EVALUATING THE ROLE OF A CaV2.2 SPLICE VARIANT IN BEHAVIORAL RESPONSES TO STRESS, NOVELTY, AND OTHER MONOAMINE-LINKED FUNCTIONS

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EVALUATING THE ROLE OF A Cav2.2 SPLICE VARIANT IN BEHAVIORAL
RESPONSES TO STRESS, NOVELTY, AND OTHER MONOAMINE-LINKED FUNCTIONS

BY

ASHTON BRENNECKE

BS Biology, Asbury University, 2017

THESIS

Submitted to the University of New Hampshire

in Partial Fulfillment of

the Requirements for the Degree of

Master of Science

in

Genetics

December, 2019
This thesis/dissertation has been examined and approved in partial fulfillment of the requirements for the degree of Master of Science in Genetics by:

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On November 8th, 2019

Original approval signatures are on file with the University of New Hampshire Graduate School.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>BACKGROUND</td>
<td>1</td>
</tr>
<tr>
<td>EVALUATING THE ROLE OF A Ca\textsubscript{v}2.2 SPlice VARIANT IN BEHAVIORAL RESPONSES TO STRESS, NOVELTY, AND OTHER MONOAMINE-LINKED FUNCTIONS</td>
<td>13</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>13</td>
</tr>
<tr>
<td>EXPERIMENTAL PROCEDURES</td>
<td>18</td>
</tr>
<tr>
<td>RESULTS</td>
<td>30</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>53</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>57</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>58</td>
</tr>
</tbody>
</table>
ABSTRACT

EVALUATING THE ROLE OF A Cav2.2 SPLICE VARIANT IN BEHAVIORAL RESPONSSES TO STRESS, NOVELTY, AND OTHER MONOAMINE-LINKED FUNCTIONS

by

Ashton Brennecke

University of New Hampshire, December, 2019

Cav2.2 (N-type) channel is a presynaptic channel that enables calcium influx into axon terminals, thereby playing a vital role in coupling action potentials to neurotransmitter release in the central and peripheral nervous systems. In vitro studies of Cav2.2 show that the channel directly modulates levels of the monoamines including dopamine, serotonin, and norepinephrine. Further, Cav2.2-null mice exhibit heightened aggression and hyperlocomotion linked to aberrant dopamine and serotonin function. All these studies suggest that Cav2.2 plays a pivotal role in monoaminergic function. Cav2.2 contains an alternatively spliced exon, e18a, which is either included (+18a-Cav2.2) or removed (Δ18a-Cav2.2) from the channel and alters calcium influx through Cav2.2 in vitro. +18a-Cav2.2 is expressed as the dominant isoform in midbrain monoaminergic areas including the substantia nigra, the ventral tegmental area, the dorsal raphe nuclei, and the locus coeruleus, as well as sympathetic system in the periphery. Its selective expression and modulation of Cav2.2 calcium current suggest that e18a could play a role in modulating midbrain monoaminergic communication and function. To study the potential in vivo effects of e18a, we utilized an e18a mouse where this exon was removed (Δ18a-only) and a battery of behavioral tests to assess monoamine function. We found that mice lacking 18a show deficits in their responses to inescapable stress. We have also demonstrated that these defects are
not attributed to alterations in basal exploratory behavior, appetitive reward, or nociception. Our results point at a novel role of Cav2.2 alternative splicing in stress responses and a possible link to monoamine function.
BACKGROUND

Voltage-Gated Calcium Channels

Voltage-gated calcium channels (VGCCs or CaVs) are transmembrane proteins which collectively function to allow selective entry of calcium ions into the cell. The calcium that enters through this channel carries out important functions including neurotransmitter release (Catterall, 1999; Dunlap, Luebke, & Turner, 1995), gene expression (Barbado, Fablet, Ronjat, & De Waard, 2009), mRNA stability (Schorge, Gupta, Lin, McEnery, & Lipscombe, 1999), muscle contraction (Bers, 2002; Kuo & Ehrlich, 2015), cardiac pacemaker activity (Mesirca, Torrente, & Mangoni, 2015; Perez-Reyes, 2003), and neurite outgrowth (Komuro & Rakic, 1992, 1998; Rusanescu, Qi, Thomas, Brugge, & Halegoua, 1995).

VGCCs are heteromultimeric complexes composed of multiple noncovalently interacting proteins encoded by different genes (Fig. 1). The largest member of the VGCC complex is the Cavα1 subunit, which forms the selectivity filter for calcium entry and serves as a target for toxins, drugs, and second messengers (Catterall & Striessnig, 1992; Diane Lipscombe & Andrade, 2015). The Cavα1 subunit is composed of four membrane-embedded domains (DIV-DIV) which are connected by intracellular linker regions (LI-II, LII-III, and LIII-IV). Each domain consists of six homologous transmembrane segments (S1-S6) and a re-entrant loop between S5 and S6. The S4 transmembrane segment of each domain contains charged arginines and lysines that are thought to act as voltage sensors (Dolphin, 2006). The Cavα1 subunit interacts with the auxiliary subunits include Cavβ and Cavα2δ. These subunits fine tune biophysical and pharmacological properties of the Cavα1 subunit (Walker & De Waard, 1998). The Cavβ subunits interact with the I-II linker region and their primarily role is to promote trafficking of the Cavα1 subunit to the membrane (Weiss & Zamponi, 2017), although they also
influence channel gating (Dolphin, 2006). The Ca\textsubscript{\(\alpha_2\)\(\delta\)} subunit also plays a role in cell surface expression and channel kinetics (Dolphin, 2013; Nieto-Rostro, Ramgoolam, Pratt, Kulik, & Dolphin, 2018); however, it is primarily known for mediating the analgesic action of the drugs gabapentin and pregabalin (Davies et al., 2007; Field et al., 2006). It is worth noting that a third, Ca\textsubscript{\(\gamma\)} subunit has been copurified with the Ca\textsubscript{\(\alpha_1\)} subunit and is thought to play an inhibitory role on calcium currents through Ca\textsubscript{\(\upsilon\)} (Snutch, Peloquin, Mathews, & McRory, 2005), however it has been mostly associated to AMPA receptors (Campiglio & Flucher, 2015).

**Figure 1.** Structural diagram of a voltage-gated calcium channel (VGCC, or Cav) with Ca\textsubscript{\(\alpha_1\)}, Ca\textsubscript{\(\beta\)}, and Ca\textsubscript{\(\alpha_2\)\(\delta\)} subunits (Ca\textsubscript{\(\gamma\)} subunit not shown). The Ca\textsubscript{\(\alpha_1\)} subunit is divided into four domains (DI-DIV) consisting of six transmembrane segments (SI-SVI) connected by SVI-SI intracellular linkers (LI-II, LII-III, LIII-IV). Each domain contains a putative voltage sensing region shown in red (S4) and a re-entrant S5-S6 loop, shown in dark blue. The Ca\textsubscript{\(\beta\)} subunit is entirely intracellular and interacts with the I-II linker, while the Ca\textsubscript{\(\alpha_2\)\(\delta\)} subunit associates with the channel via an extracellular glycosylphosphatidylinositol (GPI) anchor (Andrade et al., 2019).
**VGCC Genes**

Ten unique genes (*CACNA1A-I* and *S*) encode different Cavα₁ subunits, and they are subdivided into three subfamilies: Cav₁, Cav₂, and Cav₃ (Table 1). These subfamilies exhibit different pharmacological and gating properties (Tsien, Lipscombe, Madison, Bley, & Fox, 1988).

**Table 1.** Voltage-gated calcium channel nomenclature and classification based on channel physiology. Cav2.2 is highlighted in yellow.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Subunit Name</th>
<th>Current Type</th>
<th>Activation Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACNA1S</td>
<td>Cav1.1</td>
<td>α₁S</td>
<td>L-type</td>
<td>HVA</td>
</tr>
<tr>
<td>CACNA1C</td>
<td>Cav1.2</td>
<td>α₁C</td>
<td>L-type</td>
<td>HVA</td>
</tr>
<tr>
<td>CACNA1D</td>
<td>Cav1.3</td>
<td>α₁D</td>
<td>L-type</td>
<td>HVA</td>
</tr>
<tr>
<td>CACNA1F</td>
<td>Cav1.4</td>
<td>α₁F</td>
<td>L-type</td>
<td>HVA</td>
</tr>
<tr>
<td>CACNA1A</td>
<td>Cav2.1</td>
<td>α₁A</td>
<td>P/Q-type</td>
<td>HVA</td>
</tr>
<tr>
<td><strong>CACNA1B</strong></td>
<td>Cav2.2</td>
<td>α₁B</td>
<td>N-type</td>
<td>HVA</td>
</tr>
<tr>
<td>CACNA1E</td>
<td>Cav2.3</td>
<td>α₁E</td>
<td>R-type</td>
<td>HVA</td>
</tr>
<tr>
<td>CACNA1G</td>
<td>Cav3.1</td>
<td>α₁G</td>
<td>T-type</td>
<td>LVA</td>
</tr>
<tr>
<td>CACNA1H</td>
<td>Cav3.2</td>
<td>α₁H</td>
<td>T-type</td>
<td>LVA</td>
</tr>
<tr>
<td>CACNA1I</td>
<td>Cav3.3</td>
<td>α₁I</td>
<td>T-type</td>
<td>LVA</td>
</tr>
</tbody>
</table>

The Cav₁ family contains Cav₁.1-1.4, encoded by *CACNA1S* (Cav₁.1), *CACNA1C* (Cav₁.2), *CACNA1D* (Cav₁.3), and *CACNA1F* (Cav₁.4). Each of these channels is high-voltage activated (HVA) and L-type, exhibiting “long-lasting” currents. They are expressed in smooth, skeletal, and cardiac muscle as well as neurons, endocrine cells, cochlear hair cells, and retinal cells (Schampel & Kuerten, 2017). This family is also sensitive to dihydropyridines (DHPs), pharmacological agents that allosterically block L-type channels (Catterall & Striessnig, 1992) and are used in the treatment of cardiovascular disease (Godfraind, 2014).
The Cav2 gene family includes CACNA1A (Cav2.1), CACNA1B (Cav2.2), and CACNA1E (Cav2.3). Cav2.1 generates P/Q-type (“Purkinje” type) currents, Cav2.2 generates N-type (“neuronal” type) currents, and Cav2.3 generates R-type (“resistant” type) currents. These channels are both DHP-insensitive and HVA, although they respond to lower voltages compared to L-type channels (Nowycky, 1985). Cav2s are primarily neuronal and are located in the presynaptic terminals, playing a major role in coupling excitation to fast neurotransmitter release (Catterall, 2011; Reid, Bekkers, & Clements, 2003; Weyer et al., 2019; Wheeler, Randall, & Tsien, 1994).

The Cav3 subfamily is comprised of Cav3.1-3.3, encoded by CACNA1G (Cav3.1), CACNA1H (Cav3.2), and CACNA1I (Cav3.3). These channels are low-voltage activated (LVA) and produce T-type (“tiny” or “transient”) currents. They are called tiny because their single channel conductance is smaller than the other Cav3s and transient because they activate and inactivate faster than the rest of the Cav3s (Perez-Reyes, 2003). These channels are expressed in neuronal cells of the brain but can also be found in endocrine, muscular, and reproductive tissue (Catterall, 2011). Cav3 channels have been implicated in pacemaking activity of neurons (Perez-Reyes, 2003), oscillatory behavior of the thalamus (Destexhe, Neubig, Ulrich, & Huguenard, 1998), and maintenance of automaticity in the heart’s rhythmogenic centers (Mesirca et al., 2015).

The focus of my thesis is on Cav2.2 channels (highlighted in Table 1) for the reasons I will highlight in the following paragraphs.
Functional Importance of Cav2.2

Cav2.2 channels are primarily located in the presynaptic terminal of neurons in the central and peripheral nervous system (Chai et al., 2017; Yokoyama et al., 1995). As stated above, Cav2.2 is an HVA channel that opens at membrane potentials of roughly −30 to +60 mV (Thaler, Gray, & Lipscombe, 2004) and inactivates in response to maintained depolarizations (Hirning et al., 1988). This channel utilizes a synprint (SYNaptic PRotein INTeraction) recognition sequence within the II-III linker that binds SNARE (SNAP-25 Receptor) vesicular docking proteins to facilitate fusion of neurotransmitter vesicles with the presynaptic membrane (Harkins, Cahill, Powers, Tischler, & Fox, 2004; He et al., 2018; Szabo, Obermair, Cooper, Zamponi, & Flucher, 2006). These proteins include synaptotagmin-1 (SYT1), SNAP-25 (synaptosomal nerve-associated protein 25), and syntaxin 1A, as well as, active zone proteins such as RAB3A-interacting molecules (RIMs) and RIM-binding proteins (RIM-BPs) (Catterall, 1999; He et al., 2018). Cav2.2 channels are selectively and irreversibly blocked with the Conus geographus snail toxin, ω-conotoxin GVIA (ω-CgTx) (Dooley, Lupp, & Hertting, 1987; Ellinor, Zhang, Horne, & Tsien, 1994). Bathing nervous tissues and synaptosomes in ω-CgTx has been shown to decrease γ-aminobutyric acid (GABA), glutamate, acetylcholine (ACh), dopamine, and norepinephrine release, indicating the importance of Cav2.2 in multiple types of neurotransmission (Dunlap et al., 1995).

Cav2.2 channels have been directly linked to transmission of painful stimuli at the spinal cord level. Ziconotide, another potent Cav2.2 blocker created from ω-conotoxin MVIIA, is currently used to treat chronic neuropathic and inflammatory pain (S. Lee, 2013; Striessnig, 2018). Ziconotide is injected intrathecally and is thought to suppress signaling between the dorsal root ganglia nociceptive fibers and neurons located in the dorsal horn of the spinal cord by
blocking Cav2.2 channels (Bell, Thaler, Castiglioni, Helton, & Lipscombe, 2004). Ziconotide has been shown to be more effective than morphine with significantly less potential for abuse (Scott, Wright, & Angus, 2002); however, administration is uncomfortable, the drug can cause motor defects at high doses, and hypotension can occur if it enters the bloodstream (McGivern, 2007). Therefore, despite the existence of Cav2.2-specific drugs, further research is warranted to target cell- or tissue-specific Cav2.2 activity.

In addition to its role in pain, the medical and behavioral relevance of Cav2.2 has been studied in Cav2.2 knock-out mouse models (Cav2.2-KO). Superior cervical ganglia (SCG) neurons and sympathetic nerve terminals of mice lacking functional expression of Cav2.2 exhibit markedly diminished activity, and the mice themselves suffer from hypertension and lack of baroreflex (Ino et al., 2001). Additionally, Cav2.2-KO mice experience disturbances in their sleep-wake cycles, hyperactivity, and reductions in sensorimotor gating compared to wild type (WT) mice (Beuckmann, Sinton, Miyamoto, Ino, & Yanagisawa, 2003; Nakagawasai et al., 2010). These mice also exhibit behavioral changes which could be indicative of monoaminergic involvement—this topic will be discussed in greater detail in the Introduction. Taken together, the functional expression of Cav2.2 is an important component of nociception, maintenance of sympathetic homeostasis, and regulation of appropriate activity levels, as well as appropriate responses to monoaminergic communication.

*Alternative Splicing of Cacna1b*

Alternative splicing is a fascinating component of RNA processing that takes place in >90% of mammalian cells and is incredibly common in neuronal genes (Diane Lipscombe &
Lopez Soto, 2019). Within the nucleus, eukaryotic DNA is transcribed to immature pre-mRNA which contains both exons and introns. Small nuclear ribonucleoparticles (snRNPs) composed of disparate proteins come together to form the spliceosome which uses recognition sequences within introns to cut and fold the intron into a lariat shape. The spliceosome then connects the 3’ end of the first exon to the 5’ end of the second exon, thereby cleaving the intron out and precisely fusing the exons to one another (Y. Lee & Rio, 2015). In the same way, alternatively spliced exons can also be spliced out to further specialize the mRNA for a particular cellular function. Addition of a 5’ 7-methylguanosine cap and a 3’ poly-A tail creates a mature mRNA molecule which can leave the nucleus and be transcribed into a protein (Katahira, 2015). Through this process, cells dramatically increase protein diversity while maintaining a low genetic load.

As technological advancements make it easier to study alternative splicing, a clearer picture of its involvement in disease and genetic therapy has begun to emerge. Neuronal genes have been implicated in disorders such as Rett syndrome (Osenberg et al., 2018), autism (Lu, Dai, Martinez-Agosto, & Cantor, 2012; Quesnel-Vallières et al., 2016), spinal muscular atrophy (SMA) (Cartegni & Krainer, 2002), parkinsonism (Farrer, 2006; Satake et al., 2009), chronic pain (Manners, Ertel, Tian, & Ajit, 2016), and cancer (Fan et al., 2018). Alternatively spliced Ca-vs also modify the severity of diseases like spinocerebellar ataxia type 6 (SCA6) (Zhuchenko et al., 1997) and Timothy syndrome (Diane Lipscombe, 2005; Splawski et al., 2005). Taken together, alternative splicing is an important regulator of normal cell function and shows promise as a target for genetic therapy.

*Cacna1b* is the gene that encodes the α1 pore-forming subunit of Cav2.2 channels. The *Cacna1b* pre-mRNA undergoes a number of alternative splicing events which are both cell-
specific and developmentally regulated. In mouse, the channel contains at least five alternatively spliced exons (e18a, e20a, e24a, e31a, and e37a/e37b; **Figure 2**) and at least three more putative sites of alternate 5’ or 3’ splicing (D. Lipscombe, Qian Pan, & Gray, 2002). Additionally, studies of human brain and central nervous tissue have demonstrated presence of CaV2.2 isoforms which contain large deletions. One of these occurs in the II-III linker, where deletion of exons 19, 20, and 21 makes the channel more resistant to voltage-dependent inactivation and less responsive to ω-conotoxins GVIA and MVIIA (D. Lipscombe et al., 2002). Another splicing event gives rise to either a long channel form or a short form which is truncated by 187 nucleotides at the C-terminus (Williams et al., 1992). Only the long form of the channel binds neuronal Munc18-1-interacting protein 1 (Mint1) and calcium/calmodulin-dependent serine protein kinase (CASK) proteins, potentially implicating this event in CaV2.2 channel density and the effectiveness of synaptic transmission at nerve terminals (Kaneko et al., 2002; Maximov, Südhof, & Bezprozvanny, 1999).

**Figure 2.** Alternatively spliced exons of the mouse CaV2.2-α1 subunit gene, Cacna1b. Membrane embedded structure with approximate locations of alternatively spliced exons are shown (left) as well as the unfolded structure (right). CaV2.2-α1 contains at least five alternatively spliced exons: 18a and 20a in the II-III linker, 24a and 31a in the S3-S4 extracellular portions of DIII and DIV (respectively), and 37a/37b in the C-terminus.
Of the alternatively spliced exons, the most e37a/e37b are the most studied. These exons are mutually exclusive (either e37a or e37b is included in the final Cacna1b mRNA), and encode 33 amino acids of the C-terminus of the channel. E37a has been found to control Ca\textsubscript{V}2.2 gene expression and modulation by G-protein coupled receptors (GPCRs) (Raingo, Castiglioni, & Lipscombe, 2007). While e37b is extensively expressed in central and peripheral nervous tissues, e37a is abundantly expressed in the dorsal root ganglia (DRG) and is enriched in nociceptors, sensory receptors involved in the first step of pain sensing (Bell et al., 2004; Jiang, Andrade, & Lipscombe, 2013). In the pain pathway, e37a is linked to both thermal and mechanical hyperalgesia during transmission of nociceptive signals (Altier et al., 2007). In line with these findings, e37a also appears to enhance the analgesic effects of intrathecal morphine administration, although interestingly it is not involved in ziconotide-induced analgesia (Andrade, Denome, Jiang, Marangoudakis, & Lipscombe, 2010; Jiang et al., 2013). In the brain, e37a is localized in excitatory pyramidal neurons and is linked to behavioral responses to aversive stimuli (Bunda, LaCarubba, Bertolino, et al., 2019). These observations suggest that alternative splicing in calcium channel genes can fine tune VGCC expression, function, and have therapeutic relevance.

Exons 20a, 24a, and 31a are cassette exons, being either included or skipped in the mature mRNA. In mice, e20a is located in the II-III linker and encodes a premature stop codon, rendering the channel incomplete and nonfunctional (Raghib et al., 2001). This exon likely works to suppress Ca\textsubscript{V}2.2 protein expression via nonsense-mediated decay, particularly during development (Diane Lipscombe & Andrade, 2015). E24a and e31a are located in the extracellular IIIS3-IIIS4 and IVS3-IVS4 linkers, respectively. While e24a is preferentially expressed in the brain, e31a is preferentially expressed in the peripheral ganglia (Diane
Lipscombe, Allen, & Toro, 2013). This reciprocal expression pattern is due to e31a suppression by the splicing factor Nova-2 (Allen, Darnell, & Lipscombe, 2010; Diane Lipscombe, Andrade, & Allen, 2013). Compared to e24a, presence of e31a in Ca\textsubscript{v}2.2 leads to a more depolarized voltage of activation and slower channel opening (Lin, Haus, Edgerton, & Lipscombe, 1997; Lin et al., 1999). In this case, alternative splicing is used by cells of the central nervous system to specialize the function of neuronal populations.

**Characterization of e18a Splice Isoforms**

In my thesis work, I focused on the cassette exon 18a (e18a) of Ca\textsubscript{v}2.2. Alternative splicing of e18a generates two distinct Ca\textsubscript{v}2.2 variants, one which includes e18a (+18a-Cacna1b) and one which does not (Δ18a-Cacna1b). Several reasons motivated me to study this exon: i) e18a is evolutionarily conserved across vertebrates (Allen et al., 2017); ii) e18a encodes a 21-amino acid sequence that is rich in serine and threonine residues, indicating a potential target for protein kinases (Pan & Lipscombe, 2000); iii) e18a lies within the synprint region of the II-III linker, suggesting some involvement in Ca\textsubscript{v}2.2-mediated exocytosis (Catterall, 1999; Harkins et al., 2004); iv) e18a protects Ca\textsubscript{v}2.2 from cumulative inactivation following trains of action potentials, and it protects Ca\textsubscript{v}2.2 channels from closed-state inactivation (Thaler et al., 2004); v) e18a also shifts steady-state inactivation of Ca\textsubscript{v}2.2 to more depolarized potentials, making it more difficult for the channel to become inactivated (D. Lipscombe et al., 2002; Pan & Lipscombe, 2000); and vi) channels that include e18a have about twofold greater calcium current density than their Δ18a-Cav2.2 counterparts (Allen et al., 2017; see Figure 3). All these observations suggest that the splicing of e18a may have robust implications for Cav2.2 channel activity. However, the role of e18a is not known in vivo.
Figure 3. Impacts of e18a inclusion (+18a) and exclusion (Δ18a) on CaV2.2 channel physiology in HEK293 cells. Data is shown as mean ± SEM and n is shown in parentheses. Starting at a test potential of -20 mV, +18a-CaV2.2 channels demonstrate increased calcium current density (pA/pF) relative to cell size when compared with Δ18a-CaV2.2 channels. At peak current density, +18a-CaV2.2 exhibits approximately double the calcium current of Δ18a-CaV2.2 channels (Allen et al., 2017).

E18a demonstrates a unique expression pattern that changes with both development and tissue identity. During postnatal development, e18a increases in the central and peripheral nervous systems. In human whole brain samples, e18a is only expressed in about 12% of all CACNA1B mRNAs and is undetectable in fetal samples. In newborn mouse SCG, e18a inclusion is only about 35%, but over the course of development we see a gradual shift to >80% inclusion in adult (Allen et al., 2017; Pan & Lipscombe, 2000). The expression of e18a also depends on the tissue. In adult, e18a is only included in 10-30% of CACNA1B mRNAs from human cerebellum, dorsal root ganglia (DRG), hippocampus, and cortex (Allen et al., 2017). Similar observations have also been made in mouse tissue analyses. This nicely aligns with the conservation of this exon among humans and rodents. In a separate study, it was also
demonstrated that rat monoaminergic regions preferentially express the +18a-Cav2.2 variant. While results in other regions consistently expressed low levels of e18a, mean expression of +18a-Cav2.2 in monoaminergic regions including the ventral tegmental area (VTA), substantia nigra (SN), dorsal raphe (DR), and locus coeruleus (LC) was higher than Δ18a-Cav2.2 (Ghasemzadeh, Pierce, & Kalivas, 1999).

Because e18a has such a distinct pattern in monoaminergic areas of the brain and SCG, it is possible that the exon is involved in fine tuning peripheral and central monoaminergic function. The primary objective of my work was to perform a thorough behavioral characterization of mice lacking e18a (Δ18a-only) in order to better understand its role in baseline and monoamine-linked behaviors including mobility, stress coping, and drug response. A secondary objective was to use BaseScope™ *in situ* hybridization to determine the presence and extent of e18a and *Cacna1b* colocalization with dopaminergic neurons in VTA and SNc.
EVALUATING THE ROLE OF A Cav2.2 SPLICE VARIANT IN BEHAVIORAL RESPONSES TO STRESS, NOVELTY, AND OTHER MONOAMINE-LINKED FUNCTIONS

INTRODUCTION

Monoamines are powerful neuromodulators that are essential components of reward (Alhadeff et al., 2019; Schultz, 2013), motor coordination (Crocker, 1997; Rommelfanger et al., 2007), satiation (Augustine et al., 2019; Sandhu et al., 2018), stress (Belujon & Grace, 2015; Chaouloff, Berton, & Mormède, 1999; Habib, Gold, & Chrousos, 2001), attention (Del Campo, Chamberlain, Sahakian, & Robbins, 2011; Nieoullon, 2002; Weinberg-Wolf et al., 2018), arousal (Buchanan & Richerson, 2010; Haxhiu, Tolentino-Silva, Pete, Kc, & Mack, 2001), and motivational salience (Berridge, 2012; Puglisi-Allegra & Ventura, 2012), among others. This class of neurotransmitters includes dopamine, serotonin, and norepinephrine, small molecules which regulate these behaviors. Alterations in the signaling of these three monoamines are linked to neurological and psychiatric disorders including Parkinson’s disease (Dickson, 2012), addiction (Koob & Volkow, 2010), anxiety (de la Mora, Gallegos-Cari, Arizmendi-García, Marcellino, & Fuxe, 2010; Mohammad-Reza & Fatemeh, 2015; Nishitani et al., 2019), schizophrenia (Breier, 1995; Grace, 2016), depression (Grace, 2016; Moret & Briley, 2011; Owens & Nemero, 1994), and posttraumatic stress disorder (Krystal & Neumeister, 2009; Segman et al., 2002).

Dopamine, serotonin, and norepinephrine are primarily produced and released from groups of neurons located in the midbrain. Dopaminergic regions include the VTA and SNC; these neurons target many areas of the brain including the nucleus accumbens (NAc) and the
caudate putamen (CPu, or dorsal striatum) (Poulin et al., 2018). Serotonergic projections stem primarily from the DRN and project to much of the cortex as well as the hypothalamus, the amygdala, the VTA, and part of the hippocampus (Hornung, 2003). The largest collection of noradrenergic neurons exists in the LC (Schwarz & Luo, 2015). Despite its small size, this region plays a major role in many central nervous functions. Regions innervated by the LC include the DRN, the cerebellum, motor neurons of the brainstem and spinal cord, cortical regions in every lobe of the brain, and parts of the limbic system including the amygdala and hippocampus (Samuels & Szabadi, 2008). Taken together, these monoaminergic midbrain regions control numerous areas involved in mood and behavioral modulation.

Cav2.2 is richly expressed in the DRN, the SNc, the VTA, and the LC (Tanaka, Sakagami, & Kondo, 1995). As previously described, this channel is believed to play a vital role in neurotransmitter release by triggering the fusion of presynaptic vesicle docking proteins with the cellular membrane (Harkins et al., 2004). Calcium influx through Cav2.2 drives the release of dopamine in the VTA and SNc (Brimblecombe, Gracie, Platt, & Cragg, 2015) and blockade of Cav2.2 reduces dopamine efflux (Phillips & Stamford, 2000). Although we are not aware of direct evidence linking Cav2.2 to LC, selective blockade of Cav2.2 using ω-CgTx significantly hindered noradrenaline release in superior cervical ganglia (SCG) cultures (Hirning et al., 1988) and inhibited evoked tritium overflow in rabbit hippocampal slices labeled with [3H]noradrenaline (Dooley, Lupp, & Hertting, 1988), indicating that Cav2.2 could modulate noradrenaline release in central and peripheral tissues. Similarly, ω-CgTx significantly inhibited serotonin release in rat brain slices (Bagdy & Harsing, 1995; Rijnhout, Hill, & Middlemiss, 1990), although we are not aware of a direct connection between Cav2.2 and DRN serotonin release. Therefore, more work will be needed to elucidate the exact role of Cav2.2 in these areas.
Mice lacking the functional expression of Cav2.2 (or “knock-out” mice) also show phenotypes linked to alterations in monoaminergic signaling. These phenotypes include basal and novelty-induced hyperactivity (Beuckmann et al., 2003), spontaneous locomotor activity during the dark phase (Nakagawasai et al., 2010), vigilance state differences (Beuckmann et al., 2003), hyperlocomotion in response to apomorphine (a non-selective dopamine agonist) (Nakagawasai et al., 2010), diminished ethanol consumption and preference (Clayton & Hicks, 1994; Newton et al., 2004), alterations in dopamine and serotonin levels and turnover (Nakagawasai et al., 2010), and basal hyperaggression which also mimics behavioral response to DRN Cav2.2 blockage (Kim et al., 2009). This body of evidence suggests that Cav2.2 represents an important molecular target within all three of these monoaminergic pathways.

During Cacna1b gene expression, exon 18a (e18a) within the intracellular DII-DIII linker region is spliced to produce either +18a-Cav2.2 or Δ18a-Cav2.2 (Fig. 4). E18a falls within the synprint region, a sequence that targets Cav2.2 to the terminal membrane (Szabo et al., 2006), and is also believed to couple calcium influx to exocytosis via interactions with vesicular docking proteins (Harkins et al., 2004). Prior experiments have shown that e18a shifts the voltage dependence of closed-state inactivation of Cav2.2, it imparts the channel with resistance to closed-state inactivation, and it produces calcium conductance which is nearly double that of Δ18a-Cav2.2 channels (Allen et al., 2017; Thaler et al., 2004). Therefore, it is expected that channels without e18a (Δ18-Cav2.2) would release neurotransmitter less effectively than their +18a-Cav2.2 counterparts.

Previous studies provide strong evidence that the +18a-Cav2.2 splice variant is enriched in midbrain regions that produce dopamine (VTA and SNc), serotonin (DRN), and noradrenaline (LC). In an early study, RT-PCR of microdissected tissue from each of these areas shows a shift
to +18a-Cav2.2 isoform dominance when compared to regions such as prefrontal cortex, hippocampus, nucleus accumbens, and striatum (Ghasemzadeh et al., 1999). Work from a previous member of our lab also confirmed these findings using the BaseScope™ assay. In brief, this technique is a highly sensitive form of *in situ* hybridization in which mRNA transcripts as small as 50 bp can be visualized in tissue. Using probes which detected the 18:18a exon junction, Bunda and colleagues demonstrated that there is extensive e18a expression in all four of these midbrain monoaminergic areas. The expression pattern of e18a in these assays nicely aligned with the data from the Allen Brain Atlas showing expression patterns of *Slc6a3*, *Slc6a4*, and *Slc6a2*, encoding the dopamine transporter (DAT), serotonin transporter (SERT), and the norepinephrine transporter (NET), respectively (Bunda, LaCarubba, Akiki, & Andrade, 2019). These data in conjunction with previous studies of Cav2.2 in midbrain function implicate a potential role for e18a in monoaminergic function.

Despite its selective expression and modulation of Cav2.2 gating properties, the behavioral relevance of e18a has not been elucidated. To better understand the role of e18a *in vivo*, we developed a transgenic mouse model in which e18a is globally deleted from the genome and Cav2.2-Δ18a channels are constitutively expressed (Δ18a-only; **Figure 4**) (for details, see Methods).
Figure 4. Alternative splicing of Cav2.2-e18a in wild type (WT) animals and Δe18a-only (Δ18a) mutant mouse model. Exon 18a is a cassette exon which is 63 bp in length and lies between constitutively expressed exons 18 and 19. In the process of pre-mRNA splicing, WT Cacna1b can either include e18a (+e18a-Cacna1b) or skip e18a (Δe18a-Cacna1b), resulting in two forms of Cav2.2 (Cav2.2+18a or Cav2.2-Δ18a). In our mouse model, exons 18 and 19 are precisely fused together, resulting in global expression of Δe18a-Cacna1b transcripts and rendering all Cav2.2 channels Δe18a-only (Bunda, LaCarubba, Akiki, et al., 2019).
EXPERIMENTAL PROCEDURES

Animals

Male C57BL/6 mice (Charles River) were used as wild-type animals. The Δ18a-only mouse line was designed as follows. An 18a-CaV2.2 targeting construct was designed from the Sanger BAC clone BMQ175b12 (Adams et al., 2005) to induce targeted excision of e18a. About 12 Kb of mouse genomic DNA were cloned into a pBluescript II KS (-) vector and a series of sequential site-directed mutagenesis steps were used to delete e18a and the introns which flank it. These steps were used to generate a construct in which e18 and e19 were precisely fused together. A plasmid containing the loxP-NeoR-loxP cassette was amplified and a targeted insertion was performed to insert the cassette between e17 and e18. Finally, approximately 3 Kb of Murid beta herpesvirus dsDNA containing the e20 gene were inserted into the construct to increase the total size to ~11 Kb. This was linearized and integrated into mouse ES129 cells via electroporation, then NeoR positive clone cells were selected and confirmed using PCR and southern hybridization. These clones were microinjected into the embryos of FVB/N mice and the F1 generation was created by mating the mice produced from these embryos with animals from a C57BL/6 background. This F1 generation was mated to a line containing Cre recombinase (B6.FVB-Tg(ERLacre)C5379Lmgd/J, The Jackson Laboratory stock number 003724) to delete NeoR, and the resultant Neo- siblings were mated. Homozygous offspring were backcrossed to C57BL/6 (Charles river) mice to enhance the purity of their genetic background. These mice are recorded in the mouse genomics information database (MGI) and designated Cacna1b<sup>tm4.1DiLi</sup>.

Animals used for behavior analysis were both males and females. Unless otherwise noted, mice were group housed in rooms with a 12 h light/dark cycle and were provided food and
water *ad libitum*. All experimental procedures followed the guidelines of the Institutional Animal Care Committee of the University of New Hampshire. Animals were allowed to acclimate to housing conditions for at least one week prior to testing, and the researcher was blind to the genotype of all animals throughout testing and analysis. Prior to all behavior tests, mice were transferred and allowed to acclimate to the testing room for at least 30 minutes. In all cases, the scorer was blind to the genotype of the animal until testing and data analysis had been completed.

**Quantitative Real-Time PCR (qRT-PCR)**

Whole brain tissue was flash frozen in liquid nitrogen, and then transferred to dry ice. Individual whole brains from WT and Δ18a-only mice were pulverized with a mortar and pestle in the presence of liquid nitrogen. 30 mg of pulverized tissue was homogenized in RLT lysis buffer (Qiagen, 74134). RNA was extracted using Qiagen RNAeasy plus kit (Qiagen, 74134) according to manufacturer protocols. RNA was eluted in diethyl pyrocarbonate-treated water. 1 µg of total RNA from sorted cells was primed with oligo-dT and reverse transcribed with Superscript IV First-Strand Synthesis System (ThermoFisher Scientific, 18091050) according to manufacturer instructions. To quantify the amounts of e37a relative to total *Cacna1b* mRNA, we used two sets of primers as done previously (Bunda, LaCarubba, Bertolino, et al., 2019). Briefly, the first set of primers amplified specifically e37a-containing transcripts. This set of primers consisted of a forward primer designed to target the splice junction between e36 and e37a (e36-37aF: CTGCCTGTTGCCGGGATT) and a reverse primer to target a sequence within e37a (e36-37aR: 5’ACCTACGAGGGGCAGTTCTT). The second set of primers amplified a segment containing the constitutive exons 35 and 36 (e35-36F: 5’ GGAAACATTGCGCTGGA, e35-36R: 5’ CGACCCACTACAGTGTA).
e35-36R: 5’ CAGTGGCACTCCTGAACAATA). This amplification was used to normalize e36-37a amplification. The amplification efficiencies of both sets of primers was previously described (Bunda, LaCarubba, Bertolino, et al., 2019). All RT-qPCR reactions were run on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems) with the following conditions: 1 cycle 95 °C for 2 min, 45 cycles (95 °C for 15 s and 60 °C for 1 min). Each sample from at least five different mice per genotype (biological replicates) was run in triplicate (technical replicates). The specificity of 36-37a primers was described previously (Bunda, LaCarubba, Bertolino, et al., 2019). Ct values were determined by 7500 Software v2.3 (Applied Biosystems). Relative quantification of gene expression was performed with the \( 2^{-\Delta\Delta Ct} \) method (Livak & Schmittgen, 2001).

**Western Blot**

Brains were extracted from mice P24-P28 and flash frozen in liquid nitrogen. Brains were homogenized in 1x RIPA buffer consisting of the following: 50 mM Tris HCl (pH 7.5), 150 mM NaCl solution, 1% Igepal CA-630, 0.5% sodium deoxycholate solution (sterile filtered), and 0.1% sodium dodecyl sulfate with 1 pellet of protease inhibitors per 10 mL of buffer (cOmplete, Mini, EDTA-free; Sigma, 04 693 159 001). Homogenates were incubated on ice for 30 min and rehomogenized. Samples were centrifuged at 4 °C for 10 min at 1,000 x g to remove debris and the pellet was discarded. The supernatant was then centrifuged for 20 min at 12,000 x g. The new supernatant was transferred to a separate tube for use and analysis. Bicinchoninic acid (BCA) assay was performed for protein quantification in samples (ThermoFisher, 23227) according to manufacturer’s instruction. Samples were adjusted to 2 μg/μL with RIPA buffer. Samples were denatured for 20 min at RT using a 1:1 ratio of sample and 2x reducing buffer which consisted of
the following: 100 mM Tris-HCl (pH 6.8), 4% w/v SDS, 0.2% w/v bromophenol blue, 20% v/v stock liquid glycerol, and 200 mM dithiothreitol in Milli-Q® H2O.

Proteins were resolved using a 6% polyacrylamide resolving gel and a 4% stacking gel. The resolving gel was made fresh on the day of use from the following: 5.35 mL Milli-Q® H2O, 2.5 mL 1.5 M tris HCl (pH 8.8), 2 mL 30% acrylamide/bis (BIO-RAD, #161-0158), 100 μL 10% SDS, 70 μL 10% APS (Sigma, #A-3678), and 10 μL TEMED (Sigma, #T22500). The resolving gel was covered with H2O-saturated n-Butyl alcohol, cured for 15 min, and rinsed with dH2O. The 4% stacking gel was prepared with the following: 3 mL Milli-Q® H2O, 1.25 1M tris HCl (pH 6.8), 665 μL 30% acrylamide/bis, 50 μL 10% SDS, 40 μL 10% APS, and 5 μL TEMED. Electrophoresis run buffer stock (10x) was prepared from 250 mM tris base, 2500 mM glycine, and 1% w/v SDS. This run buffer was diluted to 1x for electrophoresis. Once the gel was cured, samples and ~10 μL of WesternSure® Pre-stained Chemiluminescent Protein Ladder (Li-Cor, #926-98000) were loaded into the BIO-RAD mini-PROTEAN 3 system. Samples were run at 10 mA per plate for 90 min, followed by 20 mA per plate for about another 30 min.

Next, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a transfer buffer. A 5x stock was prepared in Milli-Q® H2O by adding 125 mM tris base, 950 mM glycine, and 0.5% w/v SDS. This stock was diluted to 1x by combining 260 mL 5x stock, 195 mL methanol, and 845 mL Milli-Q® H2O. Prior to transfer, the PVDF membrane and gel were equilibrated for 20 min in 100% methanol and 1x transfer buffer, respectively, for 20 min. Transfer was performed on ice for 1 hr at 100 V. 1x PBS-Tween (PBS-T) was made by adding 5 mL of 20% Tween-20 solution (Sigma, P5927) to 1 L of 1x PBS in Milli-Q®. Following transfer, the membrane was rinsed with 1x PBS-T and allowed to dry overnight.
The PVDF membrane was reactivated in 100% methanol for 1 min followed by a brief PBS-T rinse step. The membrane was incubated with REVERT total protein stain (Li-Cor #926-11014) on an orbital rocker for 5 min. Stain was decanted, and the membrane was rinsed twice with wash solution for 30 s followed by a brief PBS-T rinse and subsequent storage in light-protected PBS-T. At this point, the membrane was imaged using an Odyssey CLx imaging system on the 700 nm channel.

After rinsing with PBS-T, the membrane was blocked with 5% BSA in PBS-T at RT for 1 hr. Next, it was washed three times for 3 min per wash in PBS-T. Anti-Cav2.2 primary antibody (Alomone Rabbit anti-Cav2.2, #ACC-002) was diluted in 0.425 μg/μL in Milli-Q H₂O and resuspended in 50% glycerol. Stock was prepared 1:100 with 1% BSA in PBS-T and 15 μL 10% NaN₃ for a final concentration of 0.425 μg/mL. Primary antibody incubation was carried out for 1 hr at RT with rocking, followed by three washes in PBS-T. The secondary antibody (Peroxidase-conjugated AffiniPure F(ab')2 Fragment Donkey Anti-Rabbit IgG (H+L); Jackson ImmunoLaboratories, Inc., 711-036-152) was prepared 1:15,000 in 1% BSA with PBS-T and incubated for 1 hr at RT with rocking. This was then decanted and the membrane was rinsed twice followed by three, 10-min washes in PBS-T with rocking. Signal was detected using ECL™ Western Blotting Detection Reagents (Sigma, GERPN2209) as per manufacturer’s instructions and developed on Amersham Hyperfilm ECL (Sigma, GE28-9068).

Total protein stain and chemiluminescence images were analyzed using ImageJ software (NIH, http://imagej.nih.gov/ij, v.1.51j8). Images were converted to 8-bit grayscale for analysis. Using the gel analysis tools, each lane was selected for total protein, and equally-sized boxes set for Cav2.2 at approximately the 230-260 kDa position. Areas under the curves were used for densitometry and exported for quantitative analysis. Chemiluminescence images were
normalized to total stain fluorescence, and the ratio of CaV2.2 to total protein compared. Results were compared statistically by ANOVA with sex and genotype as factors, and Tukey post-tests.

Behavior Assays

Hot Plate Test

The surface of a Hot Plate 7280 (Ugo Basile, Swenksville, PA) was heated to a constant temperature of 50, 53 and 55°C. Mice were placed on the heated plate contained in a clear acrylic cage, and video-recorded for the duration of the test. Latency to paw lick, paw flick, or jump was measured offline in the videos. Mice were removed from the hot plate and returned to their home cage immediately after responding to heat. If a mouse was unable to show a response within 60 s, the test was terminated. Scoring was done off line with a digital timer.

Open Field Test

Both males and females were used for this test. All mice were habituated in the testing room for 30 minutes prior to testing. Following habituation, mice were placed inside the open-field apparatus (a 45 x 45 cm open-top box, Harvard Apparatus, 76-0190) and activity was recorded for 5 minutes. The arena was divided into an “inner zone” which was in the center of the box and an “outer zone” which bordered one or more walls. Total distance traveled and percent of time in the inner zone were tracked using EthoVision XT version 11.5 behavioral software (Noldus Information Technologies, Inc.).
**Elevated Plus Maze**

Both males and females were tested. Mice were habituated to the testing room at least 30 minutes prior to testing. The maze was constructed from an elevated arena which contained four perpendicular arms that were 65 cm each and alternated between open (no walls) and closed (walled on 3 sides). Testing began when the mouse was dropped into the center of the arena, and each animal was tracked for 10 minutes. Total distance traveled and percent of time in the open arms were analyzed using EthoVision XT version 11.5 behavioral software (Noldus Information Technologies, Inc.).

**Saccharin Preference Test**

One week prior to preference testing, male mice were separated into single housing conditions. Drinking bottles were constructed using 50 mL conical tubes fitted with stoppers and ball bearings (VWR) and were fitted through slats in the roof of home cages. For 4 days, tap water was administered *ad libitum* in two separate drinking bottles to allow mice to acclimate to the two-bottle condition. On day 4, one of the solutions was replaced with 0.2% sodium saccharin salt hydrate (Sigma-Aldrich, S1002) in tap water. Bottles were then weighed every day for 4 days to assess consumption and compared against baseline preference measurements. The position of the bottles was counterbalanced across cages, positions, and days so as to avoid a side bias.

**Novelty-Induced Hypophagia**

This test was carried out three phases: three days of conditioning, one day of home cage testing, and one day of novel cage testing. Mice for this test were given at least one week to recover from saccharin preference testing before conditioning began and were kept singly
housed. Each day during the conditioning phase, mice were transferred in their home cages to a room with shaded lighting. Sweetened condensed milk (1:3 in tap water, Carnation®) was prepared fresh each day and delivered via leak-proof drinking bottles. Mice were allowed to drink *ad libitum* for 45 minutes. Home cage testing was performed for 10 minutes under similar conditions to create a baseline measurement of latency to drink and time spent drinking. On the final day, an empty cage was placed atop a white base and ~30 cm beneath two bright LED lights to invoke mild stress. Mice were recorded in the novel conditions for 10 minutes and latency to drink was manually scored against baseline measurements.

*Marble Burying Test*

Mice were acclimated to single housing in aspen chip bedding for 10 days in home cages prior to testing. Twenty marbles of varying colors were placed evenly across fresh beds of Aspen Hardwood Laboratory Bedding (Northeastern Products Corp.). Mice were individually placed in beds and given 1 hr to bury marbles. Irrelevant mice were given 30 minutes to dirty bedding before testing and bedding was not changed between trials. The number of marbles more than 2/3 buried was manually scored based on images taken directly following each test.

*Cocaine-Conditioned Place Preference*

The conditioning apparatus consisted of two acrylic compartments (30 cm l x 15 cm w x 15 cm h) with a removable divider separating the two and unique floors in each compartment. The “grid” floor was made from perforated stainless steel sheet with 40% open space and the “hole” floor was assembled from parallel ¼ cm stainless steel rods separated by about 1 cm open space. Cocaine hydrochloride (Sigma-Aldrich, C5776) was prepared at 2 mg/mL in sterile 0.9% sodium chloride (saline) and administered IP at 0.1 mL per 10 g body weight. This test was
divided into three phases: a baseline day, eight days of conditioning, and a final conditioned preference test. Each day of testing was separated by about 24 hrs, with a two-day break falling between the fourth and fifth days of conditioning. Mice were singly housed for at least a week prior to baseline testing.

The baseline test was conducted by giving each mouse 5 minutes to explore both compartments as desired. No injection was administered prior to baseline testing. Time spent in each compartment was assessed and an unbiased design was employed.

On days 2 through 9, mice received alternating injections of saline (vehicle) and cocaine hydrochloride (drug) as described above. Animals were injected and immediately sequestered to either the drug-paired or the vehicle-paired compartment for 15 minutes based on which injection they received. Injection schedule and the drug-paired compartment was counterbalanced across genotypes. All surfaces were thoroughly cleaned between animals.

On the day of the preference test, no injections were administered. Mice were placed on the boundary connecting the two compartments and given 30 minutes to explore both compartments freely. Time spent in each compartment and total movement were scored using EthoVision XT version 11.5 behavior software (Noldus Information Technologies, Inc.). Preference was assessed by dividing the time spent in the drug-paired compartment by the total time in the chamber.

**Forced Swim Test**

A drug solution was prepared by combining 0.167 mg/mL fluoxetine hydrochloride (Sigma-Aldrich, PHR1394) and 10 g/L sodium saccharin (Sigma-Aldrich, S1002) in tap water. Control mice received only 10 g/L sodium saccharin in tap water. Water was provided *ad libitum*
in 50 mL drinking bottles and changed every three days over an 18 day period. Mice were group housed throughout administration and testing.

On the day of testing, clear acrylic swim cylinders (30 cm x 30 cm) were filled to 18 cm with room temperature water. Mice were gently lowered into the water and recorded for 6 minutes, before being removed and placed in a fresh, pre-warmed cage. Upon completion of the study, the last 4 minutes of each test were manually scored for immobility. To assess estrous cycle stages, cells were collected posthumously via vaginal lavage with physiological saline. Solution was stained using the Dip Quick Stain Kit (JorVet, J0322) as per manufacturer’s instructions. Cytology was assessed in accordance with previously published literature (Byers, Wiles, Dunn, & Taft, 2012; Cora, Kooistra, & Travlos, 2015).

**Tail Suspension Test**

Drug preparation and administration was carried out identically to the forced swim test. The apparatus was constructed from white acrylic and consisted of four, 3-walled chambers. Each chamber was 4.5 cm x 15 cm x 55 cm (l x w x h) with 1 cm notches cut out of the top. A 1 cm x 1 cm aluminum suspension bar was fitted into the notches across the top of the apparatus. Because mice from a C56BL/6 background have a tendency to climb their tails as a means of escape, climbstoppers were made of PVC piping. Climbstoppers were fitted over the tail and tape was attached toward the end of the tail, then the tape was attached to the suspension bar. Mice were recorded for 6 minutes before being placed back in home cages. Upon completion of the test, the last 4 minutes were manually scored for immobility.
**Genotyping**

Toe samples were collected from mice P7-P9. DNA was extracted using the Phire Animal Tissue Direct kit II (ThermoFisher Scientific, F140WH) according to the manufacturer’s instructions. PCR solutions were prepared using AmpliTaq Gold® 360 mastermix (Thermo Fisher Scientific, 4398881) and primers flanking the e18a site (F-MT: 5’CCATGTCTGCTGCCAATATCT, F-WT: 5’GCAGCAGAGGTCTGTTTGC, and R-C: 5’CTCGGTGCTTTCTGTCTGTCC) (Table 2). PCR conditions were as follows: initial denaturation at 95°C for 10 min, 35 cycles (95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min), and final elongation at 72°C for 7 min. Expected bands were visualized at 880 bp for WT animals and at 395 bp for Δ18a-only mice.

**Table 2.** Endpoint PCR primers for verification of Δ18a and WT mouse genotypes.

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Primers</th>
<th>Expected products</th>
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<tbody>
<tr>
<td>Δ18a-only</td>
<td>F-MT: CCATGTCTGCTGCCAATATCT&lt;br&gt;F-WT: GCAGCAGAGGTCTGTTTGC&lt;br&gt;R-C: CTCGGTGCTTTCTGTCTGTCC</td>
<td>Hom: 395 bp&lt;br&gt;Het: 395 and 880 bp&lt;br&gt;WT: 880 bp</td>
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**BaseScope™ In-situ Hybridization**

All tissues were obtained from WT C57BL/6 male mice aged P30 or older, as e18a splicing patterns are relatively consistent from age P30. Mice were deeply anesthetized with isoflurane and injected with either 0.07 mL (mice under 40 g body weight) or 0.1 mL (mice over 40 g body weight) of Euthasol (Virbac, 200-071). Once asleep, a transcardial perfusion was performed for 10 min using ice cold 1x phosphate buffered saline (PBS; Sigma, P3813) followed
by 10 min of ice cold 10% neutral buffered formalin (NBF; Sigma, HT501128) followed by another 10 min of ice cold 1x PBS. The brain was quickly extracted and fixed in 10% NBF at 4°C for at least 24 hrs. Following fixation, the brain was rinsed with 1x PBS and cryoprotected in 15% sucrose in PBS followed by 30% sucrose in PBS until tissue was saturated. Brains were fixed in optimal cutting temperature compound (OCT; Fisher, 4585) and frozen in pre-chilled isopentane on dry ice. The OCT block was acclimated in a cryostat for at least 1 hr prior to cryosectioning. 12 μm sections were placed in Netwell™ inserts (Corning, 3478) on dry ice and transferred to 1x PBS for float mounting on positively charged microscope slides (Globe Scientific, 1358).

All BaseScope™ duplex reagents were included in a kit (Advanced Cell Diagnostics, 323800). Prior to the assay, tissues were pretreated using an optimized tissue detachment protocol and RNAscope™ pretreat reagents. Slides were air-dried for at least 1 hr followed by drying at 60°C for 30 min. Dried slides were fixed in 10% NBF pre-chilled to 4°C for 15 min. Following fixation, slides were sequentially dehydrated in 50%, 70%, and two rounds of 100% ethanol for 5 min each and allowed to dry at room temperature (RT) for 5 min. Sections were incubated with RNAscope® Hydrogen Peroxide for 10 min and rinsed twice with fresh Milli-Q® water. Slides were transferred to 99°C Milli-Q® water for ~15 s then incubated for 5 min incubation in 99°C RNAscope® 1x Target Retrieval Reagent. Slides were then rinsed in RT Milli-Q® water for ~15 s, dehydrated in 100% ethanol for 3 min, and dried at 60°C for 5 min. An ImmEdge™ hydrophobic barrier pen (Vector Laboratories, H-4000) was used to draw a barrier around each section and dried at RT overnight.

The following day, RNAscope® Protease III was applied to each section and incubated at 40°C for 30 min in the pre-warmed HybEZ™ Hybridization System (ACD, 310016) followed by
two washes in 1x RNAscope® Wash Buffer (ACD, 310091). The Channel 1 (C1) green probe for all experiments spanned 4 exon junctions within Slc6a3 mRNA for the dopamine transporter (DAT; ACD, 722171). Two Channel 2 (C2) red probes were designed: one spanned 4 exon junctions of Cacna1b mRNA (Cav2.2; ACD, 722451-C2) and the other spanned the e18:e18a exon junction of Cacna1b (e18a; ACD, 701141-C2). The C1 and one of the C2 probes were mixed together in a 50:1 ratio, dropped onto each section, and incubated as before. The rest of the BaseScope™ assay was performed as per manufacturer’s instructions, with the exception of AMP 11, which was shortened to 15 min to attenuate green signal.

RESULTS

Prior to behavioral screening, we performed a number of experiments to validate that our Δ18a-Cav2.2 mouse model contains successful deletion of e18a and does not demonstrate alterations in Cav2.2 mRNA or protein expression. Following validation, we performed an extensive behavioral screening to determine differences evoked by removal of e18a. Because Cav2.2 is involved in nociception, exploratory behavior, locomotion, we performed tasks to probe these behaviors. In addition, we tested behavioral response to appetitive stimuli, to examine the possibility of an anhedonic phenotype.

Validation of the Mouse Genetic Model to Study e18a Splice Variants

In order to study the function of e18a, we developed a mouse model in which e18a was globally deleted from the genome (Δ18a-only). We also generated another mouse that contains
constitutive inclusion of e18a (+18a-only). However, we did not perform a thorough behavioral characterization of this mouse because the forced inclusion of e18a resulted in the expression of +18a-\(\text{Cacna1b}\) in cells where it is not normally expressed, therefore complicating the interpretation of our results (Fig. 5). This mouse will be used for other type of experiments and therefore was also validated.

**Figure 5.** Mouse models developed to study impacts of restricted splice choice. WT \(\text{Cacna1b}\) contains intronic regions which flank e18a and enable its selective inclusion in or exclusion from mature \(\text{Cacna1b}\) mRNAs (and as a result, Cav2.2 channels). Using homologous recombination, we developed mouse models in which the intronic regions flanking e18a were missing. In the +18a-only mouse, e18a is fused to e18a and e19, enabling production of only +18a-\(\text{Cacna1b}\) transcripts. In the Δ18a-only model, exons 18 and 19 are fused together and e18a is removed from the genome completely, resulting in only Δ18a-\(\text{Cacna1b}\) transcripts. RT-PCR of WT, +18a-only, and Δ18a-only whole brains demonstrates that this genetic manipulation was successful—no Δ18a-\(\text{Cacna1b}\) mRNA was detected in +18a-only mice, and no +18a-\(\text{Cacna1b}\) mRNA was detected in Δ18a-only mice.
To rule out non-specific effects of our genomic manipulation we performed a thorough validation of our mouse models. We quantified the total protein levels for Cav2.2 and we also assessed if other splicing events within the Cacna1b pre-mRNA were altered. To quantify protein expression, we performed Western blots on whole brain using Cav2.2-specific antibodies. At approximately 230-260 kDa, WT samples exhibit a thick band which Cav2.2-null control brains lack (Fig. 6A, left panel). Whole protein was consistent across lanes in the loading control (Fig. 6A, right panel), indicating that this 230-260 kDa band is Cav2.2. Next, Cav2.2 expression levels were compared in WT and Δ18a-only males and females (Fig. 6B, left panel). All samples exhibited a thick Cav2.2 band in the 230-260 kDa range. Following whole protein quantification and normalization against the loading control, we found no differences in Cav2.2 protein expression across sexes or genotype (Fig. 6B, right panel).

Figure 6. Validation that restriction of e18a splice choice does not impact Cav2.2 protein expression using Western blot. (A) Western blot of proteins purified from +18a-only, Cav2.2-null, and Δ18a-only brains (left) and whole protein loading control (right). Cav2.2 appears at ~230-260 kDa in +18a-only and Δ18a-only samples but is absent from Cav2.2-null samples. Therefore, the antibody is Cav2.2-specific and detects both +18a-Cav2.2 and Δ18a-Cav2.2 proteins. Normalization against the loading control shows that protein expression is consistent. (B) Western blot of male and female WT, +18a-only, and Δ18a-only whole brain samples (left) and whole protein loading control (right). Cav2.2 antibody shows expected bands in all samples at the 230-260 kDa mark, and normalization against the loading control showed that protein expression is consistent across all genotypes and both sexes. (C) Graphical depiction of relative Cav2.2 expression in female and male WT, +18a-only, and Δ18a-only samples. Based on these data, Cav2.2 protein expression does not differ based on sex or genotype.
To ensure that restricting e18a splice choice does not impact other alternative splicing events, we quantified mRNA expression of another alternatively spliced exon within Cav2.2, exon 37a. qPCR primers were designed to detect both the constitutively expressed exon 35-36 junction and the alternatively spliced exon 36-37a junction (Fig. 7A, 7B, and 7C). Probe specificity was confirmed using an endpoint PCR reaction of cDNA that was RT-PCR amplified from whole brain samples of WT and Δ18a-only mice. Both WT and Δ18a-only samples produced bands of the expected size, ~90 bp for the 36-37a probe and ~90 bp for the 35-36 probe (Fig. 7D). qPCR of these samples and normalization against exon 35-36 expression demonstrated similar exon 37a expression levels in both WT and Δ18a-only brains (Fig. 7E). Therefore, selective exclusion of e18a does not impact splicing of other alternatively spliced exons.
Figure 7. Validation that restriction of e18a splicing does not impact expression of other alternatively spliced exons. (A) Exons 37a and 37b of Cacna1b pre-mRNA undergo mutually exclusive alternative splicing, and exons 35, 36, 45, and 46 of Cacna1b are constitutively expressed. We employed a TaqMan® assay using a probe which overlaps exons 45 and 46 to track new strand synthesis. Primer pairs spanning the 35:36 junction and the 36:37a junction were used. (B) Melt curve analysis of 35-36 and 36-37a primer sets demonstrates that each set has only one peak and that they dissociate at approximately the same temperature. (C) qPCR primer efficiency for each primer set is within acceptable limits, indicating high quality primers. (D) Endpoint PCR bands showing products amplified by 36-37a and 35-36 primers. Equally sized products were obtained in bacterial cDNA of transfected 37a and 37b clones (top) and in WT, +18a-only, and Δ18a-only mouse whole brain cDNA samples (bottom). The top panel demonstrates that primer set 36-37a cannot amplify product without the presence of e37a. Therefore, primers are acceptable for use in all three genotypes and they will recognize the 35-36 and 36-37a sequences with high specificity. (E) Mean (filled circles) and individual (empty circles) e37a fold change. E37a expression was normalized to 35:36 expression and fold change was analyzed using the 2^ΔΔCt method. Expression of e37a is consistent in WT, +18a-only, and Δ18a-only samples, demonstrating that altered e18a splicing does not impact other alternatively spliced Cacna1b exons. WT: n = 4, +18a-only: n = 4, Δ18a: n = 4. (F) Cacna1b total gene expression level comparison. Cacna1b 35-36 qPCR primer products were normalized to Gapdh levels and fold change was assessed as before. No differences were found in Cacna1b whole gene expression in WT, +18a-only, and Δ18a-only animals, indicating that altered e18a splicing does not impact Cacna1b whole gene expression. WT: n = 8, +18a-only: n = 6, Δ18a-only: n = 7.
**Exon 18a Does not Influence Nociceptive Behavior**

Previous results have shown that Ca\textsubscript{V}2.2 channels control transmitter release of sensory afferents into the spinal cord, and thereby are involved in nociceptive behavior (Heinke, Balzer, & Sandkühler, 2004). Ca\textsubscript{V}2.2-KO mice exhibit delayed responses to thermal stimuli in hot plate and Hargreaves tests (Hatakeyama et al., 2001; Kim et al., 2001). Therefore, we compared global nociceptive responses to a thermal stimulus between WT and Δ18a-only mice. We assessed the time to paw withdrawal, paw licking or jumping at three different temperatures (50, 53 and 55°C). As expected, a two-way repeated measures ANOVA revealed a significant effect of temperature (F = 161, p < 0.001), which simply demonstrates a general increase to responsiveness with heightened temperatures. There was no significant effect of genotype (F = 1.44, p = 0.27), or genotype x temperature (F = 0.92, p = 0.39), suggesting that the absence of +18a-Cacna1b does not influence nociceptive behavior (Fig. 8).
Figure 8. Thermal nociceptive responses time at 50, 53, and 55°C in the hot plate test. The same animals were used in all three trials. Black circles represent WT animals and grey circles represent Δ18a-only. Filled circles are averages while empty circles represent individuals. For both WT and Δ18a-only, average time to respond significantly decreased with increasing temperature, as expected (F = 161, p < 0.001). WT and Δ18a-only mice did not respond differently to thermal pain at any temperature tested (F = 1.44, p = 0.27). WT: n = 8; Δ18a-only: n = 8.
E18a Does not Impact Baseline Response to an Appetitive Stimulus

Preference for a sweet reward has been associated with a hedonic state, particularly monoaminergic function has been implicated in saccharin preference (Matthews, Gibson, & Booth, 1985; Sclafani, Touzani, & Bodnar, 2011). As part of our baseline screening, we used the saccharin preference test to probe for altered sweet reward response indicative of a potential anhedonic state or monoaminergic modulation. Mice were given two bottles of DI water for 4 days, followed by 4 days with one bottle of DI water and one bottle of saccharin water. As expected, mice showed no consistent baseline preference for one water bottle over the other, with average preference ranging from 35-60%. On day 1 of testing, all mice developed a preference for the saccharin water, and after day 1 of testing, all mice showed ≥90% preference for the bottle of saccharin water. Two-way repeated measures ANOVA revealed a significant effect of testing phase as expected (F = 211.7, p < 0.001) but revealed no significant effect of genotype (F = 0.457, p = 0.509) or genotype x testing phase (F = 0.463, p = 0.507) (Fig. 9). Therefore, we conclude that removal of e18a does not impact baseline appetitive preference.
Figure 9. Bottle preference during baseline (days -3 through 0) and 0.2% saccharin preference testing (days 1 through 4). Data shown as average ± SEM. Black circles are WT, grey circles are Δ18a-only. During baseline days mice were given two bottles with water only, and showed roughly equal preference for both bottles. At testing day 1, one bottle per cage was switched with 0.2% saccharin water. All mice exhibited >75% preference for the saccharin bottle during all 4 testing days and this preference was consistent in WT and Δ18a-only mice (F = 0.457, p = 0.509). WT: n = 5, Δ18a-only: n = 4.
Removal of Exon 18a Does not Impact Responses to Appetitive Stimuli under mild stress

We next assess if lack of e18a impacts the appetitive responses of mice but under a mildly stressful environment. To do this, we performed the novelty-induced hypophagia test in WT and Δ18a-only mice. In this task, mice must overcome a mildly stressful relatively novel stimulus to receive an appetitive reward. Mice were trained for three days to locate a sipper tube of sweetened milk in their home cage under dim lighting. On the fourth day home cage latency and time spent drinking were timed. On the final day, mice were placed in a bright, empty cage and latency as well as time drinking were again measured. As expected, all individuals spent less time drinking in the novel cage (Fig. 10A, left panel), and all but one individual exhibited higher latencies to find the bottle in novel conditions (Fig. 10B, left panel). Average time spent drinking in the home cage did not differ between WT and Δ18a-only mice, although Δ18a-only mice displayed a small, nonsignificant trend towards higher home cage latencies (Time spent drinking, mean ± SEM: WT home = 133.3 ± 9.6 s; Δ18a-only home = 128.4 ± 8.9 s. Student’s t-test, \( p = 0.71 \). Fig. 10A, left panel). Latency to drink, mean ± SEM: WT home = 9.5 ± 1.5 s; Δ18a-only home = 14.6 ± 2.2 s. Student’s t-test, \( p = 0.07 \). Fig. 10B, left panel). Drinking time and latency to approach in the novel cage did not differ based on genotype (Time spent drinking, mean ± SEM: WT novel = 57.3 ± 6.6 s; Δ18a-only novel = 55.3 ± 8.8 s. Student’s t-test, \( p = 0.85 \). Fig. 10A, left panel. Latency to drink, mean ± SEM: WT novel = 49.4 ± 10.9 s; Δ18a-only novel = 60.9 ± 11.3 s. Student’s t-test, \( p = 0.47 \). Fig. 10B, left panel.) Similarly, change in time spent drinking and change in latency did not differ by genotype (Δ time drinking, mean ± SEM: WT = 75.9 ± 8.8 s; Δ18a-only = 73.1 ± 6.8 s. Student’s t-test, \( p = 0.8 \). Fig. 10A, right panel. Δ latency to approach, mean ± SEM: WT = 39.9 ± 10.5 s; Δ18a-only = 46.3 ± 10.4 s.
Student’s t-test, \( p = 0.67 \). **Fig. 10B, right panel**). We conclude that e18a does not modify behavioral response to appetitive stimuli under conditions of mild stress.

**Figure 10.** Comparison of novelty-induced hypophagia in WT and Δ18a-only mice. Black circles represent WT animals and grey circles are Δ18a-only. Empty circles represent individuals and filled-in circles represent average with SEM. Days 1-3 were habituation to the new environment in the home cage where normal water bottles were replaced with sweetened milk. Day 4 was home cage (HC) testing under the same conditions, tracked for 10 min. Day 5 mice were placed in an empty, brightly-lit, novel cage (NC) and tracked for 10 min again. (A) Total time spent drinking for WT and Δ18a-only individuals (left) and change in time spent drinking (right). The novel cage consistently induced a decrease in time drinking; however, genotype did not affect drinking in either assessment (Time spent drinking: \( p = 0.85 \); Δ Time spent drinking: \( p = 0.8 \)). (B) Latency to approach the bottle (left) and change in time to approach the bottle from HC to NC (right). WT and Δ18a-only mice take longer to approach the sweet milk in most cases upon exposure to the novel environment. No difference was seen in average time to approach or change in approach time between genotypes (Time to approach (s): \( p = 0.47 \); Δ Time to approach: \( p = 0.67 \)). WT: \( n = 15 \), Δ18a-only: \( n = 16 \).
Lack of e18a-Cacna1b does not influence exploratory behavior

Previous studies have shown that the Cav2.2-KO mice displayed enhanced exploratory behavior in the open field (OF) and elevated plus maze (EPM) (Saegusa et al., 2001). Furthermore, recent evidence suggests Cacna1b alternative splicing in the e37a/e37b locus is linked to exploratory behavior (Bunda, LaCarubba, Bertolino, et al., 2019). We tested if splicing of e18a impacts exploratory behavior in OF and EPM. In OF, we tracked number of entries into the center, time spent in the center versus the outer perimeter of an open chamber, and total distance traveled. No differences were found in any parameter based on genotype (# of entries into center, mean ± SEM: WT = 14.4 ± 1.6; Δ18a-only = 17.4 ± 1.7. Student’s t-test, p = 0.22. Fig. 11A, left panel. % time in center, mean ± SEM: WT = 11.8 ± 2.3; Δ18a-only = 13.1 ± 1.5. Student’s t-test, p = 0.65. Fig. 11A, middle panel. Distance traveled, mean ± SEM: WT = 1648 ± 85.7 cm; Δ18a-only = 1670.3 ± 86.3 cm. Student’s t-test, p = 0.86. Fig. 11A, right panel).

Similar results were observed in the EPM apparatus. No differences in percent of entries into the open arms (OA), time in OA and total locomotion, were observed between WT and Δ18a-only mice (% entries into OA, mean ± SEM: WT = 35.1 ± 1.9. Student’s t-test, p = 0.99. Fig. 11B, left panel. % time in OA, mean ± SEM: WT = 18 ± 3.4; Δ18a-only = 14.7 ± 1.6. Student’s t-test, p = 0.4. Fig. 11B, middle panel. Distance traveled, mean ± SEM: WT = 1910 ± 81.9 cm; Δ18a-only = 1927.7 ± 72.5 cm. Student’s t-test, p = 0.87. Fig. 11B, right panel.) These results suggest that e18a also does not impact exploratory behavior or total locomotion.
Figure 11. Comparison of exploratory behavior and total locomotion between WT and Δ18a-only mice. Data is shown as mean (filled circles) ± SEM and individual data points (empty circles). Black circles represent WT animals and grey circles represent Δ18a-only animals. Data for males and females is provided, all animals were used in both paradigms. (A) Entries into center, time in center, and total distance traveled in the 5 minute OF paradigm did not differ between the genotypes (Entries into center: $p = 0.22$; Time spent in center (%): $p = 0.65$; Distance traveled (m): $p = 0.86$). (B) Entries into open arms, time spent in open arms, and total distance traveled in the 10 minute elevated plus maze test also did not differ based on genotype (Entries into open arms (%): $p = 0.99$; Time spent in open arms (%): $p = 0.4$; Distance traveled (m): $p = 0.87$). In all analyses, WT and Δ18a-only mice did not differ in behavioral response or basal locomotion. WT: n = 16, Δ18a-only: n = 14.
**Δ18a-only Mice Exhibit Increased Immobility during Habituation to New Environments**

We next performed a detailed analysis of locomotor behavior in both males and females. This analysis made it possible to elucidate whether mice show genotypic differences in the habituation to a novel environment. During the first 5 minutes of the OF test, we found that compared to WT mice, Δ18a-only male mice exhibited a significant reduction in overall distance traveled (Distance traveled, mean ± SEM: WT = 1927.5 ± 91.2 cm; Δ18a-only = 1572.8 ± 72 cm. Student’s t-test, *p* = 0.01. **Fig. 12A, left panel**). After the first 5 minutes, WT and Δ18a-only mice were similar in locomotor activity (**Fig. 12A, left panel**). During the first 2 minutes of EPM we noticed a trend towards Δ18a-only mice moving less, and during the 2–4 minute time period they moved significantly less (Distance traveled 0 to 2 minutes, mean ± SEM: WT = 526.6 ± 34.3 cm; Δ18a-only = 458.6 ± 16.9 cm. Student’s t-test, *p* = 0.16. **Fig. 12B, left panel**. Distance traveled 2 to 4 minutes, mean ± SEM: WT = 460.9 ± 22.8 cm; Δ18a-only = 384.7 ± 17.8 cm. Student’s t-test, *p* = 0.03. **Fig. 12B, left panel**). By contrast, female locomotion during acclimation was similar at all times analyzed (Distance traveled OF, mean ± SEM: WT = 2030.1 ± 84.4 cm; Δ18a-only = 2104 ± 48.8 cm. Student’s t-test, *p* = 0.59. **Fig. 12A, right panel**. Distance traveled EPM 0-2 minutes, mean ± SEM: WT = 592.6 ± 27 cm; Δ18a-only = 583.3 ± 39.1 cm. Student’s t-test, *p* = 0.85. **Fig. 12B, right panel**. Distance traveled EPM 2-4 minutes, mean ± SEM: WT = 490.6 ± 26.7 cm; Δ18a-only = 530.5 ± 20.6 cm. Student’s t-test, *p* = 0.41. **Fig. 12B, right panel**). No differences were found in the time spent in the center or number of transitions to the center in either males or females (data not shown). We observed similar results for both males and females in the EPM test, percentage of entries into the OA and percentage of time in the OA was similar for both genotypes at all times (data not shown). These results
suggest that Δ18a-only mice are more immobile than WT mice when acclimating to new environments in which no appetitive stimuli are present.

To determine with measure with more accuracy mouse movement in a novel environment, we performed pixel subtraction in a fear conditioning chamber (a context that mice have never been exposed). Pixel subtraction allows for measurement of freezing. In this task, males and females were combined. Δ18a-only mice spent nearly twice as much time freezing as their WT counterparts during a period of 3 minutes (% of time freezing during acclimation, mean ± SEM: WT = 15.9 ± 2.8; Δ18a-only = 34 ± 4.6. Student’s t-test, p = 0.001. Fig. 12C). The increased freezing during acclimation to the chamber is consistent with the decreased locomotion in the first 5 minutes OF and EPM. Taken together, these data suggest that e18a-Cacna1b splice variant plays a role in response to habituation to novel environments.
Figure 12. Differences in habituation to new environments in the open field (top panels), elevated plus maze (middle panels), and fear conditioning chamber (bottom panel) with males and females. Black circles represent averages for WT and grey circles are Δ18a-only averages. The same animals were used in OF and EPM (A) Distance traveled across 1 hour in an open field chamber, broken into 5-minute segments. Male Δ18a-only mice exhibit significantly less locomotor activity during acclimation (first 5 minutes) than their WT male counterparts (Distance traveled in first 5 minutes (m): p = 0.01). Females did not exhibit locomotor differences during any time period (Distance traveled (m): p = 0.59). WT males: n = 17; Δ18a-only males: n = 10; WT females: n = 21; Δ18a-only females: n = 8. (B) Distance traveled during 10 minutes in an elevated plus maze, broken into 2-minute segments. Again, Δ18a-only male locomotion is slightly attenuated (first 0-2 minutes) and significantly attenuated (2-4 minutes) during acclimation (Distance traveled in minutes 0-2 (m): p = 0.16; Distance traveled in minutes 2-4 (m): p = 0.03). Locomotion analysis in female mice shows consistent results, with no genotypic differences (Distance traveled in minutes 0-2 (m): p = 0.85; Distance traveled in minutes 2-4 (m): p = 0.41). WT: n = 17; Δ18a-only: n = 10; WT females: n = 21; Δ18a-only females: n = 8. (C) Percent of time spent freezing (immobile) during the 2.5-minute acclimation period to a fear conditioning chamber. Males and females were combined for this analysis. On average, Δ18a-only mice spend significantly more time freezing during acclimation than their WT counterparts (% Freezing: p = 0.001). WT: n = 35; Δ18a-only: n = 16.
**Removal of e18a Results in Heightened Stress-Induced Inactivity**

Based on our OF, EPM, and FC data we noted that Δ18a-only mice exhibited higher rates of immobility in response to new environments compared to WT mice. However, we did not observe differences between the two genotypes in response to novelty when there is appetitive stimulus present (Fig. 10). We next assessed the response of the mice to several stressful stimuli without reward. To test this, we used three different measures of stress: the marble burying test (MBT), the tail suspension test (TST) and the forced swim test (FST).

Marble burying is a test of anxious or repetitive behaviors and has been shown to be sensitive to a number of antipsychotics that act as monoaminergic ligands (Bruins Slot, Bardin, Auclair, Depoortere, & Newman-Tancredi, 2008). Male mice were placed on bedding that was dirtied by irrelevant male mice and given 1 hour to bury twenty marbles. After an hour, Δ18a-only mice showed a strong trend towards burying fewer marbles than their WT counterparts (# of marbles buried, mean ± SEM: WT = 11.7 ±1.8; Δ18a-only = 7.2 ± 1.3. Student’s t-test, \( p = 0.053 \). Fig. 13A).

The FST was used to measure escape-oriented behavior in response to inescapable swim stress. Mice were placed in room temperature water tanks and the last 4 minutes of the 6 minutes were scored for immobility. When male mice were tested, we saw significantly less average mobility in Δ18a-only mice compared with WT mice (Immobility, mean ± SEM: WT = 51.7 ± 18.4 s; Δ18a-only = 131.2 ± 14.3 s. Student’s t-test, \( p = 0.009 \). Fig. 13B, left panel). However, this phenotype was not observed in females (Immobility, mean ± SEM: WT = 176.3 ± 27.8 s; Δ18a-only = 157.6 ± 21.6 s. Student’s t-test, \( p = 0.6 \). Fig. 13C), and nicely correlates with our previous observations in OF and EPM.
In the TST, male mice were taped to a suspension rod and escape-oriented behavior was scored as immobility. Again, only the last 4 minutes of the 6 minute test were scored. As expected, Δ18a-only mice were more immobile on average than WT mice (Immobility, mean ± SEM: WT = 148.4 ± 14.6 s; Δ18a-only = 192 ± 7.7 s. Student’s t-test, \( p = 0.02 \). Fig. 13B, right panel). Thus, both of these paradigms are in agreement and provide evidence that removal of e18a results in heightened immobility in response to inescapable stress.

Figure 13. Black, filled circles represent averages ± SEM for WT and grey, filled circles are Δ18a-only averages ± SEM. Individual subjects are depicted as empty circles. (A) Percent of marbles buried when males were placed on bedding dirtied with competing male odor. WT animals show near-significant increases in total percent buried compared to Δ18a-only mice (% Buried marbles: \( p = 0.053 \)). WT: \( n = 11 \); Δ18a: \( n = 11 \). (B) Immobility in seconds during the FST (left panel) and TST (right panel). In both of these tests, Δ18a-only males were significantly more immobile compared to their WT counterparts (Immobility in FST (s): \( p = 0.009 \); Immobility in TST (s): \( p = 0.02 \)). WT FST: \( n = 6 \); Δ18a-only FST: \( n = 5 \); WT TST: \( n = 16 \); Δ18a-only TST: \( n = 13 \). (C) Immobility of female WT and Δ18a-only mice in the FST under the same conditions as males. No genotypic differences were detected, in line with previous findings (Immobility in FST (s): \( p = 0.6 \)). WT: \( n = 7 \); Δ18a-only: \( n = 7 \).
Cocaine-Induced Conditioned Place Preference Is Mildly Affected by e18a Removal

To narrow down the potential role of e18a in monoamine-linked behavior, we chose to test conditioned preference for a drug that has a high affinity for one specific transporter. Cocaine is a stimulant that potently blocks dopamine influx through DAT in vivo (Verma, 2015). In an unbiased design, we trained mice over a period of eight days to associate a conditioning chamber with an i.p. injection of cocaine hydrochloride and an unconditioned chamber with i.p. injection of saline. Time spent in the drug-paired chamber versus the unpaired (saline) chamber was measured after the final day of conditioning and without injection. While both genotypes spent significantly more time in the drug-paired chamber than the unpaired chamber (data not shown), Δ18a-only mice spent slightly more time in the paired chamber compared to WT mice (Time in paired chamber, mean ± SEM: WT = 1469.8 ± 58.4 s; Δ18a-only = 1607 ± 41.7 s. Student’s t-test, p = 0.09. Fig. 1A). Time spent in the paired chamber was divided by time spent in the unpaired chamber to determine the ratio of preference. Δ18a-only mice again showed a trend towards greater ratio of preference, although this trend was nonsignificant (Ratio of preference, mean ± SEM: WT = 6.8 ± 1.7; Δ18a-only = 13.4 ± 4. Student’s t-test, p = 0.12. Fig. 1B). A power analysis showed that we would need 35 animals from each genotype in order to see a significant effect, so we have decided not to further pursue these findings.
Figure 14. Preference for drug-paired chamber in cocaine-conditioned place preference post-test. Filled circles are mean ± SEM and empty circles represent individuals. Mice were given a pre-test to check for bias, then given a series of i.p. injections with either saline or cocaine and sequestered to one compartment, which was counterbalanced across genotypes and day of training. Injections were alternated each day for 8 days, and the drug-paired compartment was kept consistent each day. After a 24-hour rest, mice were post-tested with no injection for conditioned place preference, measured by the time willingly spent in the drug-paired compartment over the unpaired compartment. (A) Total time spent in the drug-paired chamber, in seconds. Both genotypes spend more time in the drug-paired chamber compared to the unpaired chamber; however, there is a nonsignificant trend for Δ18a-only mice to spend more time in the paired chamber than the WT mice (Time in paired chamber (s): p = 0.09). (B) Ratio of preference for drug-paired chamber, calculated by dividing time spent in drug-paired compartment by total time in the chamber. In this analysis, Δ18a-only mice again show a slight, nonsignificant increase in preference for the drug-paired chamber over their WT counterparts (Ratio of preference: p = 0.12). WT: n = 10; Δ18a: n = 8.
**Colocalization Studies of Cacna1b and e18a with Slc6a3 (Dopamine transporter)**

We and others have previously shown extensive expression of +18a-Cacna1b in the VTA and SNc, two major dopamine production centers in the midbrain. However, whether this splice variant is enriched in dopamine neurons is unknown. We attempted to perform colocalization studies using BaseScope™ Duplex Assay. In these experiments, we performed a dual assay experiment to determine +18a-Cacna1b colocalization with Slc6a3. Independently, we performed a dual assay experiment to determine total Cacna1b mRNA with Slc6a3. mRNA for +18a-Cacna1b and Cacna1b mRNA can be quantified by counting the red dots, since each dot is equivalent to one mRNA molecule. In line with previous findings, Cacna1b is expressed extensively in the dopaminergic midbrain (Fig. 15A). In both the VTA and the SNc, Cacna1b colocalizes with a subpopulation of Slc6a3-positive cells; however, Cacna1b is also expressed in Slc6a3-negative cells, and some cells expressing DAT do not express Cacna1b (Fig. 15B and 15C). Regarding +18a-Cacna1b, despite multiple efforts and rounds of optimization, our probe did not work for this experiment (Fig. 16A, 16B, and 16C). Therefore, we were unable to determine whether this splice variant is localized in dopamine neurons.
Figure 15. Expression of Slc6a3 (dopamine transporter) and Cacna1b (Cav2.2) mRNA in the VTA and the SNc. BaseScope™ Duplex Assay was conducted on WT mice only (but see Bunda et al., 2019 for Δ18a-only controls). Each punctate dot of red or green represents one mRNA molecule for Cacna1b or Slc6a3, respectively. Top panel (A) was taken at 10x magnification and bottom panels (B and C) were taken at 20x. We observed partial coexpression of Cacna1b and Slc6a3 in these populations, as well as independent expression of both genes.
Figure 16. Localization of Slc6a3 (dopamine transporter) and e18a of Cacna1b (Cav2.2) in the VTA and SNc. Each punctate dot of red or green represents one mRNA molecule for +18a-Cacna1b or Slc6a3, respectively. Top panel (A) was taken at 10x magnification and bottom panels (B and C) were taken at 20x. E18a can be sparsely observed within the VTA and the SNc areas and seems to colocalize with DAT in some cases; however, due to extremely faint e18a signal and overabundance of Slc6a3 in these regions, it is impossible to conclude the level of e18a and Slc6a3 coexpression.
DISCUSSION

Alternative splicing is a fascinating and nearly ubiquitous aspect of eukaryotic cellular biology. It is thought to fine-tune protein function based on tissue-, development-, and cell-specific needs. The importance of alternative splicing to Cav2.2 function has already been demonstrated with e37a/e37b, where the exons modulate nociception and behavioral responses to aversive stimuli. Exon 18a of Cav2.2 has been shown to alter channel’s calcium dynamics in vitro and exhibits a striking pattern of heightened expression within monoaminergic areas of the midbrain and sympathetic system. Based on this evidence, e18a is suspected to assist in fine-tuning the neural bases for mood.

In our baseline behavior screening, appetitive stimuli failed to produce a phenotypic difference between WT and Δ18a-Cav2.2 (Δ18a-only) mice. In the saccharin preference test, orally administered saccharin water was highly preferred (≥90%) by both genotypes after day 1 of testing. It is possible that the saccharin concentration reached a point of saturation at which differences between genotypes would be indistinguishable. However, when comparing the drinking times in their come cages in the novelty-induced hypophagia test, we observed similar results to the ones obtained in the saccharin test. These results strongly suggest that the absence of +18a-Cacna1b splice variant does not impact hedonia.

We did not detect differences in exploratory behavior in the OF and EPM. However, during the first five minutes of acclimation, Δ18a-only mice were more immobile in both tasks, indicating an altered phenotype in response to novel environment. This phenotype was observed only in males but in females. Differences in estrous cycle might explain this, and larger number of individuals would be needed to determine with more certainty if e18a is important for behavioral responses of females to a novel environment.
We next assessed the behavioral responses of mice to stressful stimuli that includes novelty in the absence of reward—MBT, FST, and TST. In MBT, an irrelevant male’s odor was used to detect differences in odor-based stress response. Δ18a-only mice, on average, buried fewer marbles under this condition than WT mice. This test is traditionally a model of anxiety- and obsessive/compulsive-like behavior (Deacon, 2006). Based on our OF and EPM data, we do not believe the differences in MBT are due to an anxiety-like state, we think our findings can be explained by differences in immobility induced by stress based on the odor of another male.

To test behavior strictly linked to stress-induced immobility, FST and TST were administered. In both cases the Δ18a-only mice showed heightened immobility compared to WT mice. While these tests are not considered to be reliable measures of depression or learned immobility (Commons, Cholanians, Babb, & Ehlinger, 2017; De Kloet & Molendijk, 2016; Molendijk & de Kloet, 2015), they have high predictive validity when testing selective serotonin reuptake inhibitors, indicating that the tests are sensitive to serotonergic modulation (Petit-Demouliere, Chenu, & Bourin, 2005). Therefore, the heightened immobility could be attributed to depletion of serotonin or decreased serotonin release from midbrain serotonergic neurons. This is in line with our previous findings showing robust expression of +18a-Cacna1b in the DRN.

Cocaine potently inhibits dopamine reuptake through the dopamine transporter (DAT) (Verma, 2015) and the predicted rewarding effects of cocaine have been associated with dopaminergic activity (Koob & Volkow, 2010; Wu et al., 2014); therefore, we used cocaine to study differences in dopaminergic function in vivo. In an unbiased design, we taught WT and Δ18a-only mice to associate cocaine with one side of a conditioned place preference chamber through a series of i.p. injections. While we noted a trend toward higher preference in Δ18a-only
mice, this finding was not significant and we saw no differences in change of preference, regardless of whether the mouse was paired with the originally preferred chamber. Based on these results, it is possible that e18a has a subtle effect on dopaminergic communication; however, a power analysis demonstrates that we would need a much higher sample size (n = 35 per genotype) to see significant results. We should also use a biased design in the future to mitigate ceiling effects and the possibility of conditioned place aversion. While cocaine is involved in reward, its potency and selectivity for the norepinephrine transporter and the serotonin transporter is similar to that of the dopamine transporter, and unlike humans, in mice its selectivity is slightly higher for the norepinephrine transporter (Han & Gu, 2006). Therefore, future tests would need to incorporate a mechanism that is more selective to the dopaminergic system, such as optogenetic inactivation paired with a biased conditioned place preference design (Zhang et al., 2015) or use of a selective DAT inhibitor like Difluoropine (Meltzer, Liang, & Madras, 1994).

Finally, we used the BaseScope™ Duplex Assay in an attempt to detect colocalization of Slc6a3 (DAT, a dopaminergic marker) with Cacna1b and Cacna1b-e18a (Cav2.2 and +18a-Cav2.2). Our data indicates that Cacna1b and Slc6a3 colocalize within a subpopulation of VTA and SNc cells. E18a seems to exhibit a similar pattern of expression; however, technical issues in either probe design or mRNA hybridization have made it impossible to accurately assess this portion of our data. In the upcoming months we hope to overcome this technical challenge and continue using the duplex assay to confirm colocalization of e18a with various cellular markers for other cell types which are expressed in the VTA and SNc. If this is not possible, we would propose using a retrograde tracer such as Fluoro-Gold™ to tag dopaminergic cells based on their projections, and use single-cell RT-PCR with e18a and Δ18a primers to identify relative
expression of channel isoforms in specific cell types. This would provide a clearer picture of +18a-Cav2.2 expression patterns, with major implications for the channel’s modulatory function.

It has been demonstrated that Cav2.2 plays a pivotal role in dopamine release from midbrain dopaminergic areas (Brimblecombe et al., 2015). The cells of the VTA and SNc are heterogeneous, releasing dopamine, GABA, and glutamate, sometimes from the same cells (Cohen, Haesler, Vong, Lowell, & Uchida, 2012; Yoo et al., 2016). It has been demonstrated that GABAergic neurons in the VTA play important roles in sleep/wake cycles, and Cav2.2-KO mice demonstrate disturbances in these cycles (Beuckmann et al., 2003; Chowdhury et al., 2019). There is also some evidence to show that somatodendritic dopamine release is Cav2.2-dependent (Ford, Phillips, & Williams, 2010). Therefore, it is possible that Cav2.2 both directly and indirectly modulates dopamine release via release of other transmitters or autoregulation of dopaminergic neurons. While it has been established that dopamine release is calcium-dependent, there is evidence to suggest that other calcium channels like Cav2.1 might aid the dominant Cav2.2 in controlling dopamine release (Dunlap et al., 1995; Phillips & Stamford, 2000). These factors should be further investigated to narrow down the role of Cav2.2 in evoked neurotransmitter release.
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