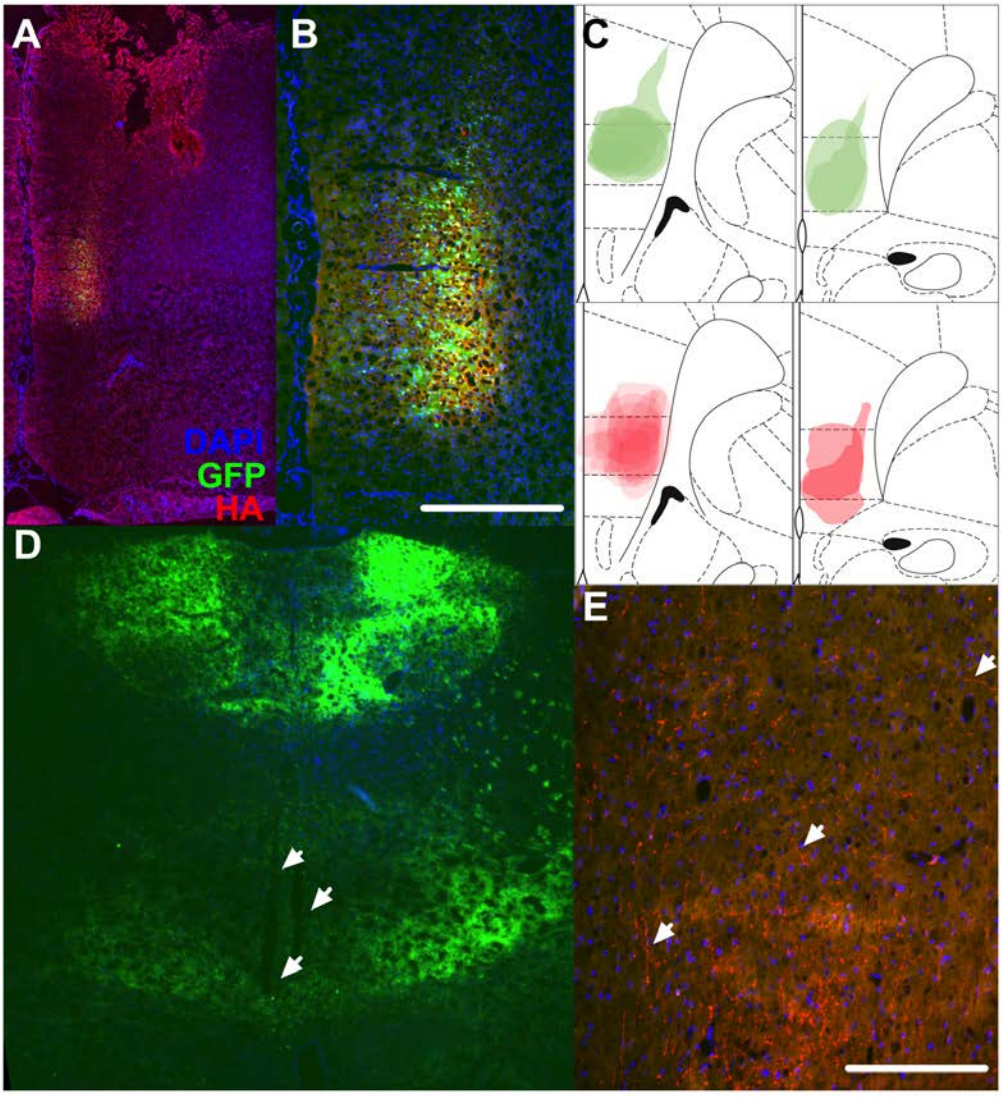

Figure 3-5: Histological validation of viral vector expression in ilPFC and RE.

AAV vector constructs containing the inhibitory DREADD hM4Di *(rAAV2-hSyn-HA-hM4Di-IRES-mCitrine*) or GFP only controls *(rAAV2-hSyn-EGFP*) were infused into ilPFC. Blue = DAPI, Green = GFP, Red = anti-HA antibody throughout, as in A. ***A,*** Representative coronal section demonstrating expression restricted to ilPFC. ***B,*** Higher magnification image of section pictured

in A. Scale bar = 300μm. ***C,*** Superimposed traces of the maximal extent of EGFP expression across EGFP-only animals (green, top) and anti-HA immunoreactivity across hM4Di animals (red, bottom) from all animals recorded. ***D,*** Low magnification image demonstrating terminal expression throughout the midline thalamus in a EGFP animal with recording electrode tracks present in RE (white arrows). ***E,*** Representative image of HA+ terminal labeling in RE (white arrows). Scale bar = 150μm.

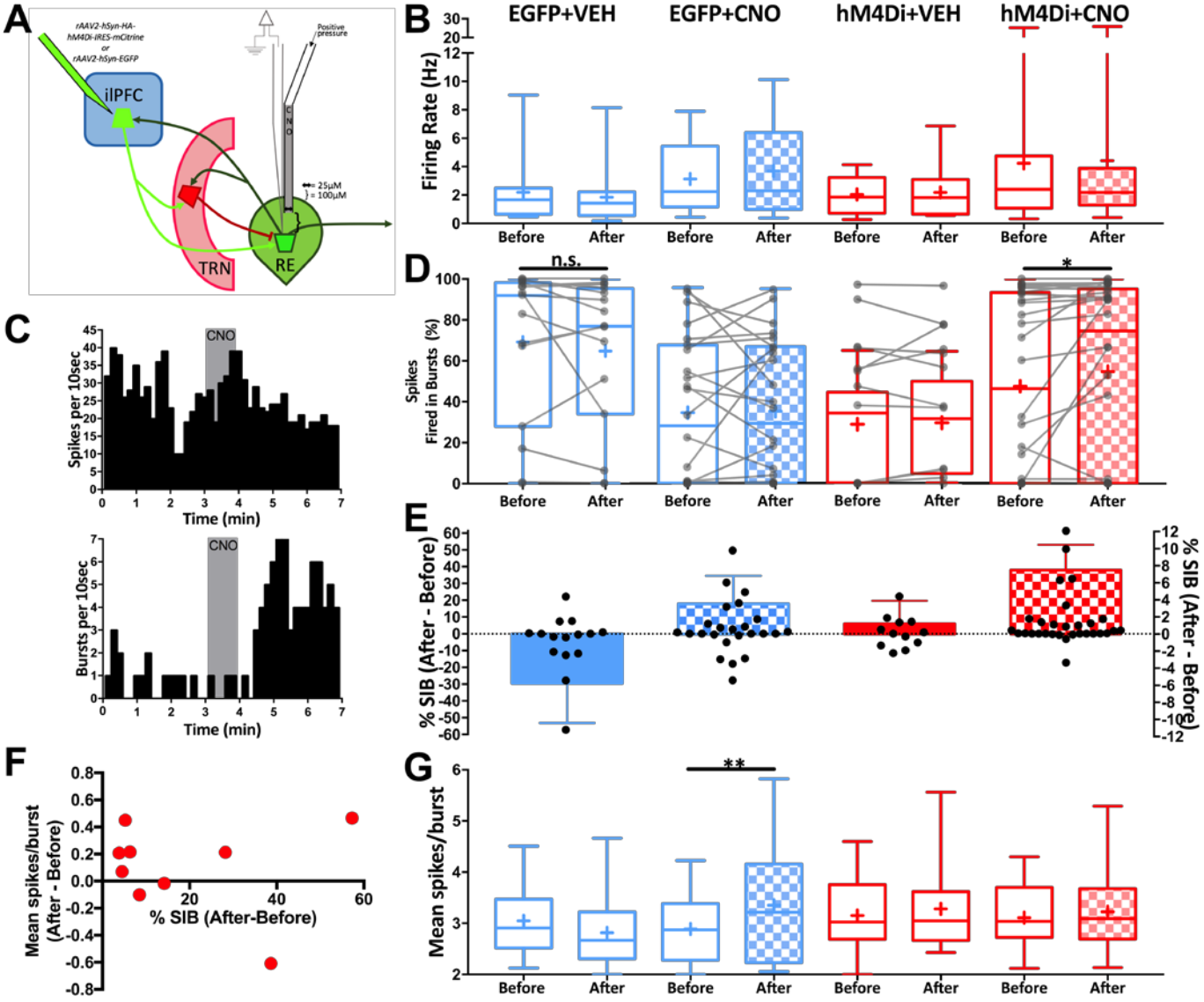

Figure 3-6: Inhibition of ilPFC terminals in RE enhances burst firing in RE neurons.

Individual RE neurons in rats expressing hM4Di or EGFP only in ilPFC and ilPFC terminals in RE were recorded before, during, and after local microinfusion of CNO (60nl of 100µM) or dPBS vehicle control (VEH) via a combined glass injection pipette-recording electrode. For panels B,D,E,G groups as indicated in B. ***A***, Schematic diagram demonstrating the virus injection site,

relevant circuitry, and combined glass injection pipette-recording electrode dimensions. ***B,*** Acute CNO or VEH application had no effect on firing rate in any group. ***C,*** Cumulative perievent histograms of firing rate (top) and burst firing (bottom) for a single RE neuron in an hM4Di animal before, during (grey box), and after CNO application. ***D,*** The percentage of spikes fired in bursts presented as individual neurons (grey lines) and grouped box-and-whiskers plots before and after acute CNO application. CNO application enhanced burst firing in RE neurons of animals expressing hM4Di, but this effect was not consistently observed in any other group. ***E,*** Data from D presented as the difference in percentage of spikes fired in bursts (% SIB) before and after vehicle or CNO application. Individual values plotted in black circles on left y-axis and group means+standard error of the mean plotted in bars on right y-axis, for clarity. ***F,*** Data from a subset of neurons in the hM4Di+CNO group that exhibited an increase in burst firing following CNO application, demonstrating inconsistent changes in mean spikes/burst. ***G,*** Acute CNO enhanced the mean number of spikes within a burst in the EGFP+CNO group, but not in any other group. *=$p<0.05$, **=$p<0.01$ (Wilcoxon paired signed-rank test). GFP+VEH n=15 neurons from 3 animals, GFP+CNO n=18 neurons from 4 animals, hM4Di+VEH n=12 neurons from 2 animals, hM4Di+CNO n=30 neurons from 9 animals.

3.4 DISCUSSION

Our findings characterize RE neuron firing patterns in an intact preparation and describe circuit dynamics underlying both the direct and indirect projections from ilPFC to RE. Pharmacological inhibition of ilPFC, while not affecting firing rate, was found to reduce the incidence of burst firing across the population of RE neurons and also reduce the mean number of spikes within a burst. In contrast, electrical stimulation of ilPFC acutely drove burst firing, and repeated stimulation was in many RE neurons able to silence almost all spontaneous activity. With respect to the distinct contributions of the TRN and monosynaptic ilPFC-RE projection to RE neuron firing, pharmacological inhibition of TRN did not affect any of the measured RE neuron firing parameters, but did reduce the number of spontaneously active RE neurons encountered in the recordings. Finally, inhibition of the direct ilPFC-RE pathway acutely enhanced burst firing in RE neurons.

The effects of acute inhibition or stimulation of ilPFC that were observed selectively affected burst firing parameters. This is consistent with prior studies that have shown that TRN-mediated feedforward inhibition is powerful, often overwhelming the monosynaptic corticothalamic projection (Swadlow and Weyand, 1987; Beierlein and Connors, 2002; Halassa et al., 2011; Paz et al., 2011; Crandall et al., 2015). Indeed, repeated stimulation of ilPFC completely silenced 75% of RE neurons after 20-22 stimuli. Following ilPFC inhibition, there was a reduction in the number of spikes within a burst, a parameter that depends on the amplitude of the T-type calcium channel-mediated current (i.e. the “low threshold spike”) that underlies thalamic burst firing (Sherman, 2001b). It should be noted that the manipulations of ilPFC were not projection-specific, and therefore these effects could be mediated in part by feedforward inhibition arising from extrathalamic sources, such as the zona incerta (Sesack et al., 1989a; Halassa and Acsády,

2016). However, these manipulations provide the advantage of assessing the combined influence of both direct and indirect pathways on RE neuron activity. Thus, these findings demonstrate that the predominant influence of ilPFC on RE neuron firing is on firing pattern, likely via feedforward inhibition from TRN.

The effects of targeted manipulation of two monosynaptic inputs to RE neurons were also evaluated: the subregion of TRN known to project to RE and ilPFC terminals in RE. Surprisingly, acute pharmacological infusion of fluorescently-conjugated muscimol into TRN did not impact firing rate or any of the measured burst firing parameters in RE neurons, but did reduce the number of spontaneously active neurons encountered in RE. This finding suggests that in the anesthetized state, closed-loop TRN input to RE might predominate over the likely sparse glutamatergic input, producing pacemaker-like activity that, when removed, results in a portion of target neurons exhibiting no spontaneous firing, as opposed to a graded change in bursting propensity. Furthermore, the effect of inhibiting TRN itself was different than that of inhibiting ilPFC, possibly because ilPFC drives TRN firing in a way that is distinct from the intrinsic firing of TRN neurons themselves (Crandall et al., 2015).

In contrast to the effects of inhibiting ilPFC cell bodies on RE neuron firing, projection-specific inhibition of monosynaptic ilPFC inputs to RE induced an acute enhancement of burst firing in a subset of RE neurons. This effect is likely attributable to the removal of a glutamatergic input causing a hyperpolarization of RE cells, leading to more de-inactivation of T-type calcium channels and a larger low-threshold spike (Sherman and Guillery, 2001). However, this manipulation did not enhance the mean number of spikes per burst, which was reduced concomitantly with a reduction in bursting seen following ilPFC inhibition. This effect suggests that the inhibition of the glutamatergic ilPFC-RE input has distinct effects on RE cell membrane

properties compared to that of removing all ilPFC input, implying the potential for highly nuanced control of RE cell firing depending on the specific inputs that are engaged. It should be noted that individual neurons in other treatment groups did exhibit changes in burst firing following application of CNO or vehicle. In particular, burst firing was often modulated up or down following CNO application in the EGFP+CNO group. Several previous studies have characterized off-target effects of CNO (MacLaren et al., 2016; Roth, 2016; Saloman et al., 2016). However, in this study no effects were apparent in any group other than the hM4Di+CNO group when examined as a population, and the combined effects of hM4Di+CNO appear to be distinct from those of CNO by itself.

One factor to be considered is that all recordings were performed in anesthetized rats. Anesthesia is known to affect the firing properties of thalamic neurons (Alkire et al., 2008). However, the current findings are likely to be relevant to the awake state for several reasons. Thalamic burst firing has been described in sleep and wakefulness across species (Jeanmonod et al., 1996; Swadlow and Gusev, 2001; Nicolelis and Fanselow, 2002; Ramcharan et al., 2005; Kim et al., 2009), suggesting that it could be used as an important mode of information transfer in the brain in various states of consciousness. Therefore, the circuit dynamics described here could potentially be highly relevant, as anesthesia is likely to impact baseline activity but it should not qualitatively impact the effects of pathway activation or inhibition. In addition, higher-order thalamic relays, such as RE, have been shown to burst more than first-order relays, suggesting that burst firing might have even greater functional relevance in these thalamic nuclei (Ramcharan et al., 2005; Wei et al., 2011). Finally, the firing pattern of individual neurons recorded in higher-order thalamic relays, including RE, is highly heterogeneous across preparations (Ramcharan et al., 2005; Morales et al., 2007; Kim et al., 2011; Zhang et al., 2012; Ito et al., 2015; Lara-Vasquez

et al., 2016; Walsh et al., 2017), and the current findings reflect this, permitting an examination of the effects of these manipulations on an RE neuron sample with various baseline firing properties.

Corticothalamic input plays a key role in modulating thalamocortcial neuron firing pattern (Godwin et al., 1996; Briggs and Usrey, 2008; Crandall et al., 2015), possibly permitting higher efficiency of information transfer and nuanced population-level coding (Mukherjee and Kaplan, 1995; Wolfart et al., 2005; Behuret et al., 2015; Whitmire et al., 2016). The current findings demonstrate that the ilPFC can modulate multiple RE neuron burst firing parameters, including percent of spikes fired in bursts and the mean number of spikes within a burst, both in tandem and independently via the monosynaptic ilPFC-RE projection. The number of spikes within a burst has recently been demonstrated to be a robust, independent mode of information transfer in thalamic neuron models (Elijah et al., 2015). In addition, the firing pattern of thalamocortical neurons can determine the manner by which these neurons engage cortical principle neurons and interneurons (Swadlow and Gusev, 2001; Bruno and Sakmann, 2006; Bayazitov et al., 2013; Hu and Agmon, 2016; LeBlanc et al., 2017). In the case of RE in particular, the modulation of RE neuron firing by the ilPFC-TRN pathway could heavily influence how RE engages its downstream targets in the hippocampus, which include pyramidal neurons and interneurons (Dolleman-Van der Weel and Witter, 2000). Future studies examining the influence of firing pattern in RE on engagement of these circuits will be critical, given the unique circuit organization of the hippocampus compared to other cortical regions targeted by thalamic input.

We have previously demonstrated that ilPFC (Patton et al., 2013), via the ilPFC-RE-vSub circuit (Zimmerman and Grace, 2016), controls the proportion of DA neurons in the VTA that are spontaneously active (“population activity”), a critical signaling parameter in the DA system (Grace, 2016). Specifically, we showed that inhibition of the ilPFC paradoxically enhanced VTA

DA neuron population activity, but concomitant inhibition of RE prevented this effect. These findings suggested that inhibition of ilPFC disinhibits RE, but the paucity of studies examining the influence of ilPFC on RE neuron firing prevented any definitive conclusions. The current work helps to clarify these effects: specifically, inhibition of ilPFC shifts the firing pattern of the RE neuron population from bursting to tonic firing, while stimulation of ilPFC was capable of silencing RE neurons. The loss of this inhibition would depolarize RE neuron membrane potential, allowing for other inputs to exert more influence on RE neuron firing. Both of these effects would then lead to enhanced RE neuron output to the ventral hippocampus that also has a more tonic character, engaging CA1 and subiculum and leading to enhanced drive of VTA DA neurons via the vSub-NAc-VP pathway (Floresco et al., 2001; 2003). From a clinical perspective disruption of corticothalamic communication in these circuits, as has been observed in schizophrenia (Woodward et al., 2012; Anticevic et al., 2014; 2015a; Woodward and Heckers, 2016), could lead to deficits in prefrontal-hippocampal-dependent behaviors (Reagh et al., 2017) and a dysregulated, hyperdopaminergic state, both of which may play a role in the disease.

4.0 GENERAL DISCUSSION

4.1 SUMMARY OF FINDINGS

The studies described in this dissertation reveal a novel role for the ilPFC-RE-vSub circuit in controlling VTA DA neuron firing and confirm that corticothalamic input from ilPFC to RE, both via direct projections and the TRN, can modulate RE neuron firing pattern in nuanced and complex ways.

In Chapter 2, we presented evidence suggesting that RE is a novel region involved in control of VTA DA neuron population activity. Pharmacological stimulation of RE enhanced DA neuron population activity, without affecting average firing rate, and mildly enhanced burst firing. The same stimulation paradigm also enhanced amphetamine-induced hyperlocomotion, a behavioral correlate of an over-responsive DA system. Furthermore, we showed that the effect of RE stimulation is prevented if vSub is also inhibited, suggesting that activity in vSub is necessary for RE to drive VTA DA neuron firing. Finally, inactivation of RE prevented the increase in VTA DA neuron population activity observed following ilPFC inhibition, suggesting that ilPFC inhibition disinhibits RE. Taken together, these findings support a model whereby inactivation of ilPFC leads to an increase in RE firing, driving vSub and enhancing VTA DA neuron population activity.

In Chapter 3, we presented findings that characterized the diverse array of RE neuron firing patterns in an intact preparation and described the circuit dynamics underlying both the direct and indirect projections from ilPFC to RE. We found that pharmacological inhibition of ilPFC, while not affecting firing rate, reduced the incidence of burst firing across the population of RE neurons and also reduced the mean number of spikes within a burst. In contrast, electrical stimulation of ilPFC acutely drove burst firing, and repeated stimulation was in many cells able to silence almost

all spontaneous activity. Next, we examined the distinct contributions of the TRN and monosynaptic ilPFC-RE projection to RE neuron firing. We found that pharmacological inhibition of TRN did not affect any of the measured RE neuron firing parameters, but did reduce the number of spontaneously active RE neurons encountered in our recordings. Finally, inhibition of the direct ilPFC-RE pathway acutely enhanced burst firing in RE neurons. Our findings show that ilPFC can modulate multiple aspects of RE neuron firing across diverse timescales.

4.2 CONTROL OF DOPAMINE NEURON FIRING BY DISTRIBUTED NETWORKS

Our findings suggest that RE drives VTA DA neuron population activity via vSub (Fig. 4-1). We have shown previously that acute infusion of NMDA into vSub also enhances VTA DA neuron population activity (Floresco et al., 2003), suggesting that RE is acting primarily by promoting ventral CA1 (vCA1)/vSub output. Previous findings support the idea that RE can increase pyramidal cell firing in vHipp (Dolleman-Van der Weel et al., 1997), but the precise manner by which this occurs is not clear. Projections from RE to the vHipp are densest in vCA1 and vSub, with almost all terminals limited to the SLM/molecular layer (Vertes et al., 2006). These fibers synapse onto apical tufted dendrites of pyramidal cells, as well as interneuron dendrites and cell bodies (Dolleman-Van der Weel and Witter, 2000). By engaging these postsynaptic targets, RE can likely modulate not only pyramidal cell firing, but also interneuron firing in a wide variety of cell types and anatomical locations in vHipp, and this has been shown experimentally (Dolleman-Van der Weel et al., 1997). In addition, afferents from perirhinal and entorhinal cortex comingle with fibers from RE in the SLM of vCA1/vSub, and RE itself projects to entorhinal and perirhinal cortex (Freund and Buzsáki, 1996; Baks-te Bulte et al., 2005; Agster and Burwell, 2013; Dolleman-Van der Weel et al., 2016). These findings suggest that the influence of RE on vHipp

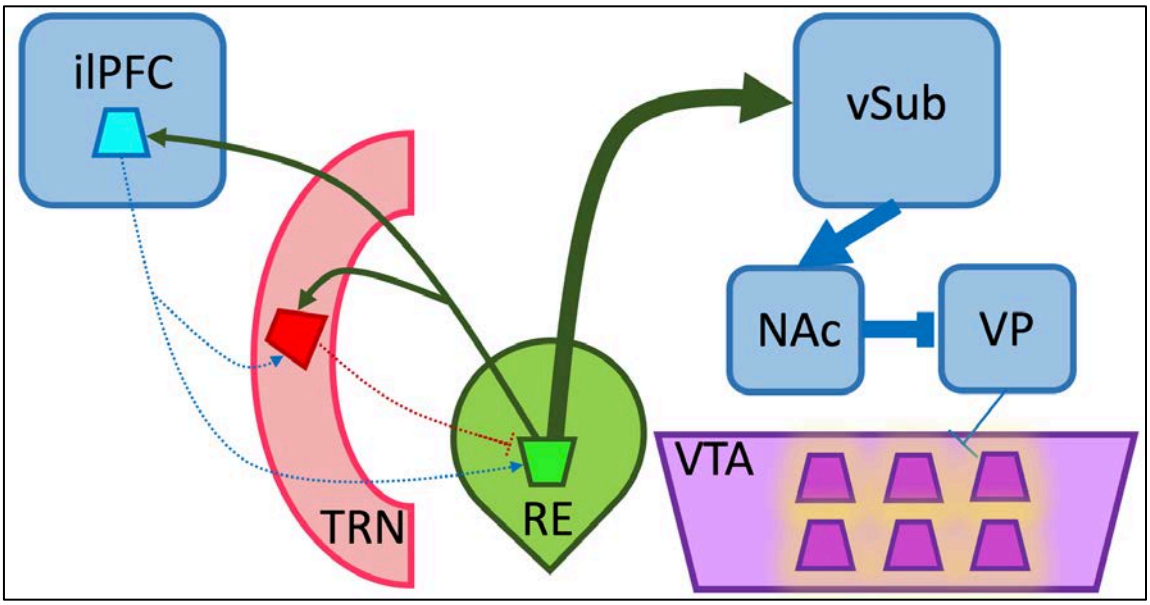

Figure 4-1: Proposed circuit model for corticothalamic modulation of VTA DA neuron population activity.

Taken together, our findings suggest that inhibition of ilPFC leads to enhanced VTA DA neuron population activity via the above circuitry. A loss of feedforward inhibition from TRN would disinhibit RE cells, which would then drive vSub activity. vSub would then engage the NAc-VP pathway, disinhibiting VTA DA cells, and enhancing population activity. The manner in which different RE neuron firing patterns engage vSub is an open but intriguing question.

could be mediated by these intervening structures. In our data we are not able to definitively rule out the potential involvement of these parallel pathways (See "*Technical Considerations and Limitations*"), necessitating further study of these circuits using projection-specific techniques.

While studies on how RE engages its diverse postsynaptic targets in vCA1/vSub are sparse, a previous study has demonstrated that the manner in which RE affects firing in hippocampus might depend on the firing pattern of RE neurons (Xu and Südhof, 2013). Xu and Südof showed that stimulating RE using "tonic" or "burst-firing" patterns during fear memory encoding had opposing effects on context-dependent memory generalization. While this behavioral effect was striking, the stimuli used were not physiologically accurate and the authors did not record the electrophysiological effects of the stimuli in hippocampus. However, the manner in which firing pattern affects thalamic engagement of cortical targets has been studied in other thalamocortical projections, specifically that of the ventrobasal thalamic nucleus (VBM) to barreloids in the somatosensory cortex (Swadlow and Gusev, 2001; Swadlow, 2002; Hu and Agmon, 2016). Specifically, these studies have shown that, like RE, VBM projections can drive firing in both pyramidal neurons and interneurons. This is true whether VBM fires/is stimulated in a tonic or burst pattern. However, it was shown that burst firing is much more efficacious at driving pyramidal neurons and somatostatin-positive interneurons, but not fast-spiking interneurons (Hu and Agmon, 2016). In our experiments, we used an acute infusion of NMDA to stimulate RE neuron firing. This manipulation likely resulted in depolarization of RE neurons, theoretically promoting tonic firing, but we did not directly measure the effect of NMDA on RE neuron firing pattern. It is possible that our exogenous, non-physiological application of NMDA resulted in an enhanced, but dysregulated firing pattern in RE. However, the finding that NMDA infusion into RE resulted in enhanced VTA DA population activity suggests that this type of input is sufficient

to drive vSub. Taken together, while these findings are intriguing, future studies will be necessary to determine how RE efferents engage the microcircuitry of the hippocampus and to what extent this depends on firing pattern.

Our findings suggest that RE control of DA neuron firing is gated by inputs from ilPFC. Specifically, we showed that inhibition of ilPFC enhances VTA DA neuron population activity, but this effect is prevented if RE is also inhibited. This suggests that removal of ilPFC input to RE disinhibits RE, permitting RE drive of vCA1/vSub and enhanced VTA DA neuron population activity. The findings from our RE recording experiments are broadly in line with this idea. We showed that the predominant influence of ilPFC on RE neuron firing is likely mediated by feedforward inhibition from the TRN. This is consistent with other studies of corticothalamic projections showing that the cortico-TRN synapse is much stronger than the monosynaptic cortico-thalamic synapse (Golshani et al., 2001; Paz et al., 2011). While this specific finding has not been shown in RE, we demonstrated that repeated stimulation of ilPFC led to predominantly burst firing in RE neurons and in many cases a complete cessation of spontaneous activity. In addition, another previous study showed that removal of GABAergic inputs to RE (the majority of which are presumably from TRN) enhances cfos expression in dCA1 (Xu and Südhof, 2013). As discussed above, the ability of differential RE firing patterns to drive activity in vSub remains an open question. However, our findings suggest that feedforward input from the ilPFC-TRN pathway causes burst firing in RE neurons, and that removal of this input is permissive for RE engagement of vCA1/vSub in a manner that promotes activity in the vSub-NAc-VP-VTA pathway.

The ilPFC-TRN-RE circuit is likely one of several pathways by which ilPFC modulates VTA DA neuron firing. Previous studies from our group have shown that acute pharmacological stimulation of ilPFC decreases VTA DA neuron population activity (Patton et al., 2013). This

effect depends on concomitant activity in BLA and glutamatergic input to the VP, suggesting that a ilPFC-BLA-VP circuit can downregulate VTA firing during various behavioral states, including chronic stress (Belujon and Grace, 2011; Chang and Grace, 2014; Moreines et al., 2017). Considered in the context of the current work, these findings suggest that activity in ilPFC can reduce VTA DA neuron population activity by both inhibiting RE and driving VP. These pathways are likely to be parallel but distinct, as RE inputs to BLA are weak (Su and Bentivoglio, 1990; Vertes et al., 2006). In addition to the ilPFC-BLA pathway, ilPFC also sends projections to the entorhinal and perirhinal cortices, although they are likely less influential than those from plPFC (Sesack et al., 1989b; Vertes, 2003). Finally, in addition to the polysynaptic circuits discussed thus far, ilPFC also sends monosynaptic projections to the VTA (Sesack et al., 1989b; Carr and Sesack, 2000b; Sesack and Grace, 2010). However, these efferents preferentially target cortically-projecting DA neurons and GABAergic long-range projection neurons, making them less likely to be involved in control of firing in striatally-projecting VTA DA neurons.

While the present work is the first to implicate a specific corticothalamic projection in the control of VTA DA neuron firing, the idea that mPFC modulates the broader, network-level impact of VTA activity, often in concert with the hippocampus, has been demonstrated previously. Stimulation of the mPFC blunts the striatal BOLD response to VTA DA neuron stimulation and attenuates behavioral seeking of VTA stimulation (Ferenczi et al., 2016). In addition, it has been shown that a 4 hz oscillation, through phase coupling with theta and gamma-band rhythms, coordinates activity in the mPFC, hippocampus, and VTA (Fujisawa and Buzsáki, 2011). Intriguingly, another study showed that inhibition of RE reduces coherence between mPFC and hippocampus in the delta (i.e. 2-5 hz) band (Roy et al., 2017). Finally, a human fMRI study showed that activity in mPFC and hippocampus predict distinct, but likely interdependent and

complementary, modes of VTA activation (Murty et al., 2017). Taken together, these findings suggest that the circuits identified in the present work are likely to impact not just VTA firing, but also broader network function in the context of several different classes of behavior.

4.3 IMPLICATIONS FOR CORTICOTHALAMIC COMMUNICATION IN NUCLEUS REUNIENS AND THALAMUS IN GENERAL

Our findings suggest that ilPFC exerts a potent influence on RE neuron firing pattern, likely via feedforward inhibition from TRN (Fig. 4-1). This motif in corticothalamic communication is well-established in other brain areas (e.g. the visual system), but not in RE. Based on previous findings in other corticothalamic projections, it is likely that the influence of ilPFC on TRN and RE firing is complex and dynamic. It has been shown that corticothalamic projections engage not only feedforward inhibition from TRN ("CT-TRN" synapse), but also intra-TRN inhibition, feedforward inhibition from extrathalamic nuclei (e.g. zona incerta), and local interneurons (Huntsman et al., 1999; Halassa and Acsády, 2016). The strength of these connections is also likely to be determined by different mechanisms than those of thalamocortical neuron-TRN synapses ("TC-TRN" synapse), e.g. GluA4-containing AMPA receptors are more highly expressed at CT-TRN synapses than TC-TRN synapses (Golshani et al., 2001; Paz et al., 2011; Ahrens et al., 2015). This also implies that the effects of CT-TRN inputs on RE firing are likely different than those of intrinsic TRN activity or TRN activity driven by other afferent inputs. Indeed, we observed disparate effects on RE neuron firing pattern following inhibition of ilPFC vs. inhibition of TRN by itself. While our findings provide a starting point, these considerations make it clear that more precise dissection of corticothalamic inputs to RE will be required in future studies.

Our Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)-based manipulations revealed an effect of the direct, monosynaptic projection from ilPFC to RE (a "CT-TC" synapse) on firing pattern in RE neurons. While the impact of these connections is likely to be weaker than those from the ilPFC-TRN pathway, our findings nonetheless demonstrated that inhibition of this projection enhanced burst firing in a subset of RE neurons. While only ~30% of recorded RE neurons were impacted by this manipulation, this could be because many of the neurons we recorded did not receive inputs from ilPFC, or from ilPFC axons expressing sufficient levels of the hM4Di protein. In addition, previous studies have highlighted the potentially potent impact of the CT-TC pathway. Following TRN lesion, inhibition, or GluA4-receptor knockout, stimulation of cortex switches from a predominantly inhibitory (as we have demonstrated) to a predominantly excitatory effect on thalamic neuron firing (Deschênes and Hu, 1990; Hu, 1993; Paz et al., 2011). Corticothalamic inputs can also paradoxically drive thalamic neuron firing if stimulated at a high enough frequency by 1) inducing short-term depression at the TRN-TC synapse and 2) facilitation at the CT-TC synapse via NMDA receptors (Crandall et al., 2015). In fact, CT-TC and CT-TRN synapses display a larger amount of NMDA-mediated currents than TC-TRN synapses (Deleuze and Huguenard, 2016; Pinault, 2017). This suggests that corticothalamic input could exert longer-term modulation of membrane potential and plasticity via NMDA receptor activation at CT-TC synapses.

In each experiment where we manipulated ilPFC cell bodies or axon terminals and recorded in RE, we observed effects specific to burst firing parameters, with less impact on overall firing rate. While this could be a result of our recordings being performed in anesthetized animals, it suggests that a primary function of corticothalamic input is to modulate the firing pattern of thalamic cells – an idea that has been discussed previously but lacks experimental verification

(Briggs and Usrey, 2008). Previous findings suggest that modulation of firing pattern might be especially important in higher-order thalamic nuclei like RE. Higher-order nuclei burst much more than first-order nuclei in the awake state, possibly due to enhanced input from extrathalamic sources of inhibition and higher levels of T-type calcium channel expression (Ramcharan et al., 2005; Wei et al., 2011). Burst firing in higher-order nuclei is not just more common than in first-order nuclei, but is also more diverse on a cell-by-cell basis. Ramcharan and colleagues demonstrated that in the awake monkey, LGN neurons rarely exhibited more than 5% of spikes fired in bursts, while MD neurons exhibited a range of percentage of spikes fired in bursts from 5% to almost 50% (Ramcharan et al., 2005). While our data were collected in the anesthetized state and therefore were skewed towards a higher incidence of bursting, we still observed a broad range of burst firing propensity between individual RE neurons. This suggests that burst firing in higher-order relays could have more functional significance than in first-order relays, as well as a more nuanced impact on cortical targets than the "wake-up call" function proposed for bursting in sensory nuclei (Sherman, 2001a). In addition, previous studies have shown that a mixed tonic and bursting pattern makes the information transfer function in thalamocortical cells more efficacious and linear (Wolfart et al., 2005; Behuret et al., 2015). Taken together, these findings highlight the potentially profound functional significance of corticothalamic modulation of firing pattern in higher-order thalamic nuclei.

4.4 TECHNICAL CONSIDERATIONS AND LIMITATIONS

4.4.1 *Anesthesia.* All of the electrophysiological recordings described here were performed in chloral hydrate-anesthetized animals. Anesthesia has been shown to profoundly impact the activity of higher brain areas (Brown et al., 2011). However, we have used chloral hydrate anesthesia

extensively in recordings of VTA (Floresco et al., 2003; Lodge and Grace, 2007; Valenti et al., 2012), as well as several other brain regions including BLA (Rosenkranz and Grace, 2001; 2002), mPFC (Lavin et al., 2005), NAc (Belujon et al., 2014), and MD (Lavin and Grace, 1998). Chloral hydrate has been shown to exert minimal effects on DA neuron firing properties compared to the awake state (Chiodo and Bunney, 1985; Freeman and Bunney, 1987). In addition, we have repeatedly demonstrated that chloral hydrate does not obscure the effects of pathway activation or inhibition (Floresco et al., 2003; Lodge and Grace, 2007; Patton et al., 2013; Chang and Grace, 2014; Moreines et al., 2017) and that opposite manipulations in the same region can result in bidirectional effects, even in higher brain areas such as mPFC (Patton et al., 2013), which are thought to be mostly silent in the anesthetized state. These principles held in the present study, where bidirectional manipulations of ilPFC produced obvious effects on RE firing, suggesting pathway integrity and the presence of baseline levels of activity that could be up- or down-modulated. It should be noted that the firing pattern of RE in our data was biased towards more bursting than has been demonstrated in other higher-order nuclei in the awake state (Ramcharan et al., 2005). However, bursting propensity in RE was still distributed across a broad range, even in the same animal in close temporal and spatial proximity, as has been reported in awake recordings in other higher-order thalamic nuclei (Ramcharan et al., 2005; Wei et al., 2011). In fact, the predominance of bursting we observed in the anesthetized state might represent an advantage of our approach, as the impact of our manipulations on bursting might have been more apparent than would be observed in the awake state. Nonetheless, future studies utilizing recent technical innovations would allow for the manipulation of the pathways assessed in the present studies in awake, behaving animals, at rest and in various behavioral contexts, and would be illuminating.

4.4.2 *Extracellular Recording.* All of the electrophysiological recordings described here were performed in vivo and were extracellular. In the VTA, this limited our identification criteria for DA neurons to waveform characteristics and firing pattern, in the absence of more intrusive/less practical approaches such as juxtacellular labeling or pharmacology (Ungless and Grace, 2012). Despite our group's extensive experience with VTA recordings, heterogeneity in the function of TH+ VTA neurons is an ongoing area of research (Stamatakis et al., 2013; Morales and Root, 2014; Mejias-Aponte et al., 2015) However, the vast majority of midbrain DA neurons (i.e. TH+ neurons) can be reliably identified using firmly established criteria (Ungless and Grace, 2012), which we have employed in the present work. We also sampled a large number of DA neurons, limiting the impact of misidentifications.

In our RE recordings, the extracellular configuration presented the limitation that we could not monitor membrane potential, a known determinant of firing pattern in thalamic neurons (Sherman, 2001b). However, the biophysical mechanisms underlying burst firing in the thalamus have been extensively characterized, and we utilized firmly established criteria to identify burst firing (Guido et al., 1992; Sherman, 1996). In addition, given that our recordings were in an intact preparation we cannot definitively rule out the possibility that some of the neurons included in our analyses were located outside the borders of RE. However, this is likely to occur only in a minority of cases, limiting the impact of misidentifications due to our relatively large sample sizes.

4.4.3 *Manipulation of Neural Activity.* To manipulate neural activity in our studies, we used acute, local infusions of drugs, electrical stimulation, and a projection-specific, DREADDs-based approach. Major limitations of pharmacologically manipulating neural activity include uncertainty about diffusion radius, dose-dependent differences in effects on target cells, and possible effects

on fibers of passage. Using drug infusions also prevents us from making any conclusions based on the function of specific projections. For example, while infusion of NMDA into RE enhanced VTA DA neuron population activity, this effect was prevented if vSub was also inhibited, and a monosynaptic projection from RE to vSub has been described, we are limited to stating that communication between RE and vSub is necessary for the effect of NMDA in RE on VTA DA neuron population activity. Limitations of electrical stimulation include the possibility of antidromic activation and tachypylaxis, although these can be avoided by limiting stimulus intensity and careful examination of response latency, as we have done in the present studies.

DREADDs offer a powerful tool for temporally-precise, projection-specific, and multiplexed manipulation of neural activity (Roth, 2016). However, multiple off-target effects of DREADDs proteins and clozapine-n-oxide (CNO) have recently been described (MacLaren et al., 2016; Saloman et al., 2016; Smith et al., 2016). In particular, the hM4Di protein has been shown to constitutively activate Gi signaling in the absence of CNO in certain preparations (Saloman et al., 2016), and CNO has been shown to affect behavior in animals not expressing DREADDs proteins (MacLaren et al., 2016), possibly because it is metabolized to clozapine in rodents (Lin et al., 1996). Germane to our studies, serotonin receptors in higher-order thalamic nuclei can modulate membrane potential (Varela and Sherman, 2009). Indeed, we did observe effects of CNO in animals expressing only EGFP. However, these effects were inconsistent, sometimes present after vehicle infusion in EGFP-only animals, and did not result in any group-level effects when data from all recorded neurons were pooled. These effects were also distinct from the effects of CNO in the hM4Di group, which were clear and consistent at the individual neuron level and in the group data. The “within-cell” design of our DREADDs experiments also rules out the possibility of any effects of the hM4Di protein by itself in our studies, other than an effect on

before-CNO firing, which was not observed as compared to EGFP-only groups. In future DREADDs-based studies, it will be useful to employ newer actuators with higher specificity for DREADDs proteins and less chemically-active metabolites, e.g. Compound 21 (Chen et al., 2015).

4.4.4 *Anatomical Homologies of Cortico-Hippocampal Relays in Rodents and Primates.* The anatomy of RE has been well-described in the rat (Cassel et al., 2013; Vertes et al., 2015). While its characterization in other species is not as thorough (Amaral and Cowan, 1980; Yanagihara et al., 1987; Giménez-Amaya et al., 1995; Sánchez-González et al., 2005), previous findings have provided some insight into the afferent and efferent connections of RE, as well as the diversity of pathways connecting the mPFC, thalamus, and hippocampus across species. According to the Paxinos Atlas of the Human Brain (Mai et al., 2015), RE lies anterior and ventral to the MD, bordering the third ventricle and contributing to the massa intermedia (with individual variation). Regarding thalamic efferents to hippocampus, a retrograde tracing study in primate reported projections to hippocampus from the paraventricular, paratenial, and laterodorsal nuclei, as well as the anterior nuclear complex and RE (Amaral and Cowan, 1980). The diversity of these projections is consistent with work in rodents, and notably doesn't include MD in either species (Wyss et al., 1979; Moga et al., 1995; Vertes and Hoover, 2008; Aggleton et al., 2010). In turn, projections from the hippocampus terminate in the anterior complex, laterodorsal nucleus, RE, and magnocellular portion of MD in primates and rodents (Aggleton, 2012; Pergola et al., 2015; Mathiasen et al., 2017). Medial temporal lobe afferents to MD mostly arise from the entorhinal and perirhinal cortices, while the anterior complex and RE are targeted primarily by subiculum (Russchen et al., 1987; Mitchell and Chakraborty, 2013).

The findings discussed thus far suggest that multiple subnuclei within the thalamus could potentially fulfill the anatomical criteria for a relay between cortex and hippocampus, a function often attributed to RE by itself (Cassel et al., 2013). Once again, studies in primates and rodents examining cortical efferents to the thalamus are informative. The anterior complex, one potential candidate for a cortico-hippocampal relay, is not densely targeted by Brodmann areas 25 or 32 in primates or ilPFC/plPFC in rodents (Freedman et al., 2000; Vertes, 2003), but does receive input from Brodmann area 23 (Yeterian and Pandya, 1988). In contrast, Brodmann area 25/ilPFC (Russo and Nestler, 2013; Delgado et al., 2016; Heilbronner et al., 2016a) projects to the majority of the midline thalamus, including the magnocellular MD, paraventricular nucleus, paratenial nucleus, and RE (Freedman et al., 2000; Vertes, 2003). Thus, anatomical findings to date across species suggest that RE is likely a key mediator of communication between the mPFC and hippocampus. However, other thalamic regions could play a role in this regard. In particular, it will be intriguing to examine the contributions of the paraventricular nucleus, as it has been shown to be important for various behaviors that could depend on coordinated action between mPFC and hippocampus (Do-Monte et al., 2015; Penzo et al., 2015).

4.5 IMPLICATIONS FOR THE PATHOPHYSIOLOGY OF SCHIZOPHRENIA

A large body of evidence suggests that the hyperdopaminergic phenotype observed in schizophrenia is caused by dysfunction in the afferent circuitry regulating the activity of VTA DA neurons (for review, see Grace, 2016). To this end, our group and others have focused on using preclinical rodent models to dissect this circuitry, in order to provide a circuit basis for translational and human subjects studies. In keeping with this motivation, the current work has identified a novel role for the ilPFC-RE-vSub circuit in controlling VTA DA neuron activity. In addition, our

studies have described the effects of a loss of prefrontal corticothalamic input on firing properties in RE. Therefore, we believe our findings have several important implications for future studies of circuit abnormalities in schizophrenia, especially when considered in light of functional imaging studies in patients.

4.5.1 *The Thalamus as a Driver of Dopamine Neuron Activity.* We have shown that pharmacological stimulation of RE is sufficient to enhance VTA DA neuron population activity and that this effect depends on activity in vSub (Fig. 4-1). This finding is somewhat difficult to interpret in light of human subjects findings, as functional imaging studies of the thalamus in patients with schizophrenia are inconsistent. While some studies report no differences in intrinsic, resting-state activity in the thalamus (Kühn and Gallinat, 2013), others report increased task-related activity (Silbersweig et al., 1995; Théberge et al., 2002; Tregellas et al., 2007b), with most studies being limited by the difficulty in resolving thalamic subnuclei using fMRI. However, decreased fractional anisotropy (FA) in the anterior thalamic radiation has been demonstrated in patients (McIntosh et al., 2008; Oh et al., 2009; Bernard et al., 2015). In particular, one study showed that ultra high risk patients exhibited a higher FA of the thalamo-hippocampal tract at baseline, which correlated with the emergence of symptoms one year later and decreased over time (Bernard et al., 2015). Another study reported reduced resting-state functional connectivity between the whole thalamus and anterior hippocampus in symptomatic psychotic patients across diagnostic categories (Samudra et al., 2015). While these findings leave thalamic function in schizophrenia an open question, we believe that it would be reasonable to speculate that hyperactivity of the anterior hippocampus, a well-documented phenomenon in schizophrenia (Heckers and Konradi, 2010; Schobel et al., 2013; Tregellas et al., 2014), could impact and/or

involve interconnected thalamic nuclei. In addition, the impact of thalamic inputs on anterior hippocampal circuits in patients with schizophrenia would likely be altered by the loss of PV-positive interneurons that has been described in the disease (Konradi et al., 2011), possibly further contributing to hippocampal hyperexcitability. Overall, these finding suggest that aberrant thalamo-hippocampal interactions could contribute to the hyperdopaminergic phenotype observed in schizophrenia (Lisman et al., 2010), but further studies will be needed to confirm this possibility.

4.5.2 *Impact of a Loss of Cortical Input to Thalamus on Dopaminergic Function.* Here we demonstrate that inhibition of ilPFC induces an increase in VTA DA neuron population activity and alters firing pattern in RE. In patients with schizophrenia, several studies have identified hypoconnectivity between the mPFC/dorsolateral PFC (DLPFC) and the anterior/MD nuclei of the thalamus, including RE (Welsh et al., 2008; Woodward et al., 2012; Anticevic et al., 2014; Pergola et al., 2015; Giraldo-Chica and Woodward, 2017). This hypoconnectivity is present in clinical high risk patients, is more severe in patients that convert to psychosis, and was also demonstrated in a separate sample of patients <2 years after their initial diagnosis (Anticevic et al., 2015a; Woodward and Heckers, 2016). While these studies did not assess DA function, previous findings support the idea that PFC dysfunction could perpetuate a hyperdopaminergic state. It has been shown that during a working memory task patients with schizophrenia exhibit lower activity in DLPFC that correlates with enhanced striatal fluorodopa uptake and SN activity (Meyer-Lindenberg et al., 2002; Yoon et al., 2013), and that markers of DLPFC dysfunction in patients predict enhanced raclopride discplacement after amphetamine challenge (Bertolino et al., 2000). In the context of the current work, these findings suggest that a loss of cortical input to thalamus could lead to dysfunction in subcortical networks that perpetuates a hyperdopaminergic state.

4.5.3 *Impact of a Loss of Cortical Input to TRN on Thalamic Circuit Function.* Our data suggest that the effects of inhibition/stimulation of ilPFC on RE neuron firing pattern depend heavily on feedforward inhibition from the TRN. Consistent deficits in TRN structure and function in patients with schizophrenia have been described, including interneuron loss (Smith et al., 2001; Burgess et al., 2016) and reduced sleep spindles (Ferrarelli and Tononi, 2011; Wamsley et al., 2012; Ferrarelli and Tononi, 2017). While TRN can intrinsically generate sleep spindle-like activity (Steriade et al., 1987), cortical input is necessary to synchronize sleep spindles across cortical areas (De Gennaro and Ferrara, 2003). Strikingly, patients with schizophrenia also show a decreased thalamic BOLD response to transcranial magnetic stimulation of cortex, which implies less effective cortical feedback and weakened TRN engagement (Guller et al., 2012). These findings suggest that intrinsic dysfunction within the TRN, along with cortical microcircuit abnormalities known to be present in schizophrenia (Lewis et al., 2005), could reduce the ability of cortex to regulate activity in thalamus. In addition, our findings show that this loss of corticothalamic-TRN input could lead to altered firing pattern in thalamic cells and therefore altered engagement of hippocampal targets. This deficit would likely be relevant for awake behaviors (Halassa et al., 2014; Wimmer et al., 2015; Wells et al., 2016) and for spindle-dependent memory consolidation in sleep, which is known to be disrupted in schizophrenia (De Gennaro and Ferrara, 2003; Wamsley et al., 2012; Ferrarelli and Tononi, 2017) Taken together, these findings suggest that the TRN should be examined in future studies of thalamic function and cortico-hippocampal communication in schizophrenia.

4.5.4 *Corticothalamic Communication and Memory-Related Deficits in Schizophrenia.* Our findings demonstrate that manipulating ilPFC can dramatically alter firing pattern in RE and

population activity in VTA. These circuit dynamics likely underlie several complex behaviors, in particular those that depend on prefrontal-hippocampal interactions. However, the behavioral relevance of the ilPFC-TRN-RE-vSub circuit is not well-described. This circuit is analogous to the ventromedial PFC (vmPFC)-TRN-RE-aCA1 circuit in humans, where vmPFC here is defined as Brodmann Area 25 and neighboring regions (Ochsner et al., 2012; Delgado et al., 2016). In this section we explore findings that suggest a unifying theory for the function of corticothalamic input to the RE-hippocampal network. In addition, we argue that dysfunction in these circuits could contribute to the psychotic symptoms of schizophrenia, in particular aberrant salience.

Emerging evidence in rodents suggests that RE is important for the encoding, consolidation, and retrieval of context-dependent fear memories (Davoodi et al., 2011; Xu and Südhof, 2013; Jin et al., 2015; Kitamura et al., 2017; Sierra et al., 2017; Vetere et al., 2017). Inhibition of RE during recall testing impairs the retrieval of extinction memories (Jin et al., 2015), while simultaneous inhibition of the ilPFC-RE and ilPFC-TRN projections during conditioning generalizes fear responding to an altered version of the training context 48hrs later (Xu and Südhof, 2013). These findings complement a robust literature demonstrating that ilPFC/vmPFC is necessary for consolidation and retrieval of context-dependent extinction memories across species (Quirk et al., 2006; Sotres-Bayon and Quirk, 2010; Milad and Quirk, 2012; Zelikowsky et al., 2013). Intriguingly, patients with schizophrenia exhibit deficits in extinction learning (Holt et al., 2009; 2012). Specifically, while patients can acquire and extinguish conditioned fear responses, they exhibit impaired extinction recall that is associated with decreased vmPFC activity compared to controls (Holt et al., 2012).

A recent fMRI study in humans has demonstrated a role for RE in the encoding of novel and familiar stimuli (Reagh et al., 2017). Subjects in the study were asked to encode stimuli

presented once or repeatedly, then asked to differentiate the stimuli based on presentation frequency. Correct recognition of repeated stimuli, but not singly-presented stimuli, was associated with enhanced functional connectivity between vmPFC/ACC and RE, and reduced functional connectivity between vmPFC/RE and aCA1. These findings are in line with studies implicating vmPFC in novelty processing (Garrido et al., 2015) and those showing that anterior hippocampus is active in response to novel stimuli and habituates as stimuli become more familiar (Knight, 1996; Murty et al., 2013; Kaplan et al., 2014). Patients with schizophrenia exhibit deficits in novelty processing in a variety of task paradigms. For example, during a recognition memory task patients failed to activate the anterior hippocampus in response to new items (Weiss et al., 2004). In addition, patients exhibit a lack of habituation of hippocampal activation in response to repeated presentation of fearful faces (Holt et al., 2005) and this finding has been correlated with memory impairment in the disease (Williams et al., 2013). During fear conditioning patients with schizophrenia show reduced physiological and neural responses to the CS+ compared to controls, as well as heightened responses to the CS- that are associated with enhanced activation of the anterior hippocampus and thalamus (Holt et al., 2012)

The findings discussed above make it tempting to speculate about a general function for the ilPFC-TRN-RE-vSub (vmPFC-TRN-RE-aCA1) circuit in normal behavior and disease states. Specifically, they suggest that thalamic input to anterior hippocampus promotes associative learning and signals novelty, both processes that could also be promoted by enhanced VTA output following RE activation (Lisman and Grace, 2005; Lisman et al., 2010; Kempadoo et al., 2016; Moreno Castilla et al., 2017). This theory is in line with the known function of the anterior hippocampus in encoding the non-spatial, emotional, and affective aspects of context (Grace, 2012b; Liberzon and Abelson, 2016). In contrast, the findings discussed above suggest that

corticothalamic input from vmPFC encodes safety and signals familiarity, both of which might be promoted by reduced VTA output following ilPFC/vmPFC activation (Schultz, 2007; Patton et al., 2013; Grace, 2016). This theory is in line with previous studies suggesting that vmPFC suppresses the retrieval of contextually-irrelevant memories (Depue, 2012; Navawongse and Eichenbaum, 2013; Delgado et al., 2016). Based on the current work, these proposed effects are likely to be mediated by gating of thalamo-hippocampal interactions by the ilPFC/vmPFC-TRN circuit. Taken together, these ideas suggest opposing roles for vmPFC and anterior hippocampus in memory and novelty processing, mediated by a thalamic relay.

In schizophrenia, a loss of vmPFC input to thalamus (Anticevic et al., 2014; Giraldo-Chica and Woodward, 2017) and intrinsic dysfunction in TRN (Ferrarelli and Tononi, 2011; Burgess et al., 2016) have been described. Based on the model outlined above, these circuit abnormalities would result in dysregulated thalamo-hippocampal communication. Combined with hyperactivity in anterior hippocampus (Heckers and Konradi, 2010), this could promote perpetual and/or inaccurate encoding of stimuli as novel or contextually relevant – i.e. salient (Lisman et al., 2010; Winton-Brown et al., 2014; Pinault, 2017). The experience of aberrant salience in schizophrenia is well-documented and has been described as "…an emotional and sensory overload, with a growing sense that things and events around them [patients] have a hidden important meaning" (Winton-Brown et al., 2014). These symptoms have traditionally been attributed to enhanced dopaminergic transmission (Howes et al., 2007). However, hyperdopaminergia is likely one component of a broader circuit deficit in schizophrenia. Thus, we propose that in schizophrenia, a loss of prefrontal regulation of thalamo-hippocampal communication contributes to the persistence of aberrant salience both by disrupting hippocampal function and disinhibiting DA signaling.

As a whole, this section describes how dysfunction in the vmPFC-TRN-RE-aCA1 circuit could account for known behavioral deficits in schizophrenia. Of course, rigorous future studies will be needed to test these predictions. We present this theory in the hopes that it will motivate studies of psychotic symptoms in schizophrenia with a focus on corticothalamic communication, the TRN, and the other distributed circuits discussed here. Indeed, inspiration could be gained from findings in other areas of psychiatric neuroscience – in the field of post-traumatic stress disorder (PTSD) research, the symptoms of hyperarousal, intrusive thoughts, and overgeneralization have long been linked to deficits in prefrontal-hippocampal circuits (Milad and Quirk, 2012; Maren et al., 2013; Liberzon and Abelson, 2016). Hopefully future studies incorporating these ideas will lead to more effective biomarkers and/or treatments for schizophrenia.

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