Beyond guidance: netrin-1 functions as a critical regulator of glutamatergic connectivity in the adult Ventral Tegmental Area

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Abstract

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The axonal guidance cue netrin-1 serves a critical role in neural circuit development by promoting growth cone motility, axonal branching, and synaptogenesis. Within the adult mouse brain, *Ntn1* expression is highly enriched in the ventral midbrain where it is expressed in both GABAergic and dopaminergic neurons, but its function in this context remains largely obscured. To address this, we performed viral-mediated, cell-type specific CRISPR-Cas9 mutagenesis of *Ntn1* in the ventral tegmental area (VTA) of adult mice. *Ntn1* loss-of-function in either cell type resulted in a significant reduction in excitatory postsynaptic connectivity. In dopamine neurons,

reduced excitatory tone had a minimal phenotypic outcome; however, reduced glutamatergic tone on VTA GABA neurons induced a hyperdopaminergic phenotype. Loss of *Ntn1* function in both cell types simultaneously largely rescued the phenotype observed in the GABA-only mutagenesis. These findings demonstrate an important role for netrin-1 in maintaining excitatory connectivity in the adult midbrain and that a balance in this connectivity within two of the major cell types of the VTA is critical for the proper functioning of the mesolimbic system.

Intriguingly, netrin-1 has not one but two receptors that have been identified as highly expressed in the adult VTA; Dcc and Unc5 homologue C (Unc5c). As the signaling cascades produced by these two receptors are in direct opposition to each other, it is possible that they are able to control differing aspects of netrin-1 circuitry regulation. To explore this, I used the same viral-mediated, cell-type specific CRISPR-Cas9 mutagenesis method to induce Dcc and Unc5c loss of function in dopamine neurons of the adult VTA. While similar to Ntn1 cKO in dopamine only VTA neurons, loss of Dcc or Unc5c produced distinct, phenotypically dissimilar, alterations in dopamine-mediated behaviors. Further research is needed to determine the individual roles of these axonal guidance receptors, and how they aid netrin-1 in modulating glutamatergic connectivity in the adult brain.

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DEDICATION

For me.

Chapter 1. INTRODUCTION

The formation of dopaminergic circuitry during early development is intrinsically dependent on the proper expression and function of axonal guidance cues and receptors[1]. Disruption in this circuitry has been linked to several neurological and developmental disorders[2,3]. Netrin-1, a bifunctional axonal guidance protein that can act as a chemoattractant or chemorepulsive cue[4], is highly expressed in dopamine producing neurons and a plays a critical role in dopaminergic circuitry development and adolescent circuitry refinement[5,6]. While netrin-1 and netrin receptor expression decreases following embryonic development, neurons of the dopamine rich adult ventral tegmental area (VTA) continue to express high levels of netrin-1 (*Ntn1*) and the netrin receptors Dcc (*Dcc*) and Unc5 homologue C (*Unc5c)*[5–7]. The function of netrin-1 and netrin receptors within this context, however, has yet to be determined.

1.1 'NETR' – THE ONE WHO GUIDES

In 1990 Hedgecock, Culotti and Hall[8] characterized a secreted protein in C. elegans directly responsible for growth cone motility and circumferential migration. Initially classified under the moniker unc-6, when the mammalian homologue was identified less than four years later this axonal guidance cue was renamed netrin-1 (from the Sanskrit *Netr*, translated to mean 'one who guides')[9]. Now, 32 years following its initial discovery, research continues to emphasize the critical importance of netrin-1 throughout neurodevelopment, synaptogenesis, and circuitry formation.

Netrins are highly evolutionarily conserved across all animals with bilateral symmetry[10], underscoring their fundamental importance as developmental regulators. In mammals, the netrin family consists of three secreted netrins (netrin-1, -2 and -4) and two membrane-bound

glycophosphatudylinositol (GPI)-linked netrins (netrin G1 and G2).[10] While all interesting in their own right, this paper will focus exclusively on the most abundant mammalian form and most well characterized member of the netrin family; netrin-1[10,11].

Netrin-1 is part of the family of laminin-related proteins, and is comprised of a laminin-like domain, a netrin-like domain and three epidermal growth like repeats (see Figure 1.1)[12]. During the initial stages of neurodevelopment, netrin-1 is secreted from floor-plate cells and acts as a axonal guidance cue[9]. Netrin-1 diffusion in the extracellular matrix creates a gradient which acts as a chemoattractant to neuronal growth cones expressing the netrin receptor Dcc on their leading edge. Extracellular netrin-1 binds to the Dcc fibronectin type 3 domain, inducing homodimerization of two Dcc receptors (Figure 1.2). This dimerization brings the Dcc cytoplasmic tails into close contact, setting off a signaling cascade which promotes actin polymerization and growth cone motility toward the site of the secreted netrin[12] (Figure 1.2 and 1.3).

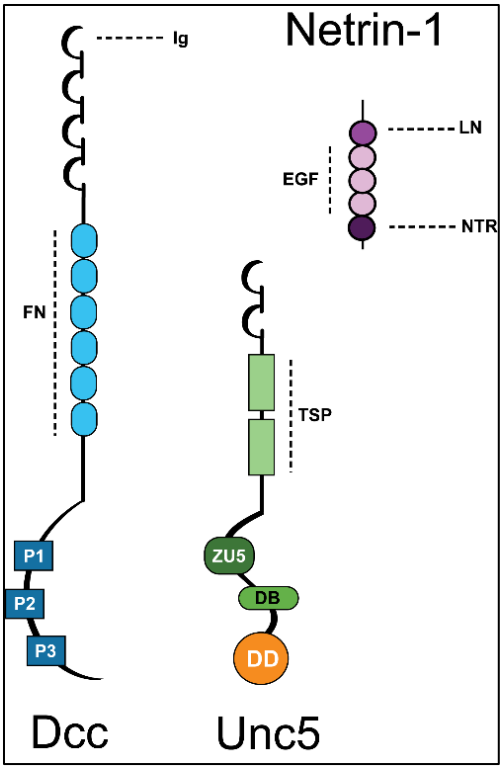


Figure 1.1 Netrin-1 and netrin receptor domains.

Netrin-1 is comprised of a laminin domain (LN), three EGF repeats and a netrin-like domain (NTR). Dcc and Unc are both single-pass transmembrane protein receptors in the Ig family. Dcc is comprised of four Ig repeats, six Fibronectin type 3 (FN) domains (necessary for ligand binding), and three conserved cytoplasmic domains (P1, P2 and P3). Unc5 receptors contain two Ig repeats, two thrombospondin type 1 (TSP) domains, and a cytoplasmic tail consisting of a zona occudens 5 (ZU5) domain, a Dcc binding domain (DC) and a death domain (DD).

While this is the canonically recognized role of netrin-1 during central nervous system development, netrin-1 is also capable of acting as a *chemorepulsive* cue in the presence of the netrin receptor Unc5[13–15]. Unc5 homologues A-D can form either a homodimer with an additional Unc5 receptor, or a heterodimer with Dcc, in the presence of netrin-1 (Figure 1.2). Either combination induces actin *depolymerization*, and growth cone motility *away* from the site of the secreted netrin, though the precise signaling mechanisms involved in this process is less understood[10]. Accordingly, netrin-1 is capable of both inducing and preventing actin polymerization and branching, depending on which receptors are present and available on the neuronal growth cone.

Netrin-1's role in neurocircitry

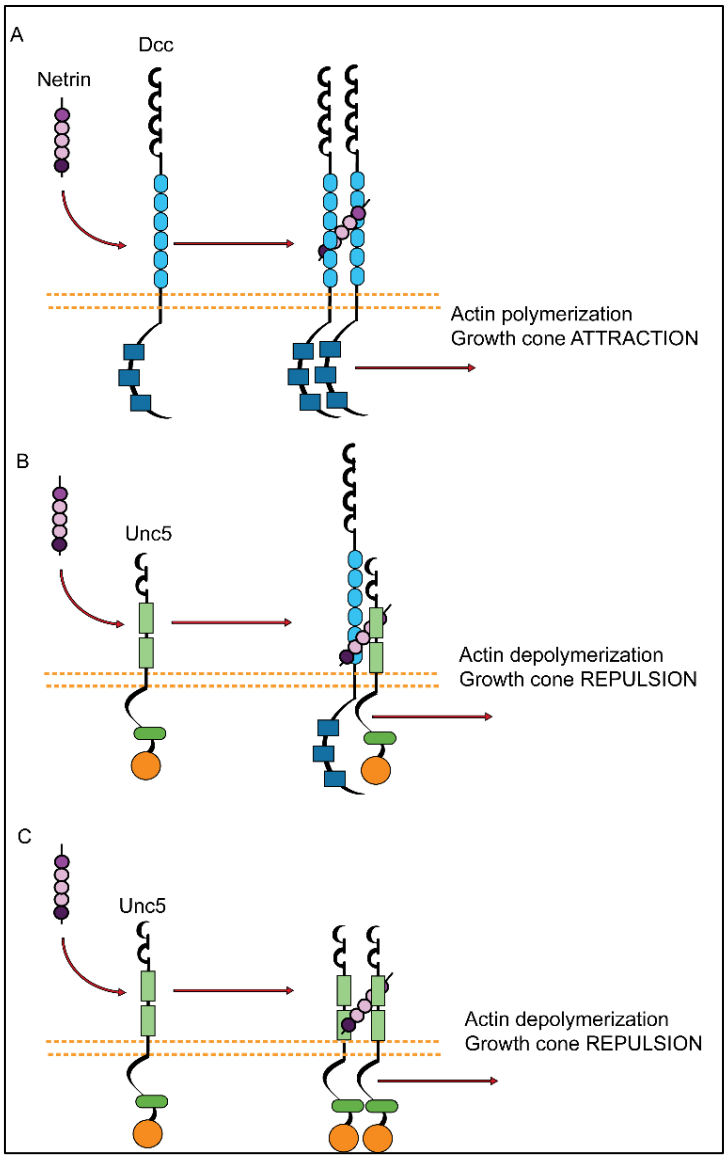


Figure 1.2 Netrin-1 as a bifunctional axonal growth cue. Netrin-1 is capable of both growth cone attraction and grown cone repulsion during neural development. Netrin-1 binding to the FN domain of Dcc (A) results in a homodimerization which induces actin polymerization and growth cone motility toward the secreted netrin. Netrin-1 binding to Unc5 can induce two dimerization; a heterodimer with Dcc in the presence of Dcc (B) and a homodimer with Unc5 in the absence of Dcc (C). Both combinations induce actin depolymerization and growth cone motility away from the source of the secreted netrin.

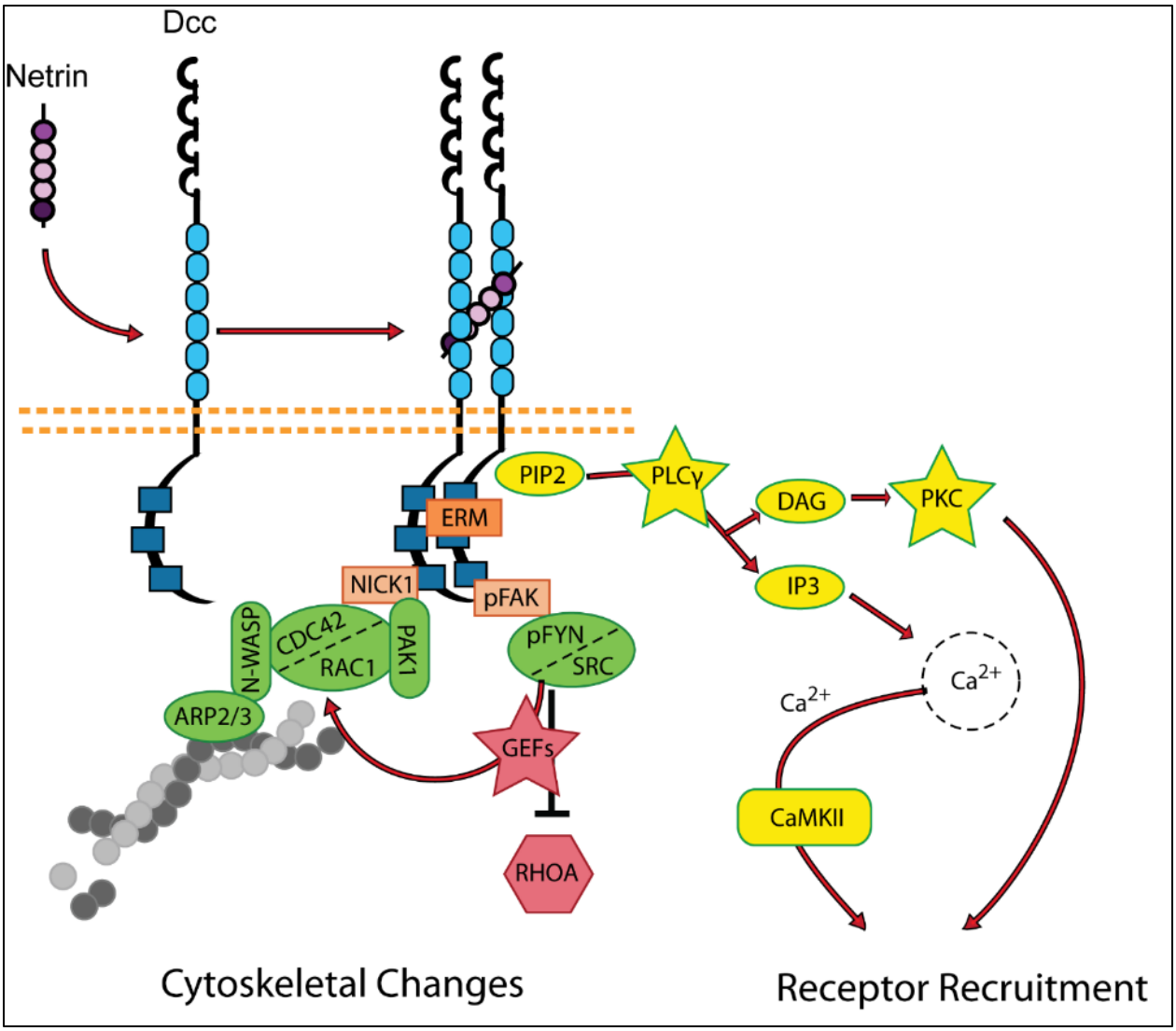


Figure 1.3 Netrin-1/Dcc cell signaling cascade hints at potential mechanisms of synaptic plasticity. Much of what is known regarding the Netrin/Dcc cell signaling cascade exists in the context of growth cone motility during neurodevelopment, but offers insights into how Netrin/Dcc may regulate synaptic plasticity. The mechanisms involved in growth cone attraction include cytoskeletal rearrangements, including the inhibition of RHOA, and the recruitment of proteins involved in actin polymerization and branching (such as ARP2/3, CDC42, and RAC1). These same signaling mechanisms have been identified as critical in the cytoskeletal rearrangements associated with hippocampal plasticity. Research from the Kennedy group has further implicated Netrin-1/Dcc signaling in adult CA1 neurons with AMPA receptor recruitment, presumably through the release of intracellular calcium (via IP3), and recruitment of CaMKII. Activation of PKC additionally results in AMPA recruitment, through the phosphorylation of GluA1-containing AMPAR at serine 831.

formation does not end at axonal guidance, however. Once neuronal growth cones have reached

their target location, netrin-1 is also involved in synaptic target recognition[16], axonal branching[17,18] and synaptogenesis[1,16,19,20]. These additional functions of netrin-1 following growth cone guidance are of particular interest to researchers investigating the potential role of netrins in synaptic plasticity within the adult brain.

1.1.1 Netrin in the adult brain

In addition to their roles in axonal guidance and neural circuitry development, research has historically supported a role for netrin-1 and netrin receptors in synaptic modification. Netrin-1 signaling has been shown to promote dendritic and axonal branching[21] and synaptogenesis[16,22,23], and both netrin-1 and the netrin receptor Dcc are enriched at mature cortical synapses[24]. Analysis of the Allen Institute mouse brain expression atlas reveals significant expression of *Ntn1* in the adult murine brain, though this expression is confined to specific anatomical regions[25]. Intriguingly, these regions of continued netrin-1 expression (the cerebellum, hippocampus, substantia nigra and ventral tegmental area) are all brain regions associated with high neuronal plasticity. This pattern of expression, coupled with what we know regarding netrin's role in neonatal circuitry development, has inspired some researchers to contemplate a potential role for netrin-1 in synaptic plasticity in the adult brain.

One of the researchers directly involved in the initial discovery and classification of the mammalian netrin-1[9], Dr. Timothy Kennedy, has since continued to investigate this axonal guidance cue in the context of both neurodevelopment and plasticity in the adult mouse hippocampus. Recent work from the Kennedy group has provided evidence indicating that netrin-1 undergoes exocytosis in an activity dependent manner from dendritic spines of CA1 hippocampal neurons in adult mice. Once released, secreted netrin acts in a cell autonomous fashion to signal through its canonical receptor Dcc (also present on CA1 dendritic spines),

increasing intracellular Ca^{2+}, and recruiting GluA1-containing AMPARs to the postsynaptic membrane through CaMKII activation[26]. Genetic deletion of either *Ntn1*[26,27] or *Dcc*[28,29] from forebrain glutamatergic neurons results in significantly reduced long term potentiation (LTP), decreased glutamatergic connectivity, and impaired spatial memory in aged mice. Relatedly, direct application of netrin-1 to these postsynaptic spaces was sufficient to induce an LTP like response similar to high-frequency stimulation[28].

This recruitment of AMPA receptors to the postsynaptic membrane is an example of synaptic scaling; a form of homeostatic synaptic plasticity in which excitatory circuitry is altered through the recruitment (or, alternatively, endocytosis) of receptors[30]. While the molecular mechanisms controlling this form of plasticity are still under investigation, impairments in synaptic scaling have been implicated in multiple forms of neurological disease, with many linking this form of plasticity and synaptic homeostasis with the inhibitory/excitatory imbalance theorized to underlie disorders such as autism spectrum disorders, schizophrenia[31], substance abuse[32], and major depressive disorder[33]. The ability of netrin-1 to recruit AMPA receptors in direct response to neuronal activation makes it a prime candidate as a potential regulator of plasticity.

1.1.2 Netrin and mental illness

Netrin-1 loss of function *in utero* is fatal in mammals[34], but genome wide association studies (GWAS) have identified several mutations in the netrin-1 signaling pathway that are potentially pathogenic. Mutations in *NTN1*, *DCC* and the *NTN1* signaling pathway in humans have now been implicated in multiple psychiatric conditions, including neurodevelopmental disorders such as schizophrenia[35–37] and major depressive disorder[36,38–41], substance use[42,43], as well as multiple neurodegenerative disorders[44–46]. These studies are particularly intriguing, as

one of the few areas *Ntn1* expression remains high in the adult brain is the dopaminergic region of the midbrain called the Ventral Tegmental Area[47]; a region associated with these same psychiatric conditions[3].

1.2 The Ventral Tegmental Area

The ventral tegmental area is a dopamine rich region of the midbrain. This heterogeneous region is comprised primarily of dopamine producing neurons (60-70%), GABA producing neurons (20-30%) with a small minority of glutamatergic producing neurons (less than 10%)[48]. This brain region is highly plastic; meaning it is capable of dynamic synaptic modifications at an individual neuronal level in response to environmental stimuli and pharmacological agents (such as cocaine and amphetamine)[49,50]. The VTA is best known for its role in motivation, reward prediction, and associative learning[51,52], though the list of VTA-dopamine and VTA-GABA mediated behaviors is expansive and includes several aspects of motor function, emotional response, and social interaction[53].

Within the VTA, the activity of dopamine producing neurons is regulated through a careful balance of inhibitory (GABAergic) and excitatory (glutamatergic) synaptic input. While the glutamatergic input originates from outside of the VTA, VTA dopamine neurons receive inhibitory synaptic input primarily from local GABA neurons[48,54]. The molecular mechanisms responsible for the regulation of this inhibitory/excitatory balance within the VTA remain poorly defined, but there is evidence to indicate axonal guidance cues may play a significant role. Our lab previously demonstrated that the axonal guidance receptor *Robo2* is necessary for the maintenance of *inhibitory* synaptic input to dopaminergic neurons in the adult VTA. Disruption of *Robo2* expression in adult VTA neurons resulted in reduced inhibitory tone onto dopamine neurons, enhanced phasic dopamine release, and profoundly altered behavioral regulation[55].

These findings suggest axonal guidance proteins have a critical function in the adult nervous system in maintaining excitatory/inhibitory balance onto dopamine neurons and may continue to contribute to circuit function/dysfunction in healthy and affected individuals.

1.2.1 The VTA and mental illness

As mentioned previously, several of the psychiatric and neurological conditions associated with the netrin signaling pathway identified by GWAS and animal studies are also associated with VTA dysfunction; most notably, major depressive disorder[56,57], substance use disorders[39], and schizophrenia[58].

Though the vast majority of VTA research has focused on the role of VTA dopamine neurons, VTA GABA neurons play a significant role in this circuitry. As direct inhibitors of VTA dopamine neurons, VTA GABA dysfunction can have devastating effects on this system[59]. Specific disruption in VTA GABA neurons can cause hyperactivity and manic like behaviors in mice[60].

1.3 NETRIN-1 AS A POTENTIAL REGULATOR OF SYNAPTIC CONNECTIVITY IN THE ADULT VTA

The expression pattern of netrin-1 and netrin receptors in the adult brain, their known role in the development and organization of midbrain dopamine circuitry [61], and netrin-1's recently established role in facilitating AMPA receptor recruitment and synaptic plasticity in the adult hippocampus [24,28,62], all hint at a potential role for netrin-1 in regulating excitatory synaptic connectivity in the adult VTA. In Chapter 2, I investigate the potential role of netrin-1 in adult VTA neurons by first identifying which VTA cell types express *Ntn1* in the adult mouse brain, and selectively mutating *Ntn1* in a cell type specific manner to isolate the direct physiological

and behavioral effects of netrin-1 loss of function.

Two netrin receptors have been identified as being present in adult VTA dopamine neurons; Dcc and Unc5c[63–65]. In Chapter 3, I utilize the same genetic techniques available to our lab to selectively disrupt *Dcc* or *Unc5c* expression in VTA dopamine neurons in adult mice, and test these mice on a range of dopamine-mediated behaviors relevant to specific behavioral domains associated with mental illness.

Elucidating the function of netrin-1 and the netrin receptors Dcc and Unc5c in adult dopamine neurons of the VTA will shed light on the molecular mechanisms responsible for regulating synaptic connectivity in the midbrain dopamine system that will advance our understanding of how this system is regulated and provide potential targets for future therapeutic development.

1.4 Cell type specific genetic mutagenesis

Until recently, the functional analysis of cell-type specific gene expression would take years to analyze through traditional gene knockout. However, advances in CRISPR/Cas9 technology have made it possible to rapidly screen the function of a large number of genes in a singular cell type. The Zweifel lab has developed a Cre-dependent adeno-associated virus (AAV) CRISPR SaCas9 vector, in which small guide RNAs (sgRNAs) specific to a gene being targeted for mutation, can be easily inserted[66]. This AAV CRISPR/Cas9 vector encodes both a Cre recombinase dependent SaCas9 endonuclease enzyme as well as a sgRNA driven by an independent (U6) promotor, all in a single viral construct. The use of such a system in combination with transgenic mice expressing Cre recombinase in specific cell types allows for the selective mutation of genes in cells of interest, without otherwise affecting non-target cells.

This approach presents two innovative advantages for the research proposed here:

1. It allows for the selective mutation of *Ntn1, Dcc* and *Unc5c* in only dopamine or GABA producing neurons of the VTA (cell-type specificity) to allow for cell specific analysis of protein loss. One limitation of our lab's previous study of Robo2 in the adult VTA was that we were unable to isolate the activity of Robo2 to a singular cell type. Thus, we were unable to determine if the loss of inhibitory connectivity on to VTA dopamine neurons that we observed was due to auto regulatory disruption or pre-synaptic GABA dysfunction. Cell type specific genetic mutation can allow us to isolate the direct physiological consequences of protein loss of function.
2. It allows for the mutation of these genes in an adult organism (temporal specificity), without otherwise affecting normal CNS development. This is especially significant and innovative since genetic deletion of either *Ntn1* or *Dcc* has proven fatal in vertebrates[34]. This has resulted in previous murine models of netrin and netrin receptor loss relying primarily on heterozygous or haploinsufficient genetic knockdown. Such models, while providing valuable insights, are unable to isolate the direct function of these genes in the adult system, as alterations in circuitry development and compensatory mechanisms cannot be discounted. The use of a virally delivered CRISPR/Cas9 directly to the VTA of adult transgenic mice allows for the selective mutation of genes without risking the disruption of circuitry development.

Chapter 2. NETRIN-1 REGULATES THE BALANCE OF GLUTAMATERGIC CONNECTIVITY IN THE ADULT VENTRAL TEGMENTAL AREA

A version of this chapter has been submitted for publication and is currently under review. A preprint is available on biorxiv.

Marcella M Cline, Barbara Juarez, Avery C. Hunker, Ernesto G. Regiarto, Bryan Hariadi, Marta E. Soden & Larry S. Zweifel.

2.1 INTRODUCTION

Proper regulation of the midbrain dopamine system is essential for numerous brain functions and behavior[67]. Disruption in the balance of midbrain dopamine neuron activity has been linked to several neurological and psychiatric conditions, including autism[68], schizophrenia[69], and substance use disorders[59]. Within the VTA, the activity of dopamine producing neurons is regulated in part by inhibitory (GABAergic) and excitatory (glutamatergic) synaptic input. The molecular mechanisms that maintain inhibitory and excitatory connectivity in the adult midbrain, however, remain poorly resolved.

Genome-wide association studies and analysis of *de novo* mutations has strongly implicated genes regulating neuronal axon guidance in neurodevelopmental disorders[70,71]. Although the impact of mutations in these genes early in development is likely critical for their role in neurodevelopmental disorders, many of the genes maintain high levels of expression in the adult brain and their functions in this context is less understood. We previously demonstrated that the axonal guidance receptor Robo2 is necessary for the maintenance of inhibitory synaptic

connectivity in the adult VTA[72], suggesting that axonal guidance proteins have a critical function in maintaining synaptic connectivity in the adult midbrain.

Netrin-1 is predominately recognized for its role in neurodevelopmental processes[7-11]. During development the gene encoding netrin-1 (*Ntn1*) is highly expressed throughout the central nervous system (CNS). Following this critical period, global expression decreases[63], but expression within the limbic system, particularly in the ventral midbrain persists. Consistent with the continued function of netrin-1 following early development, genetic inactivation of either *Ntn1*[73] or its receptor *Dcc*[26] from forebrain glutamatergic neurons in late postnatal development results in significantly impaired spatial memory in adult mice that corresponds to a loss of hippocampal plasticity. Within the VTA, *Dcc* expression levels in adult mice are significantly upregulated following amphetamine exposure[74], and *Dcc* haploinsufficient mice display blunted locomotor response to amphetamine[75], consistent with increased excitatory synaptic strength in the VTA following amphetamine treatment[76]. These results suggest a potential role for netrin-1 signaling through Dcc in regulating excitatory tone in the adult dopamine system.

To determine whether netrin-1 regulates excitatory synaptic connectivity in the VTA of adult mice, we used viral-mediated, Cre-inducible CRISPR/Cas9[66] to selectively mutate *Ntn1* in midbrain dopamine and GABA neurons. We find that *Ntn1* loss of function significantly reduces postsynaptic glutamate receptor-mediated currents in a cell-autonomous manner similar to what has been reported previously in the adult hippocampus[26]. We further show that *Ntn1* loss of function in VTA GABA neurons has a more profound effect on behavior than loss of function in VTA dopamine neurons. Intriguingly, simultaneous loss of function of *Ntn1* in both cell types of the VTA largely rescues the behavioral phenotypes observed following mutagenesis in VTA GABA neurons alone. Collectively, these data demonstrate that the balance of excitatory

synaptic connectivity onto VTA dopamine and GABA neurons is critical to the function of the mesolimbic system and that netrin-1 plays an important role in this process.

2.2 RESULTS:

2.2.1 Ntn1 expression and mutagenesis in the VTA

In situ hybridization analysis of *Ntn1* from the Allen Institute mouse brain expression atlas[47] shows diffuse and low levels of expression throughout the adult mouse brain, with moderate expression levels in the cerebellum and hippocampus (Figure 1A), and the highest level of expression in the ventral midbrain (substantia nigra and ventral tegmental area). The VTA is comprised of multiple cell types [48]; to determine the cell type specific expression of *Ntn1* within the heterogeneous VTA, we performed RNAscope *in situ* hybridization on midbrain slices from adult wild-type mice (>8 weeks of age) and probed for *Ntn1, Th* (tyrosine hydroxylase, a marker of dopamine neurons) and *Slc32a1* (vesicular GABA transporter [Vgat], a marker of GABA neurons). We found *Ntn1* expression to be present throughout the VTA, largely localized to *Th*-positive neurons but also present in GABA neurons (Figure 1C-F). *Ntn1* expression co-localized with *Th* expression in dopamine producing neurons (64% co-localization) and *Slc32a1*-expressing GABA neurons (30% co-localization) (Figure 1F). The remaining 6% of *Ntn1* expressing cells that do not co-localize with *Th* or *Slc32a1* are likely glutamatergic neurons[48]. Immunohistochemistry for netrin-1 and Th (Figure 1G) confirmed the presence of netrin-1 in dopamine and non-dopamine producing (Th-negative) cells.

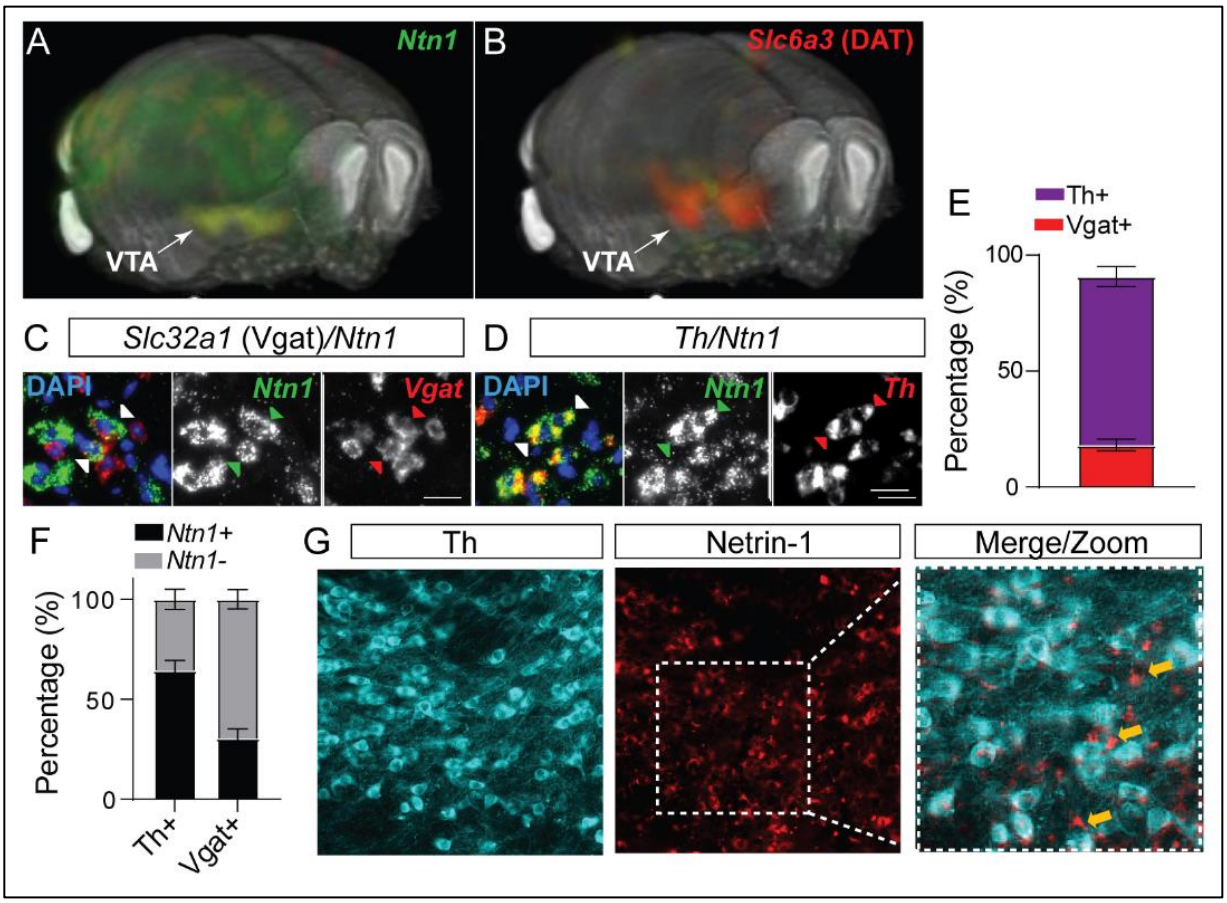


Figure 2.1 Netrin-1 is present in the adult VTA, and expressed by both dopamine and GABA neurons. 3D display of *Ntn1* (A) and *Slc6a3* (B, dopamine marker) from the Allen Brain Atlas. (C-D) 20X magnification images of in situ hybridization (RNAScope) for *Ntn1* (green) and *Slc32a1* (GABA marker; red, C) and *Th* (dopamine marker; red; D). Arrows indicate co-labeling of *Ntn1* with *Slc32a1* (C) or *Th* (D). Scale bar indicates 20μm. (E-F) Quantification of cell type expression. Of the cells expressing *Ntn1*, 72.2% were dopaminergic (*Th*+) and 18.1% were GABAergic (*Slc32a1*+*;* (E). (F) Of the total of *Th*+ identified cells, 64.5% co-expressed Ntn1 (35.6% did not expressed Ntn1), and 30.4% of *Slc32a1* identified cells co-expressed *Ntn1* (69.5% did not expressed Ntn1). (G) Immunohistochemistry confirms the presence of Netrin-1 protein (red) in both Th+ (cyan) and non-dopamine cells (Th- cells, indicated by yellow arrows).

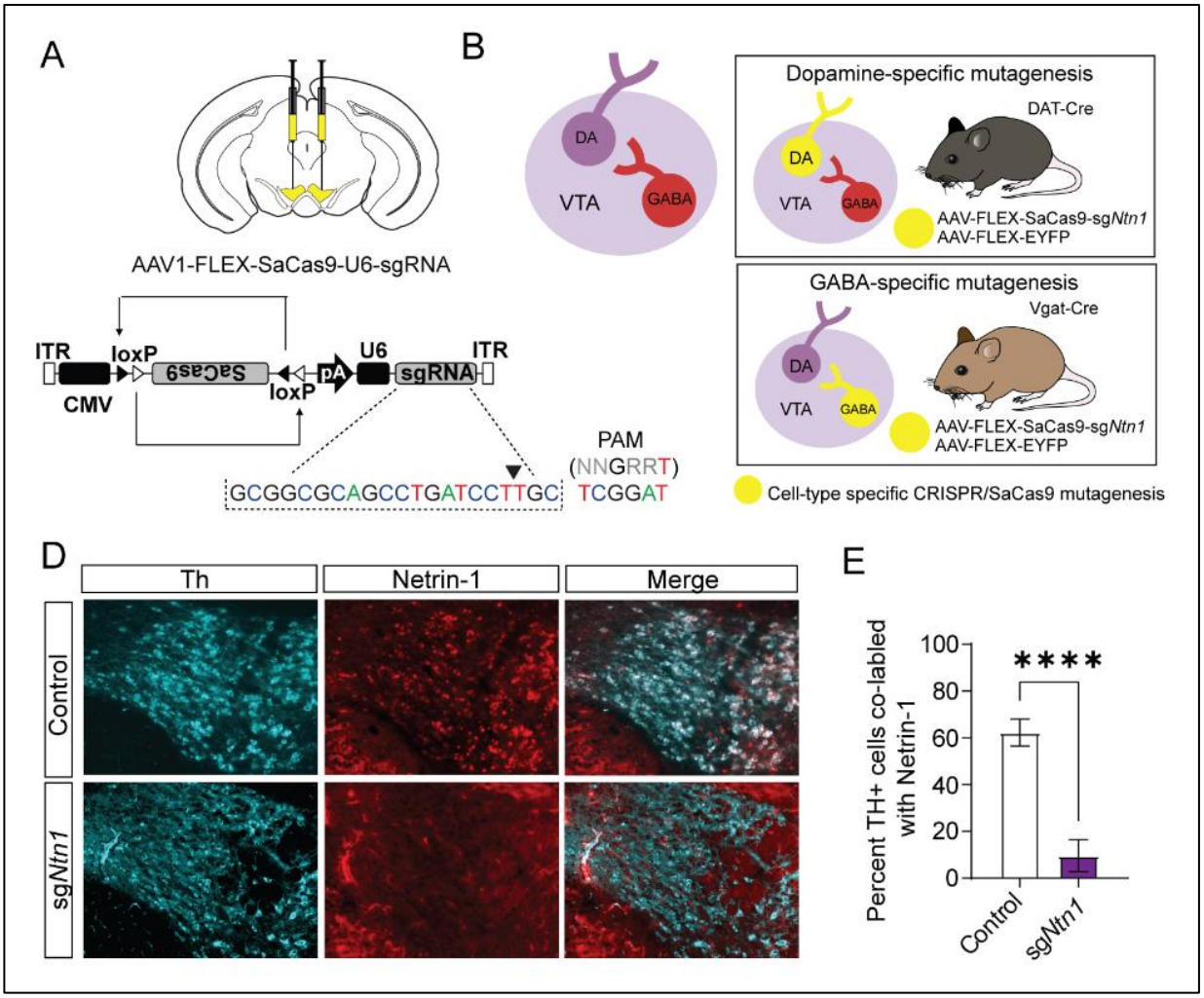


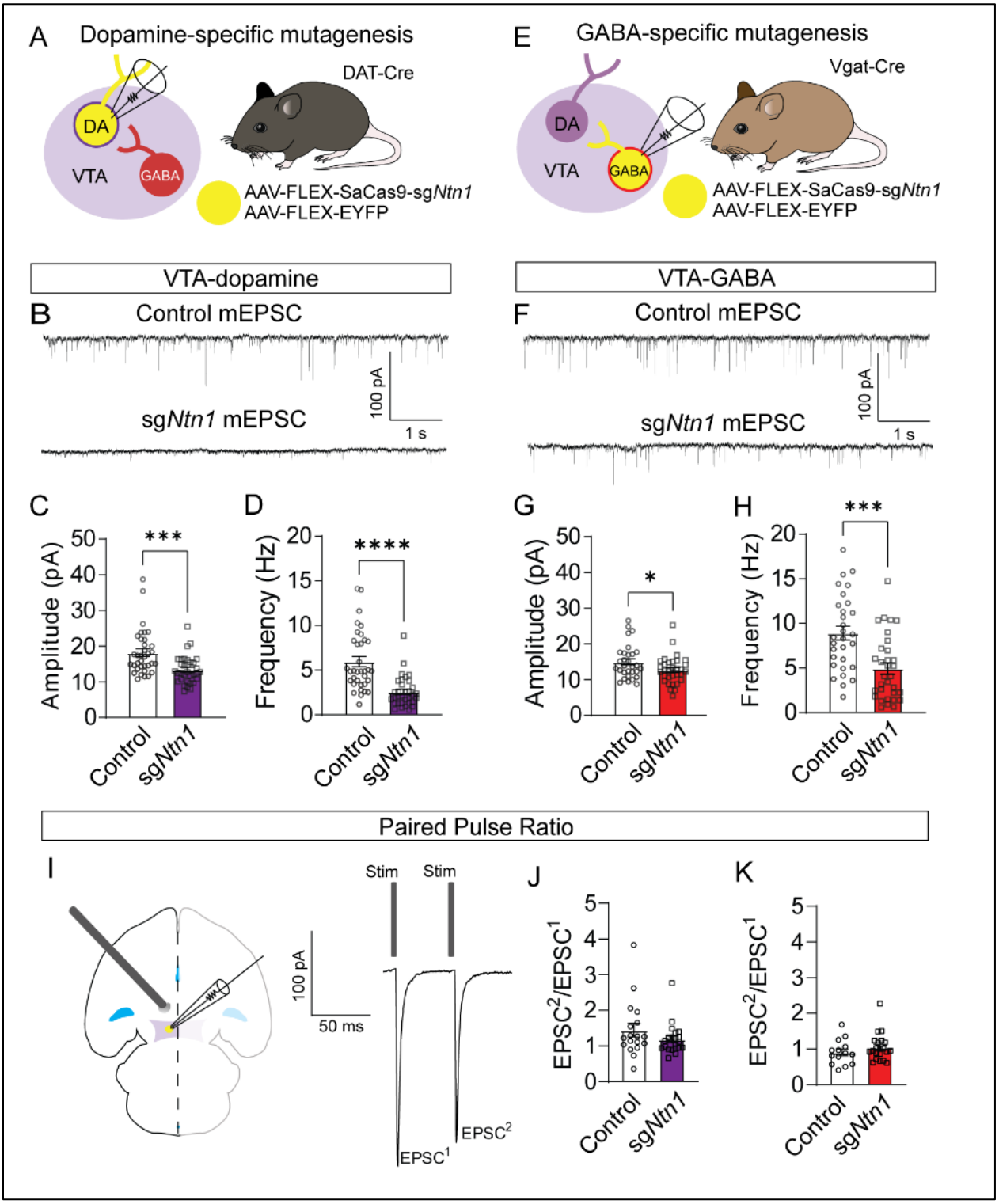
Figure 2.2 Virally delivered CRISPR-Cas9 complex targeting the *Ntn1* locus results in significant reduction in Netrin-1 antibody staining.

(A-B) Schematics summarizing cell type specific knockout procedure. (A) Adult mice were injected bilaterally into the VTA with AAV-FLEX-SaCas9-HA-sg*Ntn1* and AAV-FLEX-YFP. Control mice received an equivalent volume of -sgRosa26 and/or AAV-FLEX-YFP. SaCas9 is virally delivered into the genome in the inactive orientation and returned to the active orientation only in the presence of Cre recombinase, limiting Cas9 expression to target cells. (B) Schematic of the VTA (left) showing VTA GABA neurons project to and inhibit VTA dopamine neurons. By using transgenic Cre-driver mouse lines (right) viral delivery of SaCas9 results in gene disruption in specifically VTA dopamine neurons (DAT-Cre mice, top panel), or VTA GABA neurons (Vgat-Cre mice, bottom panel). (D) Example images for Th (cyan) and Netrin-1 (red) immuno staining in the VTA of mice injected with control or sgNtn1 CRISPR virus. (E) Quantification of the percentage of Th+ cells co-labled with Netrin-1 ($t=8.179$, $df=10$, 62.25 ±5.796 vs 9.586±2.807, ****$p<0.0001$).

To selectively mutate *Ntn1* in specific cell types in the VTA, we designed a single guide RNA (sgRNA) targeting exon 2 in mice (sg*Ntn1*; Figure 2A) and cloned it into an AAV packaging plasmid containing a Cre-recombinase dependent expression cassette for SaCas9[66]. To determine the efficiency of *Ntn1* mutagenesis, we injected DAT-Cre ($DAT^{IRES}Cre$; $Slc6a3^{Cre/+}$) mice (aged 8-10 weeks) bilaterally into the VTA with either AAV-FLEX-SaCas9-HA-sg*Ntn*1 and AAV-FLEX-YFP (DAT-Cre *Ntn1*-cKO mice) or AAV-FLEX-SaCas9-sg*Rosa26* (a gene locus with no known function; control mice). Four to five weeks following injection, we performed immunohistochemistry for netrin-1 and Th. *Ntn1* conditional knockout (cKO) resulted in a significant reduction (~80%) in the proportion of VTA Th-positive cells co-labeled with netrin-1 in DAT-Cre *Ntn1* cKO mice compared to controls (Figure 2D-E).

2.2.2 Netrin-1 regulates excitatory connectivity within the adult VTA

Previous research has shown netrin-1 regulates excitatory synaptic connectivity in the adult hippocampus[28]. To determine the impact of *Ntn1* loss of function on synaptic connectivity, DAT-Cre or Vgat-Cre mice were injected with AAV1-FLEX-SaCas9-U6-sg*Ntn1* and AAV1-FLEX–YFP (Figure 3A and 3E). After at least 4 weeks, miniature excitatory postsynaptic currents (mEPSCs) were recorded from fluorescently identified dopamine or GABA neurons of the VTA. *Ntn1* mutagenesis in dopamine neurons resulted in significantly reduced mEPSC amplitude and frequency (Figure 3B-D). Similarly, *Ntn1* mutagenesis in VTA GABA neurons



also resulted in significantly reduced mEPSC amplitude and frequency (Figure 3F-H). We did not detect significant effects on miniature inhibitory postsynaptic currents (mIPSCs) in VTA dopamine or GABA neurons following *Ntn1* mutagenesis in these cells (Figure 3 supplement 1),

Figure 2.3 Loss of *Ntn1* results in significant reduction in excitatory postsynaptic current.
(A) Schematic of DAT-Cre dopamine specific Ntn1cKO. (B) Sample traces from control (top panel) and DAT Ntn1 cKO mice (bottom panel). (C-D) mEPSC amplitude (C) and frequency (D) measured from fluorescently identified dopamine neurons (n=35 controls, n=33 cKO, t=3.744, df=66, ***p<0.001 and t=5.259, df=66, ****p<0.0001). (E) Schematic of Vgat-Cre GABA specific Ntn1cKO. (F) Sample traces from control (top panel) and Vgat Ntn1 cKO mice (bottom panel). (G-H) mEPSC amplitude (G) and frequency (H) measured from fluorescently identified GABA neurons (n=30 controls, n=32 cKO, t=2.048, df=60, *p<0.05, and t=3.966, df=60, ***p<0.001). (I) Schematic of stimulating electrode placement in horizontal midbrain slice and example EPSCs. (J-K) Paired pulse ratio in dopamine (J, n=18 controls, n=21 cKO), or GABA neurons (K, n=14 controls, n=21 cKO).

suggesting netrin-1 does not play a role in regulating inhibitory connectivity in these cells.

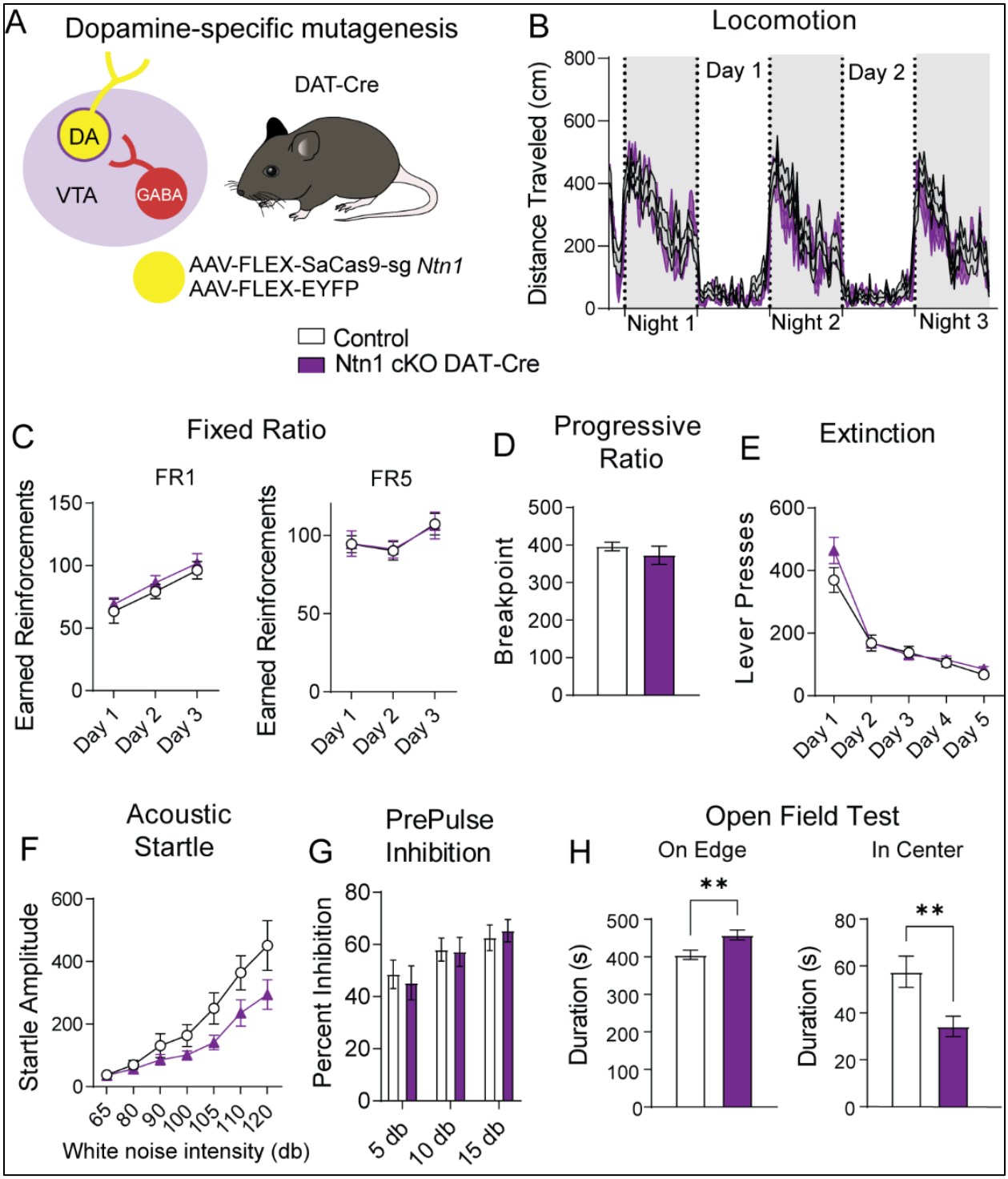
Our observed reduction in mEPSC frequency suggests that loss of *Ntn1* function could act presynaptically, potentially through postsynaptic netrin-1 secretion[28]. To test potential presynaptic changes in vesicle release probability, we analyzed the paired-pulse ratio (PPR) of electrically evoked EPSCs delivered 50 ms apart. *Ntn1* mutagenesis in either dopamine or GABA neurons did not result in a significant change in PPR compared to controls, suggesting no measurable change in presynaptic release (Figure 3J-K).

Because netrin-1 is a secreted protein, it is also possible that *Ntn1* loss of function in one cell type could affect synaptic connectivity in adjacent neurons in which the gene was not inactivated, inducing a non-cell autonomous effect. To address this, we recorded mEPSCs from non-YFP-expressing (presumptively non-dopamine) neurons in DAT-Cre mice injected with *Ntn1* CRISPR or control virus, and from non-YFP-expressing (presumptively non-GABA) neurons in Vgat-Cre injected mice. We did not observe significant non-cell autonomous effects on mEPSCs from non-targeted cells (Figure 3 supplement 2). Similarly, we also did not observe non-cell autonomous effects on mIPSCs from non-targeted cells (Figure 3 supplement 2).

2.2.3 Ntn1 loss of function in VTA-dopamine neurons has little effect on behavior

Dopamine producing neurons of the VTA regulate multiple aspects of locomotor activity, motivated behavior, and psychomotor activation. To determine whether conditional mutagenesis of *Ntn1* in dopamine neurons, and subsequent reduction in excitatory synaptic connectivity impacts these behaviors, we injected DAT-Cre mice with AAV1-FLEX-SaCas9-sg*Ntn1* or AAV1-FLEX-SaCas9-sg*Rosa26* (control) and assayed them in multiple behavioral paradigms. First, we monitored day-night locomotion in control and AAV1-FLEX-SaCas9-sg*Ntn1* injected DAT-Cre mice. No significant differences were detected (Figure 4B and Figure 4 supplement 1).

To determine whether appetitive conditioning behaviors are disrupted by loss of *Ntn1* function in VTA dopamine neurons, we assayed mice in a simple instrumental conditioning paradigm using a fixed-ratio 1 (FR1) followed by a fixed ratio 5 (FR5) schedule of reinforcement in which 1 or 5 lever presses are required to obtain a food reward, respectively. We did not observe significant differences in either of these behavioral tasks (Figure 4C). Next, we monitored motivated behavior using a progressive ratio schedule of reinforcement in which the number of lever presses required for reinforcement increases non-arithmetically (1, 2, 4, 7, 13, 19, 25, 34, 43, 52, 61, 73…), and again did not observe significant differences between control and experimental mice (Figure 4D). Following PR, we reinstated FR1 responding for 3 days followed by extinction training, and again did not detect any differences between the two groups



(Figure 4E), indicating *Ntn1* loss of function in VTA dopamine neurons did not alter appetitive conditioning behaviors.

To determine whether sensory-motor gating is altered in mice with loss of *Ntn1* function in VTA dopamine neurons, we assayed them in acoustic startle and pre-pulse inhibition (PPI)

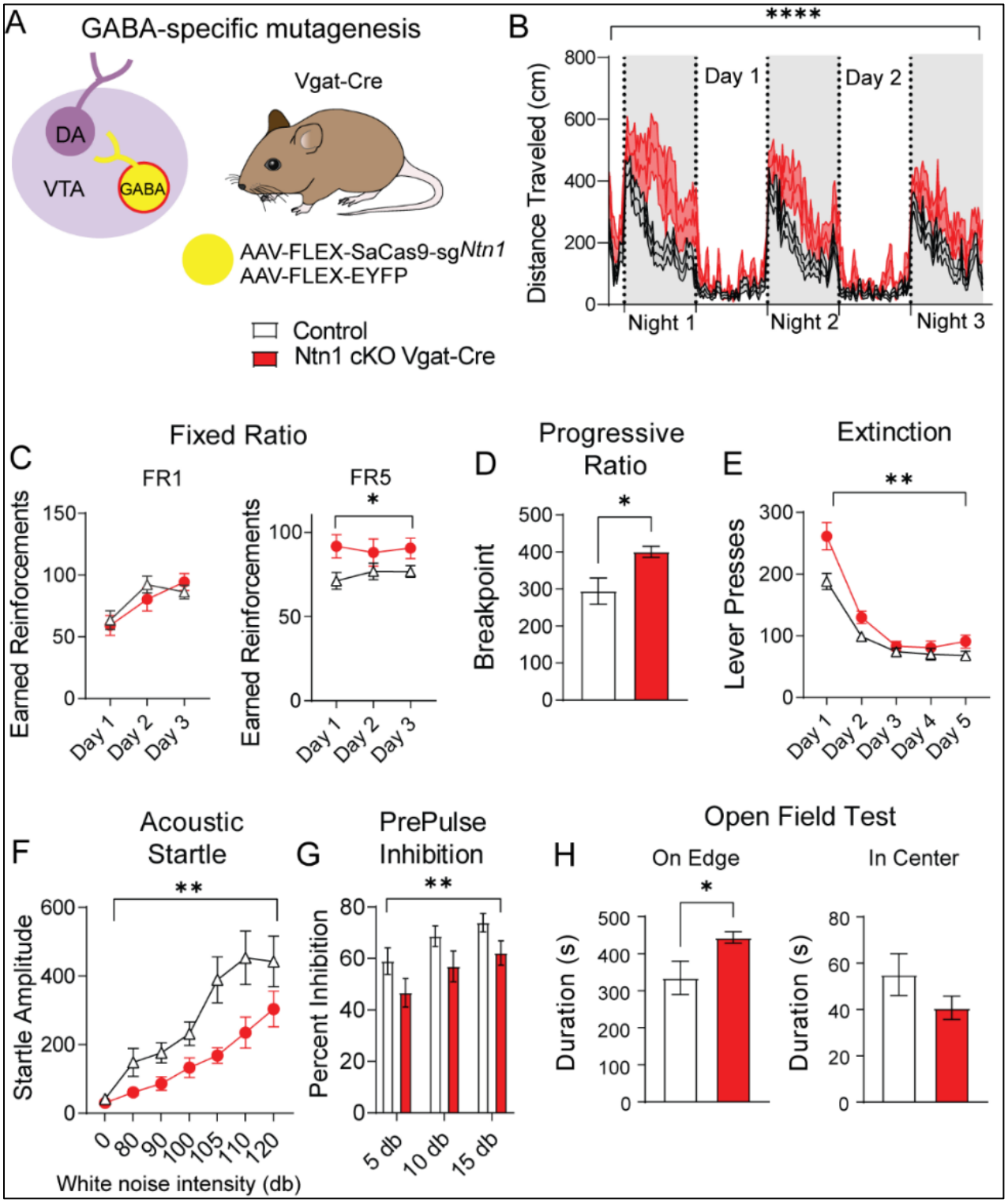
Figure 2.4 *Ntn1* cKO in DA neurons results in little behavioral alteration.
(A) Schematic summarizing cell type specific knockout procedure. (B) Distance traveled in 15 min bins over the course of 3 nights and 2 days (n=21 control; n=15 cKO). (C) Earned reinforcers during 3 days of FR1 or FR5 operant conditioning. (D) Breakpoint (maximum presses per reinforcer) on a progressive ratio task. (E) Lever presses per session during 5 days of extinction training. (F) Acoustic startle response to varying intensity white noise stimuli (G) Percent inhibition of startle response following pre-pulse at indicated intensities. (H) Time on edge or in center of open field arena during a 10 minute test session (B-H: n=19 control; n=15 cKO. H Edge: t=2.897, df=32, **p<0.01, H Center: t=2.750, df=32, **p<0.01).

paradigms. Although acoustic startle responses were reduced in AAV1-FLEX-SaCas9-sg*Ntn1* injected mice, this did not reach significance (Figure 4F). Moreover, we did not observe differences in PPI percentage inhibition (Figure 4G). These results indicate that loss of *Ntn1* function in VTA dopamine neurons does not appear to affect psychomotor activation.

In addition to reinforcement and motivation, dopamine regulates other dimensions of affective behavior. To test whether anxiety-related behavior is affected in experimental mice relative to control mice, we assayed them in an open-field test. AAV1-FLEX-SaCas9-sg*Ntn1* injected DAT-Cre mice spent significantly more time on the edge of the open field arena and significantly less time in the center of the arena, consistent with an elevation in anxiety-like behavior (Figure 4H).

2.2.4 Ntn1 loss of function in VTA-GABA neurons affects multiple behaviors

To determine whether reducing excitatory synaptic connectivity onto VTA GABA neurons through the loss of *Ntn1* function in these cells impacts behavior, we injected Vgat-Cre mice with AAV1-FLEX-SaCas9-sg*Ntn1* or AAV1-FLEX-SaCas9-sg*Rosa26* (control) into the VTA as described previously and tested these mice using the same behavioral paradigms



described above. In contrast to *Ntn1* mutagenesis in dopamine neurons, this manipulation in VTA GABA neurons resulted in a significant increase in locomotor activity (Figure 5B and Figure 5 supplement 1).

Figure 2.5 Figure 2.5: *Ntn1* cKO in GABA VTA neurons resulted in significant behavioral alterations. (A) Schematic summarizing cell type specific knockout procedure. (B) Distance traveled in 15 min bins over the course of 3 nights and 2 day (n=26 controls, n=23 cKO, Two-way ANOVA Group F (1, 11797) = 527.4, ****p<0.0001). (C) Earned reinforcers during 3 days of FR1 or FR5 operant conditioning (n=18 control; n=15 cKO; Two-way ANOVA F Group (1, 31) = 4.261, *p<0.05). (D) Breakpoint (maximum presses per reinforcer) on a progressive ratio task (t=2.577, df=31, *p<0.05). (E) Lever presses per session during 5 days of extinction training (Two-way ANOVA F Group F (1, 31) = 10.23, **p<0.01). (F) Acoustic startle response to varying intensity white noise stimuli (GABA-cKO; Group Factor F (1, 31) = 7.891, **p<0.0085, Startle x group F (6, 186) = 2.186, P=0.0462). (G) Percent inhibition of startle response following pre-pulse at indicated intensities (Group Factor F (1, 93) = 9.181, **p<0.01). (H) Time on edge or in center of open field arena during a 10 minute test session (edge: t=2.248, df=31, *p<0.05).

In the FR1 schedule of reinforcement we did not observe a significant difference between the groups; however, we observed an increase in the number of earned reinforcements in the FR5 schedule in mice with *Ntn1* loss of function in VTA GABA neurons (Figure 5C). We also observed an increase in the PR schedule of reinforcement in these mice relative to controls (Figure 5D). During extinction training, mice with *Ntn1* loss of function in VTA GABA neurons had a significant delay in the rate of extinction following reinstatement of FR1 conditioning (Figure 5E).

Analysis of sensory-motor gating in these mice revealed that Vgat-Cre mice injected with AAV1-FLEX-SaCas9-sgNtn1 had a significant reduction in the acoustic startle relative to control mice (Figure 5F) that was accompanied by a reduction in PPI (Figure 5G). Similar to mutagenesis of *Ntn1* in dopamine neurons, this manipulation in GABA neurons resulted in an increase in anxiety-like behavior as demonstrated by an increased time on edge; though we only observed a trend towards a reduction in time spent in the center of the open field arena (Figure 5H).

2.2.5 Loss of netrin-1 in dopamine neurons largely reverses the effects of Ntn1 mutagenesis in GABA neurons

A loss of netrin-1 in VTA-dopamine neurons resulted in decreased excitatory synaptic input to those cells (theoretically reducing dopamine activity) (Figure 6A), and loss of netrin-1 in VTA-GABA neurons resulted in decreased excitatory tone onto GABA neurons, which would be predicted to increase dopamine activity through disinhibition[77] (Figure 6A). Based on these observations, we asked whether a loss of *Ntn1* in both cell types would restore the balance of activity in the midbrain, or whether there is a hierarchical effect of *Ntn1* loss of function in GABA neurons. To address this, we crossed DAT-Cre with Vgat-Cre mice to develop a DAT-Cre::Vgat-Cre transgenic line, injected these mice with AAV1-FLEX-SaCas9-sg*Ntn1* or AAV1-FLEX-SaCas9-sg*Rosa26* (control)(Figure 6B), and assayed them using the previous behavioral battery.

Simultaneous *Ntn1* loss of function in VTA GABA and dopamine neurons largely reversed the hyperlocomotor phenotype (Figure 6C) observed with *Ntn1* mutagenesis in VTA

GABA neurons alone, though a modest, increase in daytime locomotion remained (Figure 6

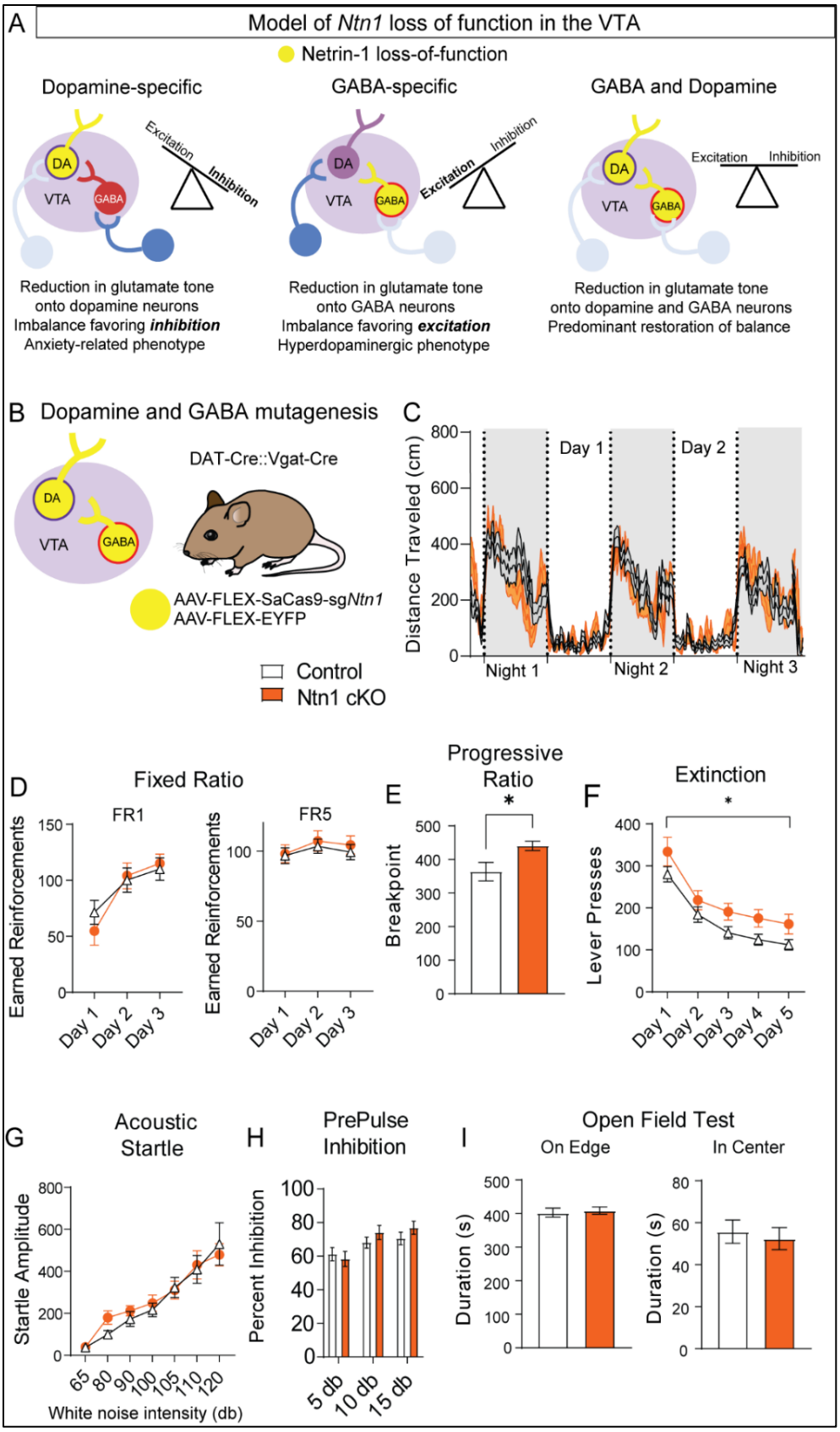


Figure 2.6 Figure 2.6: *Ntn1* cKO in DAT^{IRES}::Vgat-Cre mice partially rescues behavioral phenotype A) Model of *Ntn1* loss of function in the VTA on excitatory and inhibitory balance. B) Schematic of GABA and Dopamine Ntn1 cKO. C) Distance traveled in 15 min bins over the course of 3 nights and 2 day (Two-Way ANOVA Group Factor F (1, 45) = 0.01279, $p<0.05$). D) Earned reinforcers during 3 days of FR1 or FR5 operant conditioning. E) Breakpoint (maximum presses per reinforcer) on a progressive ratio task (n=21 controls, n=20 Ntn1 cKO, t=2.502, df=39, *$p<0.05$) F) Lever presses per session during 5 days of extinction training (Two-way ANOVA Group Factor F (1, 39) = 6.990, *p=0.0117). (G) Acoustic startle response to varying intensity white noise stimuli. G) Percent inhibition of startle response following pre-pulse at indicated intensities. (H) Time on edge or in center of open field arena during a 10-minute test session.

supplemental figure 0.3). Similarly, loss of *Ntn1* in both VTA GABA and dopamine neurons resulted in operant responding during FR1 and FR5 that was similar to controls (Figure 6D). Motivation as measured in the PR task was elevated in the double transgenic Cre line following *Ntn1* mutagenesis (Figure 6E) and extinction was impaired (Figure 6F), though these phenotypes were less robust than those observed in the VTA GABA only mice. Finally, loss of netrin-1 in both cell types resulted in acoustic startle and PPI responses (Figure 6G-H), and open field activity (Figure 6I) that was similar to control mice.

2.3 DISCUSSION:

Here we show that netrin-1 is present in both dopamine and GABA producing neurons of the adult VTA, and loss of netrin-1 function via genetic inactivation in either cell type results in a significant disruption of excitatory synaptic connectivity. The exact mechanisms by which netrin-1 regulates glutamatergic connectivity remains to be resolved. Likely mechanisms include netrin-1 regulation of the actin cytoskeleton and receptor transport vesicles through its activation of the cognate receptor DCC[78]. The latter is consistent with our observed decrease in the amplitude of mEPSCs and previous reports of netrin-1 regulating the delivery of GluA1

containing AMPA receptors to the postsynaptic density[28]. Our finding that mEPSC frequency, but not paired pulse ratio, were affected by netrin-1 loss further suggests netrin's role in modulating excitatory synaptic connectivity is likely confined to postsynaptic mechanisms.

Loss of netrin-1 in dopamine neurons had little effect on behavior; however, we did observe and increase in anxiety-like behavior and measured by the open field assay consistent with the proposed role of dopamine in the modulation of anxiety-related behavior[79]. The general lack of effect of reduced glutamatergic synaptic connectivity on appetitive behavior, locomotion, and sensory-motor gating is consistent with previous observations that reduced glutamatergic signaling in dopamine neurons largely does not affect these behaviors[80,81]. In contrast, loss of netrin-1 in VTA GABA neurons had a significant effect on multiple behaviors including locomotion, motivation, and acoustic pre-pulse inhibition, all of which are consistent with a hyperdopaminergic phenotype and with previous reports that disrupting GABA neuron function in the VTA induces similar phenotypes[54,72].

Given the robust nature of the behavioral effects observed following *Ntn1* mutagenesis in VTA GABA neurons, we were initially surprised that simultaneous loss of netrin-1 in both GABA and dopamine neurons largely rescued the observed hyperdopaminergic phenotype. These results suggest that a balance of glutamatergic signaling in these two cell types is essential for the normal functioning of the mesolimbic dopamine system (Figure 6A). This finding is similar to what has been reported previously in the striatum. Beutler et al, demonstrated that loss of NMDA receptor signaling in dopamine D1 receptor-expressing neurons prevented the development of amphetamine sensitization; however, inactivation of NMDA receptors in D1R and D2R-expressing medium spiny neurons reversed this phenotype[82].

While our findings shed light on the role of netrin-1 in adult VTA neurons, the question

remains as to which netrin-1 receptors may be involved. Indeed, though Dcc is considered to be its canonical receptor, netrin-1 is a known ligand for several additional receptors, including DSCAM, Neogenin, and Unc5 homologues A-D[10,78]. Previous work has identified the presence of both Dcc and Unc5c receptors in the adult VTA (often in the same cells)[5]. It is also interesting to note that during development of the spinal cord, netrin-1 expression in the floor plate attracts commissural axons to the midline, but following the arrival of these axons at the floor plate, Unc5 expression increases to suppress the attractive actions of DCC signaling[13]. Whether a similar relationship exists for the formation of nascent synapses and the maintenance of excitatory synapses occurs in the VTA will be important to resolve. Of further note, in addition to the role of netrin-1/Dcc/Unc5 signaling in the regulation of commissural axons crossing the midline, Slit/Robo signaling repels axons away from the floor plate[83,84] setting up a push-pull relationship between these pathways. We previously demonstrated that Robo2 maintains inhibitory synaptic connectivity in the adult VTA[72], suggesting the existence of another 'push/pull' relationship between these two pathways in which netrin/Dcc/Unc5 regulates excitation and Slit/Robo signaling regulates inhibition.

Mutations in *NTN1* (netrin-1) and *DCC* in humans and have been associated with several dopamine associated psychiatric conditions, including neurodevelopmental disorders such as schizophrenia[35–37] and major depressive disorder[36,38–40], as well as multiple neurodegenerative disorders[44–46]. Our findings that *Ntn1* plays a key role in maintaining excitatory connectivity in the adult midbrain and controlling the inhibitory/excitatory balance in this region highlights the importance of understanding these critical developmental signaling pathways in the adult nervous system that are likely important for therapeutic considerations in targeting these pathways.

2.4 METHODS:

Mice: All procedures were approved and conduced in accordance with the guidelines of the University of Washington's Institutional Animal Care and Use Committee. Mice were housed on a 12:12 light:dark cycle with *ad libitium* access to food and water, except when undergoing food restriction for operant behavioral conditioning. Approximately equal numbers of male and female mice were used. Mice were group housed (2-5 mice per cage). Mice injected with CRISPR/YFP were allowed 4-5 weeks recovery after surgery to allow for viral expression, mutagenesis, and protein turnover before any testing.

Viruses: All adeno-associated viruses (AAV) were produced in house, as previously described[66]. CRISPR viruses employed for this research: AAV1-FLEX-SaCas9-U6-sgNtn1, AAV1-FLEX-SaCas9-U6-sgRosa26, AAV1-FLEX-YFP.

Surgeries: All mice used were 8-10 weeks of age at time of surgery. Mice were inducted using isoflurane at 5.0% and held at 2% throughout the procedure. Mice were stereotaxically injected bilaterally into the VTA using the following coordinates in mm, relative to bregma: A/P: -3.25; M/L $\pm$ 0.5; D/V: (-4.9) – (-4.4), total volume 0.5 µL into each side. A/P coordinates were adjusted for Bregma/Lambda distances using a correction factor of 4.2 mm.

***In situ* hybridization:** Male and female mice (n=2 each sex, 8-12 weeks old) were used to verify mRNA expression in the VTA using RNAscope (*2*). Brains were flash frozen in 2-methylbutane and representative coronal sections that spanned the VTA were sliced at 20 µm and slide mounted for hybridization. Sections were prepared for hybridization per manufacturer's (Advanced Cell Diagnostics, Inc) instructions using probes for *Th* (Mm-*Th*), *Ntn1* (Mm-*Ntn1*-C2), and *Slc32a1* (Vgat; Mm-*Slc32a1*-C3). Slides were coverslipped with Fluoromount with

DAPI (Souther Biotech) and imaged using a confocal fluorescent microscope (University of Washington Keck Center Leica SP8X confocal) and Keyence Fluorescence Microscope (Keyence). Quantification of colabeled cells was performed using CellProfiler, with thresholding and cell identification/overlap for each channel verified for each image manually prior to quantification.

Immunohistochemistry: Mice were anesthetized with pentobarbitol and transcardially perfused with PBS followed by 4% PFA. Brains were post fixed for 24 hours in PFA at 4℃, followed by 48 hours in 30% sucrose. The VTA was coronally sectioned at 30 µm. Sections were kept in PBS with 0.3% Sodium Azide. Free floating sections were treated with 0.3% TBS-Triton-X 100 3x10 minutes, blocked in 3% Normal Donkey Serum for 1 hour, and treated overnight in primary antibody. Following 1-3 hours in secondary antibody (JacksonImmuno), sections were slide mounted and cover slipped with Fluoromount with DAPI. Images were collected on a Keyence Fluorescence Microscope (Keyence). For CRISPR validation, male and female DAT-Cre mice (8-12 weeks old) received AAV1-FLEX-SaCas9-U6-sgNtn1/ AAV1-FLEX-YFP (Ntn1-cKO) or AAV1-FLEX-SaCas9-U6-sgROSA26/AAV1-FLEX-YFP (controls) injections as described above, and quantification of colabeled cells for immunohistochemistry were performed using ImageJ 1.53 Cell Counter/Multi-point tool. Primary antibodies used: mouse anti-TH (1:1500, Millipore), chicken anti-Netrin-1 (1:1000, Abcam) and rabbit anti-HA (1:1500, Sigma)

Slice electrophysiology: Mice injected with CRISPR/YFP were allowed 4-5 weeks recovery after surgery to allow for viral expression, mutagenesis and protein turnover. All solutions were continuously bubbled with O_2/CO_2. Horizontal (200 µm) brain slices were prepared from 12-20 week old mice in a slush NMDG cutting solution[85] (in mM: 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4,

30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2$, 10 $MgSO_4$, pH 7.3–7.4. Slices recovered for ~12 min in the same solution warmed in 32°C water bath, then transferred to room temperature HEPES-aCSF solution (in mM: 92 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiouria, 5 Na-ascorbate, 3 Na-pyruvate, 2 $CaCl_2$, 2 $MgSO_4$). Slices recovered for an additional 30 -60 min in HEPES solution at room temp. Whole-cell patch clamp recordings were made using an Axopatch 700B amplifier (Molecular Devices) using 3–5 MΩ electrodes. Recordings were made in aCSF (in mM: 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 11 D-glucose, 18 $NaHCO_3$, 2.4 $CaCl_2$) at 32°C continually perfused over slices at a rate of ~1 ml/min. VTA dopamine and non-dopamine neurons were identified by fluorescence.

mE/IPSC: For miniature excitatory postsynaptic currents (mEPSCs), internal solution contained: 130 mM K-gluconate, 10 mM HEPES, 5 mM NaCl, 1 mM EGTA, 5 mM Mg-ATP, 0.5 mM Na-GTP. Picrotoxin (200 μM) was added to ACSF to block $GABA_A$ receptor-mediated events. For miniature inhibitory postsynaptic currents (mIPSCs), internal solution contained: 135 mM KCl, 12 mM NaCl, 0.05 mM EGTA, 100 mM HEPES, 0.2 mM Mg-ATP, 0.02, and Na-GTP mM. To block glutamatergic events, 2 mM kynurenic acid was bath applied in the ACSF. All mIPSCs and mEPSCs cells were recorded in the presence of 1 mM tetrodoxin (TTX) to block action potentials. Cells were held at −60 mV for a minimum of 5 minutes prior to data acquisition. Data were analyzed using Clampfit 10.3 (pCLAMP 11 Software Suite, Molecular Instruments).

Paired Pulse Ratio: For PPR, internal solution contained: 130 mM K-gluconate, 10 mM HEPES, 5 mM NaCl, 1 mM EGTA, 5 mM Mg-ATP, 0.5 mM Na-GTP. Picrotoxin (200 μM) was added to ACSF to block $GABA_A$ receptor-mediated events. Electrical stimulation was delivered

using a concentric bipolar electrode placed rostral to the VTA. Data were analyzed using Clampfit 10.3 (pCLAMP 11 Software Suite, Molecular Instruments).

Behavior:

Locomotor activity: 4 weeks after surgery, baseline locomotion was measured using locomotion chambers (Columbus instruments) that use infrared beam breaks to calculate ambulatory activity. Mice were singly housed in Allentown cages with reduced corncob bedding and provided with *ad libitum* access to food and water. Locomotion was monitored continuously for 3 nights 2 days

Open field testing: Mice were placed in a large circular arena (120 cm diameter) and activity was recorded for a period of 10 min using Ethovision software. Time in center, time on edge, and total distance were calculated.

*Operant conditioning***:** Mice were tested on an operant conditioning paradigm in Med Associates boxes in the following order: FR1, FR5, Progressive Ratio, Reinstatement and Extinction. Each fixed ratio 1 (FR1) session lasted for 60 min. Levers were extended and remained extended until a lever press. Upon a lever press, levers were retracted and a sucrose pellet was immediately delivered into the food hopper. The levers did not extend again until the mouse made a head entry into the food hopper to retrieve the pellet. Reinforced FR1 sessions lasted for 3 days, followed by 3 days of FR5 (5 lever presses required to obtain sucrose pellet), and a single day of progressive ratio where the number of lever presses necessary for sucrose pellet delivery increases non-arithmetically (i.e., 1, 2, 4, 6, 9, 13…) over the course of the session. The progressive ratio session ended after 3 consecutive min of no lever presses or after 3 hours. After progressive ratio, mice again underwent FR1 reinforced training, followed by

extinction for 60 min each session for five days. Here, levers extend and retract similarly to the FR1 reinforced paradigm, yet a sucrose pellet reward is omitted.

Acoustic startle and Prepulse inhibition: Acoustic startle responses were measured using acoustic startle chambers (San Diego Instruments). Prior to testing mice received a 10-min habituation period. Background noise was maintained at 65 dB throughout testing. After habituation, mice were presented with 5, 40-ms duration 120 dB, pulse-alone trials to obtain baseline startle responses, followed by 50 trials of either a startle pulse-alone, 1 of 3 prepulse trials, or a null trial, in which no acoustic stimulus is presented. Startle trials consisted of a 40 ms, 120-dB pulse of white noise. The 3 prepulse trials consisted of a 20-ms prepulse of 70-, 75-, or 80-dB intensity (5, 10, and 15 dB above background) that preceded 120-dB startle pulse by 100 ms. Peak amplitude of the startle response (65 ms after pulse onset) was used as the measure of startle response magnitude.

Statistics: Data were analyzed for statistical significance using GraphPad Prism. All statistical tests were two-sided and corrected for multiple comparisons where appropriate. All graphical data are presented as mean ± SEM.

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Author contributions: MMC, MS and LSZ conceived and designed experiments. MMC, ACH, and LZ designed and generated AAVs. MMC and BJ performed behavioral testing and analysis. MMC performed all surgeries and in situ hybridization experiments. MMC and MS performed and analyzed electrophysiology experiments. GE and BH performed and analyzed immunohistochemistry and viral effectiveness images. All authors provided input and approval of the manuscript.

Chapter 3. NETRIN RECEPTORS AND THEIR ROLE PRESERVING DOPAMINE MEDIATED BEHAVIORS

A version of this chapter is being prepared for potential publication.

Marcella M Cline, Barbara Juarez, Ernesto G. Regiarto, Marta E. Soden & Larry S. Zweifel.

3.1 INTRODUCTION

Neurological function requires a careful balance between excitatory and inhibitory circuitry. At the neuronal level, this balance entails precise regulation and maintenance of glutamatergic/GABAergic synaptic input. We recently showed that netrin-1 plays a critical role in regulating glutamatergic connectivity in the adult ventral tegmental area (VTA), and *Ntn1* mutagenesis specifically in VTA dopamine neurons results in significant decrease in excitatory post synaptic input and a corresponding hypodopaminergic behavioral phenotype[86]. What receptors are involved in this context, however, are yet unknown.

Since the initial discovery of netrin as an axonal guidance cue, several netrin receptors have been identified in the context of neurodevelopment. These include the canonically recognized netrin receptor deleted in colorectal cancer (DCC), Down Syndrome Cell Adhesion Molecule (DSCAM), Neogenin (a Dcc homolog), and Unc5 homologues A-D[10,78]. Of these, only two netrin receptors have been identified as being present and expressed in the adult VTA; Dcc and Unc5 homologue C (Unc5c)[63]. Intriguingly, these two receptors play diametrically opposing roles during neurodevelopment[10,15], inviting the possibility that they may work in direct opposition in the adult brain.

Netrin-1 is a bifunctional signaling protein, and while signaling through the canonical

receptor Dcc results in growth cone attraction and increased actin polymerization/complexity during neurodevelopment, netrin-1 signaling through the Unc5 family of receptors (Unc5A-D) promotes growth cone *repulsion*[87], though the mechanisms underlying Netrin/Unc5 signaling are not well understood. Immunofluorescent staining of adult mouse VTA neurons reveals extensive co-expression of Dcc and Unc5 homologue C (Unc5c) in dopamine cells[63]. The potential role of Unc5c receptors in synaptic maintenance and plasticity, however, has to date never been explored. As dopamine neurons within the adult VTA appear to co-express both Unc5c and Dcc[63], it is possible they play opposing roles in regulating synaptic connectivity at the single cell level, with Dcc *maintaining* excitatory synaptic input and Unc5c *reducing* excitatory input. While netrin-1/Dcc ligand binding results in the formation of a Dcc-homodimer, Unc5c is capable of both homodimerization and heterodimerization in conjunction with Dcc. Thus, dopamine cells could potentially regulate excitatory connectivity simply by altering Unc5c expression and receptor availability.

Based on the expression of netrin-1 and netrin receptors in VTA dopamine neurons of the adult brain, their role in the organization of the midbrain dopamine system during development and adolescence[61], and netrin-1 recently established role in maintaining excitatory synaptic connections in the adult VTA (See chapter 2) and hippocampus[24,28,62], I hypothesize the netrin receptors Dcc and Unc5c play distinct roles in regulating synaptic input onto VTA dopamine neurons. If true, disruption of Dcc or Un5c function through genetic mutagenesis would result in a measurable alteration of synaptic input to these cells, producing distinct behavioral phenotypes.

3.2 RESULTS:

3.2.1 No significant motor deficits associated with netrin receptor loss

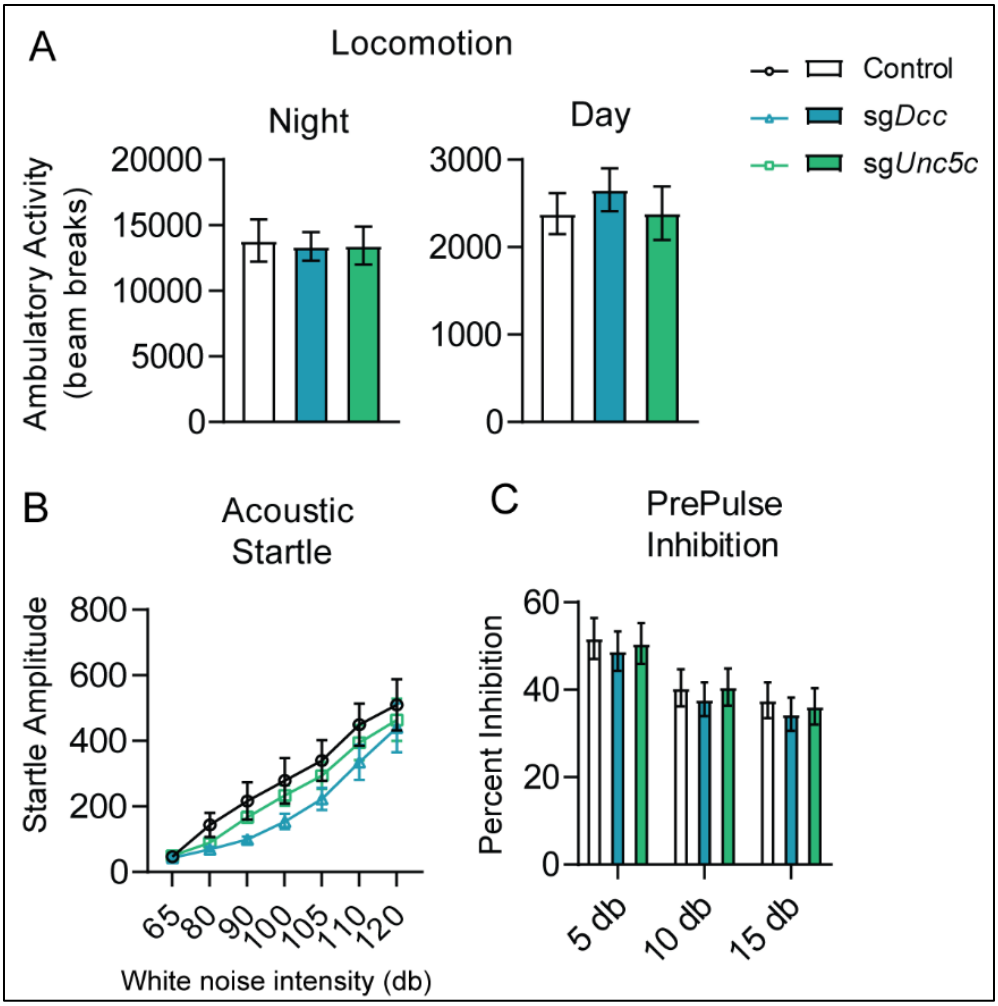

Figure 3.1 No significant motor differences with loss of either Dcc or Unc5c in VTA dopamine neurons

(A) Ambulatory activity averaged over the course of 3 nights and 2 days (B) Acoustic startle response to varying intensity white noise stimuli (C) Percent inhibition of startle response following pre-pulse at indicated intensities (n=19 controls, n=22 sg*Dcc*, n=17 *sgUnc5c)*

Dopamine producing neurons of the VTA have been shown to regulate several aspects of locomotor activity and psychomotor activation. We have previously shown that while loss of *Ntn1* from GABA producing neurons of the VTA resulted in a significant hyperlocomotive phenotype and reduction in the acoustic startle and prepulse inhibition (see Chapter 2.2.6), selective loss of *Ntn1* from dopamine producing neurons was insufficient to alter these

behaviors. To determine whether conditional mutagenesis of either *Dcc* or *Unc5c* in dopamine neurons results in alterations in these behaviors, we injected DAT-Cre mice with AAV1-FLEX-SaCas9-sg*Dcc* (sgDcc), AAV1-FLEX-SaCas9-sg*Unc5c* (sgUnc5c) or AAV1-FLEX-SaCas9-sg*Rosa26* (control) and assayed them in multiple dopamine-mediated behavioral paradigms. First, we monitored day-night locomotion in control and AAV1-FLEX-SaCas9-sg*RNA* injected DAT-Cre mice. No significant differences were detected between the three groups (Figure 3.1).

To determine whether sensory-motor gating is altered in mice with loss of either *Dcc* or *Unc5c* function in VTA dopamine neurons, we assayed each group in acoustic startle and pre-pulse inhibition (PPI) paradigms. Although acoustic startle responses were reduced in AAV1-FLEX-SaCas9-sg*Dcc* injected mice, this reduction failed to reach significance (Figure 3.2A). We did not observe any differences in PPI percentage inhibition (Figure 3.2B) in either group. These results indicate that loss of either Dcc or Unc5c function in VTA dopamine neurons does not appear to affect psychomotor activation, mirroring what we observed previously with loss of *Ntn1* in this same cell type.

3.2.2 Dcc or Unc5c loss of function in dopamine neurons of the adult VTA significantly alters appetitive conditioning

To determine whether appetitive conditioning behaviors are disrupted by loss of Dcc or Unc5c function in VTA dopamine neurons, we assayed mice in a simple instrumental conditioning paradigm using a fixed-ratio 1 (FR1) followed by a fixed ratio 5 (FR5) schedule of reinforcement in which 1 or 5 lever presses are required to obtain a food reward, respectively. We did not observe significant differences in FR1, but loss of Dcc from dopamine producing neurons did result in a significant increase in FR5 reinforcement (Figure 3.2A), suggesting the loss of Dcc in this context resulted in an altered motivational state in these mice. Next, we

monitored motivated behavior using a progressive ratio schedule of reinforcement in which the number of lever presses required for reinforcement increases non-arithmetically (1, 2, 4, 7, 13, 19, 25, 34, 43, 52, 61, 73…), and while insignificant, we did observe a trend in PR such that loss of Dcc appeared to be associated with a slight increase in PR breakpoint (Figure 3.2B). These findings (elevated FR5 and PR breakpoint) did not phenoreplicate what we saw previously with loss of *Ntn1* in dopamine VTA neurons. Curiously, these data were more indicative of loss of *Ntn1* from GABA producing neurons of the VTA (see Chapter 2.2.4), perhaps suggesting a shift in VTA circuitry favoring excitation. Following PR, we reinstated FR1 responding for one day followed by four days of extinction training (Figure 3.2 C). During extinction, both *sgDcc* and control groups engaged in significantly more lever presses over the four days of training compared to the sg*Unc5c* group. These data indicate both Dcc and Unc5c loss of function in VTA dopamine neurons is sufficient to alter appetitive conditioning behaviors, in phenotypically distinct ways.

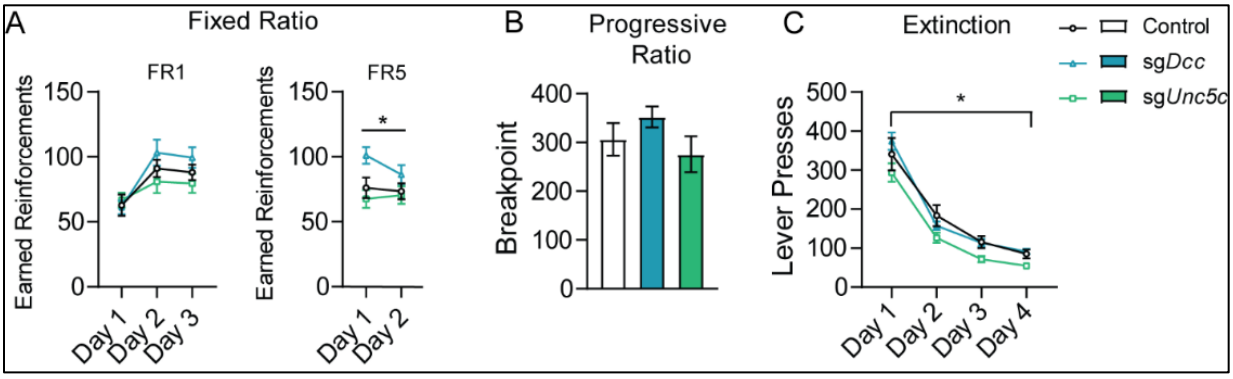

Figure 3.2 Loss of either Dcc or Unc5c results in mild alterations in instrumental learning

(A) Earned reinforcers during 3 days of FR1 or FR5 operant conditioning (n=17 control; n=24 sgDcc, n=17 sgUnc5c; Two-way ANOVA $F(2, 55) = 4.323$, $^*p<0.05$). (B) Breakpoint (maximum presses per reinforcer) on a progressive ratio task (C) Lever presses per session during 5 days of extinction training (Two-way ANOVA F Group $F(2, 55) = 3.336$, $^*p<0.05$).

3.2.3 No anxiety phenotype with loss of either Dcc or Unc5c from dopamine producing neurons of the adult VTA

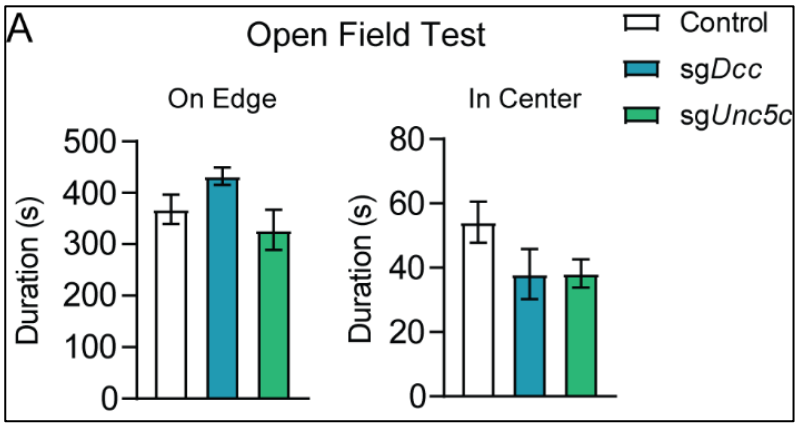

Figure 3.3 No anxiety-like phenotype associated with Dcc or Unc5c loss in VTA dopamine neurons

(A) Time on edge or in center of open field arena during a 10-minute test session (n=18 control; n=22 sgDcc, n=17 Unc5c).

In addition to reinforcement and motivation, dopamine regulates other dimensions of affective behavior. Our previous work found anxiety as measured by the open-field test to be one of the few behavioral tasks significantly altered by *Ntn1* mutagenesis in VTA dopamine neurons (See Chapter 2.2.3). To test whether anxiety-related behavior is affected in sgDcc and sgUnc5c mice relative to control mice, we assayed them in an open-field test. Unlike AAV1-FLEX-SaCas9-sg*Ntn1* injected DAT-Cre mice, loss of Dcc or Unc5c did not result in a significant anxiety-like phenotype (Figure 3.3). However, this appears to be a result of increased group deviation, as both sgDcc and sgUnc5c groups did trend toward significance with duration of time spent in OFT center.

3.2.4 Loss of Unc5c function from VTA dopamine neurons results in significant social impairments, impaired nestlet shredding and loss of novel object recognition

Nest building is a well characterized instinctual behavior in rodents, and the nestlet shredding task capitalizes on this behavior as a method of identifying potential psychiatric

disease-associated symptoms[88]. Increased or excessive nestlet shredding can be seen as a sign of compulsive and/or repetitive behavior (such as those seen in obsessive compulsive disorder, or Autism spectrum disorders), whereas reduced or limited shredding can be a sign of reduced interest in novelty and anhedonia[88,89]. Over the course of six hours, control, sg*Ntn1*, and sg*Dcc* mice successfully shredded their nestlets, and were statistically indistinguishable from each other. Sg*Unc5c* mice, however, showed impaired nestlet shredding, and after two hours were significantly distinct from all groups (Figure 3.4 A).

Novel encounters and experiences are associated with heightened dopamine activity. The recognition of common novelty (novel experiences that share appreciable commonality with previous experiences) is highly dependent on dopaminergic VTA-Hippocampal projections[90]. To measure alterations in common novelty recognition, mice were evaluated using a two-day novel object recognition task (NOR). Traditionally, mice prefer novelty, and if able to recognize each distinct object, are expected to spend significantly more time with a new, novel object compared to an object which they have encountered previously. This expected behavioral pattern was apparent in control mice (Figure 3.4 B), who spent significantly more time investigating the novel object. Loss of either Dcc or Unc5c function, however, significantly reduced this preference for novelty.

Similar to NOR, social novelty is highly motivating for mice, and it is generally expected that mice will prefer to spend time with a novel mouse. Mice were assayed for social impairments using a social interaction assay that has two parts; Social preference and social recognition (Figure 3.4 C & D). After the target mouse has acclimated to the testing arena, a novel mouse of the same sex is placed under one cup and the experimental mouse is allowed to explore each of the three chambers freely (Social Preference). After 30 minutes, a new (now

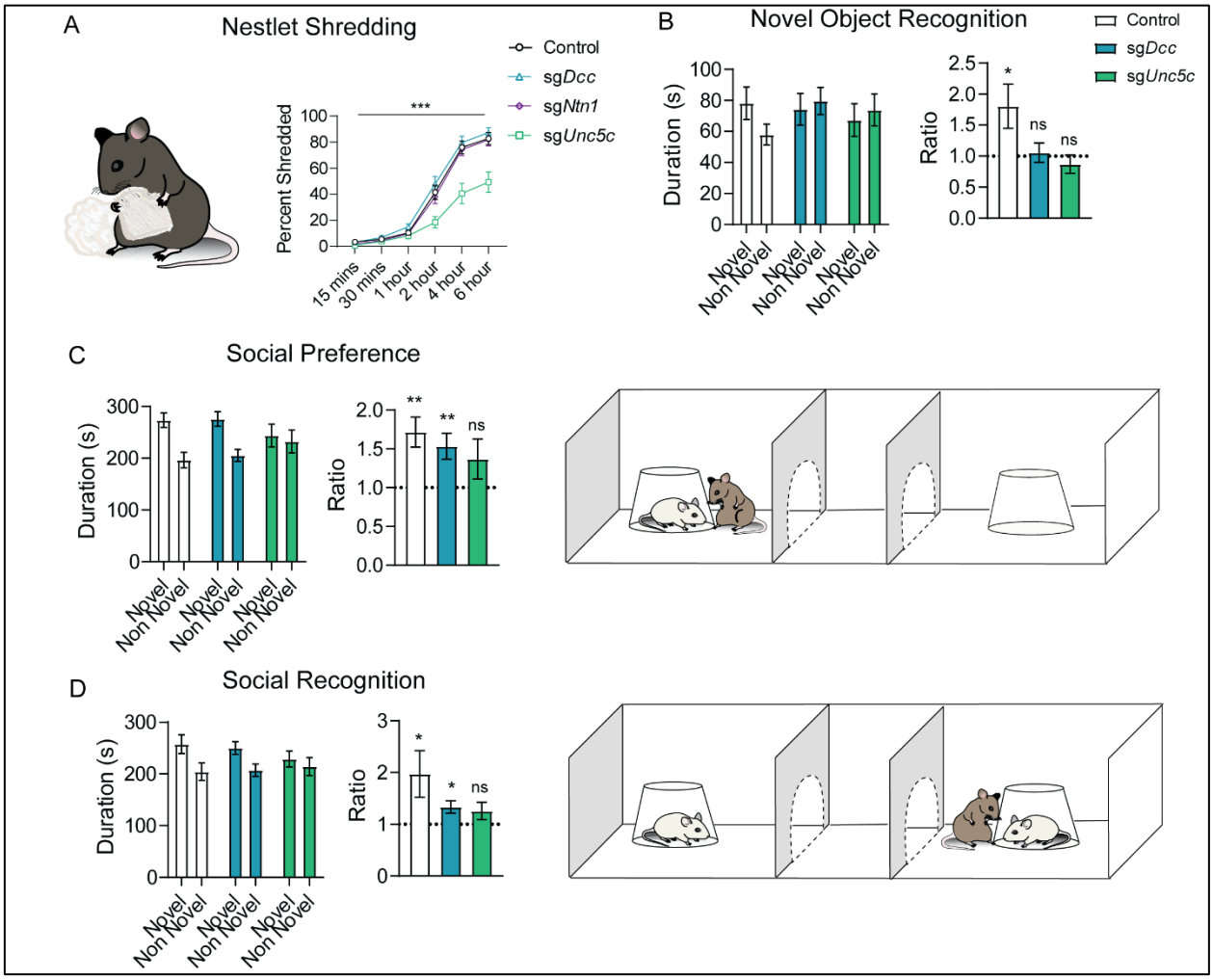

Figure 3.4 Loss of Unc5c from VTA dopamine neurons results in significant behavioral impairment (A) Nestlet shredding measured over the course of 6 hours in a novel cage (n=31 control, n=24 sgDcc, n=22 sgNtn1, n=17 sgUnc5c; Two-way ANOVA Group F (3, 90) = 7.840, ***p<0.001, Time x Group F (15, 450) = 6.168, ****p<0.0001). (B) Novel Object Recognition task duration (left) and ratio (duration of time spent investigating novel object/duration of time with non novel object) (One sample t test using a theoretical mean of 1, control t=2.261, df=24, *p<0.05). (C) Social preference task results (left) and paradigm (right). After arena acclimation mice are given the choice of interacting with an empty mesh cup or novel mouse. (One sample t test, control t=3.682, df=26, **p<0.01, Dcc t=3.178, df=23,**p<0.01). (D) Social Recognition task data (left) and paradigm (right). After 30 minutes, a new (now novel) mouse is now added to the previously empty mesh cup (control t=2.156, df=26, *p<0.05, Dcc t=2.772, df=23, *p<0.05)

novel) mouse is placed under the remaining empty cup, and the target mouse is allowed to explore for an additional 10 minutes (Social recognition). Mice will traditionally prefer to spend

time with a novel mouse over the empty cup (social preference), and a novel mouse over one with which they have interacted (social recognition). Despite their impairments in the Novel Object Recognition task, sg*Dcc* mice performed well on both Social Preference and Social Recognition assays, showing a clear preference for novelty in each case. Loss of Unc5c function, however, resulted in no preference for either the novel mouse over the empty cup, or the novel mouse over one with which they have interacted. These data point to significant social impairments associated with the loss of Unc5c in dopamine neurons of the adult VTA.

3.3 DISCUSSION

Netrin-1 functions as a regulator of excitatory synaptic input onto dopamine neurons of the adult VTA[86], but it is yet unknown which receptors mediate this activity. Here, we used advanced viral and CRISPR/Cas9 technologies to selectively inhibit the function of the netrin receptors Dcc and Unc5c specifically in adult VTA dopamine neurons, in order to determine what role they may play in the maintenance of excitatory synaptic connectivity.

Loss of Dcc function in dopamine neurons resulted in an altered motivational state, as detected by appetitive conditioning tasks, and reduced novelty seeking in a novel object task. Curiously, however, despite not being the canonical netrin-1 receptor, it was loss of Unc5c that resulted in the most significant behavioral phenotype. Loss of Unc5c resulted in a significantly impairments in several dopamine mediated behaviors. Mice with Unc5c loss of function in dopamine neurons had appetitive conditioning alterations (decreased motivation), significantly impaired nestlet shredding, reduced novelty seeking in a novel object task, and significantly altered social behaviors. Together, these results are suggestive of an autism spectrum disorder phenotype, in which novelty seeking and social interaction are significantly reduced compared to control mice.

Curiously, neither Dcc nor Unc5c loss in dopamine neurons phenoreplicated dopamine specific *Ntn1* loss, further underscoring the possibility that Dcc and Unc5c have distinct cellular roles. Thus, loss of either one is potentially capable of shifting netrin-1 signaling to exclusively favor the other receptor, whereas netrin-1 loss eliminates both signaling cascades, resulting in three distinct behavioral phenotypes. To test this theory, future research utilizing a double knockout strategy (i.e., CRISPR mutagenesis of both Dcc and Unc5c in the same cells) would be useful. If our prediction were true, we would expect loss of both Dcc and Unc5c to produce a similar, if not identical, behavioral phenotype to loss of netrin-1 alone.

One limitation of this study is the lack of physiological data. Loss of netrin-1 from dopamine neurons of the adult VTA resulted in a significant reduction in excitatory synaptic input – is this process mediated through the conical netrin receptor Dcc? If so, we would expect the sgDcc knockout to result in a similar loss of excitatory synaptic input. But what of Unc5c? If, as predicted, Unc5c were preforming an adversarial role to that of the Dcc receptor, we would expect loss of Unc5c to potentially result in an increase in excitatory synaptic input – as a direct consequence of shifting the balance of netrin signaling to solely favor the Dcc receptor.

These data, while incomplete, further our understanding of netrin-1s role in the adult nervous system. Future research focusing on the physiological impact of netrin receptor loss should be conducted in the near future to finalize these data.

3.4 METHODS:

Mice: All procedures were approved and conduced in accordance with the guidelines of the University of Washington's Institutional Animal Care and Use Committee. Mice were housed on a 12:12 light:dark cycle with *ad libitium* access to food and water, except when undergoing food restriction for operant behavioral conditioning. Approximately equal numbers of male and

female mice were used. Mice were group housed (2-5 mice per cage). Mice injected with CRISPR/YFP were allowed 4-5 weeks recovery after surgery to allow for viral expression, mutagenesis, and protein turnover before any testing.

Viruses: All adeno-associated viruses (AAV) were produced in house, as previously described[66]. CRISPR viruses employed for this research: AAV1-FLEX-SaCas9-U6-sgNtn1, AAV1-FLEX-SaCas9-U6-sgDcc, AAV1-FLEX-SaCas9-U6-sgUnc5c, AAV1-FLEX-SaCas9-U6-sgRosa26, AAV1-FLEX-YFP.

Surgeries: All mice used were 8-10 weeks of age at time of surgery. Mice were inducted using isoflurane at 5.0% and held at 2% throughout the procedure. Mice were stereotaxically injected bilaterally into the VTA using the following coordinates in mm, relative to bregma: A/P: -3.25; M/L $\pm$ 0.5; D/V: (-4.9) – (-4.4), total volume 0.5 µL into each side. A/P coordinates were adjusted for Bregma/Lambda distances using a correction factor of 4.2 mm.

Behavior:

Locomotor activity: 4 weeks after surgery, baseline locomotion was measured using locomotion chambers (Columbus instruments) that use infrared beam breaks to calculate ambulatory activity. Mice were singly housed in Allentown cages with reduced corncob bedding and provided with *ad libitum* access to food and water. Locomotion was monitored continuously for 3 nights 2 days

Open field testing: Mice were placed in a large circular arena (120 cm diameter) and activity was recorded for a period of 10 min using Ethovision software. Time in center, time on edge, and total distance were calculated.

*Operant conditioning***:** Mice were tested on an operant conditioning paradigm in Med Associates boxes in the following order: FR1, FR5, Progressive Ratio, Reinstatement and Extinction. Each

fixed ratio 1 (FR1) session lasted for 60 min. Levers were extended and remained extended until a lever press. Upon a lever press, levers were retracted and a sucrose pellet was immediately delivered into the food hopper. The levers did not extend again until the mouse made a head entry into the food hopper to retrieve the pellet. Reinforced FR1 sessions lasted for 3 days, followed by 3 days of FR5 (5 lever presses required to obtain sucrose pellet). Unfortunately, data for several controls and sgUnc5c mice were lost during equipment relocation, and so only 2 days of FR5 were analyzed. Following FR1 and FR5, mice received a single day of progressive ratio where the number of lever presses necessary for sucrose pellet delivery increases non-arithmetically (i.e., 1, 2, 4, 6, 9, 13…) over the course of the session. The progressive ratio session ended after 3 consecutive min of no lever presses or after 3 hours. After progressive ratio, mice again underwent FR1 reinforced training, followed by extinction for 60 min each session for five days. Here, levers extend and retract similarly to the FR1 reinforced paradigm, yet a sucrose pellet reward is omitted.

Acoustic startle and Prepulse inhibition: Acoustic startle responses were measured using acoustic startle chambers (San Diego Instruments). Prior to testing mice received a 10-min habituation period. Background noise was maintained at 65 dB throughout testing. After habituation, mice were presented with 5, 40-ms duration 120 dB, pulse-alone trials to obtain baseline startle responses, followed by 50 trials of either a startle pulse-alone, 1 of 3 prepulse trials, or a null trial, in which no acoustic stimulus is presented. Startle trials consisted of a 40 ms, 120-dB pulse of white noise. The 3 prepulse trials consisted of a 20-ms prepulse of 70-, 75-, or 80-dB intensity (5, 10, and 15 dB above background) that preceded 120-dB startle pulse by 100 ms. Peak amplitude of the startle response (65 ms after pulse onset) was used as the measure of startle response magnitude.

Nestlet shredding: Mice were placed in a clean cage with a previously weighed cotton fiber nestlet identical to those used in home cages and familiar to the subjects. The nestlet (or largest unshredded portion) was removed from the cage and weighed after 15, 30, 60 (1 hour), 120 (2 hours), 240 (4 hours) and 360 minutes (6 hours). After each weighing, the nestlet was placed back into the center of the cage. All testing was performed during the day cycle, in low light.

Social preference/social recall: In this social interaction task, a mouse is placed in a three-chambered arena with two metal mesh cups (one at each far chamber). After a ten-minute habituation, a target mouse of the same sex is placed under one cup and the experimental mouse is allowed to explore each of the three chambers freely (Social Preference). After 30 minutes, a novel target mouse is placed under the remaining empty cup, and the experimental mouse is allowed to explore for an additional 10 minutes (Social recall). Mice will traditionally prefer to spend time with a novel mouse over the empty cup (social preference), and a novel mouse over one with which they have interacted (social preference). Data are presented as a ratio of time spent interacting with novel target/time spent interacting with familiar target.

Novel Object Recognition: In this two-day task, mice are placed in a three chambered arena, allowed to habituate for 10 minutes, and then moved to a holding cage. Two identical objects are be placed in opposite arena chambers. Mice are then placed back into the apparatus and allowed to explore and interact with the objects for 30 minutes. The next day, mice are placed back in the three chambered arena for a 10 minute acclimation and moved to a holding cage. The object mice were exposed to the previous day is be placed in one arena chamber, and a novel object is placed in the corresponding opposite chamber. The mouse is then placed back into the apparatus and allowed to explore for 30 minutes. Data are presented as a ratio of time spent interacting with novel target/time spent interacting with familiar target.

Statistics: Data were analyzed for statistical significance using GraphPad Prism. All statistical tests were two-sided and corrected for multiple comparisons where appropriate. All graphical data are presented as mean ± SEM.

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Author contributions: MMC, MS and LSZ conceived and designed experiments. MMC, ACH, and LZ designed and generated AAVs. MMC, BJ and GE performed behavioral testing and analysis. MMC performed all surgeries and in situ hybridization experiments. All authors provided input and approval of the manuscript.

Chapter 4. WHERE WILL NETRIN GUIDE US NEXT? CONCLUSIONS AND FUTURE DIRECTIONS

Here, I have shown that the gene encoding netrin-1 is expressed in both GABA and dopamine producing neurons of the adult VTA, and netrin-1 functions in this context as a regulator of glutamatergic synaptic connectivity. *Ntn1* loss of function in adult mice through CRISPR mutagenesis in either dopamine or GABA producing neurons resulted in significant disruption to the excitatory/inhibitory balance of VTA circuitry, and caused significant alterations in dopamine-mediated behaviors. Two netrin receptors are present in dopamine neurons of the adult VTA: Dcc and Unc5c. The behavioral impact of Dcc and Unc5c loss of function was also investigated, and loss of either receptor resulted in significant impairments on a variety of dopamine-mediated tasks, though neither receptor knockout directly phenocopied the loss of *Ntn1* function from VTA dopamine neurons. Potential mechanisms, future directions and the implications of this work are addressed below.

4.1 POTENTIAL MECHANISMS

While we have shown that netrin-1 functions as a regulator of glutamatergic input in the adult VTA, the molecular and physiological mechanisms through which this regulation is achieved are unknown. Three possible explanations seem likely given what we know regarding netrin function in neurodevelopment and in the adult brain: 1) synaptic scaling, 2) actin/cytoskeletal dynamics, and 3) synaptic stabilization through transsynaptic adhesion.

4.1.1 Synaptic scaling

Previous work by the Kennedy lab has shown direct netrin-1 application is capable of recruiting GluA1 AMPA receptors to the postsynaptic membrane through CaMKII activation in the context of adult CA1 hippocampal cells[28]. If netrin-1 were performing a similar function in adult VTA GABA and dopamine neurons, loss of netrin-1 function would explain why we saw a significant decrease in excitatory postsynaptic amplitude in these cells, as decreased AMPA receptor availability results in a corresponding decrease in mEPSC amplitude[91].

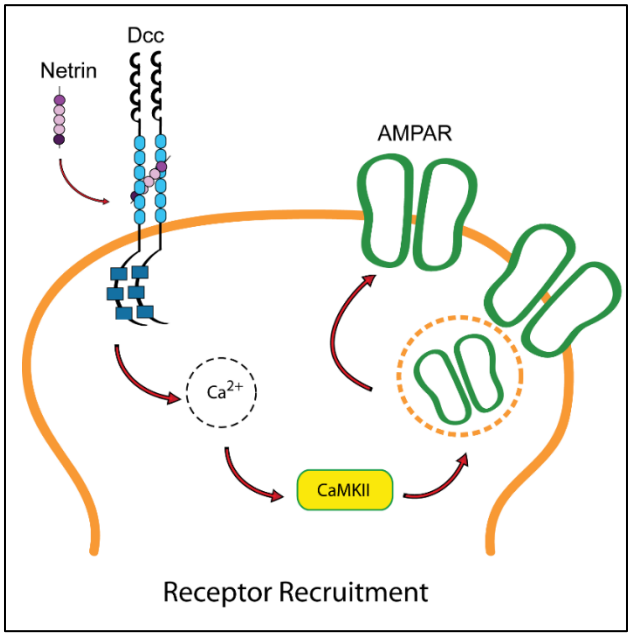

Figure 4.1 Synaptic Scaling as a potential mechanism of netrin-1 mediated regulation of excitatory synaptic connectivity

Netrin signaling through Dcc receptors could increase the recruitment of AMPA receptors to the postsynaptic membrane through the activation of CaMKII by increased intracellular calcium.

4.1.2 Actin/cytoskeletal dynamics

In addition to a reduction in mEPSC amplitude, we also recorded a significant decrease in mEPSC frequency. While postsynaptic current amplitude is typically reflective of receptor number and availability, postsynaptic current frequency is indicative of the number of active synapses. Our observed reduction in mEPSC frequency could be explained by netrin's well-characterized function as a regulator of actin dynamics. Netrin-1 signaling through Dcc induces

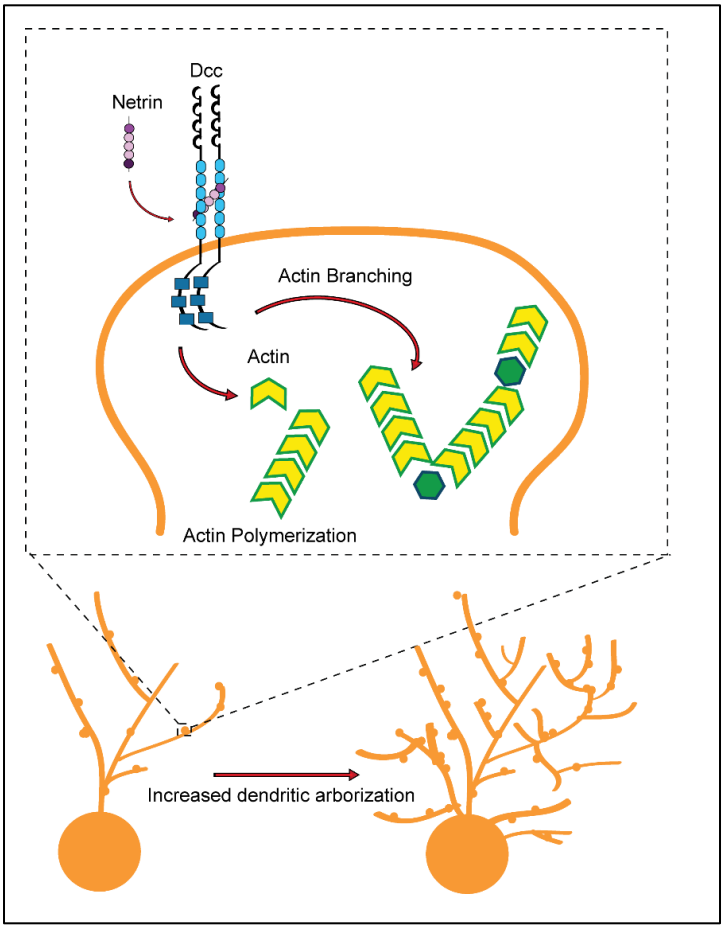

Figure 4.2 Actin/cytoskeletal dynamics as a potential mechanism of netrin-1 mediated regulation of excitatory synaptic connectivity

Netrin-1 increasing dendritic arborization and complexity through actin polymerization and branching, resulting in increased sites of synaptic connectivity.

significant cytoskeletal changes, including actin polymerization and branching (see Figure 1.3). These same mechanisms have been identified in mature cultured neurons, and are capable of increasing dendritic arborization and complexity[24]. Netrin-1 functioning in such a manner in the adult VTA would explain our findings; if netrin-1 signaling were capable of increasing dendritic arborization and potential points of synaptic contact in the adult VTA, netrin-1 loss could thus result in a decrease in available synapses compared to controls and a corresponding reduction in mEPSC frequency.

4.1.3 Synaptic stabilization through cell-adhesion:

Finally, there is existing evidence to suggest netrin-1 is capable of haptotaxis[11,92], and could potentiality stabilize synaptic connections through trans-synaptic cellular adhesion involving the Dcc receptor[93]. There exists a possibility, then, that netrin-1 is capable of forming a post and pre-

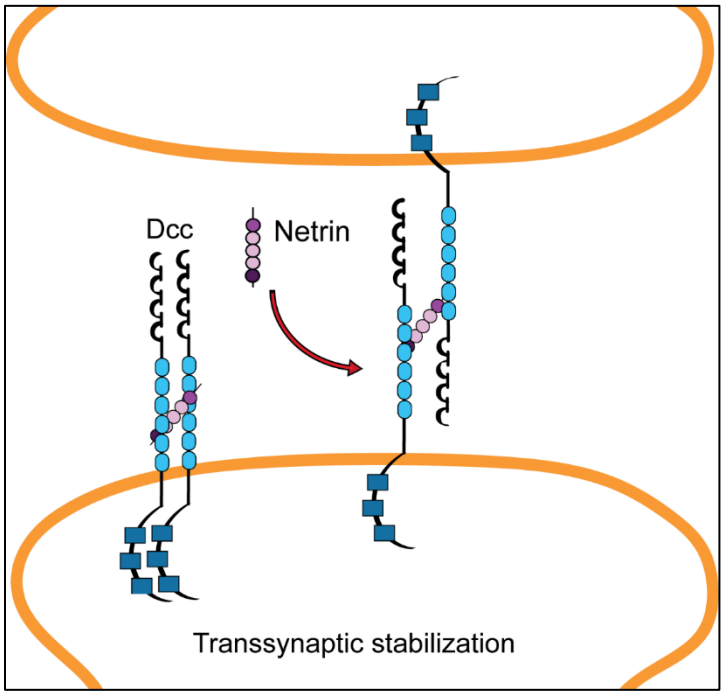

Figure 4.3 Synaptic stabilization through cell-adhesion
Netrin-1 forming a transsynaptic stabilization complex by binding pre- and postsynaptic Dcc receptors

synaptic receptor complex, stabilizing individual synaptic connections, and loss of netrin-1 results in a loss of this stabilization and subsequent reduction in synaptic connections.

Each of these three possibilities are supported by current evidence in the research field, but perhaps the most likely scenario is some combination of all three. Netrin-1 could function as s synaptic regulator through a combination of actin polymerization (increasing or decreasing axonal and dendritic complexity and synapse number), receptor recruitment (actively recruiting AMPA receptors to preexisting or nascent synapses to increase postsynaptic response to glutamatergic signaling), and synaptic stabilization (forming transsynaptic connections with pre-synaptic protein complexes to stabilize synaptic connections). Teasing apart the direct role of netrin and its receptors in each of these scenarios would require careful and precise cellular manipulations.

4.2 RECEPTOR INVOLVEMENT

While the work presented here has helped to uncover previously unknown mechanisms of netrin and netrin receptor signaling function in the adult brain, many questions remain. It is still unclear how the netrin receptors Dcc and Unc5c fit into this model. While netrin-1/Dcc interactions cause the formation of a Dcc homodimer, Unc5 receptors are capable of forming both homodimers and heterodimers in conjunction with Dcc in the presence of netrin-1 (See Figure 1.2). Netrin-1 signaling in either Unc5 configuration has been shown to have signaling mechanisms that directly *oppose* those associated with netrin-1/Dcc signaling[11,15] (i.e., chemorepulsion as opposed to chemoattraction, actin depolymerization as opposed to actin polymerization). In a brain region well known for its plasticity, it is tempting to propose a model in which netrin could simultaneously be responsible for both enhancing and reducing excitatory

VTA synaptic input depending on the availability of Unc5c receptors. In such a model, the lack of any Unc5c on the synaptic surface would favor netrin/Dcc signaling and enhance excitatory synaptic input. Conversely, Unc5c receptor availability would favor netrin/Unc5c signaling, even in the presence of Dcc, and potentially reduce excitatory input. If this model were correct, investigation into what factors are responsible for upregulating/downregulating Unc5 receptor expression would be crucial.

While netrin-1 is the only ligand for Unc5c currently identified, an additional ligand for Dcc exists and is present in the adult VTA; cerebellin 4 (*Cbln4*)[94]. Cerebellin4 is capable of forming a transsynaptic cell-adhesion complex with Dcc and neogenin-1, and could potentially act as a regulator of synaptic plasticity in its own right[94,95]. What role, if any, does cerebellin4 play in this netrin/Dcc/Unc5c model? It would seem unlikely that the two ligands are competing for binding domains in the same cell, as netrin-1 binds at a 5 fold higher affinity to Dcc[94]. Nevertheless, there exists the possibility that these two ligands and their receptors function together in some fashion to alter VTA synaptic connectivity.

4.3 CONCLUSION

More research uncovering the role of netrin and netrin receptors in the adult brain remains, but one thing is clear; netrin-1 continues to play an important role in the brain long after the central nervous system has formed. Elucidating the function of netrin-1 and the netrin receptors Dcc and Unc5c in the adult brain will not only shed light on the molecular mechanisms responsible for regulating synaptic connectivity but could provide potential targets for future therapeutic development.

BIBLIOGRAPHY

1. Flores C. Role of netrin-1 in the organization and function of the mesocorticolimbic dopamine system. *Journal of Psychiatry and Neuroscience*. 2011;36(5):296-310. doi:10.1503/jpn.100171

2. Van Battum EY, Brignani S, Pasterkamp RJ. Axon guidance proteins in neurological disorders. *The Lancet Neurology*. 2015;14(5):532-546. doi:10.1016/S1474-4422(14)70257-1

3. Dichter GS, Damiano CA, Allen JA. Reward circuitry dysfunction in psychiatric and neurodevelopmental disorders and genetic syndromes: animal models and clinical findings. *J Neurodevelop Disord*. 2012;4(1):19. doi:10.1186/1866-1955-4-19

4. Taylor AM, Menon S, Gupton SL. Passive microfluidic chamber for long-term imaging of axon guidance in response to soluble gradients. *Lab Chip*. 2015;15(13):2781-2789. doi:10.1039/C5LC00503E

5. Manitt C, Labelle-Dumais C, Eng C, et al. Peri-pubertal emergence of UNC-5 homologue expression by dopamine neurons in rodents. *PLoS ONE*. 2010;5(7). doi:10.1371/journal.pone.0011463

6. Livesey FJ, Hunt SP. Netrin and Netrin Receptor Expression in the. *Molecular and Cellular Neuroscience*. 1997;429(6):417-429.

7. Volenec A, Zetterström TSC, Flanigan TP. 6-OHDA denervation substantially decreases DCC mRNA levels in rat substantia nigra compacta. *NeuroReport*. 1998;9(16):3553-3556. doi:10.1097/00001756-199811160-00002

8. Hedgecock EM, Culotti JG, Hall DH. The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans. *Neuron*. 1990;4(1):61-85. doi:10.1016/0896-6273(90)90444-K

9. Serafini T, Kennedy TE, Gaiko MJ, Mirzayan C, Jessell TM, Tessier-Lavigne M. The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. *Cell*. 1994;78(3):409-424. doi:10.1016/0092-8674(94)90420-0

10. Sun KLW, Correia JP, Kennedy TE. Netrins: versatile extracellular cues with diverse functions. *Development*. 2011;138(11):2153-2169. doi:10.1242/dev.044529

11. Boyer NP, Gupton SL. Revisiting Netrin-1: One Who Guides (Axons). *Front Cell Neurosci*. 2018;12:221. doi:10.3389/fncel.2018.00221

12. Finci L, Zhang Y, Meijers R, Wang JH. Signaling mechanism of the netrin-1 receptor DCC in axon guidance. *Progress in Biophysics and Molecular Biology*. 2015;118(3):153-160. doi:10.1016/j.pbiomolbio.2015.04.001

13. Hong K, Hinck L, Nishiyama M, Poo M ming, Tessier-Lavigne M, Stein E. A Ligand-Gated Association between Cytoplasmic Domains of UNC5 and DCC Family Receptors Converts Netrin-Induced Growth Cone Attraction to Repulsion. *Cell*. 1999;97(7):927-941. doi:10.1016/S0092-8674(00)80804-1

14. Muramatsu R, Nakahara S, Ichikawa J, Watanabe K, Matsuki N, Koyama R. The ratio of 'deleted in colorectal cancer' to 'uncoordinated-5A' netrin-1 receptors on the growth cone regulates mossy fibre directionality. *Brain*. 2010;133(1):60-75. doi:10.1093/brain/awp266

15. Keleman K, Dickson BJ. Short- and Long-Range Repulsion by the Drosophila Unc5 Netrin Receptor. *Neuron*. 2001;32(4):605-617. doi:10.1016/S0896-6273(01)00505-0

16. Winberg ML, Mitchell KJ, Goodman CS. Genetic analysis of the mechanisms controlling target selection: Complementary and combinatorial functions of netrins, semaphorins, and IgCAMs. *Cell*. 1998;93(4):581-591. doi:10.1016/S0092-8674(00)81187-3

17. Dent EW. Netrin-1 and Semaphorin 3A Promote or Inhibit Cortical Axon Branching, Respectively, by Reorganization of the Cytoskeleton. *Journal of Neuroscience*. 2004;24(12):3002-3012. doi:10.1523/JNEUROSCI.4963-03.2004

18. Tang F, Kalil K. Netrin-1 induces axon branching in developing cortical neurons by frequency-dependent calcium signaling pathways. *Journal of Neuroscience*. 2005;25(28):6702-6715. doi:10.1523/JNEUROSCI.0871-05.2005

19. Kolodziej PA, Timpe LC, Mitchell KJ, et al. frazzled Encodes a Drosophila member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. *Cell*. 1996;87(2):197-204. doi:10.1016/S0092-8674(00)81338-0

20. Mitchell KJ, Doyle L. JL, Serafini T, et al. Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons. *Neuron*. 1996;17(2):203-215. doi:10.1016/S0896-6273(00)80153-1

21. Dent EW. Netrin-1 and Semaphorin 3A Promote or Inhibit Cortical Axon Branching, Respectively, by Reorganization of the Cytoskeleton. *Journal of Neuroscience*. 2004;24(12):3002-3012. doi:10.1523/jneurosci.4963-03.2004

22. Kolodziej PA, Timpe LC, Mitchell KJ, et al. frazzled Encodes a Drosophila member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. *Cell*. 1996;87(2):197-204. doi:10.1016/S0092-8674(00)81338-0

23. Mitchell KJ, Doyle L. JL, Serafini T, et al. Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons. *Neuron*. 1996;17(2):203-215. doi:10.1016/S0896-6273(00)80153-1

24. Goldman JS, Ashour MA, Magdesian MH, et al. Netrin-1 Promotes Excitatory Synaptogenesis between Cortical Neurons by Initiating Synapse Assembly. *Journal of Neuroscience*. 2013;33(44):17278-17289. doi:10.1523/jneurosci.1085-13.2013

25. Lein ES, Hawrylycz MJ, Ao N, et al. Genome-wide atlas of gene expression in the adult mouse brain. *Nature*. 2007;445(7124):168-176. doi:10.1038/nature05453

26. Glasgow SD, Labrecque S, Beamish IV, et al. Activity-Dependent Netrin-1 Secretion Drives Synaptic Insertion of GluA1-Containing AMPA Receptors in the Hippocampus. *Cell Reports*. 2018;25(1):168-182.e6. doi:10.1016/j.celrep.2018.09.028

27. Wong EW, Glasgow SD, Trigiani LJ, et al. Spatial memory formation requires netrin-1 expression by neurons in the adult mammalian brain. *Learning & Memory*. 2019;26(3):77-83. doi:10.1101/lm.049072.118

28. Glasgow SD, Labrecque S, Beamish I V., et al. Activity-Dependent Netrin-1 Secretion Drives Synaptic Insertion of GluA1-Containing AMPA Receptors in the Hippocampus. *Cell Reports*. 2018;25(1):168-182.e6. doi:10.1016/j.celrep.2018.09.028

29. Horn KE, Glasgow SD, Gobert D, et al. DCC Expression by Neurons Regulates Synaptic Plasticity in the Adult Brain. *Cell Reports*. 2013;3(1):173-185. doi:10.1016/j.celrep.2012.12.005

30. Turrigiano GG. The Self-Tuning Neuron: Synaptic Scaling of Excitatory Synapses. *Cell*. 2008;135(3):422-435. doi:10.1016/j.cell.2008.10.008

31. Wondolowski J, Dickman D. Emerging links between homeostatic synaptic plasticity and neurological disease. *Front Cell Neurosci*. 2013;7. doi:10.3389/fncel.2013.00223

32. Moulin TC, Schiöth HB. Excitability, synaptic balance, and addiction: The homeostatic dynamics of ionotropic glutamatergic receptors in VTA after cocaine exposure. *Behav Brain Funct*. 2020;16(1):6. doi:10.1186/s12993-020-00168-4

33. Kavalali ET, Monteggia LM. Targeting Homeostatic Synaptic Plasticity for Treatment of Mood Disorders. *Neuron*. 2020;106(5):715-726. doi:10.1016/j.neuron.2020.05.015

34. Bin JM, Han D, Lai Wing Sun K, et al. Complete Loss of Netrin-1 Results in Embryonic Lethality and Severe Axon Guidance Defects without Increased Neural Cell Death. *Cell Reports*. 2015;12(7):1099-1106. doi:10.1016/j.celrep.2015.07.028

35. Wang Z, Li P, Wu T, Zhu S, Deng L, Cui G. Axon guidance pathway genes are associated with schizophrenia risk. *Experimental and Therapeutic Medicine*. Published online 2018:4519-4526. doi:10.3892/etm.2018.6781

36. Tang J, Chen X, Cai B, Chen G. A logical relationship for schizophrenia, bipolar, and major depressive disorder. Part 4: Evidence from chromosome 4 high-density association screen. *Journal of Comparative Neurology*. 2019;527(2):392-405. doi:10.1002/cne.24543

37. Grant A, Fathalli F, Rouleau G, Joober R, Flores C. Association between schizophrenia and genetic variation in DCC: A case-control study. *Schizophrenia Research*. 2012;137(1-3):26-31. doi:10.1016/j.schres.2012.02.023

38. Zeng Y, Navarro P, Fernandez-Pujals AM, et al. A Combined Pathway and Regional Heritability Analysis Indicates NETRIN1 Pathway Is Associated With Major Depressive Disorder. *Biological Psychiatry*. 2017;81(4):336-346. doi:10.1016/j.biopsych.2016.04.017

39. Vosberg DE, Leyton M, Flores C. The Netrin-1/DCC guidance system: dopamine pathway maturation and psychiatric disorders emerging in adolescence. *Mol Psychiatry*. 2020;25(2):297-307. doi:10.1038/s41380-019-0561-7

40. Torres-Berrío A, Hernandez G, Nestler EJ, Flores C. The Netrin-1/DCC Guidance Cue Pathway as a Molecular Target in Depression: Translational Evidence. *Biological Psychiatry*. 2020;88(8):611-624. doi:10.1016/j.biopsych.2020.04.025

41. Li HJ, Qu N, Hui L, et al. Further confirmation of netrin 1 receptor (DCC) as a depression risk gene via integrations of multi-omics data. *Transl Psychiatry*. 2020;10(1):98. doi:10.1038/s41398-020-0777-y

42. Vosberg DE, Zhang Y, Menegaux A, et al. Mesocorticolimbic Connectivity and Volumetric Alterations in *DCC* Mutation Carriers. *J Neurosci*. 2018;38(20):4655-4665. doi:10.1523/JNEUROSCI.3251-17.2018

43. 23and Me Research Team, eQTLgen Consortium, International Cannabis Consortium, et al. Genome-wide association analyses of risk tolerance and risky behaviors in over 1 million individuals identify hundreds of loci and shared genetic influences. *Nat Genet*. 2019;51(2):245-257. doi:10.1038/s41588-018-0309-3

44. Lesnick TG, Papapetropoulos S, Mash DC, et al. A Genomic Pathway Approach to a Complex Disease: Axon Guidance and Parkinson Disease. Leal SM, ed. *PLoS Genet*. 2007;3(6):e98. doi:10.1371/journal.pgen.0030098

45. Lesnick TG, Sorenson EJ, Ahlskog JE, et al. Beyond Parkinson Disease: Amyotrophic Lateral Sclerosis and the Axon Guidance Pathway. Callaerts P, ed. *PLoS ONE*. 2008;3(1):e1449. doi:10.1371/journal.pone.0001449

46. Lin L, Lesnick TG, Maraganore DM, Isacson O. Axon guidance and synaptic maintenance: preclinical markers for neurodegenerative disease and therapeutics. *Trends in Neurosciences*. 2009;32(3):142-149. doi:10.1016/j.tins.2008.11.006

47. Lein ES, Hawrylycz MJ, Ao N, et al. Genome-wide atlas of gene expression in the adult mouse brain. *Nature*. 2007;445(7124):168-176. doi:10.1038/nature05453

48. Morales M, Margolis EB. Ventral tegmental area: cellular heterogeneity, connectivity and behaviour. *Nat Rev Neurosci*. 2017;18(2):73-85. doi:10.1038/nrn.2016.165

49. Thomas MJ, Malenka RC. Synaptic plasticity in the mesolimbic dopamine system. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 2003;358(1432):815-819. doi:10.1098/rstb.2002.1236

50. Bonci A, Malenka RC. Properties and plasticity of excitatory synapses on dopaminergic and GABAergic cells in the ventral tegmental area. *Journal of Neuroscience*. 1999;19(10):3723-3730.

51. Fields HL, Hjelmstad GO, Margolis EB, Nicola SM. Ventral Tegmental Area Neurons in Learned Appetitive Behavior and Positive Reinforcement. *Annu Rev Neurosci*. 2007;30(1):289-316. doi:10.1146/annurev.neuro.30.051606.094341

52. Dayan P, Balleine BW. Reward, Motivation, and Reinforcement Learning. *Neuron*. 2002;36(2):285-298. doi:10.1016/S0896-6273(02)00963-7

53. Gunaydin LA, Grosenick L, Finkelstein JC, et al. Natural Neural Projection Dynamics Underlying Social Behavior. *Cell*. 2014;157(7):1535-1551. doi:10.1016/j.cell.2014.05.017

54. Soden ME, Chung AS, Cuevas B, Resnick JM, Awatramani R, Zweifel LS. Anatomic resolution of neurotransmitter-specific projections to the VTA reveals diversity of GABAergic inputs. *Nat Neurosci*. 2020;23(8):968-980. doi:10.1038/s41593-020-0657-z

55. Gore BB, Miller SM, Jo YS, et al. Roundabout receptor 2 maintains inhibitory control of the adult midbrain. *eLife*. 2017;6:1-20. doi:10.7554/eLife.23858

56. Kaufling J. Alterations and adaptation of ventral tegmental area dopaminergic neurons in animal models of depression. *Cell and Tissue Research*. Published online 2019:59-71. doi:10.1007/s00441-019-03007-9

57. Belujon P, Grace AA. Dopamine System Dysregulation in Major Depressive Disorders. *International Journal of Neuropsychopharmacology*. 2017;20(12):1036-1046. doi:10.1093/ijnp/pyx056

58. Sonnenschein SF, Gomes FV, Grace AA. Dysregulation of Midbrain Dopamine System and the Pathophysiology of Schizophrenia. *Front Psychiatry*. 2020;11:613. doi:10.3389/fpsyt.2020.00613

59. Ostroumov A, Dani JA. Inhibitory Plasticity of Mesocorticolimbic Circuits in Addiction and Mental Illness. *Trends in Neurosciences*. 2018;41(12):898-910. doi:10.1016/j.tins.2018.07.014

60. Yu X, Ba W, Zhao G, et al. Dysfunction of ventral tegmental area GABA neurons causes mania-like behavior. *Mol Psychiatry*. 2021;26(9):5213-5228. doi:10.1038/s41380-020-0810-9

61. Flores C. Role of netrin-1 in the organization and function of the mesocorticolimbic dopamine system. *Journal of Psychiatry and Neuroscience*. 2011;36(5):296-310. doi:10.1503/jpn.100171

62. Horn KE, Glasgow SD, Gobert D, et al. DCC Expression by Neurons Regulates Synaptic Plasticity in the Adult Brain. *Cell Reports*. 2013;3(1):173-185. doi:10.1016/j.celrep.2012.12.005

63. Manitt C, Labelle-Dumais C, Eng C, et al. Peri-pubertal emergence of UNC-5 homologue expression by dopamine neurons in rodents. *PLoS ONE*. 2010;5(7). doi:10.1371/journal.pone.0011463

64. Yetnikoff L, Pokinko M, Arvanitogiannis A, Flores C. Adolescence: A time of transition for the phenotype of dcc heterozygous mice. *Psychopharmacology*. 2014;231(8):1705-1714. doi:10.1007/s00213-013-3083-z

65. Flores C, Manitt C, Rodaros D, et al. Netrin receptor deficient mice exhibit functional reorganization of dopaminergic systems and do not sensitize to amphetamine. *Molecular Psychiatry*. 2005;10(6):606-612. doi:10.1038/sj.mp.4001607

66. Hunker AC, Soden ME, Krayushkina D, Heymann G, Awatramani R, Zweifel LS. Conditional Single Vector CRISPR/SaCas9 Viruses for Efficient Mutagenesis in the Adult Mouse Nervous System. *Cell Reports*. 2020;30(12):4303-4316.e6. doi:10.1016/j.celrep.2020.02.092

67. Bissonette GB, Roesch MR. Development and function of the midbrain dopamine system: what we know and what we need to: Development and function of the dopamine system. *Genes, Brain and Behavior*. 2016;15(1):62-73. doi:10.1111/gbb.12257

68. Pavăl D. A Dopamine Hypothesis of Autism Spectrum Disorder. *Developmental Neuroscience*. 2017;39(5):355-360. doi:10.1159/000478725

69. Hietala J, Syvälahti E. Dopamine in Schizophrenia. *Annals of Medicine*. 1996;28(6):557-561. doi:10.3109/07853899608999120

70. Gilman SR, Chang J, Xu B, et al. Diverse types of genetic variation converge on functional gene networks involved in schizophrenia. *Nat Neurosci*. 2012;15(12):1723-1728. doi:10.1038/nn.3261

71. Gulsuner S, Walsh T, Watts AC, et al. Spatial and Temporal Mapping of De Novo Mutations in Schizophrenia to a Fetal Prefrontal Cortical Network. *Cell*. 2013;154(3):518-529. doi:10.1016/j.cell.2013.06.049

72. Gore BB, Miller SM, Jo YS, et al. Roundabout receptor 2 maintains inhibitory control of the adult midbrain. *eLife*. 2017;6:e23858. doi:10.7554/eLife.23858

73. Winberg ML, Mitchell KJ, Goodman CS. Genetic analysis of the mechanisms controlling target selection: Complementary and combinatorial functions of netrins, semaphorins, and IgCAMs. *Cell*. 1998;93(4):581-591. doi:10.1016/S0092-8674(00)81187-3

74. Yetnikoff L, Eng C, Benning S, Flores C. Netrin-1 receptor in the ventral tegmental area is required for sensitization to amphetamine. *European Journal of Neuroscience*. 2010;31(7):1292-1302. doi:10.1111/j.1460-9568.2010.07163.x

75. Flores C, Manitt C, Rodaros D, et al. Netrin receptor deficient mice exhibit functional reorganization of dopaminergic systems and do not sensitize to amphetamine. *Molecular Psychiatry*. 2005;10(6):606-612. doi:10.1038/sj.mp.4001607

76. Saal D, Dong Y, Bonci A, Malenka RC. Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. *Neuron*. 2003;37(4):577-582. doi:10.1016/S0896-6273(03)00021-7

77. Tan KR, Yvon C, Turiault M, et al. GABA Neurons of the VTA Drive Conditioned Place Aversion. *Neuron*. 2012;73(6):1173-1183. doi:10.1016/j.neuron.2012.02.015

78. Rajasekharan S, Kennedy TE. The netrin protein family. *Genome Biol*. 2009;10(9):239. doi:10.1186/gb-2009-10-9-239

79. Zarrindast, Mohammad-Reza. The Modulatory Role of Dopamine in Anxiety-like Behavior.

80. Zweifel LS, Parker JG, Lobb CJ, et al. Disruption of NMDAR-dependent burst firing by dopamine neurons provides selective assessment of phasic dopamine-dependent behavior. *Proceedings of the National Academy of Sciences*. 2009;106(18):7281-7288. doi:10.1073/pnas.0813415106

81. Hutchison MA, Gu X, Adrover MF, et al. Genetic inhibition of neurotransmission reveals role of glutamatergic input to dopamine neurons in high-effort behavior. *Mol Psychiatry*. 2018;23(5):1213-1225. doi:10.1038/mp.2017.7

82. Beutler LR, Wanat MJ, Quintana A, et al. Balanced NMDA receptor activity in dopamine D1 receptor (D1R)- and D2R-expressing medium spiny neurons is required for amphetamine sensitization. *Proceedings of the National Academy of Sciences*. 2011;108(10):4206-4211. doi:10.1073/pnas.1101424108

83. Kidd T, Brose K, Mitchell KJ, et al. Roundabout Controls Axon Crossing of the CNS Midline and Defines a Novel Subfamily of Evolutionarily Conserved Guidance Receptors. *Cell*. 1998;92(2):205-215. doi:10.1016/S0092-8674(00)80915-0

84. Kidd T, Bland KS, Goodman CS. Slit Is the Midline Repellent for the Robo Receptor in Drosophila. *Cell*. 1999;96(6):785-794. doi:10.1016/S0092-8674(00)80589-9

85. Ting JT, Daigle TL, Chen Q, Feng G. Acute Brain Slice Methods for Adult and Aging Animals: Application of Targeted Patch Clamp Analysis and Optogenetics. In: Martina M, Taverna S, eds. *Patch-Clamp Methods and Protocols*. Vol 1183. Methods in Molecular Biology. Springer New York; 2014:221-242. doi:10.1007/978-1-4939-1096-0_14

86. Cline M. Netrin-1 regulates the balance of glutamatergic connectivity in the adult ventral tegmental area. :28.

87. Hong K, Hinck L, Nishiyama M, Poo M ming, Tessier-Lavigne M, Stein E. A Ligand-Gated Association between Cytoplasmic Domains of UNC5 and DCC Family Receptors

Converts Netrin-Induced Growth Cone Attraction to Repulsion. *Cell*. 1999;97(7):927-941. doi:10.1016/S0092-8674(00)80804-1

88. Dorninger F, Zeitler G, Berger J. Nestlet Shredding and Nest Building Tests to Assess Features of Psychiatric Disorders in Mice. *BIO-PROTOCOL*. 2020;10(24). doi:10.21769/BioProtoc.3863

89. Angoa-Pérez M, Kane MJ, Briggs DI, Francescutti DM, Kuhn DM. Marble Burying and Nestlet Shredding as Tests of Repetitive, Compulsive-like Behaviors in Mice. *JoVE*. 2013;(82):50978. doi:10.3791/50978

90. Duszkiewicz AJ, McNamara CG, Takeuchi T, Genzel L. Novelty and Dopaminergic Modulation of Memory Persistence: A Tale of Two Systems. *Trends in Neurosciences*. 2019;42(2):102-114. doi:10.1016/j.tins.2018.10.002

91. O'Brien RJ, Kamboj S, Ehlers MD, Rosen KR, Fischbach GD, Huganir RL. Activity-Dependent Modulation of Synaptic AMPA Receptor Accumulation. *Neuron*. 1998;21(5):1067-1078. doi:10.1016/S0896-6273(00)80624-8

92. Moore SW, Zhang X, Lynch CD, Sheetz MP. Netrin-1 Attracts Axons through FAK-Dependent Mechanotransduction. *Journal of Neuroscience*. 2012;32(34):11574-11585. doi:10.1523/JNEUROSCI.0999-12.2012

93. Meijers R, Smock RG, Zhang Y, Wang JH. Netrin Synergizes Signaling and Adhesion through DCC. *Trends in Biochemical Sciences*. 2020;45(1):6-12. doi:10.1016/j.tibs.2019.10.005

94. Haddick PCG, Tom I, Luis E, et al. Defining the Ligand Specificity of the Deleted in Colorectal Cancer (DCC) Receptor. Key B, ed. *PLoS ONE*. 2014;9(1):e84823. doi:10.1371/journal.pone.0084823

95. Wei P, Pattarini R, Rong Y, et al. The Cbln family of proteins interact with multiple signaling pathways. *Journal of Neurochemistry*. 2012;121(5):717-729. doi:10.1111/j.1471-4159.2012.07648.x

APPENDIX A

5.1 SUPPLEMENTAL FIGURES

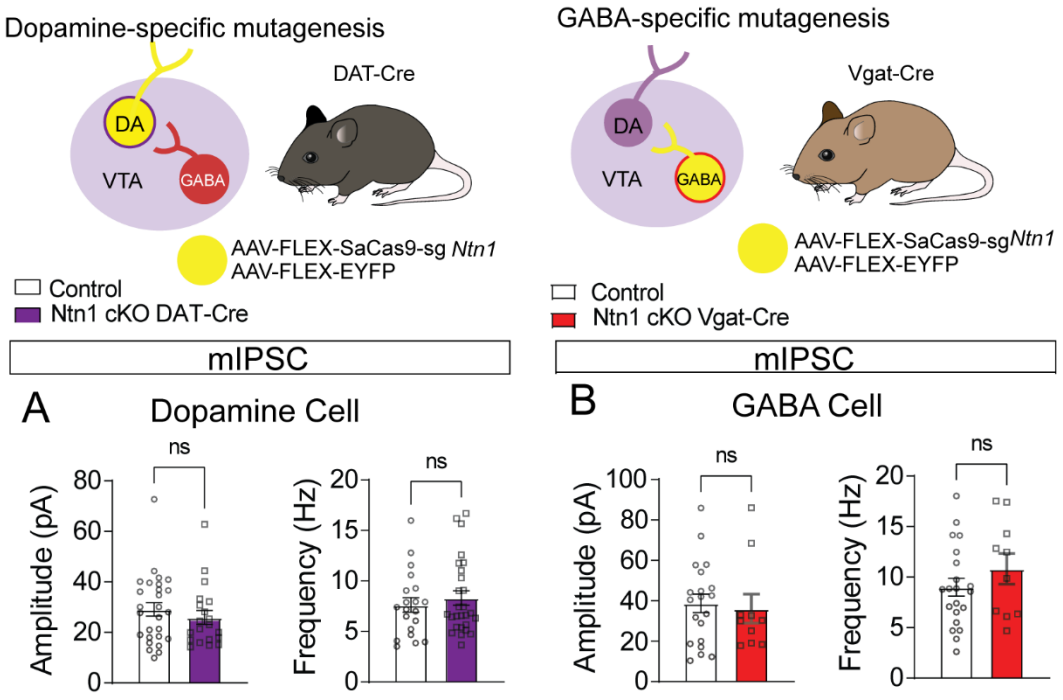

Figure 0.1, 2.3 supplemental 1: No significant differences in inhibitory synaptic connectivity associated with *Ntn1* loss of function.

A) mIPSC amplitude and frequency of DAT-Cre fluorescently identified dopamine neurons (n=20 controls, n=27 cKO). B) mIPSC amplitude and frequency of Vgat-Cre fluorescently identified GABA neurons (n=20 controls, n=10 cKO).

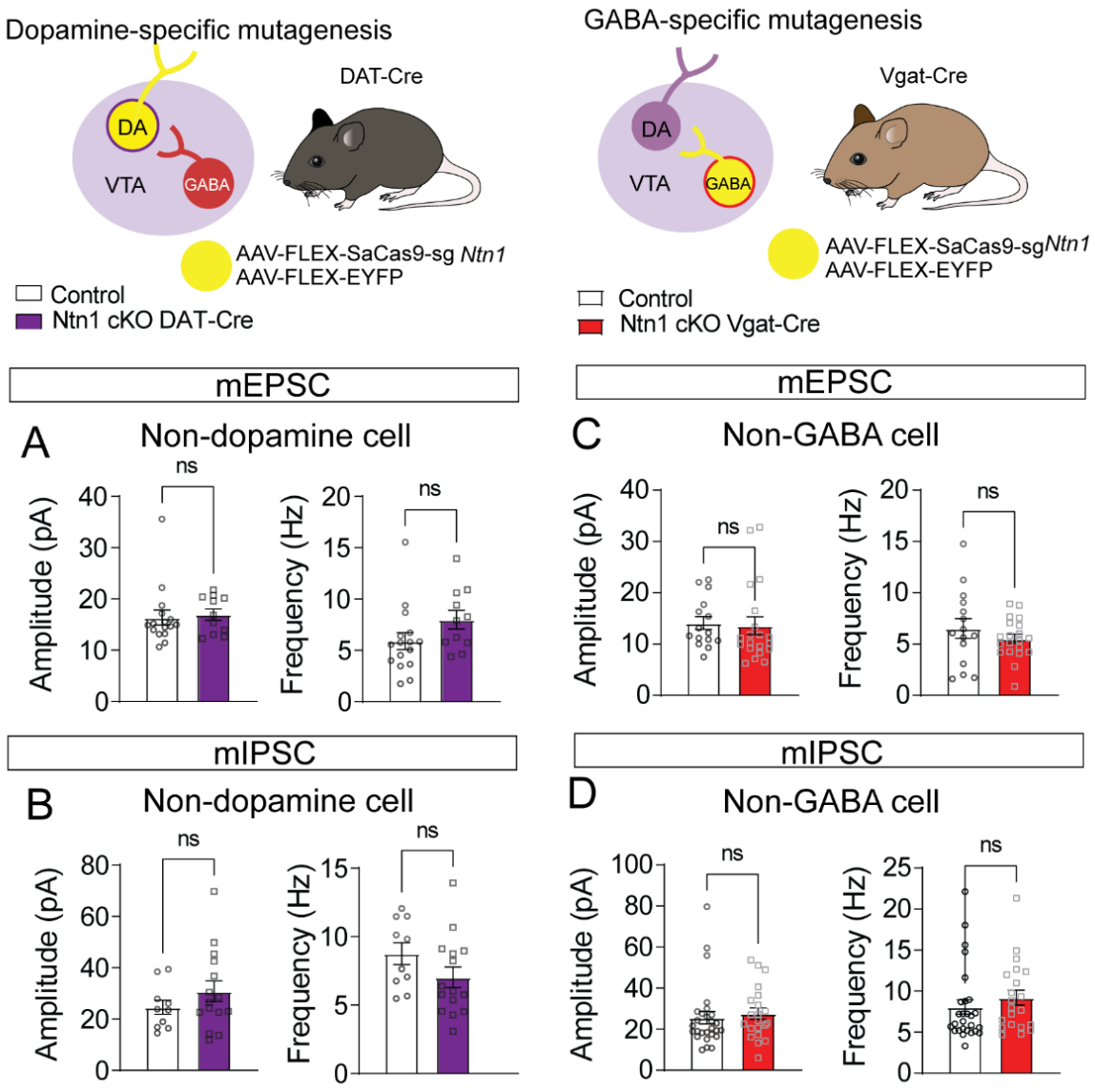

Figure 0.2, 2.3 supplement 2: No significant differences in excitatory or inhibitory synaptic connectivity in non-targeted cell types.

A) mEPSC amplitude and frequency recorded from non-fluorescent cells in DAT-Cre mice (presumptively non-dopamine neurons) (n=16 controls, n=11 cKO). B) mIPSC amplitude and frequency recorded from non-fluorescent cells in DAT-Cre mice (n=10 controls, n=15 cKO). C) mEPSC amplitude and frequency recorded from non-fluorescent cells in Vgat-Cre mice (presumptively non-GABA neurons) (n=18 controls, n=20 cKO). D) mIPSC amplitude and frequency recorded non-fluorescent cells in Vgat-Cre mice (n=27 controls, n=21 cKO).

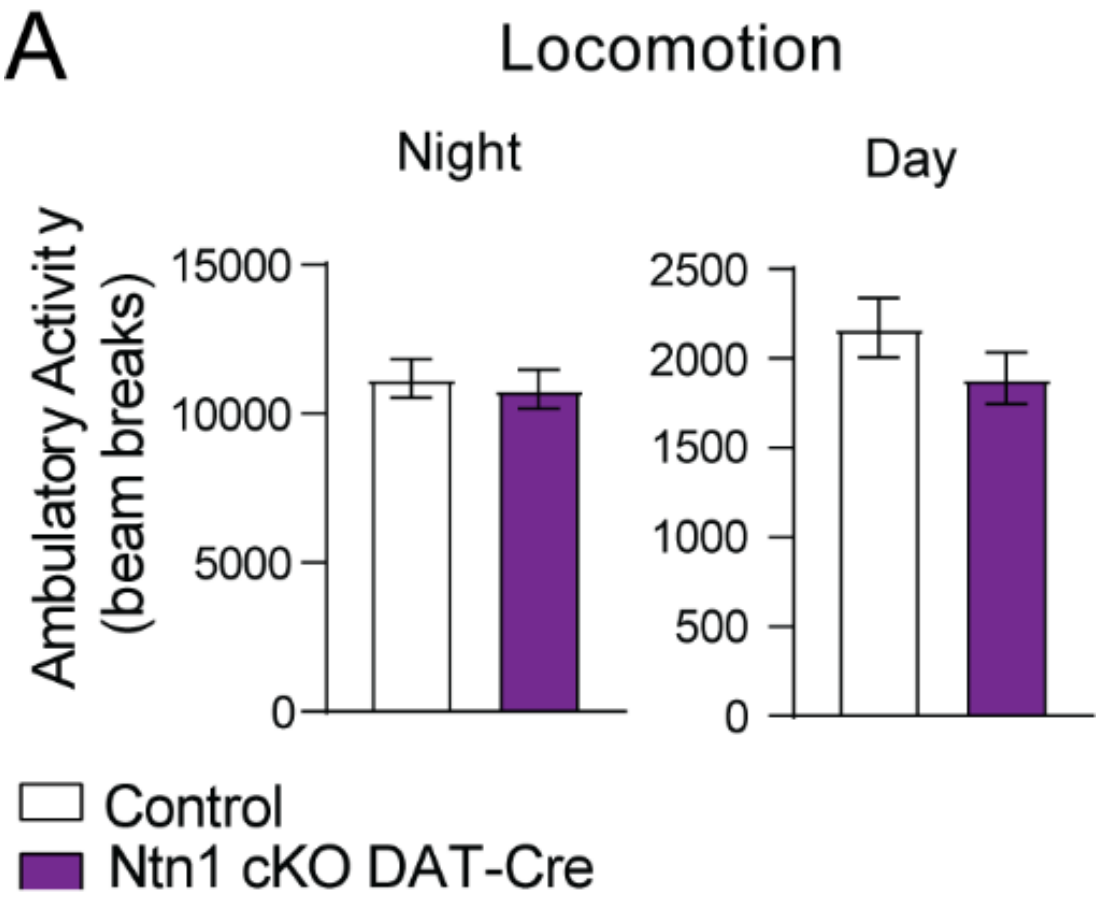

Figure 0.3 , 2.4 supplement 1: Average day and night locomotion in DAT-Cre mice.

A) Ambulatory activity (beam breaks) averaged across 3 nights and 2 days (n=21 control; n=18 cKO).

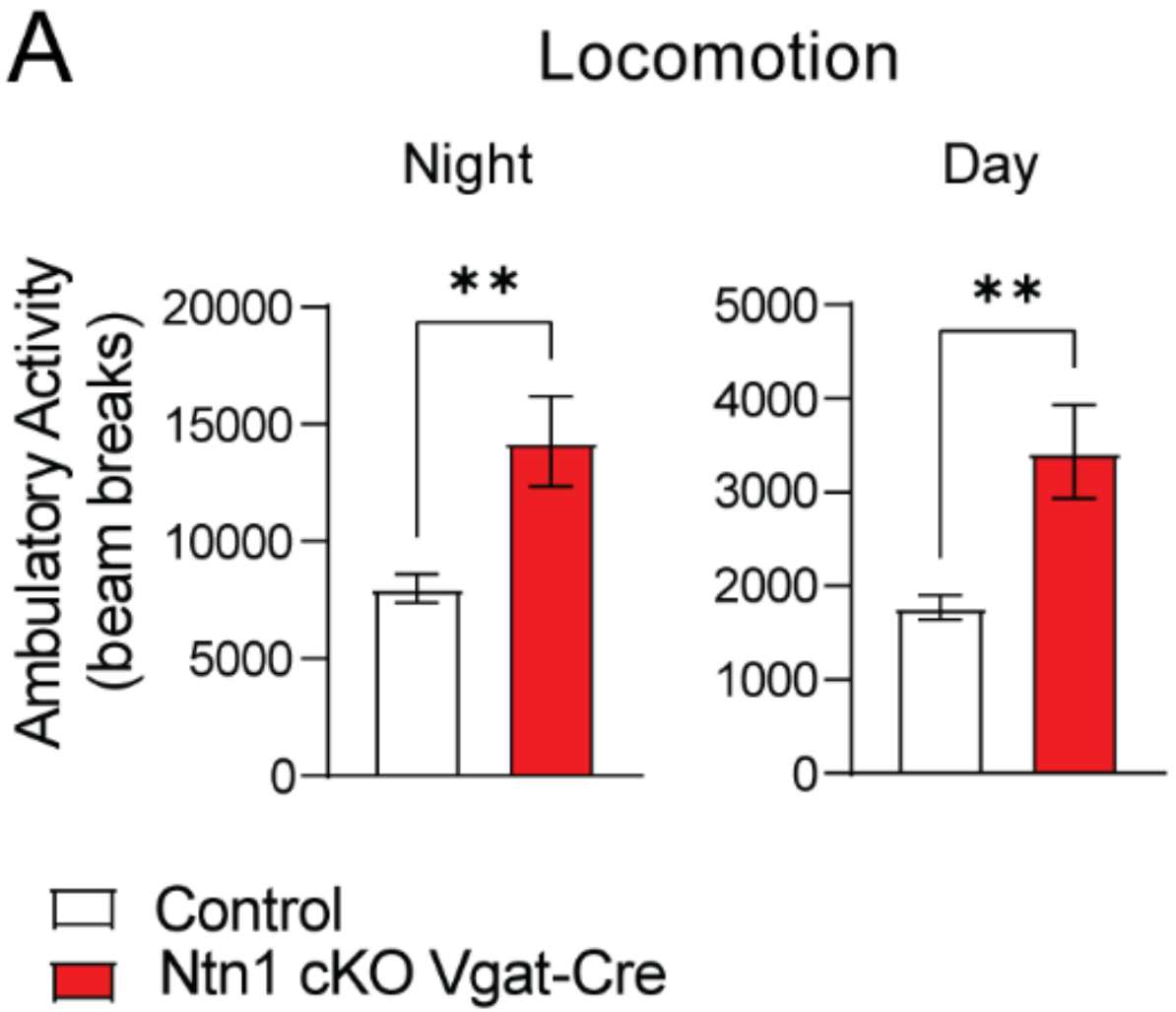

Figure 0.4, 2.5 supplement 1: Average day and night locomotion in Vgat-Cre mice.

A) Ambulatory activity (beam breaks) averaged across 3 nights and 2 days (n=26 control; n=26 cKO, (t=3.109, df=50 **p=0.01 and t=3.227, df=50 **p=0.01).

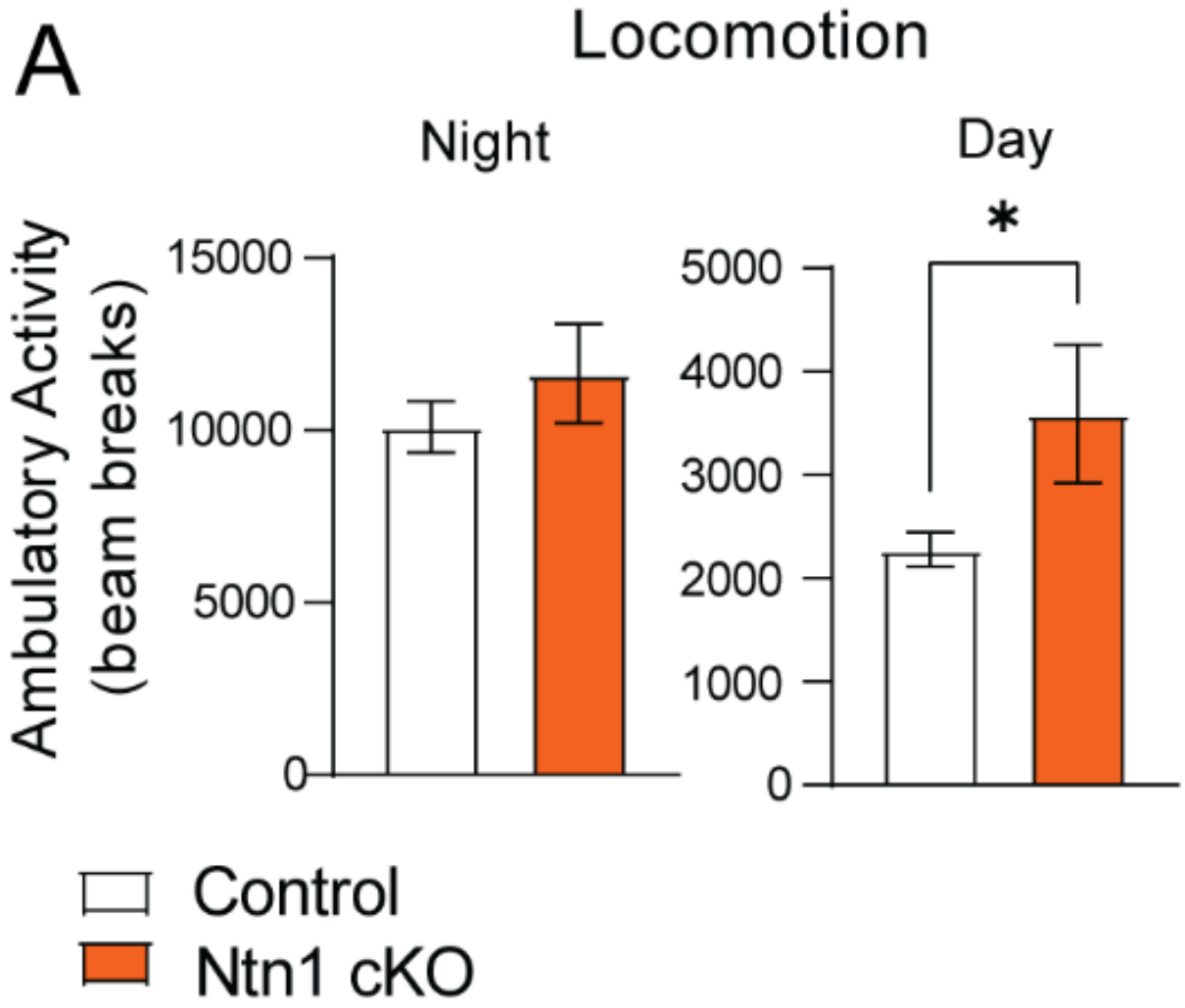

Figure 0.5, 2.6 supplement 1: Average day and night locomotion in DAT-Cre::Vgat-Cre mice.

A) Ambulatory activity (beam breaks) averaged across 3 nights and 2 days (n=26 controls n=21 cKO, t=2.091, df=45 *p<0.05).