

# **CACNA1C: association with psychiatric disorders, behaviour and neurogenesis**

*Running Title: CACNA1C: from genetic association to biological function*

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## Introduction

### *Genetic association*

The growth of psychiatric genetics has heralded in a new era of knowledge about psychiatric and neurodevelopmental disorders. Genome-wide association studies (GWAS) have been highly influential in identifying common variation in genes that are over or underrepresented in individuals with a certain disorder. These studies identify single-nucleotide polymorphisms (SNPs) that occur throughout the genome that increase risk for neuropsychiatric disorders. One of the first, and now well replicated, GWAS finding in psychiatry was the association of SNP rs1006737 within the *calcium voltage-gated channel subunit alpha1c* (*CACNA1C*) gene with bipolar disorder<sup>1</sup>. This association was confirmed in a larger data set<sup>2</sup>, and subsequent studies showed a further association of this SNP with schizophrenia, major depressive disorder (MDD) and autism (Table 1). Further SNPs within *CACNA1C* have since been associated with these disorders in multiple studies (Table 1).

The majority of these SNPs are in known linkage disequilibrium with each other, except rs7297582 and rs12898315, potentially due to the fact they are less studied. The SNPs lie within introns, within predicted enhancers which can interact with the *CACNA1C* transcription start site<sup>25</sup> and therefore may determine gene expression<sup>26,27</sup>. rs1006737 has been shown to be an expression quantitative trait loci (eQTL) for *CACNA1C* expression: associated with decreased expression<sup>27</sup>.

*CACNA1C* SNPs were found to have shared effects across attention deficit hyperactivity disorder (ADHD), autism, BPD, SCZ and major depressive disorder<sup>22</sup>, implying that common variation in *CACNA1C* may be associated with particular symptom clusters instead of one particular disorder.

In addition to GWAS findings, large exome sequencing studies have shown that rare disruptive mutations within calcium ion channels are enriched in patients with schizophrenia<sup>28</sup> and autism<sup>29,30</sup>. Furthermore, missense mutations in exon 8, or the alternatively spliced exon 8a, of *CACNA1C* can cause an autosomal dominant genetic disorder named Timothy Syndrome (TS)<sup>31</sup>. TS is a multisystem channelopathy characterised by cardiac defects, craniofacial abnormalities, autism and cognitive impairments. There are two common types of Timothy Syndrome characterised by mutation; TS1 (G406R in exon 8a) and the more severe form TS2 (G406R or G402S in exon 8). Both TS1 and TS2 are characterised by gain-of-function mutations in *CACNA1C*<sup>32</sup>.

#### *Cacna1c, gene transcription and synaptic plasticity*

*CACNA1C* encodes for the alpha1c subunit of the Cav1.2 L-type voltage-gated calcium channel (LTCC). This subunit forms the pore through which calcium influxes into a cell and initiates downstream signalling cascades<sup>33</sup>. LTCCs have a prominent role in controlling gene expression through coupling membrane depolarisation with cAMP response element-binding protein (CREB) phosphorylation via local Ca<sup>2+</sup>/calmodulin-dependent protein kinase II

(CaMKII) signalling<sup>34</sup>. CREB can bind to a critical Ca<sup>2+</sup> response element within brain derived neurotrophic factor (BDNF) to trigger its transcription<sup>35,36</sup>. This pathway, and particularly CREB and BDNF, are thought to be essential for learning and memory processes. Synaptic plasticity, which is thought to underlie learning and memory, can be modulated by LTCCs<sup>37,38</sup>; LTCC antagonists reduce induction of long-term potentiation (LTP) in the CA1 of the rat hippocampus<sup>39</sup>. Cav1.2 knockdown models have shown reduced CREB transcription and hippocampal LTP<sup>40,41</sup>, implicating the important of these channels in gene expression and plasticity.

This review aims to give a brief overview of the current phenotypes relevant to psychiatric and neurodevelopmental disorders studied so far in animal models of *Cacna1c/Cav1.2* dysfunction, including new findings on impacts on neurogenesis in a rat model of reduced gene dosage of *Cacna1c*.

### Models

Genetic *Cacna1c/Cav1.2* rodent models have mostly concentrated on reduced gene dosage. Some studies utilise a constitutive heterozygote model (*Cacna1c*<sup>+/-</sup>) to study gene dosage effects as the homozygote model is embryonically lethal. However other studies have utilised region-specific complete knockouts of *Cacna1c* (*Cacna1c*<sup>-/-</sup>) driven by specific promoters to disentangle the neuronal contribution of this gene compared to the cardiac properties. Bader and colleagues (2011) developed a genetic mouse model based on TS2<sup>42</sup>. While both homozygote and heterozygote knockout of exon 8a were lethal, a heterozygote model that included an inverted neomycin cassette was viable (TS2<sub>neo</sub>)<sup>42</sup>. An overview of the genetic *Cacna1c/Cav1.2* mouse models and their associated phenotypes are presented in Table 2.

### *Motor function*

Neurodevelopmental disorders, particularly autism, can present with neurological disturbance of the motor system resulting in abnormal gait<sup>54,55</sup> and dysfunctions in movement planning and execution<sup>56</sup>. Bader et al (2011) reported that TS2\_neo mice had similar motor abilities and reflexes in their home cage, however had decreased locomotion when placed in a novel environment<sup>42</sup>. Consistently, another study reported that whilst TS2\_neo mice had no deformities in gait, they had reduced locomotion in social tests such as reciprocal social interaction, urine open field test and increased freezing in the Smartcube platform challenge<sup>43</sup>. *Cacna1c*<sup>+/-</sup> mice were reported to be markedly hypoactive in both a home cage and novel environment<sup>42</sup>, however studies on *Cacna1c*<sup>+/-</sup> mice using a rotarod paradigm<sup>40,44,48</sup> did not report any differences in motor ability or co-ordination. A prefrontal cortex specific elimination of *Cacna1c* also did not result in any different basal locomotor behaviour<sup>49</sup>. Dao et al (2010) also reported no genotype differences in motor activity in the home cage however did report a slight hypoactivity in females in the open field test, as well as reduced exploratory activity in the holeboard test<sup>44</sup>.

The role of Cav1.2 in motor activity thus requires further clarification, models suggest that dysfunction in *Cacna1c* may lead to elements of hypoactivity. It is important to consider that this reduced locomotion could in part reflect an indication of anxiety in contrast to a motor deficit *per se*.

### *Sociability*

Social interactions, and the perceptions of them, are often altered in psychiatric patients. TS2\_neo mice show no sociability defects in the 3 chamber test<sup>43</sup> and maintained social memory<sup>42</sup>, however present decreased activity in social interactions. They also initiate less social events, but maintain them longer<sup>42,43</sup>. The *Cacna1c*<sup>+/-</sup> knock out mouse did not show any differences in social behaviour<sup>42</sup>, however a *Cacna1c*<sup>+/-</sup> excitatory neuron knockout showed decreased sociality<sup>53</sup>. This suggests that some subtle elements of social interactions may be affected in Cav1.2 dysfunction, but no global social deficits are present.

### *Fear conditioning*

Aversive associative learning processes such as fear conditioning can be used to investigate learning, memory and cognitive processes in animal models. They can give us an understanding on the neural circuitry that is affected in a wide range of psychiatric disorders. Interestingly it has been shown that Cav1.2 levels are increased in the amygdala following fear conditioning<sup>57</sup>. In genetic models, deletion of Cav1.2 in the anterior cingulate cortex results in decreased observational fear learning, where unconditioned mice develop freezing behaviour by observing conditioned mice receiving foot shocks<sup>58</sup>. TS2-neo mice can acquire cued fear conditioning correctly, however demonstrate increased freezing in context and cue recalls, as well as reduced extinction<sup>42</sup>. The authors suggest that this is due to an enhanced perseverance of both tone and context memory. However, other models of *Cacna1c* knockdown do not show alterations of fear memory. Animals with neuron specific knockout of *Cacna1c*<sup>-/-</sup> show no impairments in acquisition, consolidation or recall of auditory<sup>48</sup> or contextual<sup>50</sup> fear conditioning paradigms. However, Temme et al (2016) did show significant context discrimination deficits in their neuronal knockout model<sup>50</sup>. The *Cacna1c*<sup>-/-</sup> (forebrain excitatory neurons only) model also maintained successful consolidation and extinction of conditioned fear<sup>46</sup>. This disparity between the *Cacna1c* knockdown models and TS models is interesting and may suggest that there are some compensatory adaptations<sup>48</sup>. Future studies on *Cacna1c*<sup>+/-</sup> models would be beneficial to further investigate Cav1.2's influence over fear memory.

### *Anxiety and depressive phenotypes*

*Cacna1c*<sup>+/-</sup> mice have shown decreased depressive-related phenotypes as assessed by the tail suspension test<sup>45</sup> at 5-7 days following a chronic stress. *Cacna1c* heterozygosity has also been associated with protection against depressive-like phenotypes in the forced swim, sucrose preference and tail suspension tests<sup>44,52,53</sup>. However, *Cacna1c*<sup>+/-</sup> deletion during development increases susceptibility to chronic social defeat stress<sup>53</sup>. In addition, a gene x environment human study revealed that SNPs in *CACNA1C* interact with trauma to predict

depressive symptoms<sup>53</sup>, suggesting that depressive-phenotypes may be subject to environment factors interacting with *CACNA1C*.

Dao et al (2011) reported increased anxiety-related phenotypes in female *Cacna1c*<sup>-/-</sup> mice only<sup>44</sup>. Increased anxiety-like phenotypes in males has been reported in *Cacna1c*<sup>+/-</sup> mice in an annex test<sup>42</sup>, dark-light box<sup>53</sup> and in the open field<sup>49</sup> however these findings are not consistent across all models<sup>40,45</sup>. The TS2-neo model has not been associated with alterations in anxiety<sup>42,43</sup>.

The association between Cav1.2 and anxiety is still not fully understood. However the current literature seems to suggest that *Cacna1c* heterozygosity may result in increased anxiety and this effect may be stronger in females.

### *Cognition*

Elements of cognitive dysfunction, such as working memory, are common in psychiatric disorders and may represent core features of these conditions<sup>59</sup>. The SNP rs1006737 was associated with increased prefrontal activity during executive cognition in healthy humans<sup>60</sup> and impaired logical memory performance<sup>14</sup> in those with schizophrenia. SNP rs2007044 was also associated with poor working memory in schizophrenia patients, potentially through decreased prefrontal cortex connectivity to other cortical regions<sup>61</sup>.

No significant differences were seen between TS2-neo mice and wild-types in the procedural T-maze<sup>43</sup>, however increased preservative behaviour was observed in the Y maze<sup>42</sup>.

Elements of spatial memory have been shown to be affected in *Cacna1c*<sup>-/-</sup> conditional forebrain knockout mice<sup>40,47,50</sup>. In the Morris water maze knockout mice could learn the spatial task correctly<sup>46,47</sup>, but they display spatial memory impairments when tested 30 days later<sup>47</sup>. In a neuronal specific *Cacna1c* knockdown, mice could successful learn a simple Morris water maze but had profound deficits in the acquisition of spatial learning within a more complex maze when visual cues around the room were limited<sup>50</sup>. Impairments in a water maze spatial-discrimination task have also been reported<sup>40</sup>.

These findings have implications for understanding how genetic variants can have an impact on underlying cognitive impairments in psychiatric disorders, however more research is needed to clarify the primary domains affected. It will also be important to test animal models on tasks with a high degree of translational potential, such as rodent analogues of human touchscreen tasks, in order to facilitate future integrative research and drug development.

### *Neurogenesis*

Cav1.2 may be required for more complex cognitive behaviours such as limited cued Morris water mazes where allocentric spatial representations are required. Data has shown that adult hippocampal neurogenesis is required for formation of complex forms of spatial representations but not simple<sup>62</sup>, mirroring results seen in cognitive tasks following Cav1.2 knockdown. This suggests a possible deficit in adult hippocampus may be responsible for elements of behaviour dysfunction in these models.

Psychiatric disorders, and in particular mood disorders, have been linked to alterations in adult neurogenesis<sup>63</sup>. In rodents and humans, neurogenic niches have been found in the ventricular-subventricular zone in the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus<sup>64</sup>. These neurogenic cells of the hippocampus have been commonly associated with psychiatric and affective disorders<sup>63</sup> although this is still controversial in the current literature. Adult hippocampal neurogenesis is a complex multistep process that is necessary for the generation of new neurons from neural stem cells (NSCs). A range of psychotropic medications have been associated with increasing neurogenesis in rodent models (including SSRIs, selective SNRIs and tricyclic antidepressants)<sup>65</sup>. There is also increasingly evidence that hippocampal neurogenesis contributes to some forms of hippocampus-dependent learning and memory<sup>66</sup>. There are many factors regulating this process: environmental cues, growth factors such as BDNF, glucocorticoids and neurotransmitters<sup>63</sup>. As the literature suggests that stress may interact with *CACNA1C* to cause depressive symptoms, and *CACNA1C* is known to mediate BDNF



production, it may be hypothesised that Cav1.2 has a role in neurogenesis, through interacting with stress or BDNF.

LTCCs have been shown to regulate the conversion of adult hippocampal neural precursors to immature neurones in a bidirectional manner<sup>67</sup>. This agrees with findings in genetic models; *Cacna1c*<sup>-/-</sup> deletions in the forebrain and neurones both show decreases in immature neurons (Table 3). In the forebrain-Cav1.2 knockout, this was attributed to increased cell death of young neurons, correlated with decreased BDNF levels<sup>51</sup>. However, in a pan-neuronal *Cacna1c* deletion marked decreases in cell proliferation were seen which is likely to be the cause of decreased numbers of immature neurons<sup>50</sup>. Völkening et al (2017) deleted *Cacna1c* on Type 1 cells and reported decreased proliferation and immature neuron production<sup>68</sup> (Table 3). These mice also showed deficits in a pattern separation paradigm – a type of learning thought to require intact hippocampal neurogenesis<sup>68</sup>.

We have used a novel *Cacna1c* heterozygote (*Cacna1c*<sup>+/-</sup>) rat model to investigate if these findings could be replicated in another rodent species<sup>69</sup>. We show a marked decrease in cells incorporating 5-bromo-2-deoxyuridine (BrdU)– a nucleotide analogue that marks dividing cells- suggesting that proliferation is significantly decreased in the SGZ in this model, confirming a key role for *Cacna1c* in neurogenesis across species. However, we do not see any difference in the number of immature neurons, contrasting with the findings in the mouse models (Table 3, Figure 1). This may be due to compensatory mechanisms such as decreased apoptosis resulting in increased cell survival. Further studies assessing long term survival, over following the proliferation, survival, differentiation and integration of newly born neurons following BrdU incorporation, would give valuable insight into the functional consequences of *Cacna1c* knockdown on psychology and behaviour related to this process.

### *Conclusions*

Associations of psychiatric disorders with the *CACNA1C* locus has been one of the most robust findings from genetic studies in mental health. This has led to the investigation of a number of animal models of genetic variation in *Cacna1c* to study potential risk pathways.

These models have yielded some clues as to functional impacts – including potential alterations in motor behaviours, social interactions as well as increased anxiety and preservative behaviour. Interestingly, there may also be a subtle anti-depressive effect of a reduced gene dosage of *Cacna1c*, although interactions with stress may alter this phenotype.

Cav1.2 also appears to play an essential role in elements of hippocampal neuron production, suggesting that the alterations seen in neurogenesis in rodent models have also play a part in other phenotypes seen. This is of interest as disruptions in SGZ neurogenesis have been associated with both psychiatric disorders and treatment response. However, more work is needed to determine if this, in fact, a causative effect.

There are, of course, limitations to the work so far. The majority of the *Cacna1c*<sup>+/-</sup> models have focused on reduced gene dosage, whereas some of the genetic literature suggests that both loss and gain-of-function phenotype may be relevant to disease. Additionally, it is important to note that there is an imprecise relationship between rodent behavioural tests and human psychiatric disorders. Further studies using translational tasks and assessments in both animal models and human subjects with specific genetic variants in *CACNA1C* will be needed to build up the knowledge required for potential therapeutic targeting of LTCCs and associated pathways in psychiatric disorders.

### **Laboratory Animals**

All procedures were carried out in accordance with local ethics guidelines, the UK Home Office Animals Act 1986 and the European Communities Council Directive of 24 November 1986 (86/609/EEC). For further details on methods, please see supplementary material.

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## Figure Legends

**Figure 1:** *Cacna1c*<sup>+/-</sup> rats show decreased BrdU, a marker of cell proliferation, in both suprapyramidal and infrapyramidal blades of the dentate gyrus (F=11.9133, p =0.0043, One-way ANOVA). There are no differences in doublecortin positive cells between *Cacna1c*<sup>+/-</sup> rats and wild-type littermates. Bars represent normalised mean per mm<sup>2</sup> +/- SEM, n=8/genotype, all males.

**Figure 2:** Representative immunofluorescent image of BrdU+ cells (green) and DCX+ cells in the dentate gyrus of the hippocampus.

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## Supplementary Material

### Materials and Methods

#### Animals

The *Cacna1c*<sup>+/-</sup> rat model was obtained from Sage Research Labs on a Sprague Dawley background (TGRA6930, Sage Research Labs, Pennsylvania, USA). Sixteen rats, 8 *Cacna1c*<sup>+/-</sup> rats and 8 wild-type littermates were housed in mixed genotype groups of 2-4 with ad libitum access to food and water. All animals were handled and tail-marked prior to experiments proceeding. *Cacna1c*<sup>+/-</sup> animals were indistinguishable from wild-type littermates in weight, development and general health. On day of experimentation rats were administered with a single intraperitoneal injection of 50mg/kg BrdU in 0.1 M phosphate buffered saline (PBS). 6 hours following BrdU injection rats were euthanised via intraperitoneal injection of Euthatal (200mg/ml) and transcardially perfused with PBS, followed by 4% paraformaldehyde (PFA). Brains were cryoprotected in 30% sucrose prior to sectioning.

#### Fluorescence immunohistochemistry

Brains were sectioned using a cryostat (Leica Microsystems CM1860UV) to produce 40µm coronal sections spanning the hippocampus (Bregma -2.04mm to -4.68mm), and transferred to PBS. To unmask BrdU, sections were first incubated at 37°C for 30 minutes in 2M HCl. Sections were thoroughly washed and blocked using 1% Triton-X and 10% donkey serum in PBS for 2 hours at room temperature. Primary antibodies were diluted in 0.1% Triton-X and 0.2% donkey serum in PBS to appropriate concentrations (BrdU: 1:500 (Roche), doublecortin: 1:100 (SC8066 - Santa-Cruz)) and allowed to bind sections overnight at 4°C. Sections were washed in 0.1 M PBS at least three times and incubated with donkey anti-goat Alexa 647 and donkey anti-rat Alexa 555 (1:1000) for 2 hours at room temperature in

the dark. Sections were washed with 0.1 M PBS and incubated for ten minutes in the dark with DAPI counterstaining (50ug/ml) for nuclei staining. Sections were washed twice more in 0.1 M PBS before being mounted on standard microscopy slides using Mowiol aqueous mounting medium and standard cover slips. Sections were imaged using an epifluorescent microscope (Leica DM6000B Leica Application Suite Advanced Fluorescence 3.0.0 build 8134 software, Leica Microsystems). For every animal, one in seven sections through the hippocampus were stained to be counted. Immunopositive cells were quantified through visual counting, giving a cell count per mm<sup>2</sup>. Cell counts were checked for normality and homogeneity of variances and transformed in appropriate. One-way ANOVAs were used to compare counts between genotypes.

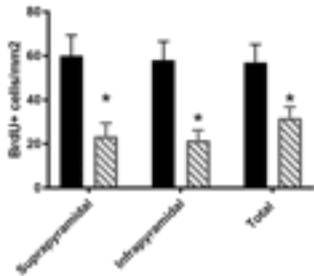
Table 1: Summary of published association studies of SNPs within CACNA1C with psychiatric/neurodevelopmental disorders (BPD = bipolar disorder, SCZ = schizophrenia, MDD = major depressive disorder, ADHD = attention deficit hyperactivity disorder)

SNP	Disorder	Risk allele	Main references
rs1006737	BPD	A	Ferreira et al, 2008 <sup>2</sup> Sklar et al, 2008 <sup>1</sup> Green et al, 2013 <sup>3</sup> Gonzalez et al, 2013 <sup>4</sup> Liu et al, 2011 <sup>5</sup> Ruderfer et al, 2014 <sup>6</sup> Lett et al, 2011 <sup>7</sup>
	SCZ	A	Green et al, 2010 <sup>8</sup> Nyegaard et al, 2010 <sup>9</sup> He et al, 2014 <sup>10</sup> Ivorra et al, 2014 <sup>11</sup> Guan et al, 2014 <sup>12</sup> Zheng et al, 2014 <sup>13</sup> Hori et al, 2012 <sup>14</sup> Ruderfer et al, 2014 <sup>6</sup>
	Autism	G	Zhao et al, 2015 <sup>15</sup>
	MDD	A	Liu et al, 2011 <sup>5</sup> Green et al, 2010 <sup>8</sup> Wray et al, 2010 <sup>16</sup> Casamassima et al, 2010 <sup>17</sup>
rs4765905	SCZ	A	Hamshere et al, 2013 <sup>18</sup> Takahashi et al, 2015 <sup>19</sup>
	Autism	G	Zhao et al, 2015 <sup>15</sup>
rs4765913	BPD	A	Ripke et al, 2014 <sup>20</sup> Muhlesisen et al, 2014 <sup>21</sup>
	SCZ	A	Ripke et al, 2014 <sup>20</sup>
	MDD	A	Ripke et al, 2013 <sup>20</sup>
rs1024582	BPD, SCZ, ADHD, MDD, Autism	A	Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013 <sup>22</sup>
rs2007044	SCZ	A	Ripke et al, 2013 <sup>20</sup> Pardiñas et al, 2018 <sup>23</sup>
rs7297582	BPD	T	Liu et al, 2011 <sup>5</sup>
	MDD	T	Liu et al, 2011 <sup>5</sup>
rs12898315	SCZ	A	Pardiñas et al, 2018 <sup>23</sup>
rs10744560	BPD	T	Stahl et al, 2018 <sup>24</sup>

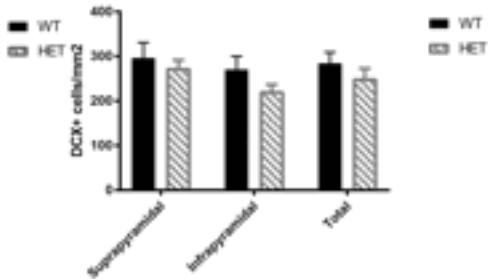
Table 1: Overview of the currently studied mouse models of *Cacna1c* dysfunction and their associated psychiatric and mood phenotypes

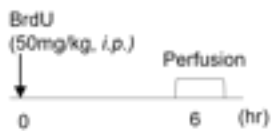
Study	Model	Phenotype
Bader et al, 2011 <sup>42</sup>	TS2_neo <sup>+/-</sup>	↓ novelty induced locomotion ↓ sociability ↑ cued and contextual fear memory ↓ extinction of fear memory ↑ preservation in Y maze
Kabitzke et al, 2018 <sup>43</sup>	TS2_neo <sup>+/-</sup>	↓ social-induced locomotion ↓ sociability
Dao et al, 2010 <sup>44</sup>	<i>Cacna1c</i> <sup>+/-</sup>	↓ exploratory activity in females ↓ locomotion in females ↓ depressive phenotype ↑ anxiety in females
Bader et al, 2011 <sup>42</sup>	<i>Cacna1c</i> <sup>+/-</sup>	↓ basal and novelty induced locomotion ↑ anxiety
Bavley et al, 2017 <sup>45</sup>	<i>Cacna1c</i> <sup>+/-</sup>	↓ depressive phenotype
Moosmang et al, 2005 <sup>40</sup>	<i>Cacna1c</i> <sup>-/-</sup> (forebrain only)	↓ spatial discrimination
McKinney et al, 2008 <sup>46</sup>	<i>Cacna1c</i> <sup>-/-</sup> (forebrain excitatory neurons only)	No effect on contextual fear memory
White et al, 2008 <sup>47</sup>	<i>Cacna1c</i> <sup>-/-</sup> (forebrain only)	↓ long-term spatial memory
Langwieser et al, 2010 <sup>48</sup>	<i>Cacna1c</i> <sup>-/-</sup> (CNS only)	No effect on cued fear memory
Lee et al, 2012 <sup>49</sup>	<i>Cacna1c</i> <sup>-/-</sup> (prefrontal cortex only)	↑ anxiety
Temme et al, 2016 <sup>50</sup>	<i>Cacna1c</i> <sup>-/-</sup> (neurons only)	↓ context discrimination ↓ spatial memory (complex task) ↓ neurogenesis
Lee et al, 2016 <sup>51</sup>	<i>Cacna1c</i> <sup>-/-</sup> (forebrain only)	↓ neurogenesis
Kabir et al, 2017 <sup>52</sup>	<i>Cacna1c</i> <sup>-/-</sup> (prefrontal cortex only)	↓ depressive phenotype
Dedic et al, 2018 <sup>53</sup>	<i>Cacna1c</i> <sup>+/-</sup> (excitatory neurons only)	↓ sociability ↓ depressive phenotypes ↑ susceptibility to chronic social defeat stress ↑ anxiety

### BrdU



### DCX





DAPI/DCX/BrdU

