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IMMUNOHISTOCHEMICAL CHARACTERIZATION OF A NOVEL POPULATION OF RETICULAR THALAMIC NEURONS EXPRESSING CHOLECYSTOKININ AND TYPE 1 CANNABINOID RECEPTORS

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IMMUNOHISTOCHEMICAL CHARACTERIZATION OF A NOVEL POPULATION OF RETICULAR THALAMIC NEURONS EXPRESSING CHOLECYSTOKININ AND TYPE 1 CANNABINOID RECEPTORS

BY

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THESIS

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This thesis has been examined and approved in partial fulfillment of the requirements for the degree of Master of Science in Integrative and Organismal Biology by:

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Figures and Tables

ABTRACT

The thalamic reticular nucleus (TRN) filters somatosensory stimuli by providing inhibition to the excitatory thalamic relay nuclei, which then communicate with the cortex to appropriately integrate and respond to sensory stimuli. The TRN is also responsible for generating and maintaining sleep spindles, which are important for memory and cognition, and are disrupted in various psychiatric disorders. Previous studies have shown that the TRN is comprised of several neuronal subpopulations that carry out specific functions. These neurons can be distinguished using peptide markers such as parvalbumin (PV), somatostatin (SST), and calbindin (CB). In this thesis, I identified a novel neuronal population in the TRN that expresses the peptide cholecystokinin (CCK) and distal-less homeobox gene 5/6 (Dlx5/6). *CCK;Dlx5/6* neurons have been widely studied in the hippocampus and amygdala where they have been shown to highly express the type 1 cannabinoid receptor (CB1R) and produce a long-lasting inhibition of pyramidal neurons. However, basic characterization of *CCK;Dlx5/6* neurons has been missing. Through immunohistochemical assays, we found that *CCK;Dlx5/6* neurons of the TRN express CB1R and the CCK peptide. Our results show that *CCK;Dlx5/6* are highly concentrated in the dorsal areas of the TRN relative to the ventral ones, dorsal areas of the TRN are associated with processing of visual cues. Our preliminary results also show that dorsal and ventromedial areas of the thalamus receive more inputs from *CCK;Dlx5/6* neurons than ventral and medial thalamic areas. Furthermore, some of the *CCK;Dlx5/6* neurons establish perisomatic synapses with neurons of the lateral dorsal nucleus of the thalamus, this type of connectivity is consistent with previous observations for CCK neurons in amygdala and hippocampus. Taken together our results provide insights into the anatomy of *CCK;Dlx5/6* neurons that suggest a potential role of these neurons in visual processing.

1. INTRODUCTION

1.1 Sensory processing in both physiological and pathological conditions

Sensory processing depends on the proper functioning of peripheral sensory receptors that transform environmental cues into electrical signals that can then be interpreted at the central level. Sensory information passes through the thalamus before reaching the cortex where they ultimately lead to a response (Sherman & Guillery, 2002). Sensory processing is the foundation for selective attention and the proper perception of reality, which depends on a balance of excitation and inhibition within the brain as too much excitation and/or too little inhibition can lead to the perception of non-salient stimuli (Foss-Feig et al., 2017; Zheng, Matsuo, Miyamoto, & Hoshino, 2014). The inability to properly process sensory information, whether that be hypersensitivity or hyposensitivity, is at the core of many neurological and neurodevelopmental disorders and is thought to be a consequence of excitation-inhibition imbalance (Kang et al., 2019;Rubenstein & Merzenich, 2003; Foss-Feig et al., 2017).

Attention deficit disorder with or without hyperactivity (ADD and ADHD) are defined by cognitive rigidity, impulsivity, and inattention according the American Psychological Association (American Psychiatric Association, 2013). Children with ADHD often suffer from comorbid autism spectrum disorders (ASD) (Naaijen et al., 2017), which are largely characterized by having deficits with social communication and the presence of repetitive behaviors (American Psychiatric Association, 2013). Another common symptom of ASD is sensory over-responsivity, which is associated with higher severity of social and repetitive behaviors (Ben-Sasson et al., 2008; Green et al., 2018). Attention deficits and disrupted sensory processing seen in ADHD and ASD are also common in patients with schizophrenia (McGhie & Chapman, 1961;Krol et al., 2018). Schizophrenia (SCZ) is a debilitating psychiatric disorder with symptoms grouped into 3

categories: positive and negative symptoms. Positive symptoms include hallucinations, delusions, and disorganized thoughts while negative symptoms refer to anhedonia, impaired social behavior, and lack of emotional expression (Curley & Lewis, 2012). Cognitive processes such as working memory are also dramatically impaired in schizophrenia (Carter et al., 2008;Curley & Lewis, 2012). In sum, a common factor amongst these disorders is some type of deficit in the numerous circuits that contribute to proper sensation and perception including the cortico-thalamic circuitry.

1.2 Role of the thalamus in sensory processing

The thalamus was once thought to simply relay information from the periphery and sub-cortical structures to the proper cortical destination has since been expanded upon. Before terminating in the cortex, sensory information goes through circuits involving the thalamus such as thalamocortical (TC), corticothalamic (CT), thalamo-thalamic circuits. The thalamus is made up three functionally distinct types of nuclei: relay nuclei, intralaminar nuclei, and thalamic reticular nucleus (TRN) (Veres, Linley, & Hoover, 2015). The relay nuclei represent the majority of thalamic nuclei, are comprised of glutamatergic neurons, and are function/modality specific (**Figure 1**). "Relay" refers to nuclei that are passing information between brain regions as opposed to being the main drivers themselves or the ultimate destination of information (Crabtree, 2018). Depending on the source of their driver input, thalamic relay nuclei are classified as first order (FO) or higher order (HO). FO nuclei receive afferents from subcortical and peripheral sensory structures (i.e. first in line of the sensory integration process), while HO nuclei receive afferents from the cortex, mainly layer 5, and act as a means of communication between cortical areas (Lam & Sherman, 2011;Crandall, Cruikshank, & Connors, 2015). The majority of these driver inputs are excitatory (Pinault, 2004). The intralaminar nuclei process information from the brainstem

reticular formation and are involved in sensory-motor integration (Fisher & Reynolds, 2014). Finally, and the main focus of my thesis is the TRN. In the section below, I will describe in detail the neurobiology of this nucleus.

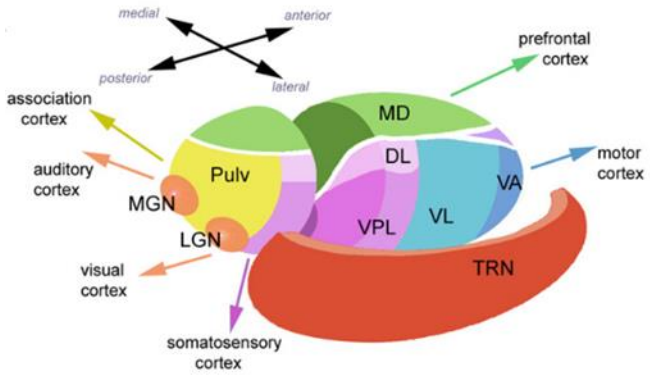

Figure 1. Anatomy of nuclei in the dorsal thalamus. The TRN wraps around FO and HO relay nuclei. FO nuclei include the lateral geniculate nucleus (LGN), medial geniculate nucleus (MGN), ventroposterolateral nucleus (VPL), ventral lateral nucleus (VL), and ventral anterior nucleus (VA). HO nuclei include the medial dorsal nucleus (MD), pulvinar (Pulv), and dorsolateral or laterodorsal nucleus (DL). (Pratt et al., 2016)

1.3 Functional organization of the thalamic reticular nucleus and its role in sensory processing

In addition to driver inputs that carry specific information to relay nuclei, FO and HO nuclei receive inhibitory and excitatory modulatory afferents that fine tune the information arriving to these nuclei before it is relayed further. The excitatory modulator afferents originate from layer 6 of the cortex, whereas the inhibitory afferents originate from the TRN (Sherman & Guillery, 2002). The TRN is the only thalamic nucleus comprised of entirely GABAergic neurons and is crucial in controlling the excitation and gain of the thalamocortical transmission (Pinault, 2004). Both

descending afferents from the cortex to the thalamus (corticothalamic or CT) and ascending efferents from the relay nuclei to layer 4 of the cortex (thalamocortical or TC) converge on the TRN (**Figure 2**) (Crabtree & Isaac, 2002). This not only allows a way for cortical areas to communicate with each other without direct innervation with one another, but also allows the same for thalamic nuclei. The relay and interpretation of stimuli can go awry in either the CT or TC pathways, but dysfunction in the TRN has the potential to disrupt both of these pathways. Also, sensory processing relying on a delicate balance of excitation and inhibition puts a lot of focus on the TRN. The importance of the TRN in sensory processes was established long ago when Francis Crick said that if the thalamus is the gateway to the cortex then the TRN is considered the guardian of that gateway (Crick, 1984).

As described above, the TRN participates in both feedforward and feedback processing of sensory information in the form of LVI → TRN → TC neurons and TC neurons → TRN → TC neurons, respectively (Crabtree, 2018). Aside from being the only GABAergic nucleus of the thalamus, the TRN also differs from the other nuclei in the sense that it receives afferents from cortical and sub-cortical structures (so it is neither FO or HO) and only innervates thalamic relay nuclei (Pratt et al., 2016). An example of these circuits working together to respond to stimulus is that of a visual cue and is shown in Figure 2. The retina sends the visual information to the first-order lateral geniculate nucleus (LGN). The LGN is innervated by the TRN and will provide inhibitory feedback before the LGN passes this information to the visual cortex. The cortex then sends this information back down the higher-order Pulvinar (Pulv) within the thalamus, also innervated by the TRN (Pratt et al., 2016). This is of course a simplistic version of the circuit as it ignores other sub-cortical modulatory inputs that contribute to determining the significance of the visual information. In addition, the TRN is divided into sectors that correspond to specific sensory

modalities that are connected to different relay nuclei associated with that sensory modality. In particular, the dorsocaudal TRN is the visual sector, ventrocaudal is the auditory sector, ventrocentral is the somatosensory sector, and lastly the least studied rostral motor sector (Crabtree, 2018). A single TRN neuron can innervate more than one thalamic nucleus, but these multiple thalamic nuclei are often functionally related to each other (Pinault & Deschênes, 1998). This aspect is particularly important when considering attention to competing stimuli. An example is the suppression of the visual TRN (connected to LGN) and activation of the auditory TRN (connected to MGN) during a visual task with an auditory distractor (Wimmer et al., 2015).

Aside from receiving glutamatergic projections from layer 5 of the cortex (Zikopoulis & Barbas, 2007) and thalamic relay nuclei (Pinault, 2004), the TRN also receives GABAergic signals from the lateral hypothalamus to regulate arousal (Hou et al., 2016), the globus pallidus (Gasca-

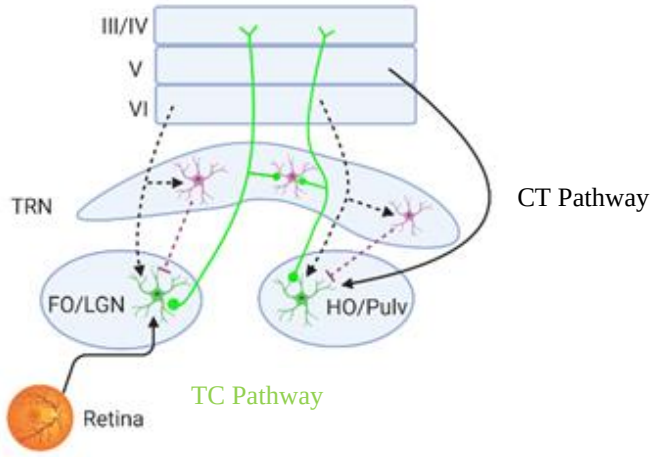

Figure 2. Simplified illustration of thalamocortical and corticothalamic pathways that converge on the TRN when processing visual information from the eye. The LGN (FO nucleus) receives driver input about visual stimuli from the retina as well as modulatory afferents from cortical layer VI and the TRN. The LGN then terminates in cortical layers III and IV. The pulv (HO nucleus) receives its driver input from cortical layer IV corresponding to the visual stimuli, as well as modulatory afferents from cortical layer VI and the TRN. The pulv then innervates cortical layers III and IV of the cortex. Created with BioRender.com

Martinez et al., 2010), as well as cholinergic inputs from the basal forebrain (Pita-Almenar et al., 2014), monoamergic and cholinergic afferents from the brain stem (Jones, 1991), and dopaminergic afferents from the substantia nigra (Picazo et al., 2009). The amygdala has also been shown to project directly to the TRN, with 70% of axons terminating in the rostral TRN, and be important for emotional attention in monkeys (Zikopoulis & Barbas, 2012).

Cortical areas and associated specific relay nuclei corresponding to different somatosensory and motor information form a topographic map onto the TRN (Pinault, 2004). Therefore, although FO and HO thalamic relay nuclei may not communicate directly, the same sensory input driving them will converge and be integrated in the TRN. To make matters even more complicated, the TRN and relay nuclei can participate in both open and closed feedback loops. The closed loop consists of a thalamic relay neuron projecting onto the TRN and the TRN then sending inhibitory input to that thalamic relay neuron. The open loop is when a thalamic relay neuron projects onto the TRN and then the TRN sends inhibitory input to a different thalamic relay neuron (Barbas & Zikopoulis, 2007). Additionally, TRN neurons innervate themselves in what is called lateral inhibition, and this is thought to play a significant role in convergent sensory information from different sources competing for attention (John et al., 2016). The complex role of the TRN in the integration of information that is sent back and forth to the cortex makes this an exciting focus of study. I will describe below the pathophysiology of the TRN.

1.4 The thalamic reticular nucleus in health and disease

Given the key role of the TRN in the thalamus, it is not surprising that this nucleus has been linked to the physiological functions related to sensory processing and to neurological conditions in which this sensory processing is altered. The TRN has been reported to play a role in attention (Wells et al., 2016; McAlonan et al., 2000;Weese et al., 1999; Zikopoulos & Barbas, 2006;Wimmer et al., 2015), arousal (Lewis et al., 2015), pain sensitivity (Liu et al. 2017), memory consolidation via the induction of sleep spindles (Latchoumane et al. 2017;), cognition and working memory (Whissell et al., 2019), and motor processes (Villalobos et al., 2016).

Dysfunction of the TRN has been observed or strongly implicated to play a role in various psychological disorders such ADD and ADHD (McAlonan et al., 2000;Wells et al., 2016), ASD (Green et al., 2017;Wells et al., 2016), bipolar disorder (BPD) (Ferrarelli & Tononi, 2017;Steullet et al., 2018), and SCZ (Ferrarelli & Tononi, 2017; Baran et al., 2019;Steullet et al., 2018; Young & Wimmer, 2017). The TRN's strategic position between the thalamus and cortex sets it up to be a common denominator in these diseases.

Another characteristic that the disorders mentioned above share is abnormal sleep spindles. Sleep spindles are synchronized oscillations in the frequency range of 9-16 Hz that occur in nonrapid eye movement (NREM) sleep and are thought to support neuroplasticity and memory consolidation (Latchoumane, Ngo, Born, & Shin, 2017). The TRN is well known to be the original source of sleep spindles by changing their firing mode to bursts, deinactivating low-threshold calcium channels and leading to rebound burst firing that ultimately activates the TRN (Piantoni et al., 2016). Spindles can vary across samples in duration, amplitude, and frequency depending upon the overall communication of the entire thalamocortical circuit, which then impact waking brain activity (Bandarabadi et al., 2020).

The fact that sleep spindles can easily be measured makes them a promising biomarker for disease. Lower sleep spindle intensity and amplitude in adolescents with ASD are associated with severity of autistic traits (Merikanto et al., 2019). There are increased slow-spindle activity in children diagnosed with ADHD (Saito et al., 2019). Many studies have found impairments in sleep spindle density, amplitude, and duration in patients with chronic SCZ (reviewed in Kaskie et al., 2019). Interestingly, patients experiencing the first-episode of psychosis, and therefore unmedicated, had reduced spindle duration and intensity, but not amplitude (Kaskie et al., 2019). In addition, the severity of spindle dysfunction in these first-episode in patients with SCZ predicted the severity of the negative symptoms they would go on to form, indicating that quality of sleep spindles is present as early as the onset of schizophrenia as opposed to be a product of chronic schizophrenia (Kaskie et al., 2019). What exactly initiates these spindle abnormalities in psychiatric disorders is unknown.

1.5 Neuronal populations within the thalamic reticular nucleus

Although all neurons in the TRN are GABAergic, different populations of cells have been identified through peptide markers. The majority of neurons express the calcium-binding protein parvalbumin (PV^+) (Steullet et al., 2018; Alberi et al., 2013). The peptide somatostatin (SST^+) and calbindin (CB^+) are also expressed in cells of the TRN (Pinault, 2004;Martinez-Garcia et al., 2020). The neuropeptide cholecystokinin (CCK) was shown to be expressed in the TRN decades ago (Sohal, Cox, & Huguenard, 1998), but little research has been done to understand function of cells that express CCK (CCK^+) in this area of the brain compared to PV^+, CB^+ and SST^+ interneurons. The importance of investigating these interneuron populations separately becomes clear when

considering the individual differences of among them, with emphasis on different firing properties, co-expressed receptors, and their ability to modulate one another (**Table 1**).

Protein/Peptide	Electrophysiological Properties	Receptor Subtypes	Function
Parvalbumin (PV)	. Fast-spiking . Non-adapting . Synchronous release . Controlled by P/Q type Ca channels	. NMDA . 5-HT1A . D_2	. Inhibit pyramidal neurons via perisomatic compartment . Contribute to synchronization of oscillations
Cholecystokinin (CCK)	. Unknown	. Unknown	. Unknown
Somatostatin (SST)	. Regular spiking . Slow, weak, less precise than PV	. Erbb4	. Inhibit pyramidal neurons at distal dendrites
Calbindin (CB)	. Low-threshold . Regular Spiking	. Unknown	. Inhibit pyramidal neurons typically at dendritic sites

Table 1. Properties of neuronal populations classified according to peptide markers in the TRN. Although these interneuron populations are well-defined in other areas of the brain, they still remain relatively elusive in the TRN. N-methyl-D-aspartate receptor (NMDA), serotonin receptor 1A (5-HT1A), dopamine receptor 2 (D_2), receptor tyrosine-protein kinase subtype (Erbb4).

The function of CCK^+ interneurons within the TRN has not yet been determined. Part of this thesis is to lay the groundwork to characterize the role of CCK^+ interneurons in the function of the TRN. Furthermore, because CCK^+ interneurons in other brain areas are hugely enriched with the cannabinoid receptor type 1 (CB1R), we also sought to generate preliminary evidence of the potential role of these neurons in the regulation of the TRN by CB1R agonists.

1.6 Cholecystokinin-expressing interneurons and cannabinoid receptor type 1 in the thalamic reticular nucleus

Endocannabinoids have been linked to the regulation of sleep states (Pava et al., 2014;Perescis et al., 2020), pain sensitivity (Smith et al., 2017;Woodhams et al., 2017), anxiety (Smith et al., 2017;Ruehle et al., 2012; Patel et al., 2017), and memory deficits (Smith et al., 2017; Volk & Lewis, 2016). The two main endocannabinoids produced in the brain are anandamide and 2-arachidonoyl glycerol (Cristino et al., 2020). These endocannabinoids bind to CB1 and CB2 receptors. The distribution of cannabinoid receptors across brain regions is highly conserved in different species (Katona & Freund, 2012), further demonstrating the importance of this system.

CB1R has previously been shown to be expressed in the TRN (van Rijn et al., 2010), but the cell type in which it is expressed has not been determined. In the rest of the brain CB1R is expressed primarily on CCK^+ interneurons (Katona et al., 1999;Omiya et al., 2015;Neu et al., 2007;Bowers et al., 2014). And our lab replicated these results CCK+ interneurons express 100 fold more CB1R transcript than pyramidal neurons (Bunda, LaCarubba, Akiki, & Andrade, 2019). Although, PV^+ interneuron axon terminals have been found to express CB1R in the infralimbic prefrontal cortex in small amounts (Liu, Dimidschstein, Fishell, & Carter, 2020) and excitatory terminals of pyramidal neurons also express small amounts of CB1R (Liu et al., 2020). Activation of CCK/CB1R neurons ultimately leads to suppression of neurotransmitter a process called depolarization-induced suppression of inhibition or excitation depending on whether the neurotransmitter is GABA or glutamate (DSI and DSE, respectively). Stimulation of the post-synaptic cell results in synthesis of endocannabinoids that travel back to the CCK/CB1R pre-synaptic neuron where it binds to CB1R and blocks calcium channels needed for neurotransmitter release. In this sense, CB1R acts as a type of autoreceptor.

1.7 Function of cholecystokinin-expressing interneurons in other areas of the brain

CCK^+ interneurons have been widely studied in other areas of the brain, thus we can gain insight about their function by reviewing the information about these neurons from other areas such as the amygdala and hippocampus. In contrast to PV^+ interneurons, CCK^+ interneurons display regular-spiking activity and asynchronous release of GABA via N-type calcium channels, leading to long-lasting inhibition (Hefft and Jonas, 2005). Activation of CCK^+ interneurons in the mouse brain results in enhanced memory and cognition with no effect on emotional processing (Whissell et al., 2019). A more region-specific activation of this same interneuron population within the basal amygdala (BA) leads to modulation of fear extinction (Rovira-Esteban et al., 2019). CCK^+ interneurons within the hippocampus express vasoactive intestinal polypeptide (VIP), vesicular glutamate transporter type 3 (VGLUT3), somatic $GABA_B$ receptors, and 5-HT3 receptors (Freund, 2003). Furthermore, CCK+ interneurons in hippocampus and amygdala are enriched with large amounts of the cannabinoid receptor 1 (CB1R) (Bowers & Ressler, 2014; Iball & Ali, 2011; Katona et al., 1999; Lee, Földy, & Soltesz, 2010). Roughly 90% of CB1R expressing interneurons within the CA1 region of are CCK+ (Jappy et al., 2016).

2.0 RESEARCH QUESTIONS

In this thesis, I performed a thorough histochemical characterization of genetically identified CCK interneurons in the TRN. To do this, we labeled CCK interneurons using intersectional labeling (**Figure 3**). An intersectional approach was necessary because the CCK promoter is active in both excitatory and inhibitory neurons. In order to restrict the expression of the red fluorescence protein, tdTomato (tdT) to CCK expressing interneurons we took advantage of the activity of the distal-less homeobox 5/6 (Dlx5/6) promoter, which is specific of forebrain interneurons. In this approach, two recombinases (Cre and Flpe) which expression depends on the CCK and Dlx5/6 promoters respectively, excise two stop codons present before the start site of tdT. Thus, it is condition that both promoters are active in the same cell to induce the expression of tdT (Taniguchi et al., 2011). I will hereafter name this novel cell population as *CCK;Dlx5/6* neurons.

I pursued the following research questions: Are *CCK;Dlx5/6* cells in the TRN neurons? How are *CCK;Dlx5/6* neurons distributed across the anatomical regions of the TRN? Do *CCK;Dlx5/6* neurons express the CCK peptide? Do *CCK;Dlx5/6* neurons express the PV peptide? Do *CCK;Dlx5/6* neurons express CB1R similar to what has been observed in this neuronal type in the amygdala and hippocampus? What are the likely synaptic targets of *CCK;Dlx5/6* interneurons? We expect that by answering these questions, we provide insights into the role of this novel cell-population in sensory processing at the TRN level.

3.0 METHODS

3.1 Animals

All procedures were approved by the Institutional Animal Care and Use Committee at the University of New Hampshire (APPENDIX). To label *CCK;Dlx5/6* neurons in the TRN, we performed intersectional labeling. We obtained a triple transgenic mouse line containing alleles for *CCK-Cre*, Dlx5/6-Flpe and tdT via breeding (**Figure 3**). Initially, *Cck-Cre* mice (012706; The Jackson Laboratory) were crossed with *Dlx5/6-Flpe* mice (010815; The Jackson Laboratory). Cre

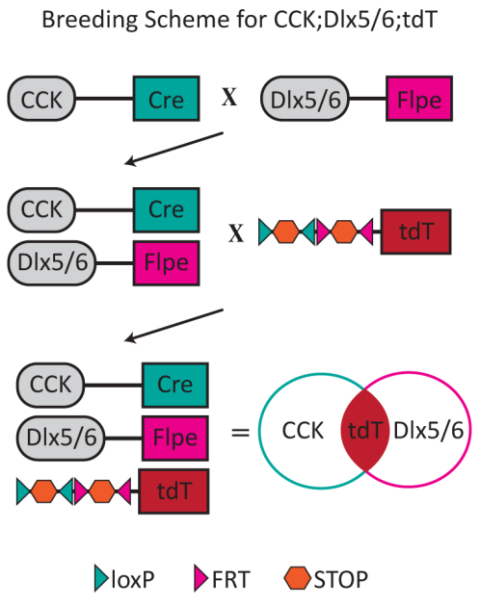

Figure 3. Breeding scheme to label *CCK;Dlx5/6* cells in the TRN. Top panel: homozygous *CCK-IRES-cre* mice are first mated with *Dlx5/6;Flpe* mice. Middle panel: The offspring of this mating produce *CCK;Dlx5/6* mice, which are then mated with tdT homozygous mice to produce *CCK;Dlx5/6;tdT* progeny. Recombination of Cre-loxP and Flpe-FRT remove both STOP cassettes and result in tdT expression in GABAergic CCK^{+} cells.

and Flpe are recombinases expressed under the control of the CCK promoter and the Dlx5/6 promoter, respectively. Progeny from this initial cross, *Cck-Cre; Dlx5/6-Flpe* (abbreviated *CCK-Dlx5/6*), were dual transgenic mice with both alleles. Because mice with the *Dlx5/6* alleles could

not be bread to homozygosity, we selected for *CCK-Dlx5/6* mice that were homozygous for *CCK-Cre* and heterozygous for *Dlx5/6-Flpe*. To optimize the recombination efficiency, male *CCK-Dlx5/6* mice were bred to female mice containing a tdT allele downstream of two recombinase target-flanked STOP cassettes (*Ai65(RCFL-tdT)-D*;021875; The Jackson Laboratory). The first STOP cassette was flanked by loxP sites (recognized by Cre) and the second was flanked by FRT sites (recognized by Flpe) (**Figure 3**). The resulting progeny had individuals with two distinct genotypes: A) heterozygous for *CCK-Cre* and *Ai65(RCFL-tdT)-D*, but WT for *Dlx5/6-Flpe*, and B) heterozygous for the three alleles (abbreviated *CCK;Dlx5/6;tdT*). In the latter mice, Cre-Lox and Flpe-FRT recombination removed the two STOP cassettes, resulting in tdT fluorescence in cells that expressed both CCK and Dlx5/6.

3.2 Genotyping

To differentiate between mice with genotype A and genotype B, we performed three PCR reactions in genomic DNA. This was an extensive amount of work because entire litters needed to be genotyped to select individuals with proper tdT expression. Briefly, genomic DNA was extracted from P7-P9 animal tissue using Phire Animal Tissue Direct kit II (ThermoFisher Scientific, F140WH) according to manufacturer instructions. PCR was performed with AmpliTaq Gold® 360 mastermix (Thermo Fisher Scientific, 4398881) using the following conditions: a hot start of 95^0 C for 10 min, followed by 35 cycles (95^0 C, 30 s; 60^0 C, 30 s; 72^0 C, 1 min), and a final step of 72^0 C for 7 min. Primers and expected products are shown in **Table 2**. Primers were added to the same mixture for genotyping. **Figure 4** shows typical PCR for mice with genotype A (lanes 1, 3, and 5), and genotype B (lanes 2, 4, and 6). Note the presence of ~ 400 bp band in lane 2 that correspond to the *Dlx5/6-Flpe* allele, a band of this size is absent in WT mice.

Mouse line	Primers	Expected products
CCK-Cre	F-WT1: GGGAGGCAGATAGGATCACA F-MT1: TGGTTTGTCCAAACTCATCAA R: GAGGGGTCGTATGTGTGGTT	Hom: 180 bp Het: 180 bp and 468 bp WT: 468 bp
Dlx5/6-Flpe	F-T1: CAGAATTGATCCTGGGGAGCTACG R-T1: CCAGGACCTTAGGTGGTGTTTTAC F-C: CAAATGTTGCTTGTCTGGTG R-C: GTCAGTCGAGTGCACAGT TT	Transgene: 406 bp PCR positive control: 200 bp *PCR conditions do not differentiate between heterozygous and homozygous mice
Ai65(RCFL-tdT)	F-WT2: AAGGGAGCTGCAGTGGAG TA R-WT2: CCGAAAATCTGTGGGAAG TC R-MT1: GGCATTAAAGCAGCGTAT CC F-MT2: CTGTTCCTGTACGGCATGG	Hom: 196 Het: 196 and 297 WT: 297
All primers are reported in 5' to 3' direction		

Table 2. Primers and expected products

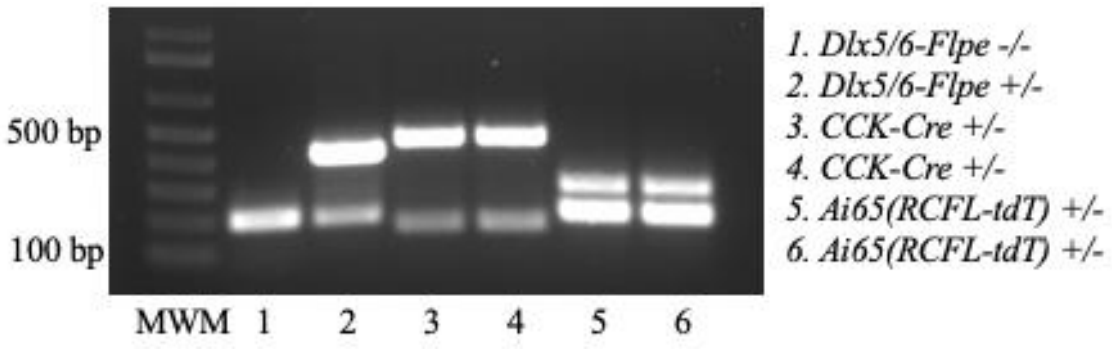

Figure 4. PCR results from *CCK;Dlx5/6;tdT mice.* Mouse A genotype regarding each gene is found in lanes 1 (200 bp), 3 (180 and 468 bp), and 5(196 and 297 bp). This indicates that mouse A is *Dlx5/6-Flpe* -/-, *CCK-Cre* +/-, *tdT* +/-. Mouse B genotype can be found in lanes 2 (200 and 406 bp), 4 (180 and 468 bp), and 6 (196 and 297 bp). In contrast to mouse A, mouse B contains the transgene for *Dlx5/6-Flpe* in lane 2 at 406 bp, and therefore is *Dlx5/6-Flpe* +/-.

3.3 Immunohistochemistry

Adult *CCK;Dlx5/6;tdT* mice of both sexes were deeply anesthetized in a chamber of isoflurane (07-893-1389; Patterson Veterinary). After ensuring the absence of rear-foot reflex, an intraperitoneal injection of 0.07 mL Euthasol® euthanasia solution (Virbac Animal Health, Co) was given in the lower right abdomen with a 26-gauge needle. I next performed cardiac perfusion with ice cold 1X PBS (1002657611; Sigma Aldrich), followed by ice cold 4% formalin solution (HT501128; Sigma Aldrich), and followed again by ice cold PBS. The brains were then extracted and stored in 4% formalin solution at 4˚ C for 24-48 hours. 50 µm coronal slices were prepared using a vibratome (VT1000S; Leica) between -0.5 mm and -1.9 mm relative to Bregma to ensure the entirety of the TRN was included. The slices were then kept in 12 well plates containing 1X PBS and stored at 4˚ C overnight. The following day, slices were rinsed with PBS 3 times for 10 minutes while rotating (Roto Mix Type 50800; Thermolyne) to ensure removal of formalin solution.

For CCK, PV and NeuN staining: Slices were then incubated in a blocking buffer consisting of 0.2% Triton-X (1002214179; Sigma Aldrich) in 1X PBS, 10% normal goat serum (ab7481; Abcam), and 10% BSA (A9418; Sigma Aldrich) for 30-60 minutes while lightly rotating. The slices were then rinsed with PBS for 10 minutes while rotating 3 times. The primary antibodies rabbit polyclonal anti-CCK-8 (C2581; Sigma Aldrich), rabbit polyclonal anti-PV (ab11427; Abcam), and/or mouse monoclonal anti-NeuN (MAB377; Sigma Aldrich) were added to the slices sitting in blocking buffer mentioned above at a concentration of 1:1000. For CB1R staining, permeabilization detergents were not used: slices were incubated in a blocking buffer that consisted of 10% NGS in PBS for 2 hrs. They were then incubated in the same blocking solution

containing the primary antibody extracellular rabbit polyclonal anti-CB1R (ACR-001; Alomone labs) at 1:1000.

Slices were covered and left in 4˚C for 18-24 hours while rotating. The slices were then rinsed again in PBS 3X, while lightly rotating, before adding the secondary antibodies Alexa488 (ab150077; Abcam) for rabbit primary antibodies, Alexa647 (ab150115; Abcam) for mouse primary antibodies, and/or FITC (ab97239; Abcam) for mouse primary antibodies at a concentration of 1:1000 in PBS to incubate for 2 hours in the dark, at room temperature, while rotating. Slices were then rinsed again with PBS before adding the nuclear stain To-Pro-3 iodide (T3605; inVitrogen) or SYTO-13 (S7575; Thermofisher) at a concentration of 1:1000 in PBS for 20 mins at room temperature. Slices were then rinsed another 3 times with PBS in the dark, while rotating before being mounted on to positively charged glass slides(1358W; Globe Scientific) with Vectashield hard set mounting medium (H-1400; Vector Labs) and micro glass cover slips(48393; VWR). Slides were allowed to dry before sealing with clear nail polish.

To visualize the red fluorescence from tdT in the *CCK;Dlx5/6* neurons and green fluorescence from antibodies against the proteins mentioned above, a Nikon A1R laser-scanning confocal fluorescence microscope was used. Fluorophores and their spectra were as follows (excitation/emission (nm)): tdTomato (554/581), Alexa 488 (495/519), FITC (493/528), Alexa 647 (652/668), SYTO-13 (488/509, and TO-PRO-3 (642/661). Slices were never co-stained with fluorescent proteins that had significant overlapping emission spectra. Images were taken at 10X to view the TRN in its entirety, 20X to visualize individual tdT^+ cells in the TRN. Brain slices with multiple fluorescent proteins with overlapping wavelengths were scanned and imaged using a spectral unmixing tool provided by the Nikon software package, NIS Elements. Pictures of one or

both TRNs per brain slice were taken, often in the form of 10X z stacks at slice thickness ranging from 2 µm to 4 µm.

Confocal images were originally saved in .ND2 format on to a flash drive and later opened with ImageJ Fiji for analysis. Cell counts were done manually using the Cell Counter plug in for FIJI (https://imagej.nih.gov/ij/plugins/cell-counter.html). Cell counts were done for tdT^+ cells (*CCK;Dlx5/6* neurons) within the TRN, and tdT^+ cells that colocalized with the proteins of interest (NeuN, PV, CCK, and CB1R). Colocalization of 2 different fluorescent proteins was also done manually. To keep consistency across brain slices and individual mice, cell counts were done on the max intensity projection of the Z stack collected. This combines all individual pictures taken within the z stack to give a better representation of fluorescence throughout the entire slice.

To image larger brain regions when analyzing potential target areas of TRN *CCK;Dlx5/6* neurons throughout the thalamus, variations in surface focal point across the slice were adjusted accordingly using the NIS software before scanning the whole image, with each picture sized at 1024. Individual z stacks taken at each focal point were then stitched together with 10 % overlap. We measured corrected total area fluorescence (CACF) associated to different thalamic nuclei. Areas that correspond to thalamic nuclei were drawn based on the Allen Brain Reference Atlas for the adult mouse (Lein et al., 2007). All areas were drawn relative to the localization of the TRN, which was easily distinguishable thanks to the strong tdT signal from *CCK;Dlx5/6* neurons. CACF was corrected for background fluorescence. CACF was calculated with the following formula: CACF = Integrated Density – (Area x mean fluorescence of background). Integrated density is the product of mean fluorescence by the area for each drawn for each nuclei (https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html).

The tdT fluorescence signal in all of our images was pseudcolored with magenta instead of red to facilitate the interpretation of our results by people with color blindness.

3.4 Statistical analysis

Cell counts of tdT-expressing cells of the TRN manually counted using the ImageJ FIJI Cell Counter Plugin were collected for every individual slice. To determine if tdT^+ cells of the TRN co-expressed any of the proteins of interest (NeuN, PV, CB1R, and CCK), the different channels were merged with the tdT images for each slice and the cells that appeared to show co-localization, often producing a yellow color as a result of red tdT and green secondary antibody of protein of interest. The number of cells counted as expressing both tdT and the secondary antibody were then divided by the total number of tdT cells to give an average of co-localization. If a mouse had multiple brain slices stained and imaged, those averages would then be combined to give an overall average of co-localization for that individual mouse. Averages and S.E.M. were calculated using excel and plots were created with R programing language (or statistical software).

4.0 RESULTS

4.1 Novel *CCK;Dlx5/6* neurons identified in the TRN

Previous studies have shown the presence of mRNA for CCK in neurons as well as non-neuronal tissue (Hökfelt T et al., 1991). Furthermore, some promoters can exhibit basal activity regardless of the presence of their transcription factors leading to low unregulated gene expression (Bittner-Eddy, Fischer, & Costalonga, 2019), Because in the *CCK;Dlx5/6;tdT* mouse line we are utilizing two promoters to label CCK;Dlx5/6 neurons, thereby increasing the possibility of finding expression of tdT in non-neuronal tissue. To rule out this possibility, we first confirmed that *CCK;Dlx5/6* cells in the TRN are indeed neurons. We stained slices of *CCK;Dlx5/6;tdT* mice with the neuronal marker NeuN. Next, we quantified the number of cells exhibiting fluorescent signals for tdT and NeuN. We found that approximately 94.4% of tdT cells also express NeuN (mean ± s.e.m. = 94.5 ± 1.06 %, n = 4, 2-4 slices per mouse. **Figure 5A and 5B**). No differences were observed between males and females, and the tdT in the TRN labeling was consistent among multiple litters and generations. This result strongly suggests that the vast majority of *CCK;Dlx5/6*

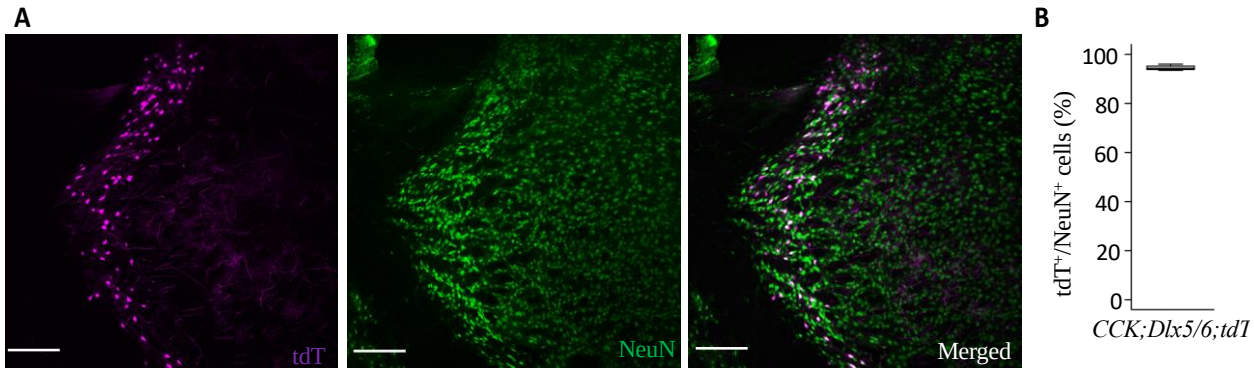

Figure 5. Staining of *CCK;Dlx5/6;tdT* slices with NeuN identifies tdT+ cells within the TRN as neurons. A. 10x confocal images of the TRN**.** Left, tdT in the TRN (magenta); Middle, NeuN expression (green); Right, tdT and NeuN merged. Scale = 200 μm. B. On average, 94.4% of *CCK;Dlx5/6* neurons in the TRN are positive for expression of NeuN across all samples. Data are represented using a box plot with interquartile range and 95% confidence interval.

cells in the TRN are neurons. Furthermore, our images show that CCK;Dlx5/6 neurons are strikingly localized to the TRN because none of the cell bodies for these neurons was observed in none of the relay or intralaminar nuclei (**Figure 5A**). Our results unveiled a novel neuronal population that has not been characterized before in the TRN and that is distinguished with the activity of CCK and Dlx5/6 promoters.

4.2 Distribution of the *CCK;Dlx5/6* neurons in the TRN

As indicated above, the TRN is subdivided into three regions in head, body and tail (Perez et al., 2017). Interestingly, head is associated to the processing of visual information, whereas cells in

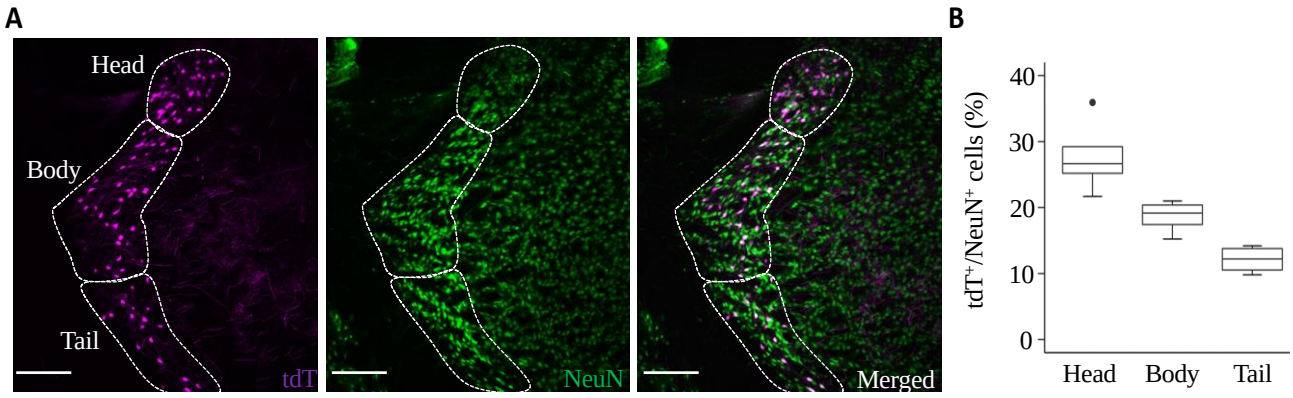

Figure 6. Relative distribution of *CCK;Dlx5/6* neurons across the head, body, and tail regions of the TRN. A. 10x confocal images of the TRN with the head, body, and tail regions segregated. Left, tdT expression in the TRN (magenta). Middle, NeuN expression (green) across regions and the dorsal thalamus. Right, tdT and NeuN expression merged. Scale = 200 μm. B. Quantification of *CCK;Dlx5/6* neurons of the TRN across regions reveal that approximately 27.7% in the head, 18.6% in the body, and 12.1% in the tail of $NeuN^+$ neurons in the TRN express tdT, with the head region having the highest concentration. Data are represented using a box plot with interquartile range and 95% confidence interval. Outliers were calculated as Q1 - (1.5 X IQR) and Q3 + (1.5 X IQR).

the body process somatosensory information, and the tail motor information (Pinault & Deschênes, 1998). To gain insights into the potential function of *CCK;Dlx5/6* neurons in the TRN we determined their relative abundance in each of the three regions. To determine the relative

distribution of *CCK;Dlx5/6* neurons , the TRN was separated into 3 regions: head, body, and tail (**Figure 6A**). All confocal images used for data analysis were in the form of a single image that combined every image within the z-stack for that given slice. By doing this, we disregarded differences in distribution in terms of rostral vs caudal as some previous studies have done. We quantified the amount of cells with signals for tdT and NeuN relative to the total number of $NeuN^+$ neurons throughout the TRN in each region (**Figure 6A**).

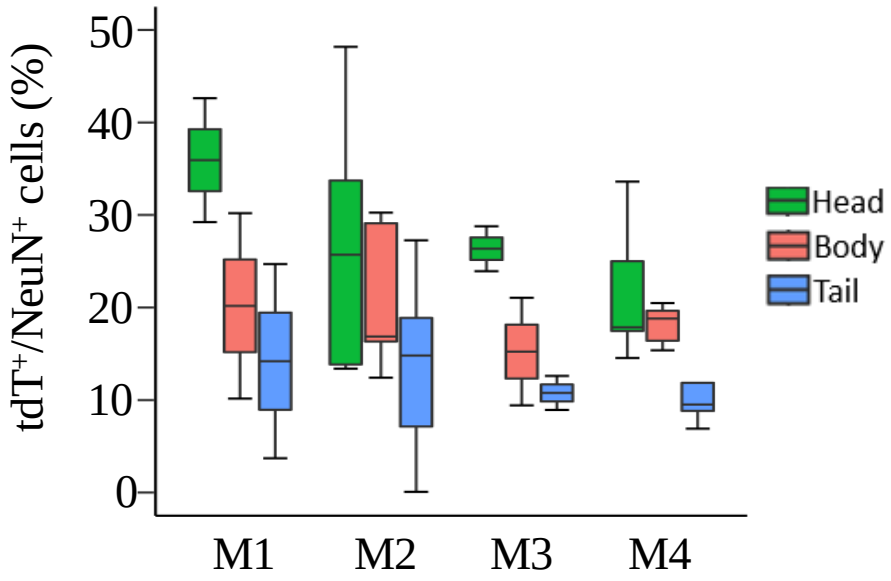

Figure 7. Distribution of *CCK;Dlx5/6* neurons in regions of the TRN per mouse. Comparison of the relative proportion of *CCK;Dlx5/6* neurons per region among individual mice. At least 3 slices per mouse were analyzed. Data are represented using a box plot with interquartile range and 95% confidence interval.

Approximately 27.7 % of neurons within the head region express tdT, 18.6% of neurons in the body express tdT, and 12.1% of neurons in the tail express tdT (% of CCK;Dlx5/6 neurons per TRN region, mean ± s.e.m. head = 27.7 ± 3.0 %, body = 18.6 ± 1.3 %, tail = 12.1 ± 1.1 %. **Figure 6B**). Three individual t tests were run to compare concentration of neurons between the 3 regions. The t test comparing the head and body region was $p = 0.044$, for the head and tail region was $p = 0.0055$, and body and tail was $p = 0.0044$. To correct for multiple comparisons, I used the

Bonferroni correction and α was set at 0.016, thus all p values below 0.016 were considered significant. The relative abundance of *CCK;Dlx5/6* neurons was higher in the head than tail and the body relative to the tail (**Figure 6B**). These results show that there is pattern of distribution of *CCK;Dlx5/6* neurons, where there is higher abundance of them in the dorsal region (head) relative to the ventral region (tail). Our results were also consistent among individual mice (**Figure 7**).

4.3 *CCK;Dlx5/6* neurons in the TRN express the CCK peptide

The DNA recombination and thus tdT expression by Cre and Flpe recombinases is irreversible, this opens the possibility that a transient expression of either or the two enzymes resulted in the production of tdT at some point in development, without necessarily labeling neurons that express the CCK peptide (Abremski & Hoess, 1984;Metzger & Feil, 1999). To rule out this possibility, we

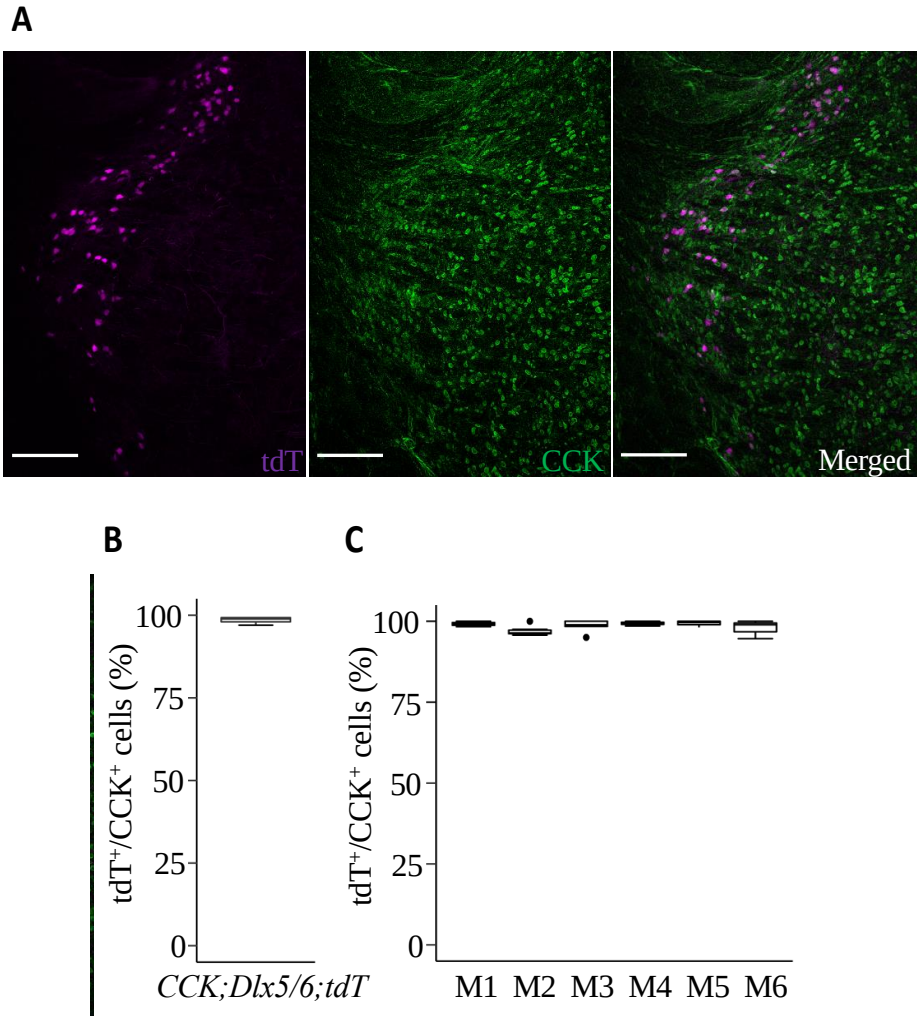

Figure 8. Expression of CCK peptide in *CCK;Dlx5/6* neurons of the TRN. A. 10x confocal images of coronal slices of the TRN. Left, tdT in the TRN (magenta); Middle, CCK peptide expression (green); Right, tdT and CCK peptide merged. B. Quantification *CCK;Dlx5/6* neurons expressing CCK peptide in the TRN. Scale = 200 μm. C. Percentage of *CCK;Dlx5/6* neurons in the TRN positive for expression of CCK peptide is consistent across mice. Data are represented using a box plot with interquartile range and 95% confidence interval. Outliers were calculated as Q1 – (1.5 X IQR) and Q3 + (1.5 X IQR).

performed immunohistochemistry experiments using an antibody against CCK. This experiment is essential to provide a thorough characterization of the *CCK;Dlx5/6* neurons in the TRN. Brain slices from *CCK;Dlx5/6;tdT* mice were stained for the CCK peptide. We found that approximately 98.5% of *CCK;Dlx5/6* neurons within the TRN expressed the CCK peptide (% of CCK;Dlx5/6 expressing CCK, mean ± s.e.m. = 98.5 ± 0.4%, n = 6 mice, 3-5 slices per mouse. **Figure 8A-B**). This observation was also consistent across different mice (**Figure 8C**). We observed wide expression of CCK in the TRN and thalamus, which is not surprising given that CCK is the most abundant neuropeptide in the brain (**Figure 8A**). Our results strongly suggest that *CCK;Dlx5/6* neurons in the TRN belong to a class of interneurons that express the CCK peptide in adulthood and our intersectional strategy to label them is adequate.

4.4 *CCK;Dlx5*/6 neurons in the TRN partially express parvalbumin

As noted previously, it has been suggested that all neurons within the TRN expresses PV (Hou, Smith, & Zhang, 2016). Although recent evidence suggests that there is another non-overlapping population of interneurons expressing SST in the TRN (Perez et al., 2017). In the hippocampus CCK^+ and PV^+ interneurons represent two non-overlapping classes of interneurons (Hefft & Jonas, 2005). Therefore, we tested if *CCK;Dlx5/6* express PV in the TRN. We stained for PV using antibodies against this protein. We found that approximately 58 % of *CCK;Dlx5/6* neurons in the TRN also express PV (% of *CCK;Dlx5/6* neurons expressing PV, mean ± s.e.m. = 58.1 ± 5.7 %, n = 4. **Figure 9A and B**). However, this observation was not consistent among different mice (**Figure 9C**). Our results suggest that in terms of PV expression, there are two subpopulations *of CCK;Dlx5/6* neurons, one that expresses the PV peptide and one that does not.

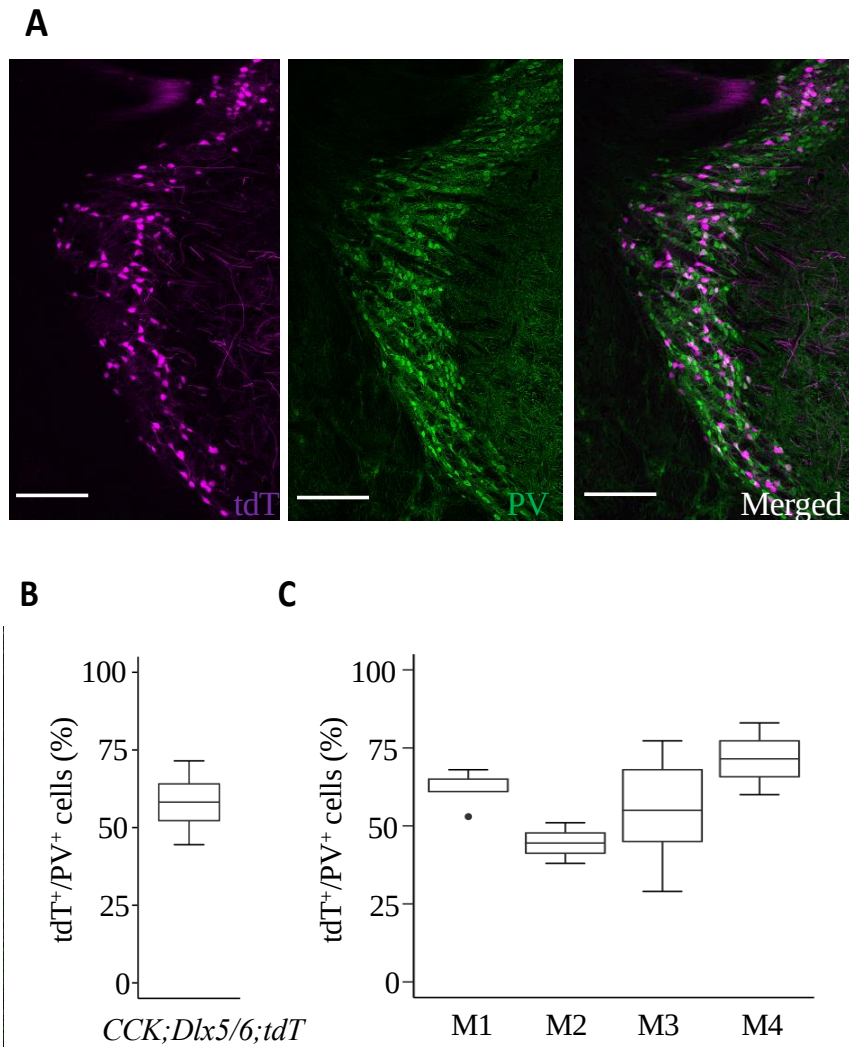

Figure 9. Expression of the PV peptide in *CCK;Dlx5/6* neurons in the TRN. A. 10x confocal images of the TRN. Left, tdT expression (magenta) in *CCK/Dlx5/6* neurons. Middle, PV protein (green) expression in the TRN and dorsal thalamus. Right, tdT and PV protein staining merged. B. Approximately 58% of *CCK;Dlx5/6* neurons in the TRN express the PV peptide when averaged across 4 mice. Scale = 200 μm. C. The percentage of *CCK;Dlx5/6* neurons that also express PV varied across mice. Data are represented using a box plot with interquartile range and 95% confidence interval. Outliers were calculated as Q1 – (1.5 X IQR) and Q3 + (1.5 X IQR).

4.5 *CCK;Dlx5/6* neurons in the TRN express the cannabinoid receptor type 1

CCK^+ interneurons from the hippocampus and amygdala have been shown to be enriched with CB1R (Bowers & Ressler, 2014; Iball & Ali, 2011; Katona et al., 1999; Lee et al., 2010). Our

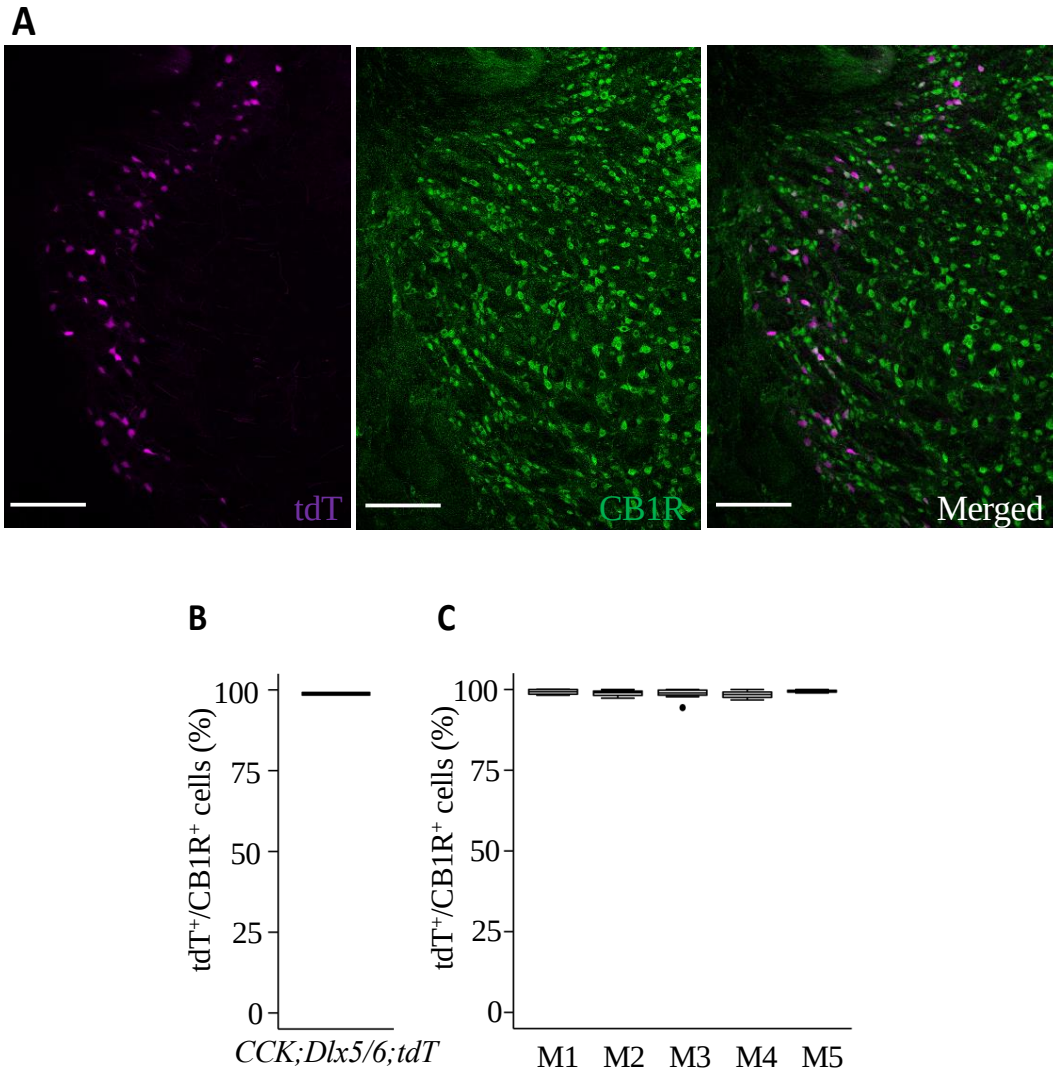

Figure 10. Expression of CB1R in *CCK;Dlx5/6* neurons of the TRN. A. 10x confocal images of the TRN. Left, tdT (magenta) expression in *CCK;Dlx5/6* neurons. Middle, CB1R (green) protein staining. Right, CB1R expression merged with tdT expression. B. Approximately 98.8% of *CCK;Dlx5/6* neurons in the TRN express the CB1R protein averaged from 5 mice. Scale = 200 μm. C. Percentage of tdtom neurons in the TRN that express CB1R showed little variability between 5 mice. Data are represented using a box plot with interquartile range and 95% confidence interval. Outliers were calculated as Q1 – (1.5 X IQR) and Q3 + (1.5 X IQR).

laboratory confirmed these results in the *CCK;Dlx5/6* mice using fluorescence activated cell-sorting coupled to qRT-PCR (Bunda et al., 2019). Thus, we tested if *CCK;Dlx5/6* neurons also express CB1R in the TRN by staining for the CB1R. We found that approximately 98.8% of *CCK;Dlx5/6* neurons also express CB1R (% of *CCK;Dlx5/6* neurons expressing CB1R, mean ± s.e.m = 98.8 ± 0.19, n = 5 mice, 3-5 slices per mouse. **Figure 10A and B**). Furthermore, this observation was consistent across different mice (**Figure 10C**). Our results suggest that similar to CCK^+ interneurons from other areas of the brain, *CCK;Dlx5/6* also express the CB1R.

4.6 *CCK;Dlx5/6* neurons target multiple areas of the thalamus

To start elucidating the potential target areas of the *CCK;Dlx5/6* neurons in the TRN, we took advantage of the ability of tdT to travel to via axons to the synaptic terminals(Besser et al., 2015; Fujita, Nakanishi, Ueno, Itohara, & Yamashita, 2020; Kim et al., 2020), as well as from previous knowledge showing that TRN is the only thalamic nuclei that does not project outside of the thalamus (Pinault, 2004). Next, using as reference the Adult Mouse Brain Atlas from the Allen Institute (Lein et al, 2007), we estimated the subdivisions of the thalamus and the approximate position of each area relative to the TRN (**Figure 11A and B**). We readily identified the following nuclei: medial habenula (MH), lateral habenula (LH) (**Figure 11C**), lateral dorsal (LD) (**Figure 11D**), mediodorsal (MD) and paracentral (PCN) (**Figure 11E**), ventral posterolateral (VPL) and ventral posteromedial (VPM) (**Figure 11F**), ventral anterior-lateral (VAL), ventral medial (VM) and submedial nucleus (SMT) (**Figure 11G**), and the posterior complex (PO) (**Figure 11H**). Next, we quantified the fluorescence in each of these putative regions of the thalamus. It is important to note that the fluorescent signal from tdT is associated with axons and synaptic terminals, although

not ideal to determine synaptic targets, this represents a first step to assess the thalamic areas that are likely be targeted by *CCK;Dlx5/6* neurons.

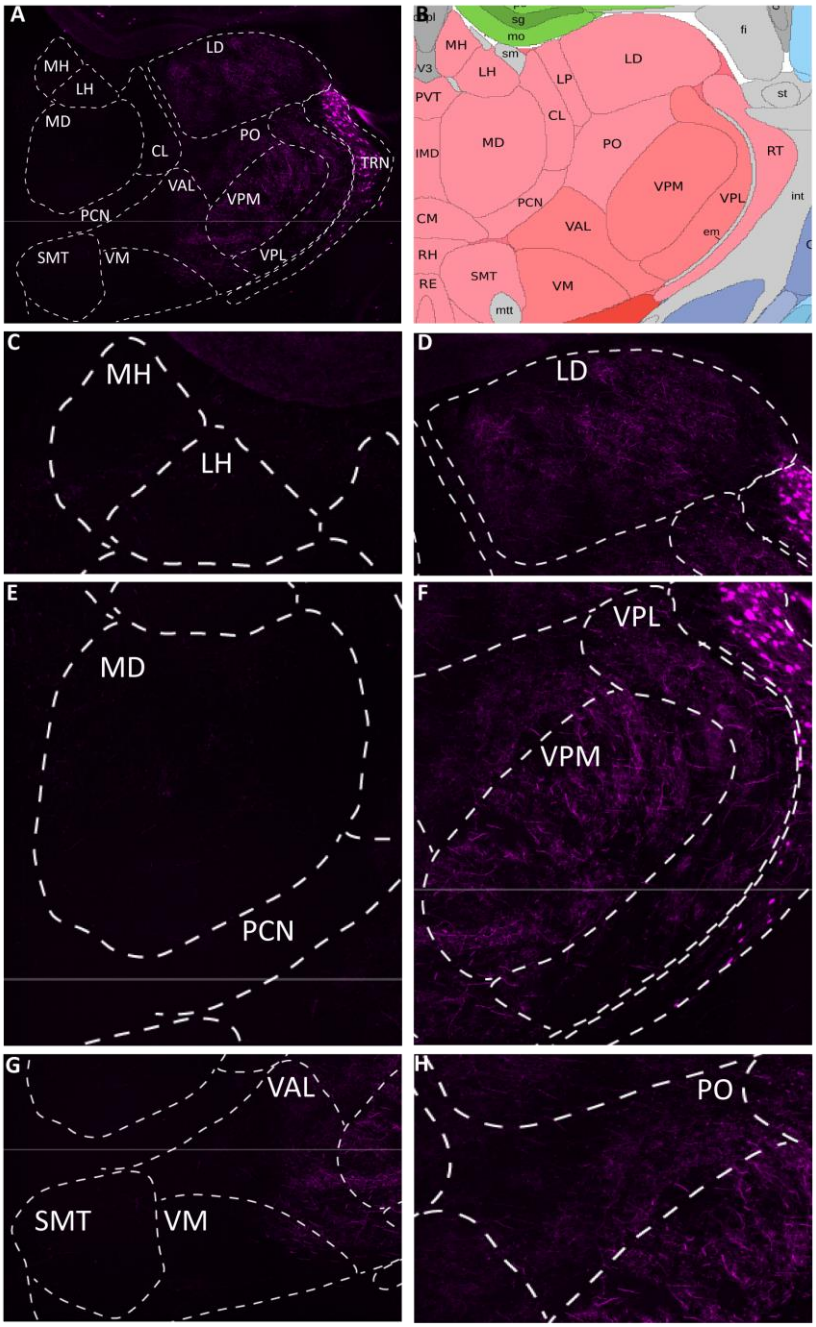

Figure 11. Target thalamic areas of *CCK;Dlx5/6* neurons. A. Confocal image of the TRN and adjacent relay nuclei outlined from *CCK;Dlx5/6* slices. **B. A** visual representation of the thalamic nuclei seen in **A.,** according to Allen Brain Atlas **C.** Relatively small amount of tdT seen in the medial habenula (MH) and lateral habenula (LH) **D.** tdT appears to be highly expressed in the laterodorsal nucleus (LD) **E.** tdT expression in the mediodorsal (MD) and paracentral nucleus (PCN) **F.** Abundant tdT axons expressed in both the ventral posterolateral (VPL) and ventral posteromedial nucleus (VPM) **G.** tdT expression in the ventral anterior-lateral (VAL), ventral medial (VM), and submedial nucleus (SMT) **H.** tdT axon collaterals in the posterior complex (PO).

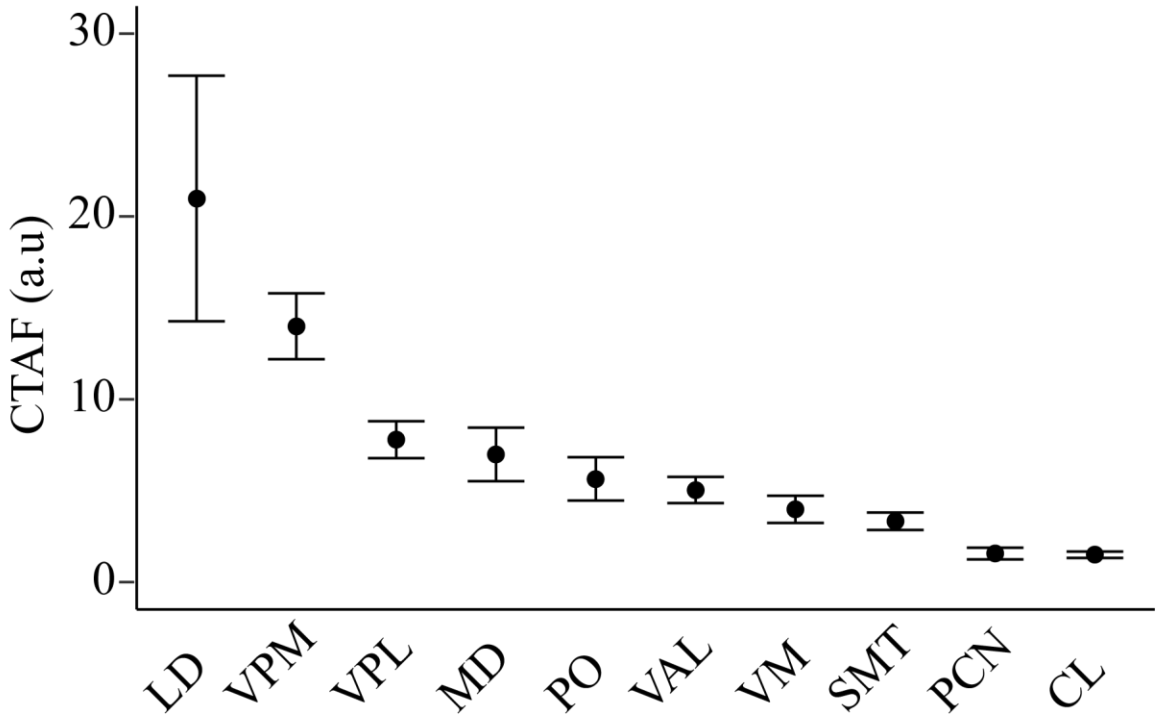

Figure 12. Fluorescence corrected by background in different thalamic areas. Quantitative data of tdT axons and synaptic terminals expressed as fluorescence are presented as mean and s.e.m. At least three images from different mice were used for this analysis.

We found that thalamic areas with the highest fluorescence corrected by background were the LD, VPL, and VPM, whereas MD, VM, SMT and CL showed less fluorescence (**Figure 12**). These preliminary observations suggest that *CCK;Dlx5/6* neurons are more likely to expand their axons and terminals to dorsal and ventral posterior areas rather than medial and ventral medial areas.

Finally, a high magnification in the LD of brain slices stained with SYTO-13 (a cell body marker) shows that *CCK;Dlx5/6* neurons form perisomatic synapses around target neurons (**Figure 13**, *insets*). This is in accordance with previous studies of CCK interneurons from the hippocampus

and amygdala. However, our current analysis does not allow to discard the possibility that these neurons also form synapses.

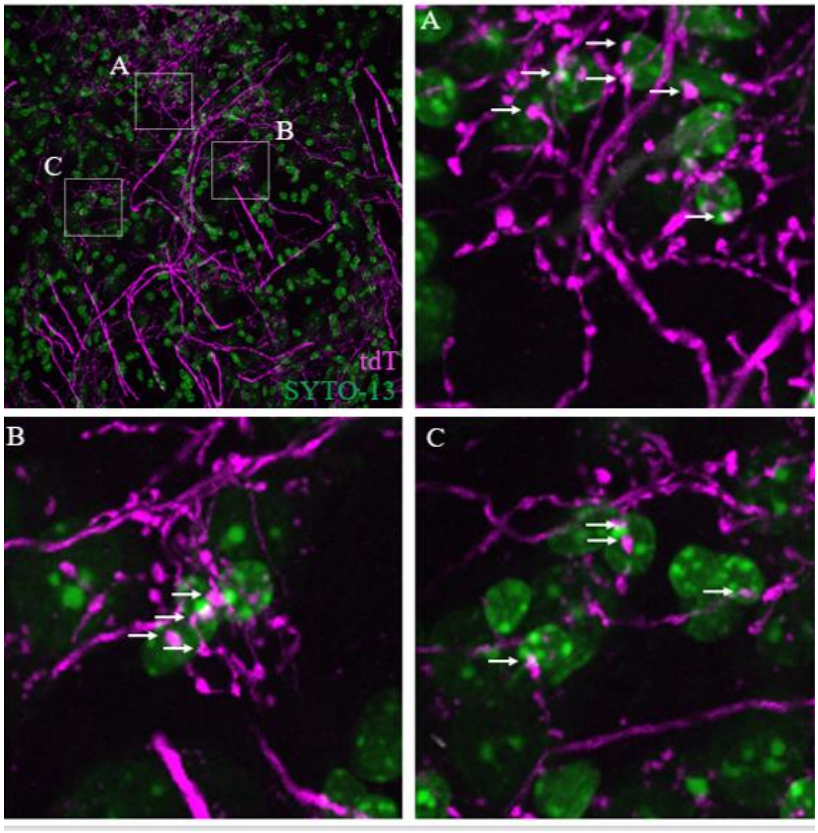

Figure 13. High magnification image of the LD in slices of *CCK;Dlx5/6;tdT* mice. Several insets were digitally magnified to observe putative synaptic buttons (magenta) around cell bodies (green) of target cells located in the LD. Axons are clearly seen in the non-digitally magnified image (top left panel, 60X). **A-C:** White arrows point at putative synaptic buttons, characterized by their morphology.

5.0 DISCUSSION

An easy way to drive home the function of TRN in sensory processing is referring to research that has completely removed it from the brain. However, this does not tell the whole story and isn't quite applicable to sensory disorders in humans where the TRN is never completely absent. Removing the TRN and therefore removing this inhibitory feedback and integration of signals is not nearly the same thing as the TRN being present but integrating signals in the wrong way. It needs to be remembered that the TRN is right in the middle of this bigger circuit where multiple parts are contributing to it working properly. For example, if the cortical neurons/driver inputs are dysfunctional, do we know that the TRN is able to compensate for this? It is manipulation of specific cell types, gene expression, neurotransmitters/neuromodulators that give us the best insight.

My results show that more than a quarter of neurons in the TRN that are part of the head and tail are *CCK;Dlx5/6* neurons. The head and body of the TRN are two regions associated with processing of visual and somatosensory information, respectively (Clemente-Perez et al., 2017; Crabtree, 2018; Pinault & Deschênes, 1998). This opens the possibility that *CCK;Dlx5/6* neurons in the TRN fine tune visual and somatosensory information on its way or back from the cortex.

My immunohistochemistry analysis for the PV staining in brain slices from *CCK;Dlx5/6;tdT* mice show two *CCK;Dlx5/6* population one expressing PV and another without the PV peptide. This potential difference could arise from incomplete staining with the PV antibody. Previous reports have shown that more than 95% of the TRN neurons express PV, however this may heavily depend on the antibody and incubation conditions. We utilized a shorter incubation time than previously reported, thus it is possible that our staining conditions did not capture *CCK;Dlx5/6* neurons with low PV expression.

My results also show that almost all *CCK;Dlx5/6* neurons express the CCK peptide. This is important because suggest that the labeling of these neurons by tdT reflects the adult phenotype of *CCK;Dlx5/6* neurons and is not the result of transient expression of Cre and Flpe recombinases during development. The CCK peptide within itself is an exciting peptide to study because it has been implicated in anxiety(Bowers, Choi, & Ressler, 2012; Keimpema, Straiker, Mackie, Harkany, & Hjerling-Leffler, 2012; Lach, Schellekens, Dinan, & Cryan, 2018) and manic-like behaviors(Arey et al., 2014). However, its role in the thalamus has not been previously described and the mechanisms of release for CCK from *CCK;Dlx5/6* neurons is not known.

Exogenous cannabinoids in humans cause symptoms such as hypersensitivity to visual and auditory stimuli sometimes leading to hallucinations, reduced working memory, trouble coordinating movements, impulsivity, difficulty concentrating, altered sense of time, and general changes in mood (NIDA, 2019). As my results indicate, almost all *CCK;Dlx5/6* neurons in the TRN also express the CB1R. This can be significant to explain the enhanced sensitivity to sensory cues induced by cannabinoids. The activation of CB1R inhibits release of neurotransmitters including GABA, thus it is predicted that CB1R agonists such cannabinoids would suppress *CCK;Dlx5/6* neurons leading to disinhibition (lack of filtering) of sensory information traveling to and back from the cortex.

Interestingly, our preliminary evidence shows that cells in the LD receive perisomatic connections from *CCK;Dlx5/6* neurons, likely from the head. The LD is a HO thalamic nucleus that process sensory information arriving from the visual cortex, this nucleus also sends connections to the limbic system (Aggleton, Saunders, Wright, & Vann, 2014; Perry & Mitchell, 2019). It is thought that the LD is involved in assessing the emotional component of visual stimuli, mainly aversive stimuli, by relaying information between the amygdala and higher brain structures (Zanoveli et al.,

2007). This observation allows to formulate the idea that *CCK;Dlx5/6* neurons are finetuning emotional responses to sensory cues. However, behavior studies to target *CCK;Dlx5/6* neurons connecting to the LD will be necessary to determine their role in visual processing. Furthermore, our initial evidence also showed that *CCK;Dlx5/6* connects to the VPL and VPM, both nuclei relay somatosensory information including nociceptive information. The VPL relays spinothalamic connections, whereas the VPM receives trigeminothalamic inputs. Thus, it is possible that *CCK;Dlx5/6* neurons in the TRN are also linked to processing of pain at the central level (Andrew, May, & Warren, 2020; Youssef et al., 2019). Interestingly, CCK interneurons in the hippocampus and cortex have been linked to emotional processing, and CCK interneurons in the spinal cord have been linked to nociception(Bowers & Ressler, 2015; Brown et al., 2014; Truitt, Johnson, Dietrich, Fitz, & Shekhar, 2009;Freund, 2003).

6.0 FUTURE DIRECTIONS

6.1 What are the electrophysiological properties of *CCK;Dlx5/6* neurons in the TRN?

To better understand the function of these neurons within the TRN, we need to determine their electrophysiological properties. This is particularly important for this area of the brain where the other 2 major cell populations in the TRN, PV and SST, have been described as having firing properties that are either fast-spiking or regular spiking and typically synchronous release of NT. *CCK;Dlx5/6* neurons in other areas of the brain appear to be regular-spiking with asynchronous release of NT, which is thought to create long-lasting inhibition onto post-synaptic targets. The TRN controls activity of the relay thalamic nuclei in part by switching between tonic and burst firing and therefore these *CCK;Dlx5/6* neurons may play an important in this process that is not accomplished by other cell populations previously established in the TRN.

6.2 What are the synaptic properties of *CCK;Dlx5/6* neurons in the TRN?

CCK neurons in other areas of the brain have been shown to release NT in an asynchronous manner, which leads to long-lasting inhibition. This feature of this cell type could help us understand the impact of these neurons within the TRN onto post-synaptic targets. Other synaptic properties to explore could be number of the number of release sites. In other areas of the brain, CCK neurons are also shown to rely on N-type calcium channels as opposed to P/Q-type channels that PV neurons rely on to release NT. We could stain *CCK;Dlx5/6;Tdt* brain slices with a marker for axonal terminals. Relative distribution and concentration of these terminals in specific areas could correlate to the amount of impact these neurons have on those post-synaptic targets.

6.3 What neurons do *CCK;Dlx5/6* neurons in the TRN target?

Identifying post-synaptic target sites of these neurons will provide an important missing piece to the puzzle, especially when sectors of the TRN correspond to specific functional modalities and the relay nuclei that they target are also specific and functionally distinct from one another. It has been established that individual TRN neurons that target more than one relay nuclei are targeting areas that are associated with the same modality. Exploring if *CCK;Dlx5/6* neurons of the TRN target other TRN neurons would also give us insight into how intra-TRN activity affects the output of these neurons. To accomplish establishing these post-synaptic targets, it may be necessary to develop an anterograde tracer capable of specifically targeting *CCK;Dlx5/6* neurons of the TRN.

6.4 What behaviors are under the control of or are influenced by *CCK;Dlx5/6* neurons in the TRN?

Once electrophysiological and synaptic properties of these neurons, as well as their post-synaptic targets are established, it will be easier to speculate what behaviors they may be specifically controlling and allow for the design of relevant behavioral experiments. For example, if these neurons preferentially target the LD of the thalamus that has been suggested to play a role in limbic function, it would make sense to design an experiment that confirm what this means on a behavioral level. Ways to accomplish that may be to have mice participate in a task that involves the proper recognition of stimuli that is novel vs something that has been conditioned to induce an emotional response (or some other task associated with LD function), such as fear. Then by selectively activating or silencing *CCK;Dlx5/6* neurons of the TRN and having these mice once again complete the same task, we would expect to see a difference in behavior.

Although PV interneurons of the TRN are thought to drive spindles during NREM sleep (Thankachan et al., 2019), the activity of *CCK;Dlx5/6* neurons on PV+ neurons should be considered. Release of CCK from CCK+ neurons preferentially target PV neurons, leading to inhibition of PV neurons. The delicate balance between tonic and burst firing is required for spindles, as is synchronous oscillations. It could be hypothesized that in order for PV neurons to maintain spindles, *CCK;Dlx5/6* neurons would need to be inhibited somehow- leading to disinhibition of TRN PV neurons.

6.5 How do *CCK;Dlx5/6* neurons interact with cannabinoids to control TRN's interaction with relay thalamic nuclei?

It has been well established that CB1R and cannabinoids play a significant role in behaviors such as sleep maintenance, sensory processing, working memory. The effect that cannabinoids have on electrophysiological and synaptic processes of *CCK;Dlx5/6* neurons of the TRN could first be investigated with slice electrophysiology and the application of CB1R antagonists and agonists. Once the impact of cannabinoids on these electrical properties is determined, it could then be applied at the in vivo level during behavioral tasks that have been established to be related to the function of these neurons within the TRN. For example, using the behavioral experiment mentioned above, at each stage: no manipulation, silencing, and activating these neurons, cannabinoids could be introduced to provide greater detail into how this cell population is coordinating with cannabinoids to control output and subsequently behavior.

6.6 What role do *CCK;Dlx5/6* neurons of the TRN play in disease?

Many diseases that have been shown or suggested to be related to dysfunction of the TRN are those of neurodevelopmental disorders previously discussed. Once properties of these neurons and

the behaviors in which they have an influence over have been established, it should be easier to narrow down what specific symptoms of these disorders that these neurons may contribute to.

The next step may be to investigate changes in this cell population within the TRN over development, particularly at critical periods involved in the development of spindle activity and sensorimotor circuits. This could be done by either evaluating connections of *CCK;Dlx5/6* neurons onto dorsal thalamic nuclei in post-mortem tissue at different points in development or selectively silencing these neurons at different periods in development and observe behavioral phenotypes compared to littermates who did not have these neurons silenced.

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Appendix

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

04-Oct-2018

Andrade, Arturo S
Dept. of Biological Sciences
Rudman Hall
Durham, NH 03824

IACUC #: 180803
Project: Study of Calcium Channel Splice Isoforms in Monaminergic Neurons
Approval Date: 20-Sep-2018

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category E in Section V of the Application for Review of Vertebrate Animal Use in Research or Instruction - *Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are not used.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Rebecca Rowe

Rebecca Rowe, Ph.D.
Chair

cc: File

IACUC **IACUC**

University *of* New Hampshire
Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585

29-May-2019

Andrade, Arturo S
Dept. of Biological Sciences
Rudman Hall
Durham, NH 03824

IACUC #: 180708
Project: Breeding Protocol for the Study of Calcium Channel Splice Isoforms in Neurons of Hippocampus and Monaminergic Neurons of Central Nervous Systems
Approval Expiration Date: 25-Jul-2019
Protocol Three-Year Expiration Date: 25-Jul-2021

Institutional Animal Care and Use Committee (IACUC) Annual Review Form

Federal regulations require annual review of all approved projects involving vertebrate animal care/use. Accordingly, please supply the information requested below in questions 1-5, and return to the IACUC at the above address **at least 4 weeks prior to the approval expiration date listed above.** Please contact Dean Elder at (603) 862-4629, Susan Jalbert at (603) 862-3536, or Julie Simpson at (603) 862-2003 with questions. Thank you.

1. Is this project still active? If NO, please sign and return. Yes_X_ No___

2. Will there be modifications of procedures described and approved in the original application? Yes___ No_X_

   If YES, please submit as a separate document, following the guidelines at https://unh.app.box.com/s/lhyhle6jnd18h6qnq6q2kstbw770fcla. Modifications must be approved by the IACUC prior to implementation.

3. Animal Numbers (If your project involves non-target species/by-catch, please provide the information requested about these animals separately as an attachment):

SPECIES	CURRENT INVENTORY (live animals on-hand)	USED TO DATE (since start of project)	TOTAL # APPROVED (3-year total)
__mus musculus_____	_____102_____	_____85_____	_____2944_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

4. The IACUC is required to ensure that animal care personnel regularly engage in continuing education activities. Please identify the methods you used to provide continuing education to animal care personnel (including students) on this project during the past year (as applicable to the project).

___ Not applicable to this project.
___ No project personnel other than PI.
__X Direct supervision: PI works closely with personnel during procedures/activities involving animals.

__X Discuss and address issues relating to animal care and use during lab/staff meetings or regular meetings with individuals
__X Organize lab/staff training sessions focused on animal care and use (e.g., as part of orientation for new staff or students).
_X_ Encourage or require personnel to participate in preparation of IACUC applications, annual reports, reviews, inspections or similar activities.
_X_ Other (please describe): _We work directly with our veterinarians Dr. Morley and Dr. Elder to establish new protocols_____

5. Please indicate on the list below any staff who are no longer affiliated with this project. If reporting new staff, please provide the information requested in the table below.

Currently-affiliated protocol staff ("X" indicates person has completed Occupational Health Program [OHP] *; NAC = no animal contact):

Andrade, Arturo S	X	Bain, Ashton	X
Londrigan, Laura	X	Czepiel, Natalie	X

Monahan, Angela ~ no OHP approval on file

Reporting new project staff:**

First & Last Name	UNH Faculty/ Staff/Student?	Role on Protocol	Yrs Experience w/Animal Model**	Date OHP Form Completed/to be Completed*
___Shayna Mallat_____	Student _	maintaining the colony	_____2_____	January/2018___
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____

Principal Investigator/
Instructor Signature: _____ Date: _3/7/2019_____

*Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. **Participation is mandatory for all principal investigators/instructors and their affiliated personnel, employees of the University and students alike.** Principal investigators/instructors are responsible for ensuring that all new staff on projects are provided with and complete a Medical History Questionnaire (the questionnaire only needs to be completed once during a person's time at UNH unless he/she change risk categories, or approval expires during an individual's tenure at UNH). Forms are available on-line at https://www.unh.edu/research/occupational-health-program-animal-handlers. Completed questionnaires should be sent to Suzanne Chalmers at UNH Health & Wellness.

** The IACUC requires all project-affiliated personnel to possess knowledge appropriate for the animal model and procedures used in the project. Further, each person working with animals in this project must complete training for individuals who use/care for vertebrate animals (personnel need only complete the training once during their time at UNH). See http://unh.edu/research/training-animal-care-and-use-personnel for details or contact Susan Jalbert at (603) 862-3536.