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The Molecular and Behavioral Characterization of Cav1.3 Over-Expressing Mice

Jamie Nicole Slater

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THE MOLECULAR AND BEHAVIORAL CHARACTERIZATION OF CaV1.3 OVER-
EXPRESSING MICE

by

Jamie Nicole Slater

Thesis

Submitted to the Department of Biology

Eastern Michigan University

In partial fulfillment of the requirements for the degree of

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in

Molecular and Cellular Biology

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December 5, 2011

Ypsilanti, Michigan
DEDICATION

To my grandmother, who always told me I would write a book one day.

Louise Morelli

March 25, 1922 – April 20, 2008
Many people were involved in helping me with this project over the past few years. I would like to thank my project supervisor, Dr. Geoff Murphy, for his continuous support and patience throughout the duration of the project. I would like to thank my committee chair, Dr. Robert Winning, for his support and advice over the past year and a half. Additionally, I would like to thank my committee members, Dr. Daniel Clemans and Dr. Aaron Liepman, for their encouragement.

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ABSTRACT

Age-related cognitive decline refers to the memory impairment and difficulty learning new tasks that occurs during the normal process of aging. There are many possible changes that occur at the neuronal level that could account for age-related cognitive decline. One hypothesis suggests that a dysregulation of neuronal intracellular calcium concentration contributes to age-related cognitive deficits. Previous research suggests that an increase in neuronal L-type voltage-gated calcium channels (LVGCCs) occurs in the brain during aging. This increase could account for altered intracellular calcium concentration and lead to age-related cognitive decline. A line of transgenic mice that over-express the LVGCC $\text{Ca}_V1.3$ in the forebrain was developed in order to investigate the relative contribution of this change to age-related cognitive decline. These animals were also characterized on both on a molecular and behavioral level. Molecular characterization revealed approximately 20% over-expression of $\text{Ca}_V1.3$ in the forebrain of transgenic animals, while neurobehavioral experiments indicate these mice have unimpaired motor function and exploratory behaviors. Based upon these results, this new line of mice will be an excellent model for studies of age-related cognitive decline.
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INTRODUCTION

The Aging Population

In America, the population of individuals over the age of 65 is expected to rise from 37.8 million to between 99 and 108 million within the next forty years. The average life expectancy at birth is expected to rise from 76 years of age to between 82 and 89 years of age (Olshansky et al., 2009). Currently, forty percent of people over the age of 65 are affected by deficits in cognition related to aging (Grundman et al., 2004). Based on these data, the number of individuals experiencing age-related cognitive decline is also likely to rise. Age-related cognitive decline is primarily characterized by memory impairment and difficulty learning new tasks. These impairments are a part of the normal process of aging and occur in the absence of overt pathology, such as Alzheimer’s disease or dementia (Grundman et al., 2004). Due to the increasing number of individuals experiencing age-related cognitive decline, development of novel and effective treatments for cognitive loss has become increasingly important. Therefore, it is critical to determine the underlying molecular mechanisms of age-related cognitive decline.

The Calcium Dysregulation Hypothesis of Aging

Many changes occur at the neuronal level during the process of normal aging that may lead to age-related cognitive decline. One change at the molecular level that occurs during aging is a dysregulation of calcium homeostasis. Khachaturian’s calcium hypothesis of aging suggests that an alteration in intracellular calcium concentration compromises downstream homeostatic functions (Khachaturian, 1987). The effects of dysregulation of calcium homeostasis, including dysfunction in mechanisms that eliminate oxidative stress,
altered synaptic plasticity, and altered gene transcription, may contribute to premature neuronal death and cognitive decline (Khachaturian, 1987).

Results of calcium imaging experiments were consistent with Khachaturian's hypothesis that changes in intracellular calcium levels accompany aging. Intracellular calcium imaging was performed on hippocampal slices of both young adult (3-5mo.) and aged (24-27mo.) rats (Thibault et al., 2001). The hippocampus is an area of the brain thought to play a role in learning and memory that is also sensitive to aging. Ratiometric pseudocolor images were obtained using the fluorophore Indo-1, which fluoresces when bound to calcium in the presence of ultraviolet light. Confocal imaging was performed on single hippocampal neurons while at rest and during trains of action potential firing. At rest, hippocampal neurons from both young adult and aged rats showed similar low levels of fluorescence, indicating a low intracellular calcium concentration. Both young and aged neurons showed an activity-dependent increase in fluorescence, indicating an increase in intracellular calcium concentration with action potential firing (Thibault et al., 2001). This increase was significantly larger in aged neurons than in young neurons. Taken together, these results demonstrate an age-related activity-dependent increase in intracellular calcium, consistent with Khachaturian’s calcium dysregulation hypothesis of aging.

**Calcium and Normal Cell Function**

Calcium plays a critical role in normal cell function. It acts as a signaling molecule for energy production, cell proliferation, gene regulation, and apoptosis (Case et al., 2007). Many of the cellular functions regulated by calcium influx are performed via second-messenger signaling cascades. When intracellular calcium levels increase even slightly,
downstream effects can be greatly amplified. Therefore, it is very important that intracellular levels of calcium are closely maintained. Intracellular calcium concentrations are regulated through a series of membrane channels that regulate both influx and efflux, as well as via intracellular calcium buffering systems (Figure 1). As calcium enters the cell, it is quickly buffered by calbindin and other buffering proteins (Mattson et al., 2007). Calcium can also be removed from the cell via the Na+/Ca+ exchange channel in order to maintain a low intracellular concentration (Mattson et al., 2007). Changes in any of these calcium regulation processes may lead to altered neuronal function.

In neurons, calcium holds an additional function as a regulator of neuronal activity. Calcium is critical for signaling at synapses and plays an important role in membrane excitability and synaptic plasticity (Mattson et al., 2007). Calcium entry into a neuron activates many kinases and results in the production or activation of many downstream signaling molecules. One specific example of an important calcium signaling pathway is the activation of calcium/calmodulin kinase signaling cascades that eventually lead to brain-derived neurotrophic factor expression (West et al., 2001). Brain-derived neurotrophic factor (BDNF) acts as a neuronal survival factor as well as a regulator of synaptic activity (West et al., 2001). Therefore, changes in intracellular calcium may lead to altered expression of BDNF, leading to alterations of neuronal signaling. Calcium also induces neurotransmitter release when voltage-dependent and ligand-gated calcium channels open at presynaptic terminals (Mattson et al., 2007). In a normal state, intracellular calcium concentration is significantly lower than the extracellular concentration, causing rapid influx when calcium-permeable ion channels are open. In conclusion, calcium plays an important role in many vital functions within neurons. Therefore, changes in calcium channels themselves can lead
Figure 1. Regulation of intracellular calcium homeostasis. Intracellular calcium influx occurs via L-type voltage-gated calcium channels as well as NMDARs, whereas efflux occurs via the sodium-calcium transporter. Calcium that has entered a cell can also be buffered via binding proteins such as calbindin and calnexin, or by the endoplasmic reticulum and mitochondria.
to dysregulation of intracellular calcium concentrations, contributing to aberrant neuronal function and cognitive decline.

**L-Type Voltage-Gated Calcium Channels and Aging**

Alterations in calcium buffering, influx, or efflux could account for the dysregulation of intracellular calcium that accompanies aging. One possible explanation for the dysregulation of calcium concentration is the up-regulation of neuronal calcium channel expression. There are a number of routes for neuronal calcium influx, including calcium influx through L-type voltage-gated calcium channels (LVGCCs).

Several subunits make up a complete LVGCC, including the $\alpha_1$, $\alpha_2$, $\beta$, and $\gamma$ subunits (Figure 2). The ion-conducting pore-forming subunit of the complex is the $\alpha_1$ subunit (Lipscombe et al., 2004). The remaining subunits interact with other proteins and calcium channel antagonists, as well as modulate activity of the $\alpha_1$ subunit (Lai et al., 2006). Two types of LVGCCs are expressed in the brain: Ca$_{V}$1.2 and Ca$_{V}$1.3 (Lipscombe et al., 2004). These ion channels are characterized by their activation via strong depolarization of the neuron. L-type voltage-gated calcium channels exhibit slow activation kinetics and calcium-dependent inactivation. The nomenclature "L-type" refers to the "large and long-lasting" current originally described during LVGCC activation. Dihydropyridines are calcium channel antagonists that block calcium flow through the LVGCCs. The dihydropyridines nimodipine and nifedipine are commonly used as antagonists to inactivate LVGCCs (Lipscombe et al., 2004).

LVGCCs are involved in control of neuronal excitability. Neuronal excitability refers to the ability of a neuron to evoke action potentials. This excitability is, in part, controlled by rises in intracellular calcium that occur after periods of neuronal activity. After an action
Figure 2. Diagram of L-type voltage-gated calcium channel. An LVGCC is made up of four subunits, the $\alpha_1$, $\alpha_2$, $\beta$, $\gamma$ and $\delta$ subunits. The $\alpha_1$ subunit forms the ion-conducting pore, while the remaining subunits are responsible for interactions with other proteins and calcium-channel antagonists, as well as regulate the $\alpha_1$ subunit (Lai et al., 2006).
potential or series of action potentials, known as a "burst," has occurred, there is a rise in intracellular calcium concentration. Calcium-activated potassium channels then open, causing a rapid efflux of potassium into the neuron. The efflux of potassium hyperpolarizes the cell, causing what is known as the post-burst afterhyperpolarization (AHP) (Toescu et al., 2010). The neuron must return from the hyperpolarized state to resting potential before again activating, which suppresses neuronal excitability.

There are three phases of the AHP: the fast AHP (fAHP), the medium AHP (mAHP), and the slow AHP (sAHP). The fAHP occurs immediately after an action potential, lasting for about 10ms. The mAHP occurs approximately between 10ms and 100ms after a neuron has fired. The sAHP occurs after a series of action potentials and can last from around 100ms up to 1s post-burst (Toescu et al., 2010). It is known that the sAHP is calcium-dependent, as a sAHP decrease has been seen after blocking of LVGCCs using dihydropyridines (Campbell et al., 1996). The sAHP plays a role in slowing the discharge rate of action potentials (Nicoll et al., 1988). A large sAHP greatly lowers neuronal excitability, due to the longer duration of time before the neuron re-polarizes to normal resting potential.

**The Afterhyperpolarization and Aging**

One experiment describing the AHP in aged animals was performed on hippocampal slices of young (3mo.) and aged (36+ mo.) rabbits (Moyer et al., 1992). The experiment found that the slow AHP in the hippocampus of aged rabbits was significantly larger than that of young rabbits. Furthermore, an addition of nimodipine decreased the AHP in an age-dependent manner in the hippocampus of aged rabbits. The addition of nimodipine also increased neuronal excitability in an age-dependent manner (Moyer et al., 1992; Disterhoft et...
al., 1996). These experiments indicate that both the increase in AHP and decrease in neuronal excitability that occur in the hippocampus of aged animals are LVGCC-dependent.

A correlation has also been found between learning impairments and decreased neuronal excitability in aged rats. Young (3-4 mo.) and aging (29-31 mo.) rats were trained in trace eyeblink conditioning, a task used to study learning in both animals and humans. Rats were conditioned to a tone paired with an air puff to the eye. Animals that learned the task were animals that blinked within 100ms preceding the air puff. After conditioning, brain slices were prepared from the rats and the AHP was recorded in the hippocampus. Aged rats that were unable to learn the task had a large sAHP, whereas aged rats that learned the task had a sAHP of similar size to that of young animals (Matthews et al., 2009).

LVGCCs have been studied in aged rats. A LVGCC agonist, BayK8466, was used on hippocampal slices to augment the L-type current. The neurons were depolarized to 10mV from a holding potential of -70mV. The average total current was recorded in hippocampal slices of young adult, middle-aged, and aged rats. During both depolarization and repolarization, the L-type current was increased in aged animals (Thibault and Landfield, 1996). This result supports the hypothesis that during aging there is an increase in LVGCC current. Furthermore, the density of available LVGCCs was calculated during the experiment and was found to be significantly higher in aged rats than in young adult or middle aged rats (Thibault and Landfield, 1996).

The previously discussed experiments strongly suggest that LVGCCs play a role in decreased neuronal excitability in aged animals. However, these experiments did not determine which LVGCC, Cav1.2 or Cav1.3, was over-expressed during aging and caused an
increase in the AHP. This ambiguity is due to the similar pharmacology of CaV1.2 and CaV1.3 (Gamelli et al., 2011). While dihydropyridines block LVGCCs in general, no specific antagonist is available to target either CaV1.2 or CaV1.3 individually. In order to circumvent non-specificity of LVGCC antagonists, knockout (KO) mice of each brain-specific LVGCC were developed. Brain slices were prepared from knockout mouse models of CaV1.2 or CaV1.3. Experiments were performed in the hippocampus to determine which LVGCC contributes to the sAHP (Gamelli et al., 2011). When CaV1.2 was knocked out, the sAHP in KO animals vs. wild-type animals was not diminished. This result indicates that CaV1.2 is not involved in the sAHP in the hippocampus of young mice. However, the sAHP in CaV1.3KO animals was significantly diminished compared to wild-type counterparts, suggesting that CaV1.3 plays a role in the slow afterhyperpolarization (Gamelli et al., 2011).

One caveat to this study is that it was not performed on aged animals, therefore, it has been extrapolated that knocking out CaV1.3 in aged mice would result in a diminished sAHP in the hippocampus compared to that of age-matched wild-type counterparts.

Based on the preceding evidence, a transgenic mouse model was developed with the purpose of studying the relative contribution of CaV1.3 to the increase in AHP and cognitive deficits that accompany the process of normal aging. This mouse model was designed to over-express CaV1.3, mimicking LVGCC over-expression previously found in aged rats. This model is referred to as CaV1.3HA because of an HA tag located on the CaV1.3 transgene. A forebrain-specific transgenic promoter, alpha calcium/calmodulin kinase II, was exploited to localize transgene overexpression to the area of the brain affected by aging that is also associated with learning and memory (Mayford et al., 1996). It is hypothesized that young CaV1.3HA mice will exhibit cognitive deficits similar to the deficits found in wild-
type aged mice. Before utilizing this new line of mice as a model of aging, it is imperative to examine the molecular characteristics of CaV1.3 over-expression. Transgenic mice also need to be examined in basic neurobehavioral tests to determine if transgene presence affects normal brain function and behaviors. The work presented herein describes the development as well as a molecular and basic neurobehavioral characterization of this new mouse model of aging.
MATERIALS AND METHODS

Transgene Construction

Two plasmids contained the DNA components necessary for creating the Ca\textsubscript{V}1.3HA transgene. The first plasmid (pMM403; a gift from M. Mayford, SCRIPPS, CA) (Mayford et al., 1996) contained a sequence of the 5’ UTR (approximately 8.5kb) isolated from the upstream region of rat alpha Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II, αCaMKII, which contained the promoter element (Mayford et al., 1996). A characterization utilizing this 8.5kb 5’UTR demonstrated that the αCaMKII promoter effectively localizes transgene expression in the forebrain (Mayford et al., 1996). Furthermore, protein expression of αCaMKII begins postnatally, with expression beginning around day four and leveling off around day 35 (Burgin et al., 1990). Plasmid pMM403 also contained a pBS backbone region of approximately 3,000 base pairs (bp). The pBS region contained an ampicillin-resistance gene, which was taken advantage of during cloning procedures. This plasmid was linearized using NotI (NEB, Ipswich, MA) and dephosphorylated with Antarctic Phosphatase (NEB, Ipswich, MA) in order to aid in ligation of the Ca\textsubscript{V}1.3HA sequence. All enzymes were used according to standard protocol. The linearized and dephosphorylated promoter sequence was then gel-purified according to kit instructions using the SNAP UV-Free Extraction Kit (Invitrogen, Carlsbad, CA).

The transgene was located on plasmid pBCM04, flanked by synthetic 3’ and 5’ UTRs. The Ca\textsubscript{V}1.3 transgene, a generous gift from I. Bezprozvanny (University of Texas, TX), was derived from rat Ca\textsubscript{V}1.3 cDNA and tagged on an extracellular loop of the second transmembrane domain with an HA epitope as previously described (Zhang et al., 2006).
Characterization of the epitope-tagged Cav1.3 sequence demonstrated that the recombinant protein is functional (Zhang et al., 2006). Synthetic 5’ and 3’ UTRs were inserted flanking the Cav1.3HA transgenic cDNA in order to help stabilize mRNA and increase transgene expression during the transcription process (Callis et al., 1987).

The transgene-synthetic UTR sequence was extracted from pBCM04 by digesting with NotI (NEB, Ipswich, MA) and gel-purifying the 7218bp fragment using the SNAP UV-Free Extraction Kit (Invitrogen, Carlsbad, CA). The purified DNA fragments from pMM403 and pBCM04 were ligated using T4 DNA Ligase (NEB, Ipswich, MA) and transformed into Escherichia coli DH5α Subcloning Efficiency Competent Cells (Invitrogen, Carlsbad, CA). The transformation was performed according to the Invitrogen protocol. Colonies from transformations were plated and grown overnight on LB-ampicillin (100µg/mL) plates and were then grown overnight in 3mL LB-ampicillin (100µg/mL). DNA was extracted using the Qiaprep Spin Miniprep Kit (Qiagen, Valencia, CA) and analyzed using NotI (NEB, Ipswich, MA) to check for transgene insertion, and HindIII (NEB, Ipswich, MA) digestions to check for insertion orientation. All mini-preps were performed according to the Qiaprep protocol. Additionally, plasmid DNA was sent to the University of Michigan Sequencing Core with plasmid-specific primers to ensure the final DNA sequence was correct.

The resulting plasmid, containing the αCaMKII promoter, 3’ and 5’ synthetic UTRs, as well as the Cav1.3HA sequence, was called pJNS01 (Figure 1A). The 50µg of transgene needed for injection was obtained from approximately 50 Qiaprep Spin mini-preps as opposed to a single maxi-prep, due to the large size of pJNS01 (18kb), which inhibited bacterial growth. DNA was linearized for injection using SfiI (NEB, Ipswich, MA), and the injection sequence of approximately 15kb was purified using the SNAP UV-Free Extraction
Figure 3. CaV1.3HA transgenic construct and diagnostic cuts. A. Diagram of plasmid pJNS01, containing the 5’ UTR region of the αCaMKII promoter, 5’ intron (UTR), CaV1.3 transgene, 3’ intron and pBS backbone. Locations of NotI and SfiI restriction sites are labeled. The NotI sites indicate where the CaV1.3 transgene was inserted into the αCaMKII promoter-containing plasmid, pMM403 (not shown). The SfiI sites indicate where the pBS backbone was removed to linearize the transgene. B. Diagnostic cuts of the final transgenic construct (pJNS01). (Left) The NotI cut shows the 12.8kb band containing the αCaMKII promoter fragment, as well as the 7.2kb band containing the CaV1.3 transgene fragment. (Right) Uncut pJNS01 (U) and a SfiI cut of pJNS01 showing the linearized transgene at 15kb, with the pBS backbone at 3kb. A Lambda-HindIII digest high-base pair ladder was used in both gels (Takara, Japan).
Kit (Invitrogen, Carlsbad, CA). 50µg of DNA was sent to the University of Michigan Transgenic Core for injection into C57/B6 female mice. Transgenic injection occurs at random; therefore, the locus of transgene integration is unknown in the resulting transgenic mice.

Genotyping

Transgenic mice were maintained on a C57/B6NTac background (Taconic Laboratories, Cambridge City, IN). Tail samples were digested overnight at 55°C in One-Step Tail Buffer (50mM KCL, 10mM Tris-HCL pH 9.0, 0.1% Triton X-100, 0.15mg/mL Proteinase K). Polymerase chain reaction (PCR) was performed using Titanium Taq Polymerase (Clontech Laboratories, Mountain View, CA) at the following parameters: initial denaturation for 3:00 at 94°C, 30 cycles (0:45 at 94°C, 0:45 at 62°C, 0:45 at 72°C), and final elongation of 5:00 at 72°C. Genotyping was performed using the transgene primers Fg3 (CCCCGAGGCTACCATCACC) and R2 (AGCCATGTACGTAGCCATCC) (~1000bp) (Figure 2A), as well as wild-type primers β-actin Right (CTCTCAGCTGTGGTGGTGAA) and β-actin Left (GCTCCCATTTCATCATGTCCTCATGGA) (~250bp). Transgene primers were specifically designed on two separate exons of the CaV1.3 sequence, in order to ensure that endogenous CaV1.3 DNA would not be detected during the PCR process.

RNA Analysis

Mice were anesthetized using Isoflurane (MWI, Meridian, ID), and brains were removed and dissected into cortex, hippocampus, and cerebellum. RNA was extracted from the brain regions using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s directions. RNA purity and concentration were determined using a NanoDrop
**Figure 4. PCR primers and HA-tag locations on the CaV1.3HA transgene.** A. Diagram of the linearized CaV1.3HA transgenic construct, showing the locations of the forward and reverse PCR primers used for reverse transcription PCR (HA RT F and HA RT R), and genotyping (Fg3 and R2). B. Diagram of CaV1.3, showing the location of the HA tag on the second extra-cellular loop of domain two in the four-domain transmembrane calcium channel.
spectrophotometer (Thermo Scientific, Waltham, MA). Any residual DNA in each sample was eliminated using DNasel (Invitrogen, Carlsbad, CA). Contamination of residual DNA was tested using reverse-transcription controls.

Reverse transcription was performed using SuperscriptIII First-Strand Synthesis System according to manufacturer’s directions (Invitrogen, Carlsbad, CA). PCR on cDNA samples was performed using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) and the following parameters: initial denaturation for 2:00 at 94°C, 40 cycles (0:15 at 94°C, 0:30 at 55°C, 1:00 at 70°C). Genotyping for cDNA used the transgene primers HA RT F (ACACAGACGCGTCACTATCC) and HA RT R (TGACACCAGAGACACCCCAACGAC) (~1000bp) (Figure 2A), as well as wild-type primers β-actin Right (CTCTCAGCTGTGGTGTTGGA) and β-actin Left (GCTCCCATTCATCAGTTCCATAGGT) (~250bp). Reverse transcription PCR primers were designed to include the 3’ synthetic intron, so that they would not detect endogenous CaV1.3 reverse-transcribed mRNA.

**Western Blot Analysis**

Western blotting was performed using membrane fractions in order to examine proteins embedded in the neuronal membrane. To obtain membrane fractions, mice were anesthetized using Isoflurane (MWI, Meridian, ID) and the brain was removed, glued to the bottom of a Petri dish, and then covered with ice-cold phosphate-buffered saline (PBS: 0.02M phosphate, 0.15M NaCl, pH 7.4). The brain was dissected into the hippocampus, the cortex, and the cerebellum. Each sample was covered with 500µL of ice-cold HEPES sucrose EDTA (HSE: 10mM HEPES, 350mM Sucrose, 5mM ETDA) with one tablet of Compete
Mini Protease Inhibitor (Roche, Indianapolis, IN). Samples were homogenized with a pestle and centrifuged at 4°C for 5 minutes at 2000xg. The supernatant and a high-sucrose buffer, HSE, were placed in a 10.4mL Nalgene Oakridge ultracentrifuge tube (Thermo Fisher Scientific, Rochester, NY). Samples were spun at 100,000xg for 1 hour. The supernatant was removed and the pellet resuspended in 200µL HSE with one tablet of Complete Mini Protease Inhibitor (Roche, Indianapolis, IN) and 1% Triton X to solubilize membranes and release membrane proteins.

Protein quantification was performed using the Quick Start Bradford Protein Assay (BioRad, Hercules, CA) according to the manufacturer’s directions. Samples were prepared for Western Blotting by mixing 1:2 with Laemmli Buffer (BioRad, Hercules, CA) containing β-mercaptoethanol. Samples were then heated for five minutes at 95°C to denature the protein. Between 20µg and 30µg of protein were loaded per lane in a 7.5% SDS-polyacrylamide gel (BioRad, Hercules, CA). For detection of the recombinant protein, mouse anti-HA (Covance, Princeton, NJ) was used at dilution of 1:1000 in water. Endogenous CaV1.3 was detected using rabbit anti-CaV1.3 (1:3000) as previously described (Jenkins et al., 2010). The CaV1.3 antibody was a kind gift from A. Lee (University of Iowa, IA). The loading control used in all Western blots was mouse anti-Transferrin (Invitrogen, Carlsbad, CA) used at a dilution of 1:500. Secondary antibodies used were horseradish-peroxidase conjugated (HRP) goat anti-mouse and HRP goat anti-rabbit (Roche, Indianapolis, IN). Western blots were visualized using Amersham Prime ECL (GE Healthcare, Waukesha, WI) and exposed for between 10s and 5min. Film was then developed using a Kodak developer (Eastman Kodak, New Haven, CT).
Behavioral Subjects

Mice used for behavioral experiments were moved to a holding room near behavior testing facilities at least a week before the start of testing. All animals were fed *ad libitum* and kept on a schedule of 14 hours light: 10 hours dark. Testing was performed at approximately the same time each day, during the animals' light cycle. CaV1.3HA line 102 transgenic mice and age-matched wild-type siblings were between the ages of 4 and 4.5 months. CaV1.3HA line 103 transgenic mice and age-matched wild-type siblings were between the ages of 2 and 5 months. All animals were housed and cared for according to University of Michigan and the University Committee on Use and Care of Animals (UCUCA) guidelines. All experiments were approved by UCUCA on protocol #08768 (Appendix E).

Rotarod

Mice were placed on a rotating rod (UGO Basile Accelerating Rotarod, Collegeville, PA) in squads of five. Mice were separated by dividers attached to the rotarod. The rotarod accelerated from 4 to 40rpm over the course of five minutes. Latency to fall was recorded as the time when the mouse made a full rotation or fell off the rotating rod. Mice were given one trial per day over a five-day period, with a five-minute maximum time of trial.

Open Field

The open field was a 71x71x30cm white acrylic box placed in a room lit by indirect white light. Mice were singly placed in the center of the box and allowed to explore for five minutes. The field was divided into a center and a peripheral zone. Exploration was recorded using a video camera (Olympus, Center Valley, PA). The total distance traveled as well as
the distance traveled in each zone was calculated using LimeLight 2 tracking software (Actimetrics, Evanston, IL).

**Light/Dark Box**

The light/dark box was a 46cm box placed in a room lit by indirect white light. The box was divided into two zones, a dark zone, made of black acrylic with a black lid, and a light zone, which was open at the top. The light zone was two times the length of the dark zone. A small opening was cut in the center of the black acrylic wall separating the two zones. Mice were singly placed in the center of the light zone and allowed to explore for five minutes, and exploration was recorded using a video camera. The total distance traveled in each zone as well as the number of transitions between the light and dark zones was calculated using LimeLight 2 tracking software (Actimetrics, Evanston, IL).

**Data Analysis**

Kodak 1D software (Eastman Kodak, New Haven, CT) was used to analyze pixel density of Western blot images. Data collected from behavioral experiments were exported from LimeLight 2 software and imported into StatView (SAS Institute Inc., Cary, NC) or GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). All graphs were made using GraphPad Prism 5. Statistical significance ($\alpha = .05, p < 0.05$) was obtained using Student’s $t$-test or repeated measures ANOVA, as appropriate.
RESULTS

Molecular Characterization

Transgene construct assembly

The CaV1.3HA transgene was created from two separate plasmids, pBCM04 and pMM403. The 5’UTR-CaV1.3 Transgene-3’UTR was excised from pBCM04 using NotI and ligated into pMM403, the promoter-containing plasmid. As shown in Figure 1B, a NotI diagnostic cut of pJNS01 contains the 5’UTR-CaV1.3 Transgene-3’UTR fragment at 7,200bp and the αCaMKII promoter-pBS fragment at 12,800bp. The transgene was linearized via a SfiI cut, removing the pBS backbone fragment at 3,000bp and linearizing the CaV1.3HA transgene at 15,000bp (Figure 3B).

Mice

The linearized transgene construct was sent to the University of Michigan Transgenic Core for injection into pronuclear oocytes of a C57/B6 female mouse to generate transgenic pups. As shown in Figure 5, six of eighteen mice obtained from the transgene injection were genotyped as positive for the CaV1.3HA transgene. The six mice positive for the transgene, hereafter referred to as founders, were each given a number (100-105) and paired with a C57/B6Tac mouse of the opposite sex. Four of the initial founding mice were male, and two were female. Female transgenic mice were unable to produce viable progeny, whereas all four founding males produced pups that lived to be weaned. This result may indicate a high copy number of transgene or unviable locus of transgene injection in the female transgenic mice. The CaV1.3HA transgene was detected in the progeny of three of the four founding
Figure 5. Multiplex PCR of CaV1.3HA founding mice. A band at 1000bp indicates the presence of the CaV1.3HA transgene in tail DNA from six of the eighteen live pups obtained from transgene injection at the University of Michigan Transgenic Core. Each positive mouse was labeled with a founding line number (100-105). A single copy of the CaV1.3HA transgene-containing plasmid, pJNS01, was used as a positive control (+). The band at 230bp indicates the presence of β-actin in all tail samples. A 1000bp ladder was used for reference.
males. Upon further molecular characterization, only two founding lines (102 and 103) expressed the recombinant protein. Further analysis of lines 102 and 103 determined that both lines exhibit normal motor performance and exploratory behavior. These founding data are summarized in Table 1. Initial molecular characterization of the Ca\textsubscript{v}1.3HA mice focused on line 102 based on results of concurrently performed behavioral experiments (not shown). Molecular characterization of Ca\textsubscript{v}1.3HA line 103 will be performed in the future.

**RT-PCR (Reverse-Transcriptase Polymerase Chain Reaction)**

To determine if the Ca\textsubscript{v}1.3HA transgene inserted into the genome of founding line 102 was functional and transcribed into RNA, RT-PCR was performed on RNA samples from brain tissue. Reverse-transcriptase PCR is used to synthesize and amplify cDNA from messenger RNA (mRNA) via the enzyme reverse-transcriptase. RNA was isolated from cortex, hippocampus, and cerebellum tissue samples obtained from a mouse positive for the Ca\textsubscript{v}1.3HA transgene, as well as a wild-type counterpart. After reverse-transcription, the resulting cDNA samples from each brain region were amplified in a PCR reaction using the primers shown in Figure 4A. As shown in Figure 6, PCR amplification of cDNA in brain tissue samples revealed Ca\textsubscript{v}1.3HA transgene expression in the cortex, hippocampus, and cerebellum of the Ca\textsubscript{v}1.3HA positive animal. There was no Ca\textsubscript{v}1.3HA transgene expression in the wild-type animal.

**Western blot analysis**

Western blot analysis was performed on membrane fractions of mouse cortex, hippocampus, and cerebellum to determine Ca\textsubscript{v}1.3HA recombinant protein presence. Membrane fractions were used to examine the parts of the cell containing Ca\textsubscript{v}1.3HA protein.
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**Table 1: CaV1.3HA Founding Mice.** This table presents the breeding, molecular and behavioral characteristics of each founding line of mice. Each line was examined for successful breeding, which included having progeny and the progeny living until the age of weaning, twenty-one days. Progeny of each founding line were genotyped to determine if the transgene was present. The progeny of founding lines containing the transgene were then tested for recombinant protein presence using Western blot analysis. Founding lines containing the recombinant protein were examined for a normal behavioral phenotype using a series of neurobehavioral tests. Results are marked as + (yes/positive), - (no/negative) and NA (not applicable).
Figure 6. RT-PCR of CaV1.3HA expression in different brain regions. The bands on the left indicates the presence of the CaV1.3HA at 1000bp in the cortex (ctx), hippocampus (hip) and cerebellum (cbm) of the line 102 CaV1.3HA positive (+) mouse. No bands are present in the line 102 CaV1.3HA age-matched wild type sibling (-). The bands on the right control for reverse transcription, indicating the presence of β-actin at 250bp in each sample. Controls were conducted by excluding reverse-transcriptase from each sample (not shown). A 100bp ladder from Promega was used for reference.
The location of the HA-tag on the Ca\textsubscript{V}1.3 protein is shown in Figure 4B. The tag is located on the extracellular side of second domain of the protein (Zhang et al., 2006). As seen in Figure 7, a Western blot probing for the HA epitope on the Ca\textsubscript{V}1.3 gene shows Ca\textsubscript{V}1.3HA expression in the cortex and hippocampus of transgenic animals. The recombinant protein is found at 250kD and is approximately the same size as endogenous Ca\textsubscript{V}1.3. A lack of Ca\textsubscript{V}1.3HA expression in the cerebellum is accounted for by the forebrain specificity of the \(\alpha\)CaMKII promoter. The blot was also probed with anti-transferrin as a control for protein loading.

Figure 8 shows a Western blot using an antibody against endogenous Ca\textsubscript{V}1.3. The blot indicates the presence of Ca\textsubscript{V}1.3 in the cortex, hippocampus, and cerebellum of both a transgenic and wild-type animal. However, there appears to be significantly more Ca\textsubscript{V}1.3 protein in the cortex and hippocampus of the transgenic animal. This is presumably due to the Ca\textsubscript{V}1.3 antibody detecting both the endogenous as well as overexpressed protein.

To further examine the overexpression of the Ca\textsubscript{V}1.3 protein in transgenic animals, cortex samples were analyzed from four transgenic and four wild-type animals (Figure 9A). Pixel density of each protein band (Ca\textsubscript{V}1.3 and transferrin) was calculated using Kodak1D software. The resulting blot was subjected to densitometric analysis, normalizing the pixel density of Ca\textsubscript{V}1.3 to the pixel density of the loading control in each animal to generate a ratio of Ca\textsubscript{V}1.3 expression. The ratio for all wild-type animals was averaged, and then each Ca\textsubscript{V}1.3:transferrin ratio was normalized to this value. Figure 9B shows the comparison of average data from the transgenic group of animals and wild-type animals. Animals positive for the Ca\textsubscript{V}1.3HA transgene were found to have approximately twenty percent more \(p = \)
Figure 7. Hybrid Western blot of CaV1.3HA and Transferrin expression. The Covance mouse antibody against the HA tag (anti-HA) was used initially, then the membrane was stripped and reprobed with mouse anti-transferrin (anti-Tfer), a transmembrane protein found throughout the brain. The presence of the 250kD CaV1.3HA protein can be noted in the cortex (ctx) and hippocampus (hip) of the line 102 CaV1.3HA positive animal (+). Human embryonic kidney (hek) cells were transfected with CaV1.3HA DNA and were used as a control for the HA antibody.
Figure 8. Western blot of CaV1.3 expression. A rabbit anti-CaV1.3 (anti-1.3) antibody was used initially, then the membrane was stripped and reprobed using mouse anti-transferrin (anti-Tfer). A CaV1.3 knockout mouse (ko) was used as a control for the antibody. The line 102 mouse positive for CaV1.3HA (+) had more CaV1.3 in its cortex (ctx) and hippocampus (hip) than its wild-type counterpart (-). An equal amount of CaV1.3 was found in the cerebellum (cbm) of both animals. The band at 250kD indicates the presence of CaV1.3 and the band at 95kD indicates the presence of transferrin, a ubiquitous membrane protein.
Figure 9. Relative CaV1.3 expression in CaV1.3HA mice. A. Membrane fractions from the cortex of four line 102 CaV1.3HA positive (+) and four CaV1.3 negative (-) were used to examine relative CaV1.3 overexpression in transgenic mice. A rabbit anti-CaV1.3 (anti-1.3) antibody was used initially, then the membrane was stripped and re-probed with mouse anti-transferrin (anti-Tfer). Underlined +/− pairs indicate age-matched littersmates. B. The normalized band density (percentage of WT) of the CaV1.3:Transferrin bands were obtained for each of the four CaV1.3 HA positive (HA+) and the four control (WT) mice from (A). The normalized band density of HA+ mice was significantly more (*, p-value 0.02) than the normalized band density of wild-type mice. Data are plotted as mean ± standard error of mean (SEM).
0.02) Ca\textsubscript{V}1.3 in their cortex than their wild-type counterparts. A membrane fraction sample from a Ca\textsubscript{V}1.3KO mouse was also probed with anti-Ca\textsubscript{V}1.3 to ensure antibody specificity.

**Neurobehavioral Analysis of Ca\textsubscript{V}1.3HA Line 102**

**Rotarod**

Mice were tested on the accelerating rotarod to analyze motor function. The test was given for 300 seconds once a day over the course of five days. Mice with normal motor function are anticipated to show an increased latency to fall during the training period. Figure 10 shows the data acquired over a five-day testing period for six Ca\textsubscript{V}1.3HA positive mice from the 102 line and four age-matched wild-type control animals. While both groups of mice had relatively low performance on day one, by day five both groups had reached an average of about 180 seconds on the rotarod before falling. There was no significant difference between transgenic and wild-type animals (\(p = 0.12\)) during each trial. However, performance of each group improved daily, and performance was significantly better on the final day of testing than on the first (\(p < 0.0001\)). These results suggest that there is no deficit in motor function due to Ca\textsubscript{V}1.3 over-expression in founding line 102.

**Open Field**

The open field test was performed to determine overall exploratory and anxiety-like behavior in Ca\textsubscript{V}1.3HA mice. Eight Ca\textsubscript{V}1.3HA-positive mice from line 102 and six wild-type mice were individually placed for five minutes in the open field and allowed to freely explore. As seen in Figure 11A, there was no difference in the total distance traveled per group (\(p = 0.29\)). The distance traveled in the center versus the distance traveled in the periphery was also examined, as shown in Figure 11B. Again, there was no significant
Figure 10. Rotarod performance in line 102 CaV1.3HA mice. Six CaV1.3HA positive (HA+) and four wild-type controls (WT) were trained for five days (300 second trials) on the Rotarod. The latency to fall (s) was recorded for both groups of mice each day. Data are plotted as mean ± standard error of mean (SEM).
Figure 11. Open field performance in Line 102 CaV1.3HA mice. Five wild-type (WT) and eight CaV1.3HA+ (HA+) mice were placed individually in the open field for five minutes. A. The total distance traveled (cm) for WT and HA+ mice. B. The average distance traveled per zone (cm), center and edge, for CaV1.3HA Line 102. Data are plotted as mean ± standard error of mean (SEM).
difference between the distances traveled in either zone between the groups \((p = 0.22)\), suggesting that the \(\text{Ca}_V1.3\)HA mice do not express an anxiety-like phenotype. However, both groups traveled a significantly farther distance around the edge of the open field \((p < 0.0001)\). Due to a tracking error, one wild-type mouse from group 102 was excluded in the open field analysis. These results suggest that overexpression of \(\text{Ca}_V1.3\) did not affect exploratory and anxiety-like behaviors of transgenic mice.

**Light/Dark Box**

The final behavioral test performed was the light/dark box. This test assessed anxiety-like and exploratory behavior in the \(\text{Ca}_V1.3\)HA mice. Eight \(\text{Ca}_V1.3\)HA positive mice from line 102 and six wild-type counterparts were individually placed in the box for 300 seconds. Data from the light/dark box are represented in Figure 12. Both transgenic and wild-type animals avoided the light side of the box, spending significantly more time in the dark zone \((p < 0.0001)\) (Figure 12A). There was no difference between the genotypes in time spent in either zone \((p = 0.87)\). The number of crossings between the zones was also similar between the transgenic and wild-type animals \((p = 0.73)\) (Figure 12B). These results suggest that \(\text{Ca}_V1.3\) overexpressing mice exhibit normal behavior in the light/dark box.

**\(\text{Ca}_V1.3\)HA Line 103 Neurobehavioral Analysis**

**Rotarod**

Six \(\text{Ca}_V1.3\)HA positive mice from line 103 and seven wild-type controls were trained for five days on the accelerating rotarod. Figure 13 shows the average latency to fall for each group over five days of training. There was no statistically significant difference between
Figure 12. Light dark box performance in line 102 CaV1.3HA mice. Six wild-type (WT) and eight CaV1.3HA positive (HA+) mice were individually placed for 300 seconds in the light dark box. A. The total time in zone (s) by each group. B. The number of transitions between the light and dark zones per group. Data are plotted as mean ± standard error of mean (SEM).
Figure 13. Rotarod performance in line 103 Cav1.3HA mice. Six Cav1.3HA positive (HA+) and seven wild-type controls (WT) were trained for five days (300 second trials) on the RotaRod. The latency to fall (s) was recorded for both groups of mice each day. Data are plotted as mean ± standard error of mean (SEM).
genotypes on each day ($p = 0.86$). All mice performed significantly better over the course of the five-day training period ($p = 0.0005$). These results suggest that overexpression of $\text{CaV}1.3$ does not affect motor function in $\text{CaV}1.3\text{HA}$ line 103.

**Open Field**

Six $\text{CaV}1.3\text{HA}$ positive mice from line 103 and seven wild-type controls were tested in the open field. The total distance traveled for each group is shown in Figure 14A. There was no significant difference in the distance traveled between the genotypes ($p = 0.30$). The distance traveled in each zone is shown in Figure 14B. All mice traveled significantly more in the edge zone than in the center zone ($p < 0.0001$). However, there was no difference between the genotypes within each zone ($p = 0.79$). These results suggest that exploratory and anxiety-like behaviors in $\text{CaV}1.3\text{HA}$ line 103 are similar to that of wild-type mice.

**Light Dark Box**

Six $\text{CaV}1.3\text{HA}$ positive and seven wild-type controls were tested for 300 seconds in the light dark box. The total time spent per group in either the light or dark zone is shown in Figure 15A. $\text{CaV}1.3\text{HA}$ positive mice spent significantly less time in the dark zone than their wild-type counterparts ($p = 0.01$). Both groups spent significantly more time in the dark zone than in the light zone ($p < 0.0001$). The number of transitions between zones is shown in Figure 15B. There was no difference in the number of transitions between zones per group ($p = 0.46$). These results suggest that there is a slight difference in light dark box behavior in $\text{CaV}1.3$ over-expressing mice compared to wild-type counterparts.
Figure 14. Open field performance in line 103 Ca$_V$1.3HA mice. Seven wild-type (WT) and six Ca$_V$1.3HA positive (HA+) mice were placed in the Open Field for 300s. A. The total distance traveled (cm) for Ca$_V$1.3HA Line 103. B. The average distance traveled (cm) by WT and HA+ animals in each of the two zones of the open field, edge and center. Data are plotted as mean ± standard error of mean (SEM).
Figure 15. Light/dark box performance in line 103 Ca\textsubscript{V}1.3HA mice. Seven wild-type (WT) and six Ca\textsubscript{V}1.3HA positive (HA+) mice were individually placed for 300 seconds in the light dark box. A. The total time (s) spent in each zone of the light dark box. B. The number of transitions between the light and dark zones per group. Data are plotted as mean ± standard error of mean (SEM).
DISCUSSION

Biochemical and Molecular Characterization of Ca\textsubscript{v}1.3HA Mice

Ca\textsubscript{v}1.3HA transgenic mice were developed in order to mimic the putative increase in Ca\textsubscript{v}1.3 expression that may occur during aging in young animals. The first step in characterization of these animals was to characterize transgenic Ca\textsubscript{v}1.3HA expression.

The molecular characterization of Ca\textsubscript{v}1.3HA animals explicitly examined the Ca\textsubscript{v}1.3HA transgene in RNA as well as in Ca\textsubscript{v}1.3HA protein expression (Figures 6 and 7). Furthermore, endogenous Ca\textsubscript{v}1.3 expression in transgenic animals was also examined via Western blot analysis, in order to determine the effect of Ca\textsubscript{v}1.3 over-expression on endogenous protein levels.

The αCaMKII promoter was used in the Ca\textsubscript{v}1.3HA transgenic construct for two reasons. Primarily, previous research using the promoter has shown localization of transgenic proteins to the forebrain of transgenic animals (Mayford et al., 1996). Forebrain expression of the transgenic protein marginalized the possibility that transgene expression in the cerebellum would interfere with normal motor function. An additional benefit to using the αCaMKII promoter is that its expression begins post-natally (Burgin et al., 1990). Therefore, it was anticipated that transgenic mice would not experience problems that may arise from transgenic expression during fetal development.

RNA expression of Ca\textsubscript{v}1.3HA was anticipated in only the cortex and hippocampus of transgenic brain tissue samples. RNA samples were treated with DNase\textsubscript{I}, then reverse-transcribed into cDNA and analyzed via PCR for transgene expression. The results obtained from reverse-transcriptase PCR analysis demonstrated RNA presence in all three areas of the
brain examined: the cortex, hippocampus, and cerebellum. In experiments characterizing αCaMKII localization using in-situ hybridization, some expression of αCaMKII RNA was discovered in the cerebellum of rats (Burgin et al., 1990). Expression of αCaMKII in the cerebellum was very low compared to the expression in the forebrain. Interestingly, expression in all areas of the brain increased over time (Burgin et al., 1990). In order to determine if there is less expression of Cav1.3HA RNA in the cerebellum than in the forebrain of transgenic mice, a quantitative analysis could be performed using real-time PCR.

After RNA analysis, localization of Cav1.3HA recombinant protein within the brain was determined. In membrane fraction samples, Cav1.3HA recombinant protein was found in the cortex and hippocampus of transgenic mice, but not in the cerebellum (Figure 7). This result is similar to that of the initial characterization of transgenic protein localized to the forebrain using the αCaMKII promoter (Mayford et al., 1996). This result further supports the explanation that the expression of Cav1.3HA RNA found in the cerebellum of transgenic animals was negligible. In addition, motor performance in both Cav1.3HA lines 102 and 103 was unimpaired, further indicating that any Cav1.3HA expression in the cerebellum was insignificant.

Because Cav1.3HA transgenic mice were developed to overexpress Cav1.3 in the forebrain, it was important to determine the effect on endogenous Cav1.3 protein expression in the presence of the Cav1.3HA transgene. A possible outcome of Cav1.3 overexpression was a down-regulation of endogenous Cav1.3 in areas where the transgene was expressed. One caveat assessing endogenous Cav1.3 levels in Cav1.3HA overexpressing mice was that the anti-Cav1.3 antibody detected the same epitope in both Cav1.3HA and Cav1.3 in transgenic animals. This is because rat Cav1.3 (as used in the Cav1.3HA transgenic
and mouse Ca\textsubscript{V}1.3 have similar protein sequences. Therefore, the Western blot obtained using an anti-Ca\textsubscript{V}1.3 antibody showed an increase in Ca\textsubscript{V}1.3 protein expression in the forebrain but not cerebellum of a transgenic mouse (Figure 8). This result supports that the Ca\textsubscript{V}1.3HA transgene was forebrain specific, and leads to a Ca\textsubscript{V}1.3 over-expression in transgenic animals. In the future, brain tissue samples from additional mice will be examined to ensure results can be replicated. In addition, when cortical membrane fractions of four Ca\textsubscript{V}1.3HA positive and four wild-type mice were examined for the relative overexpression of Ca\textsubscript{V}1.3, the samples from transgenic mice had approximately 20% more Ca\textsubscript{V}1.3 than wild-type mice (Figure 9).

Expression of Ca\textsubscript{V}1.2 was not examined in this study. There could be down-regulation in Ca\textsubscript{V}1.2 expression compensating for the overexpression of Ca\textsubscript{V}1.3. Although down-regulation has not been examined directly, one experiment suggesting otherwise examined the sAHP in both Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 knockout mice. The sAHP was diminished in a knockout model of Ca\textsubscript{V}1.3. However, the sAHP was not affected in a knockout model of Ca\textsubscript{V}1.2 (Gamelli et al., 2011). The change in the sAHP in the knockout model of Ca\textsubscript{V}1.3 indicates that Ca\textsubscript{V}1.2 expression is not up-regulated in order to compensate for the absence of Ca\textsubscript{V}1.3. This lack of compensation by Ca\textsubscript{V}1.2 in Ca\textsubscript{V}1.3 knockout models suggests that there is no interaction between expression levels of Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3. Therefore, it seems unlikely that Ca\textsubscript{V}1.2 expression would be down-regulated in order to maintain homeostatic balance in the occurrence of Ca\textsubscript{V}1.3 overexpression. In the future, protein expression of Ca\textsubscript{V}1.2 will be analyzed in Ca\textsubscript{V}1.3 overexpressing mice in order to determine if Ca\textsubscript{V}1.2 expression is affected in the up-regulation of Ca\textsubscript{V}1.3.
Another potential complication is that Ca\textsubscript{V}1.2 expression may be up-regulated in Ca\textsubscript{V}1.3 over-expressing mice. This possibility would limit definitive conclusions made about the specific role of Ca\textsubscript{V}1.3 overexpression. However, any observed impairments found in learning and memory tasks in these animals would be due to an overall up-regulation of LYGCC expression, and thus would be in accordance with previous conclusions regarding LYGCCs and aging.

**Ca\textsubscript{V}1.3HA Mice Exhibit Normal Motor Performance**

One possible outcome of Ca\textsubscript{V}1.3 overexpression in Ca\textsubscript{V}1.3HA mice was a motor deficit. To examine this possibility, mice were given five days of testing on the accelerating rotarod. Both wild-type and Ca\textsubscript{V}1.3 over-expressing animals from founding lines 102 and 103 improved in rotarod performance during the five days of testing. This result indicates that Ca\textsubscript{V}1.3HA mice do not have a motor deficit associated with over-expression of Ca\textsubscript{V}1.3. This result is encouraging and indicates that these mice may be used in the future in tasks requiring unimpaired motor performance, such as the Morris water maze and the radial arm maze (Murphy et al., 2006).

**Ca\textsubscript{V}1.3HA Mice Exhibit Normal Exploratory Behavior**

In addition to data provided from rotarod performance, mice from both Ca\textsubscript{V}1.3HA line 102 and line 103 exhibit normal exploratory behaviors. In the open field, mice from both lines traveled the same distance as their wild-type counterparts. This indicates a lack of overt fear, anxiety-like or hyperactive behavior in the transgenic animals (McKinney et al., 2008). However, it should be noted that transgenic mice as well as wild-type mice spent significantly more time traveling along the edges than traveling in the center zone. The lack
of travel in the center zone of the open field should not be considered a deficit or unusual behavior in mice, as these animals are naturally fearful of open spaces (Murphy et al., 2006).

The final behavioral test performed in CaV1.3HA mice was the light/dark box. This test is another examination of exploratory and fear behavior (McKinney et al., 2008). Both CaV1.3HA lines 102 and 103 performed similarly in the test. While all mice spent significantly more time in the dark side of the box, they all also traveled between the light and dark sides between thirteen and eighteen times. This result demonstrates that the mice are fearful of the more open space provided by the white side of the box but that they still exhibit exploratory behavior and travel willingly between the two halves of the box.

Interestingly, CaV1.3HA line 103 transgenic animals spent significantly more time in the light side of the light dark box than did their wild-type counterparts. However, this difference was not large. Because other measures of CaV1.3HA line 103 demonstrate that the line of mice does not exhibit overtly exploratory behavior, it is likely that this difference is due to a small sample size.

**CaV1.3HA Mice can be used as a Model of Aging**

Current paradigms for studying age-related cognitive decline in animals have several limitations. Aging studies are typically very lengthy, taking up to two years to obtain "aged" rodents. The time course of aging is extremely costly in terms of both care and resource use. In addition, there is no guarantee that animals will survive to reach an age appropriate to use for these experiments.

Many age-associated changes occur simultaneously. It is difficult to assess the effects of each change when studying a typical model of aging, because results can be confounded
by other age-associated changes. For example, aged mice experience age-related hearing loss and therefore cannot be tested in a fear learning paradigm using tone as a conditioned stimulus (Keithley et al., 2004).

The transgenic mice that overexpress CaV1.3 address the above concerns of current models of aging. Because transgene expression begins shortly after birth, the problems associated with the length of time needed to conduct an aging study would be virtually nonexistent. These mice only express a single change that appears to be associated with aging. Therefore, any age-associated changes unrelated to learning and memory, such as motor impairments or hearing loss, are unlikely to occur because these transgenic mice can be studied at relatively young ages. This study has shown that CaV1.3HA mice do not exhibit impaired mobility or motor function that often occur in typical models of aging. They also appear to have similar exploratory behaviors as wild-type mice.

The results reported in this study indicate that in the future, CaV1.3HA mice can be used successfully in various tasks examining learning and memory. Two tasks previously demonstrated to be sensitive to aging are the radial arm maze and the Morris water maze (Murphy et al., 2006). It is hypothesized that CaV1.3HA mice will exhibit deficits in these memory and learning tasks similar to that of aged mice. Preliminary experiments have found an altered behavioral phenotype in the Morris water maze in CaV1.3HA line 102 mice (Moore, S., personal communication 2011).

In addition, CaV1.3HA mice can be used to determine the relative contribution of CaV1.3 to the sAHP and neuronal excitability. Previous research indicated a decrease in the sAHP of CaV1.3 knockout animals (Gamelli et al., 2011). A separate study demonstrated an
increase in neuronal excitability in Ca\textsubscript{V}1.3 knockout animals (McKinney et al., 2009). It is anticipated that opposite effects on the sAHP and neuronal excitability will occur in Ca\textsubscript{V}1.3 overexpressing animals. Electrophysiological experiments will be performed to determine if Ca\textsubscript{V}1.3HA mice exhibit an increase in the sAHP as compared to their age-matched counterparts. It is hypothesized that an increase in the sAHP in Ca\textsubscript{V}1.3HA mice will be comparable to the sAHP increase observed in aged animals. Furthermore, it is likely that Ca\textsubscript{V}1.3HA mice will demonstrate a decrease in neuronal excitability similar to that previously observed in aged animals.

The development of Ca\textsubscript{V}1.3 overexpressing mice could expand the calcium hypothesis of aging by demonstrating that an up-regulation in expression of a specific L-type voltage-gated calcium channel, Ca\textsubscript{V}1.3, contributes to altered intracellular calcium homeostasis. By determining if Ca\textsubscript{V}1.3 overexpression in young animals mimics the increased sAHP and cognitive deficits found in aged animals, this line of mice could present a significant contribution towards understanding the role of calcium dysregulation in the underlying mechanisms of age-related cognitive decline.
REFERENCES


Gamelli AE, McKinney BC, White JA, Murphy GG (2011) Deletion of the L-type calcium channel Ca(V) 1.3 but not Ca(V) 1.2 results in a diminished sAHP in mouse CA1 pyramidal neurons. Hippocampus 21:133-141.


APPENDICES

Appendix A: Chemicals and Reagents

A

Agarose, Genepure LE

Ampicillin

ISC, Kaysville, UT

Sigma, St. Louis, MO

B

Beta-mercaptoethanol

Blotto, Non-fat Dry Milk

Bovine Serum Albumin (BSA)

Bradford Assay One-Step Reagent

BioRad, Hercules, CA

BioRad, Hercules, CA

C

Complete Mini Protease Inhibitor

Roche, Indianapolis, IN

D

DNase/RNase Free Distilled Water

DNaseI

Invitrogen, Carlsbad, CA

Gibco, Grand Island, NY

dNTPs, 10mM

Promega, Madison, WI

E

EDTA, 0.5M

AccuGENE, Rockland, ME
Enhanced Chemiluminescence Kit, ECL Plus | Amersham, UK
Ethidium bromide | ISC, Kaysville, UT

**G**

Glycine | Roche, Indianapolis, IN

**H**

HEPES, 1.0M | Sigma, St. Louis, MO

*HindIII* | NEB, Ipswich, MA

**I**

Isoflurane | MWI, Meridian, ID

**K**

Kwik-Stop | Arc Labs, Atlanta, GA

**L**

Ladder, 100bp | Promega, Madison, WI
Ladder, 1kb | Promega, Madison, WI
Ladder, *Lambda-HindIII* digest | Takara, Japan

LB Broth | Invitrogen, Carlsbad, CA
LB-Agar | Invitrogen, Carlsbad, CA
<table>
<thead>
<tr>
<th>Letter</th>
<th>Description</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Methanol</td>
<td>Fisher, Fair Lawn, NJ</td>
</tr>
<tr>
<td>N</td>
<td>NotI</td>
<td>NEB, Ipswich, MA</td>
</tr>
<tr>
<td>P</td>
<td>Phenol:Chloroform:Isoamyl Alcohol</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td></td>
<td>Primers</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td></td>
<td>Proteinase K</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td></td>
<td>PVDF Membranes</td>
<td>BioRad, Hercules, CA</td>
</tr>
<tr>
<td>S</td>
<td>SDS-PAGE 7.5% Gels</td>
<td>BioRad, Hercules, CA</td>
</tr>
<tr>
<td></td>
<td>SfiI</td>
<td>NEB, Ipswich, MA</td>
</tr>
<tr>
<td></td>
<td>S.N.A.P UV-Free Gel Extraction Kit</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td></td>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td></td>
<td>Sodium chloride, NaCl</td>
<td>Fisher, Fair Lawn, NJ</td>
</tr>
<tr>
<td></td>
<td>Sodium phosphate dibasic, Na$_2$HPO$_4$</td>
<td>Fisher, Fair Lawn, NJ</td>
</tr>
<tr>
<td></td>
<td>Sodium phosphate monobasic, NaH$_2$PO$_4$</td>
<td>Fisher, Fair Lawn, NJ</td>
</tr>
<tr>
<td>Item</td>
<td>Supplier</td>
<td>Location</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>-------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Sucrose, C_{12}H_{22}O_{11}</td>
<td>Fisher, Fair Lawn, NJ</td>
<td></td>
</tr>
<tr>
<td>SuperScriptIII First-Strand Synthesis System</td>
<td>Invitrogen, Carlsbad, CA</td>
<td></td>
</tr>
<tr>
<td><strong>T</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>NEB, Ipswitch, MA</td>
<td></td>
</tr>
<tr>
<td>TAE 50X Buffer</td>
<td>Fisher, Fair Lawn, NJ</td>
<td></td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>Clontech, San Jose, CA</td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td>Fisher, Fair Lawn, NJ</td>
<td></td>
</tr>
<tr>
<td>Tris base</td>
<td>Fisher, Fair Lawn, NJ</td>
<td></td>
</tr>
<tr>
<td>Tris-HCL</td>
<td>Fisher, Fair Lawn, NJ</td>
<td></td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Merck, Batavia, IL</td>
<td></td>
</tr>
<tr>
<td>Trizol</td>
<td>Invitrogen, Frederick, MD</td>
<td></td>
</tr>
<tr>
<td>Tween</td>
<td>Invitrogen, Frederick, MD</td>
<td></td>
</tr>
</tbody>
</table>
### Appendix B: Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse HA Antibody</td>
<td>Covance, Battle Creek, MI</td>
</tr>
<tr>
<td>Anti-rabbit CaV1.3 Antibody</td>
<td>UofI, Iowa City, IA</td>
</tr>
<tr>
<td>Anti-mouse Transferrin Antibody</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>HRP Goat anti-mouse</td>
<td>Roche, Indianapolis, IN</td>
</tr>
<tr>
<td>HRP Goat anti-rabbit</td>
<td>Roche, Indianapolis, IN</td>
</tr>
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</table>
### Appendix C: Buffers and Solutions

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Components</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LB Broth</strong></td>
<td>LB broth base</td>
<td>20g</td>
<td>H₂O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bring to 1L</td>
</tr>
<tr>
<td><strong>LB-Amp Plates</strong></td>
<td>LB agar</td>
<td>32g</td>
<td>H₂O</td>
</tr>
<tr>
<td></td>
<td>Ampicillin (100mg/mL)</td>
<td></td>
<td>1mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bring to 1L</td>
</tr>
<tr>
<td><strong>Tail Digestion Buffer 1-Step</strong></td>
<td>KCl</td>
<td>1.86g</td>
<td>H₂O</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl pH 9</td>
<td></td>
<td>1.55g</td>
</tr>
<tr>
<td></td>
<td>Triton X-100</td>
<td></td>
<td>0.5mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bring to 1L</td>
</tr>
<tr>
<td><strong>HEPES-Sucrose EDTA (HSE)</strong></td>
<td>1M HEPES buffer</td>
<td>1mL</td>
<td>H₂O</td>
</tr>
<tr>
<td></td>
<td>0.5M EDTA</td>
<td></td>
<td>0.5mL</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td></td>
<td>12g</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bring to 100mL</td>
</tr>
<tr>
<td><strong>HSE + protease inhibitor</strong></td>
<td>HSE</td>
<td>10mL</td>
<td>H₂O</td>
</tr>
<tr>
<td></td>
<td>Protease Inhibitor Complete</td>
<td></td>
<td>1 tablet</td>
</tr>
<tr>
<td><strong>Resuspension Buffer</strong></td>
<td>HSE + protease inhibitor</td>
<td>10mL</td>
<td>Triton X-100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10µL</td>
</tr>
<tr>
<td><strong>10X Phosphate-Buffered Saline</strong></td>
<td>NaH₂PO₄</td>
<td>2.28g</td>
<td>H₂O</td>
</tr>
<tr>
<td></td>
<td>Na₂HPO₄</td>
<td></td>
<td>11.5g</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td></td>
<td>43.84g</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bring to 500mL</td>
</tr>
<tr>
<td><strong>1X PBS-0.1% Tween</strong></td>
<td>10X PBS</td>
<td>25mL</td>
<td>H₂O</td>
</tr>
<tr>
<td></td>
<td>Tween</td>
<td></td>
<td>250µL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>225mL</td>
</tr>
<tr>
<td><strong>Blocking Solution</strong></td>
<td>1X PBS-Tween</td>
<td>40mL</td>
<td>Blotto</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.0g</td>
</tr>
<tr>
<td><strong>Laemmli Buffer: 950µL 2:1</strong></td>
<td>Laemmli Buffer</td>
<td>950µL</td>
<td>β-Mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50µL</td>
</tr>
<tr>
<td><strong>10X Western Blot Running Buffer</strong></td>
<td>Tris</td>
<td>30.3g</td>
<td>H₂O</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td></td>
<td>144g</td>
</tr>
<tr>
<td></td>
<td>SDS</td>
<td></td>
<td>10g</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bring to 1L</td>
</tr>
</tbody>
</table>
10X Western Blot Transfer Buffer
Tris 30.3g
Glycine 144g
H₂O Bring to 1L

1X Western Blot Transfer Buffer
10X Transfer buffer 100mL
Methanol 100mL
H₂O 800mL

10% SDS
SDS 10g
H₂O 100mL

Stripping buffer
Glycine 144g
10% SDS 10mL
H₂O Bring to 1L
pH to 2.0
## Appendix D: Software and Databases

<table>
<thead>
<tr>
<th>Software and Databases</th>
<th>Company and Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adobe Acrobat</td>
<td>Adobe Systems, San Jose, CA</td>
</tr>
<tr>
<td>Adobe Photoshop CS</td>
<td>Adobe Systems, San Jose, CA</td>
</tr>
<tr>
<td>EndNote 5.0</td>
<td>ISI Research Software, Berkely, CA</td>
</tr>
<tr>
<td>Filemaker Pro 7.0</td>
<td>Filemaker Inc., Santa Clara, CA</td>
</tr>
<tr>
<td>GraphPad Prism 5.0</td>
<td>GraphPad, San Diego, CA</td>
</tr>
<tr>
<td>Kodak 1D Scientific Imaging Software</td>
<td>Kodak, Rochester, NY</td>
</tr>
<tr>
<td>Limelight</td>
<td>Actimetrics, Wilmette, IL</td>
</tr>
<tr>
<td>Microsoft Office 10</td>
<td>Microsoft, Redmond, WA</td>
</tr>
<tr>
<td>Statview 5.0</td>
<td>SAS Institute, Cary, NC</td>
</tr>
</tbody>
</table>
Appendix E: IACUC Approval

This project was approved for the use of animals by the University Committee for the Use and Care of Animals (UCUCA) at the University of Michigan under approval number 08768 as of 11/20/2009. The University’s Animal Welfare Assurance Number on file with the National Institute of Health’s (NIH) Office of Laboratory Animal Welfare (OLAW) is A3114-01.