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14. ABSTRACT

Autism Spectrum Disorders (ASDs) are characterized by problems with social engagement and communication, as well as inappropriate restrictive and repetitive behaviors. It has been reported that as many as 1 in 70 children are diagnosed with autism; therefore, it represents a major health problem that also profoundly impacts a sizeable number of military families. ASDs have a strong genetic heritability component, but only in a small proportion of cases has the genetic basis been identified, and there is large heterogeneity in the genetic causes. Recently several mutations were identified in individuals with ASDs in genes that code for important Ca^{2+} channels. These ion channels are known to affect neuronal and synaptic development, and therefore are likely causal to autism diagnosed in these patients. More specifically, because these mutations are known to cause a gain-of-function phenotype, increasing Ca^{2+} influx through the channel, they provide a unique opportunity to model the disorder in a mouse and establish a "molecules to behavior" understanding of how brain circuits are functionally altered in ASDs. The two partnering laboratories have collaborated to create a novel mutant mouse with the human mutation engineered into the genome. The mice display several aberrant repetitive and social behaviors that are correlates of the altered behaviors in the human disorder. Therefore, these mice are potentially valuable models for understanding the alterations in brain activity that underlie ASDs. In this proposal we will use these mice to determine the extent of the alteration in synapses, neural circuits and behavior and ask the following three questions: 1) how does the mutation in this ion channel affect the development of neurons in a region of the brain known to be important for repetitive and restricted behaviors? 2) what are the alterations in naturalistic behaviors in these mice that correlate with the symptoms of ASDs, and can we detect this by imaging activity of neurons as mice perform basic behaviors? 3)can we fix the problems in these mice by using drugs that target this ion channel? This proposal directly addresses one of the "Areas of Interest" by assessing novel therapeutics in valid preclinical models. These studies are designed to understand a critical problem in the ASD field, address important knowledge gaps, and ultimately will determine whether we can find ways to rectify the activity in brain circuits that contribute to the altered behaviors in ASDs. Our experimental design will employ cutting-edge techniques to record from neurons in regions of the brain associated with ASDs, and is designed to incorporate the complementary expertise of the partnering laboratories. The ultimate outcome will be in identifying the network basis for repetitive and restricted behaviors, which are a hallmark of ASDs, and will inform the future development of novel treatments.

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Introduction:

Autism Spectrum Disorders (ASDs) are a group of prevalent neurodevelopmental disorders. They are characterized by problems with social engagement and communication, inappropriate repetitive actions, perseverative behaviors, and a range of associated symptoms, including sensory and motor abnormalities, intellectual disability, and mood disorders (Delorme et al., 2013). Studies in families demonstrate that ASDs have a strong genetic heritability component. Single gene mutations are associated with approximately 5% of cases (De Rubeis et al., 2014) and approximately 10% of cases are associated with copy number variations; but in the vast majority of cases the genetics remain unknown. The application of whole exome sequencing of patient DNA has identified many rare *de novo* mutations associated with autism but establishing the effect of these mutations on brain development and function is still at an early stage. Many of these genetic mutations associated with autism converge upon synaptic and neuronal development abnormalities that are the basis for the aberrant behavioral phenotypes and other symptoms of the disorder (De Rubeis et al., 2014; Delorme et al., 2013).

Recently, a group of mutations in Cav1 Ca^{2+} channels have been linked to neurodevelopmental disorders including autism (Gargus, 2009; Pinggera et al., 2015). In particular, seven separate *de novo* missense mutations in *CACNA1D* have been discovered in individuals with autism (Iossifov et al., 2012). All of these mutations occur within intracellular domains of the pore forming subunit of the ion channel. Three of these mutations have been functionally characterized in heterologous expression systems, including G407R, A749G, and V401L (Pinggera et al., 2015).

We have created a new mouse model in which we have engineered the G407R mutation in the alpha 1 subunit of Cav1.3 (*Cacna1d* G407R) providing a model with construct validity for autism. Using this model we propose to identify the synaptic and circuit basis for the core symptoms of autism that contribute to many of the aberrant behaviors, focusing primarily on alterations in function of the striatum.

Key Words:

Autism, Ca2+ Channels, behavior, synapses, striatum

What were the major goals of the project as stated in the approved SOW?

- Major Goal 1- *To determine the effects of a gain-of-function mutation on the formation and function of synaptic connections in the striatum*
- Major Goal 2 - *Determine the alteration in naturalistic behaviors that correlate with ASD symptoms*

What was accomplished?

Aim 1 was to *Determine the effects of the Cacna1d G407R mutation on striatal circuits and plasticity* and *major task 1* in the Statement of Work was to: “determine the effects of a gain-of-function mutation on the formation and function of synaptic connections in the striatum” To determine the effects of a gain-of-function mutation on the formation and function of synaptic connections in the striatum.

As we have reported previously reported we were using in vitro electrophysiology and imaging of Ca2+ activity to determine how the this “gain of function” mutation had effects particularly in striatal synapses. This hypothesis was based upon the finding that not only are striatal dependent behaviors affected in the *Cacna1d* mutant mice but also that specific forms of plasticity of corticostriatal synapses are dependent upon (Rafalovich et al., 2015). Thus the goal of his aim was to directly measure both how Ca2+ signaling in synapses and dendrites is affected by the G407R mutation in Cacna1d as well as to determine whether the presence of this mutant channel specifically affected the induction of NO-LTD of corticostriatal synapses. We have reported some of this data in the progress report last year but we have now expanded and completed these experiments and report them here in their final form. In the first series of experiments, we measured the Ca2+ currents in dendrites of direct pathway (d1 expressing) spiny projection neurons (SPNs) in the dorsolateral striatum. Striatal slices were made from wildtype (WT) and mutant (G407R) mice and whole cell recordings made from labeled (D1-tdTom) D1 SPNs (Figure 1A). The Ca2+ indicator dye Fluo4 was included in the electrode along with a Ca2+ insensitive dye to be able to visualize the neurons dendrites. Backpropagating action potentials were stimulated by depolarizing the neuron using current steps injected through the patch electrode and Ca2+ transients recorded in the primary dendrites using 2 photon Ca2+ imaging (Figure 1). As multiple channels can contribute to this Ca2+ response

and we are interested in the Cav1.3 Ca2+ channel we distinguished this using a selective antagonist isradipine (Figure 1 B and C). We found that the isradipine sensitive Ca2+ response in the dendrites was elevated in the G407R in line with what would be expected from this mutation in the Cav1.3 Ca2+ channel. This is the first

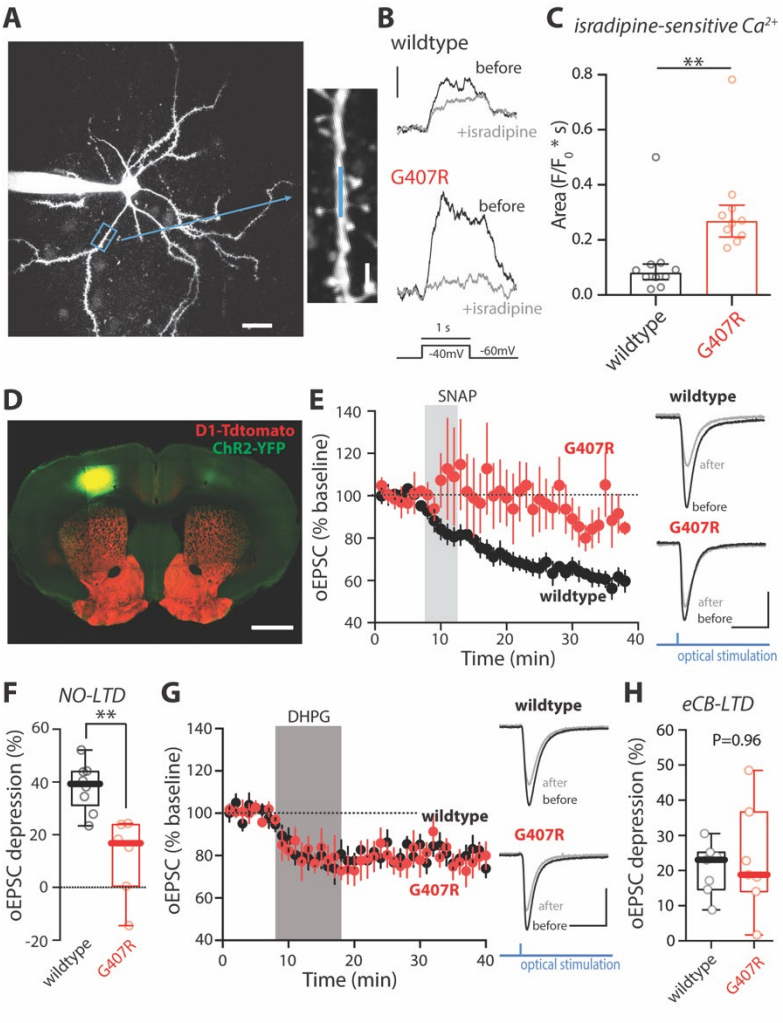

Figure 1. In mice with an ASD-associated G407R mutation in Cav1.3 channel, abnormal Ca^{2+} signal leads to impaired NO-LTD
(**A**) Schematic depicting the Ca^{2+} imaging assay. Top, a dSPN was patched in the whole-cell configuration, dialyzed with Ca^{2+}-insensitive Alexa 568 and Ca^{2+}-sensitive Fluo4. Dendritic Ca^{2+} transients were triggered by somatic depolarization (-60 mV to -40 mV for 1s) through the patch pipette. Two-photon line-scan imaging was performed at segments of dendrites ~45 µm from the soma (yellow box). Bottom, a high magnification image of a segment of dendrite. Line scan was indicated by the yellow line. Scale bars, 20 µm (top) and 2 µm (bottom).
(**B**) Top, Ca^{2+} transients before and after application of isradipine (2 µM) in G407R heterozygous mutant or wildtype siblings. Bottom, voltage step protocol.
(**C**) Box plot summary of isradipine-sensitive component of Ca^{2+} transient in wildtype and mutant (both n = 10 cells). G407R mutation significantly increases depolarization-evoked Ca^{2+} transients in the dendrites. p = 0.0011, Mann-Whitney test.
(**D**) Representative confocal image of ChR2 expression in M1 motor cortex of D1-tdTomato mouse.
(**E**) SNAP-LTD in wildtype (black) and G407R mutant (red). EPSC was evoked by wide-filed blue LED illumination (0.3 ms duration). LTD was induced bath application of SNAP (5 µM) for 5 min (indicated by a grey bar). Plot shows EPSC amplitude as a function of time. Data are mean ± SEM. n = 8 dSPNs from wildtype and 6 dSPNs from G407R mutant. Scale bars in (E) and (G) are 200 pA x 20 ms.
(**F**) Box plot summary of LTD amplitudes from the last 10 min of recordings shown in (E). In G407R mutant mice, SNAP-induced LTD was significantly impaired. **p < 0.01, Mann-Whitney test.
(**G**) DHPG-LTD was similar in wildtype and G407R mutant mice. Here, DHPG-LTD was induced by S-DHPG (50 µM) application for 10 min at a holding potential of -60 mV in the presence of 1.2 mM Ca^{2+} in the bath solution.
(**H**) Box plot summary of LTD amplitudes from the last 10 min of recordings shown in (G). The near-threshold DHPG-LTD protocol induced similar synaptic depression in wildtype and mutant mice. p = 0.97, Mann-Whitney test.

characterization of this mutant channel in vivo and is consistent with the biophysical properties of the mutant channel. Next we sought to determine whether NO-LTD was affected in these neurons. Prior work has demonstrated that NO (nitric oxide) can induce a postsynaptic LTD and interacts with Cav1.3 channels by reducing their activity (Rafalovich et al., 2015). Based upon this model, a channel with elevated Ca2+ influx would counteract this form of LTD. To test this we use mutant mice in electrophysiological recordings. In these mice we injected ChR2 into the cortex to allow the selective activation of corticostriatal synapses (Figure 1D). Recording optically activated EPSCs (oEPSCs) from labeled D1 SPNs we applied the NO donor SNAP ((*S*)-nitroso-*N*-acetyl-D,L-penicillamine) which is known to induce NO-LTD (Figure 1E)(Rafalovich et al., 2015). We found in WT mice there was a strong depression of the oEPSC but little or no depression induced in the recordings from D1-SPNs in the G407R mice (Figure 1E). This is the first evidence that this form of synaptic plasticity is altered in the striatum of G407R mutant mice. As a control we also measured mGluR dependent LTD in these mice. This form of plasticity is mediated by Group I mGluRs which are coupled to signaling pathways that result in the release of a retrograde signaling molecule that acts on presynaptically localized endocannabinoid receptors and causes a presynaptic reduction in transmitter release. We found that this form of plasticity was not affected in the G407R mutant mice. Together we have established how the mutation in this Ca2+ channel affects Ca2+ signaling in the dendrites and leads to an imbalance in striatal specific forms of long term synaptic plasticity.

Aim 2 was to *Determine whether Cacna1d G407R mice display core features of autistic-like behavior* . Again we had reported some of this data in prior reports but here we show the completed data set. These experiments focused on behavioral paradigms in mice that are used to test behavioral flexibility and perseveration. The operant task requires mice to touch one of two lit panels. If a correct choice is made the mouse receives a water reward. After overtraining the rule is reversed and mice are tested to determine how long it takes them to learn the new rule. We had previously reported primary finding demonstrated that G407R mice had normal behaviors

in the acquisition phase but that they were impaired during the reversal phase, consistent with a decrease in behavioral flexibility. Here we report the full data set in which we performed several experiments on separate cohorts of mice and varied the length of the overtraining phase. This demonstrated that there was a clear dependency of the mutant mice reversal deficit on the length of overtraining with 7 and 14 days causing effects on the reversal, while a short 2 day overtraining resulted in no difference in the reversal phase (Figure 2). This complete data set clearly demonstrates that G407R mice have increased perseveration that is induced by the overtraining period in the operant task.

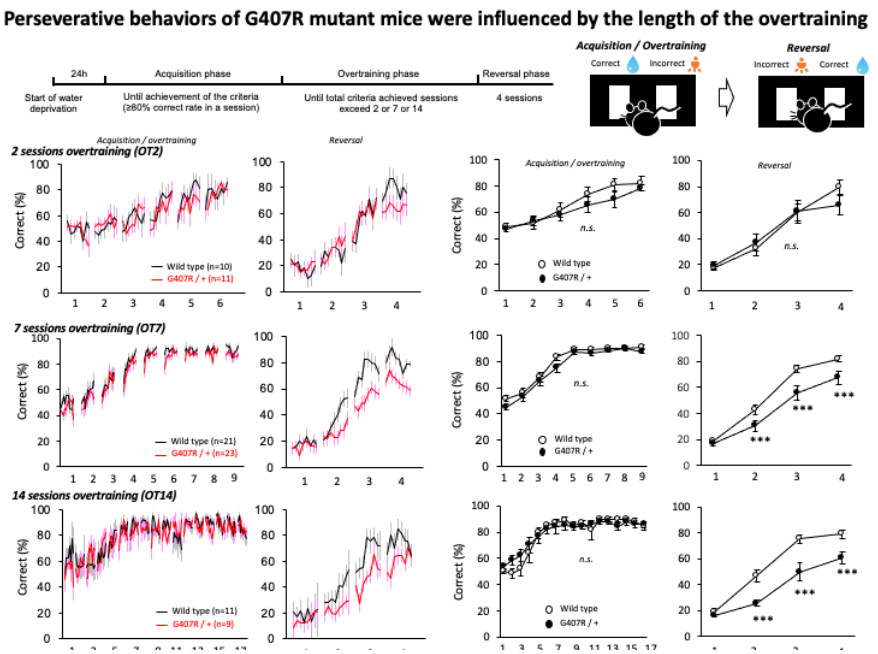

Figure 2 The overtraining duration affects the response of G407R mice in the reversal task
Top shows timeline of the experiment and cartoon representation of the task. Separate cohorts of mice were overtrained for 2 sessions (top row). In this case there was no difference in the reversal phase between genotypes. However when mice where overtrained for 7 days or 14 days (middle and bottom rows) there were significant effects during the reversal phase so that the G407R mice did not learn the new rule as quickly.

In a second set of experiments linking these behavioral paradigms to the mechanisms of NO-LTD we have also demonstrated that pharmacological manipulations that block the induction of NO-LTD using nNOS inhibitors in WT mice also impairs mice in the reversal task (these data are complete but not shown here). Together these data are being prepared for inclusion in a manuscript that will be submitted in the coming weeks.

Citations:

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Impact:

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report

What was the impact on other disciplines?
Nothing to report

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

Changes/Problems:
There have not been any problems and no changes to the Aims.

Products:
Nothing to report

7. PARTICIPANTS:

Name: Anis Contractor
Project Role: PI
Researcher Identifier (e.g. ORCID ID) :
Nearest person month worked: 0.6
Contribution to Project: Overall lead for the project, provides scientific direction, mentors postdocs, analyses data and performs administrative duties
Funding Support: None (Complete only if the funding support is provided from other than this award.)

Name: Jian Xu
Project Role: Research Assistant Professor
Researcher Identifier (e.g. ORCID ID) :
Nearest person month worked: 0.6
Contribution to Project: Performed experiments and analyzed data
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Appendices

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