The *Drosophila* ortholog of the schizophrenia-associated *CACNA1A* and *CACNA1B* voltage-gated calcium channels regulate memory, sleep and circadian rhythms

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A B S T R A C T

Schizophrenia exhibits up to 80% heritability. A number of genome wide association studies (GWAS) have repeatedly shown common variants in voltage-gated calcium (Ca$\text{v}$) channel genes *CACNA1C*, *CACNA1I* and *CACNA1G* have a major contribution to the risk of the disease. More recently, studies using whole exome sequencing have also found that *CACNA1B* (Ca$\text{v}$,2.2 N-type) deletions and rare disruptive variants in *CACNA1A* (Ca$\text{v}$,2.1 P/Q-type) are associated with schizophrenia. The negative symptoms of schizophrenia include behavioural defects such as impaired memory, sleep and circadian rhythms. It is not known how variants in schizophrenia-associated genes contribute to cognitive and behavioural symptoms, thus hampering the development of treatment for schizophrenia symptoms. In order to address this knowledge gap, we studied behavioural phenotypes in a number of loss of function mutants for the *Drosophila* ortholog of the Ca$\text{v}$,2 gene family called cacophony (cac). cac mutants showed several behavioural features including decreased night-time sleep and hyperactivity similar to those reported in human patients. The change in timing of sleep-wake cycles suggested disrupted circadian rhythms, with the loss of night-time sleep being caused by loss of cac just in the circadian clock neurons. These animals also showed a reduction in rhythmic circadian behaviour a phenotype that also could be mapped to the central clock. Furthermore, reduction of cac just in the clock resulted in a lengthening of the 24 h period. In order to understand how loss of Ca$\text{v}$,2 function may lead to cognitive deficits and underlying cellular pathophysiology we targeted loss of function of cac to the memory centre of the fly, called the mushroom bodies (MB). This manipulation was sufficient to cause reduction in both short- and intermediate-term associative memory. Memory impairment was accompanied by a decrease in Ca$^{2+}$ transients in response to a depolarizing stimulus, imaged in the MB presynaptic terminals. This work shows loss of cac Ca$\text{v}$,2 channel function alone causes a number of cognitive and behavioural deficits and underlying reduced neuronal Ca$^{2+}$ transients, establishing *Drosophila* as a high-throughput in vivo genetic model to study the Ca$\text{v}$, channel pathophysiology related to schizophrenia.

1. Introduction

Schizophrenia is a common and debilitating neuropsychiatric disorder characterised by positive symptoms such as delusions and hallucinations and negative symptoms such as anhedonia, social impairments and depression (Berrios, 1985; Cornblatt et al., 1985). Schizophrenia also involves cognitive symptoms including problems with language, executive function, verbal memory, spatial memory and working memory (Hoff et al., 1999; Takano, 2018; Tripathi et al., 2018). Several studies have found that decreased cognitive performance can be

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**Abbreviations:** Ca$\text{v}$, channel, Voltage-gated calcium channel; cac, cacophony; CS+, conditioned stimulus; DD, Continuous darkness; DAM, *Drosophila* Activity Monitors; GWAS, Genome wide association study; ITM, intermediate-term memory; KCl, potassium chloride; MB, mushroom body; LD, 12 h light:12 h dark conditions, hour (h); NREM, Non-Rapid Eye Movement; REM, Rapid Eye Movement; RS, Rhythmicity statistic; SCN, suprachiasmatic nucleus; SEM, Standard error of the Mean; STM, short-term memory; US, unconditioned stimulus; WES, whole exome sequencing.

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observed in a vast majority of first-episode psychosis (also known as first-episode schizophrenia) patients and follow up reports indicate that these impairments can be still observed up to five years later, even on medication (Hoff et al., 1999; Keshavan et al., 2004; Mohamed et al., 1999). Behavioural symptoms also include disrupted sleep and circadian rhythms (Cosgrave et al., 2018; Waters and Manoach, 2012) with people with schizophrenia showing changes in locomotor patterns of daily activity, non-24 h periods and sleep phase changes (Cosgrave et al., 2018). Sleep latency (i.e. the time to fall asleep) and the number of awakenings (i.e. times when the sleep state is disrupted) episodes are also increased in schizophrenia compromising sleep quality and resting levels in these individuals (Ferrarelli et al., 2007; Wulff et al., 2012). Interestingly, although these sleep and circadian disruptions are observed in up to 80% of schizophrenia cases (Cosgrave et al., 2018; Wulff et al., 2012), the underlying mechanisms are not fully understood.

Schizophrenia exhibits high heritability showing 65–80% inheritance (Harrison, 2015; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014) with recent GWAS identifying a number of common variants for the disorder (Harrison, 2015; Ripke et al., 2013; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The most highly represented group of genes associated with schizophrenia encode voltage-gated Ca\(^{2+}\) (Ca\(_v\)) channels (Curts et al., 2011; Heyes et al., 2015; O’Connell et al., 2019; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Ca\(_v\) channels can be classified into L-type (Ca\(_v1.1–1.4\)), P/Q-type (Ca\(_v2.1\)), N-type (Ca\(_v2.2\)), R-type (Ca\(_v2.3\)) channels that assemble with one Cα1 and Cα2δ accessory subunits and T-type (Ca\(_v3.1–3.3\)) channels (Nanou and Catterall, 2018; Simms and Zamponi, 2014). Multiple GWAS have replicated associations between Ca\(_v\) channel and increased risk of schizophrenia (Moskivina et al., 2009; Ripke et al., 2013; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), bipolar disorder and autism (Dedic et al., 2018; Purcell et al., 2009; Smeland et al., 2020; Willems et al., 2018). Historically, the strongest associations of Ca\(_v\) genes with schizophrenia have been found for the CACNA1C (Ca\(_v1.2\)) and CACNA1I (Ca\(_v3.3\)) genes (Heyes et al., 2015; Nanou and Catterall, 2018). Throughout the years several population studies have been consistent in describing mutations or allelic variations on these two genes as risk factors for schizophrenia (Bhat et al., 2012; Heyes et al., 2015; Li et al., 2018; Moskivina et al., 2009; Ripke et al., 2013; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Recently whole exome sequencing has highlighted the association of CACNA1G and schizophrenia through the presence of rare coding variants in the gene contributing substantial risk for the disease (Singh et al., 2020). Moreover, functional studies have found that Ca\(_v1.2\) schizophrenia risk variants disrupt human synaptic transmission, working memory (Cosgrove et al., 2017) and sleep (Kantolarj et al., 2017), further supporting a role of Ca\(_v1.2\) and Ca\(_v3.3\) in schizophrenia pathophysiology. Nonetheless, within the past few years, new studies have uncovered the association of other Ca\(_v\) genes with schizophrenia, namely the Ca\(_v2.2\) gene family. In an attempt to improve extraction of information from GWAS, Moskivina et al. (2009) used two large schizophrenia and bipolar-disorder data sets. The authors uncover an association of CACNA1B (Ca\(_v2.2\)) alleles with schizophrenia, and suggested that similar Ca\(_v2\)-dependent mechanism might drive bipolar disorders and schizophrenia pathology (Moskivina et al., 2009). Similar findings were obtained using whole exome sequencing (WES), showing that large deletions (Glissner et al., 2010) and mutations in CACNA1B are among the causative factors of disruptive mutations in Ca\(_v\) genes associated with schizophrenia (Purcell et al., 2014). Also, a recent study have suggested that damaging mutations in another Ca\(_v2\) gene, namely CACNA1A (Ca\(_v2.1\)), are associated with schizophrenia in Xhosa population of South Africa (Gulsuner et al., 2020) and allelic variants on CACNA1B in South African cohorts were associated with treatment outcome in first-episode schizophrenia patients (O’Connell et al., 2019). These findings support a possible genetic and functional role of Ca\(_v2\) family in schizophrenia pathophysiology.

In terms of how Ca\(_v\) channels may lead to the behaviour deficits associated with the disorder, mouse models mostly focusing on heterozygous CACNA1C mutant mice revealed memory, circadian and sleep deficits (Bhat et al., 2012; Dedic et al., 2018; Heyes et al., 2015; Kumar et al., 2015; Zamponi, 2016). Less is known about CACNA1B. However homozygous knock-out mice (Ca\(_v2.2\) −/−) showed several schizophrenia related phenotypes such as reduced spatial and long-term memory (Jeon et al., 2007), and differences in vigilance state, measured by the time spent in rapid eye movement (REM), non-rapid eye movement (NREM) sleep and awake states (Beuckmann et al., 2003).

The fruit fly, Drosophila, offers a genetically tractable high-throughput in vivo system to perform genetic screens and characterisation of the function of genes associated with psychiatric diseases (Senturk and Bellen, 2018; Van Alphen and Van Swinderen, 2013), including schizophrenia (Hidalgo et al., 2020; Shao et al., 2011; Ueoka et al., 2018; Wise et al., 2015). Drosophila are molecularly and functionally conserved with humans, with about 80% of disease-causing genes having a strong fly ortholog (Senturk and Bellen, 2018). Fly models of human disease can be based on mutations in the fly ortholog of the human disease gene or by expressing the human disease causing gene (Senturk and Bellen, 2018; Van Alphen and Van Swinderen, 2013). A recent GWAS for differential size of human brain regions, identified genes involved in neurodevelopment, synaptic signalling and neurological disease susceptibility. Most of these genes exhibit Drosophila orthologs associated with neurodevelopmental phenotypes, which is consistent with the idea that these genes are involved in evolutionarily conserved mechanisms (Satizabal et al., 2019). Furthermore schizophrenia genes are particularly highly conserved in Drosophila and are enriched for essential genes with similar pathways and developmental stages being affected (Kasap et al., 2018). A recent Drosophila forward genetic screen identified 165 neurodevelopmental and neurodegenerative mutants including orthologs of schizophrenia associated CACNA1A, 1B, 1E, DLG2 and with over 55% of these genes found to be associated with human disease (Yamamoto et al., 2014).

Flies have a single gene ortholog for human Ca\(_v\), called cacophony, mediating N/P/Q/R-type Ca\(_{2+}\) currents in neurons (Kawasaki et al., 2004, 2002; Ryelewski et al., 2012). Presynaptic cac controls synaptic depression (Gavino et al., 2015) and homeostatic potentiation of neurotransmitter release (Müller and Davis, 2012), and CRISPR mediated tagging of endogenous cac showed its localization to active zones (Gratz et al., 2019). Moreover, schizophrenia associated Rim (Graf et al., 2012), Dlg (Astorga et al., 2016) and Cα2δ (Wang et al., 2016) localised cac to active zone controlling neurotransmitter vesicle release, short term and homeostatic plasticity, suggesting a common mechanism for schizophrenia based on Ca\(_{2+}\) signalling (Simms and Zamponi, 2014; Zamponi, 2016).

In order to determine how loss of CACNA1A/CACNA1B function may contribute to the behavioural deficits associated with schizophrenia, we tested loss of function mutants of the fly ortholog of the gene, cac in sleep, circadian rhythm and memory assays.

2. Materials and methods
2.1. Fly stocks and husbandry

Flies were raised at 25 °C on 12 h light: 12 h dark (LD) cycles and a standard yeast meal diet. CSw and CSw’ wild type control flies were gifts from Dr. Scott Waddell, University of Oxford, UK. CSw were crossed with flies bearing GAL4 (GAL4/+) and UAS (UAS/+) transgenes and used as control genotypes. Tim(27)-GAL4 (Buhl et al., 2019; Hedge and Stanewsky, 2008) flies were a gift from Dr. Ralf Stanewsky (University of Münster, Germany). The following strains were obtained from Bloomington Drosophila Stock Center (BDSC; stock number provided in brackets): cacHSk (42245) (Smith et al., 1998; Tong et al., 2016), c309Gal4 (6906) (Joiner et al., 2006; Ueno et al., 2013), OK107-Gal4 (854) (Higham et al., 2019a; Su and O’Dowd, 2003) and UAS-GCaMP6f
In this study, the first two days allowed proper entrainment to the LD cycle (Higham et al., 2019a; Malik and Hodge, 2014). Groups of RS rhythmicity characteristic of a normal wild type fly (Buhl et al., 2019; Curran et al., 2019). Flies with RS values of fresh air exposure separated CS+ and the CS−. Memory was evaluated 2 min or 1 h post-conditioning to test short-term memory (STM) or intermediate-term memory (ITM), respectively. A performance index (PI) was calculated using the following equation:

$$PI = \frac{(N_{CS^+} - N_{CS^-})}{(N_{CS^+} + N_{CS^-})}$$

where $N_{CS^+}$ and $N_{CS^-}$ is the number of flies choosing CS+ and CS−, respectively. The CS− odour was reversed in alternate groups of flies to account for any possible trial to trial innate bias toward one odorant. The average of the performance between these two consecutive trials was considered as a n = 1 (i.e. 40–100 flies). Sensorimotor controls were performed to test that all strains used in this work have similar levels of olfactory acuity and shock reactivity (Table S1).

2.4. Calcium imaging

GCaMP imaging was performed as previously described (Higham et al., 2019a; Malik et al., 2013). Brains dissected from anesthetised flies were held anterior side upwards, in a perfusion chamber using a custom-made anchor. Extracellular saline containing (in mM): 101 NaCl, 1 CaCl2, 4 MgCl2, 3 KCl, 5 n-glucose, 1.25 NaH2PO4, and 20.7 NaHCO3 at pH 7.2, was used to perfuse the brains at 3 mL/min. Visualization was carried out using a 40× water-immersion lens on an upright microscope (Zeiss Examiner Z1). Images were acquired at 4 frames/s with 100 ms exposure using a charge-coupled device camera (Zeiss AxioCam) and a 470 nm light-emitting diode light source (Thor Labs). Transient (12 s) bath application of 100 mM potassium chloride (KCl) extracellular solution was performed to depolarize and transiently stimulate the neurons (Higham et al., 2019a, 2019b).

Baseline fluorescence (F0) was calculated as the averaged fluorescence during the first 80 frames (20 s), prior to the start of KCl bath application. A change in fluorescence relative to baseline was calculated as [(F − F0)/F0], where F is fluorescence at any given time. The change in the peak amplitude following high KCl exposure was used as a metric of transient Ca2+ increase. Data processing was carried out using RStudio version 1.1.463 (RStudio, Inc., Boston, MA).

2.5. Statistical analysis

Data were analysed using GraphPad Prism (version 8.00 for Windows, GraphPad Software, La Jolla California USA). Normality was assessed in all datasets using Shapiro-Wilk’s test, prior choosing the appropriate parametric or non-parametric statistical test to be used. The description of the tests used and the number of experiments/animals (n) for each dataset are indicated in the corresponding figures. Data is presented as Mean ± Standard error of the Mean (SEM). Statistical levels are denoted as follows: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

3. Results

3.1. cac regulates locomotor activity and sleep

Sleep phenotypes are widely observed in schizophrenia patient (Benson, 2006; Wulff et al., 2012), and changes in circadian locomotor activity occur in the Cac−/− mouse (Beuckmann et al., 2003). To determine if loss of function of the fly ortholog cac also disrupts sleep we analysed the locomotor activity under LD of a cac hypomorphic loss-of-function chromosomal cacH18 mutant which is a nonsense mutation resulting in a truncated transcript and reduced cac expression (Smith et al., 1998; Tong et al., 2016). cacH18 flies displayed increased total locomotor activity under LD conditions compared to control flies (1360.27 ± 48.83 counts/24 h vs 1073.48 ± 48.83 counts/24 h, respectively, p = 0.0002; Fig. 1A-B). The overall increase in total locomotion was explained by an increase in the night-time locomotor activity (CSw: 507.38 ± 32.16 counts/12 h vs cacH18 735.43 ± 55.86 counts/12 h, p = 0.0031; Fig. 1B) with no difference in the activity during the day (CS+: 566.10 ± 26.46 counts/12 h vs cacH18 624.84 ± 42.28 counts/12 h, p = 0.4114; Fig. 1B). To further dissect these effects from the visual impairments described by previous studies in cacH18 (or nightblind) mutants (Smith et al., 1998), we knocked down cac throughout the clock circuit which is independent of visual inputs, using the timeless (tim)-Gal4 promoter line (Kaneko et al., 2000) and a previously described RNAi line: cac-RNAi KK (Brusich et al., 2015; Limptitkul et al., 2018; Weiss et al., 2019). Tim > cac-RNAi flies (987.89 ± 37.72 counts/24 h) displayed no changes in total locomotor activity compared to Tim/+ control (1086.48 ± 38.45 counts/24 h, p = 0.1871), while a small but statistically significant difference with the cac-RNAi KK/+ control was found (866.00 ± 34.48 counts/24 h, p = 0.0184; Fig. 1C-D). Further analysis of the locomotor activity during the day as opposed to night demonstrated that Tim > cac-RNAi KK (407.40 ± 14.14 counts/12 h) showed a decrease in locomotion at daytime compared to Tim/+(634.97 ± 23.74 counts/12 h; p < 0.0001) and cac-RNAi KK/+ (536.79 ± 23.01 counts/12 h; p = 0.0099). In contrast Tim > cac-RNAi KK
(580.50 ± 27.82 counts/12 h) showed increased night-time activity than Tim+/+(451.51 ± 20.22 counts/12 h, p = 0.0306) and cac-RNAi KK/+ (329.21 ± 15.73 counts/12 h, p < 0.0001) controls (Fig. 1D).

Compared to control animals, cac<sup>H18</sup> and Tim > cac-RNAi flies showed disrupted sleep profiles (Fig. 2A). cac<sup>H18</sup> flies showed less total sleep than controls (CSw<sup>−</sup> 1017.00 ± 14.83 min/30 min vs cac<sup>H18</sup> 784.00 ± 44.82 min/30 min; t(86) = 6.320, p < 0.0001; 2B, left panel). This was explained by a reduction in both, day-time (CSw<sup>−</sup> 456.90 ± 9.92 min/30 min vs cac<sup>H18</sup> 390.60 ± 21.82 min/30 min; t(86) = 3.188, p = 0.002; Fig. 2B, middle panel) and night-time sleep in cac<sup>H18</sup> mutants (CSw<sup>−</sup> 560.00 ± 7.02 min/30 min vs cac<sup>H18</sup> 393.40 ± 25.50 min/30 min; t(86) = 8.469, p < 0.0001; Fig. 2B, right panel). Analysis of the sleep time in the Tim > cac-RNAi KK flies (488.80 ± 6.92 min/30 min) revealed an increase (F(2,219) = 26.44, p < 0.0001; Fig. 2C, middle panel) in daytime sleep compared to Tim+/+(448.90 ± 8.16 min/30 min) and cac-RNAi KK/+ (412.00 ± 8.39 min/30 min). Conversely at night, Tim > cac-RNAi KK (482.20 ± 9.10 min/30 min) slept less (H = 49.00, p < 0.0001; Fig. 2C, right panel) compared to cac-RNAi/+(570.60 ± 5.64 min/30 min) and Tim+/+(532.20 ± 6.61 min/30 min) controls. Therefore, as it was observed in the locomotor activity analysis, day and night behaviour are oppositely affected. Thus, when added across the 24 h period these defects cancel each other out, resulting in no overall change (F(2,219) = 0.2259, p = 0.7980; Fig. 2C, right panel) in total sleep or total locomotor activity in Tim > cac-RNAi KK (971.00 ± 13.91 min/30 min) compared to Tim+/+(981.00 ± 11.89 min/30 min) and cac-RNAi KK/+ (982.60 ± 11.79 min/30 min) controls.

3.2. cac is required for circadian locomotor rhythmicity and free-running period

Under constant darkness cac<sup>H18</sup> flies showed arrhythmicity with the normal morning and evening peaks of activity disappearing due to the hyperactivity of these animals, which is observed regardless of time of day (Fig. 3A). This resulted in only 46% of cac<sup>H18</sup> flies being rhythmic as opposed to 77% of the control flies (Table 1). In order to map the circuit or neurons where cac deficiency plays a bigger role in defining the mutant phenotype, we expressed a cac RNAi transgene in all clock mutants and this was explained by an increased activity during the night (Two-way ANOVA with Holm-Sidak’s post hoc test). n (CSw<sup>−</sup>) = 62, n (cac<sup>H18</sup>) = 26, n (Tim+/+) = 46, n (Tim > cac-RNAi KK) = 112 and n (cac-RNAi KK/+ = 64 flies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
CAC-RNAi KK 24.56 ± 0.15 h) caused a slight increase in free-running period (H = 87.51, p < 0.0001; Fig. 4D) compared to Tim/+ (24.15 ± 0.08 h) and CAC-RNAi KK/+ (23.40 ± 0.03 h) controls. In contrast, the period of cacH18 (23.58 ± 0.25 h) was indistinguishable (U = 266.5, p = 0.5501; Fig. 4B) from control (CSw+ 23.64 ± 0.07 h).

3.3. cacophony regulates mushroom body mediated short- and intermediate-term memory

Cognitive functions such as working memory are impaired in schizophrenia (Petit et al., 2017; Pogue-Geile and Harrow, 1984; Strac et al., 2016) with Ca2.2−/− knock-out mice displaying reduced associative memory (Joon et al., 2007). In order to determine if the role of Ca2 channels in cognition is conserved in flies we knocked-down cac in the MBs, the memory centre of the fly brain (Busto et al., 2010; Davis, 2005; Waddell, 2013) and then assessed STM and ITM using aversive olfactory conditioning. Knocking-down cac throughout the MBs using OK107-GAL4 (OK107 > cac-RNAi KK; 0.31 ± 0.08) caused a reduction in STM compared to OK107/+ (0.66 ± 0.05) and cac-RNAi KK/+ (0.54 ± 0.07) control lines (F(4,38) = 7.669, p = 0.0001; Fig. 5A). Similar results were obtained when using a second independent GAL4 promoter (c309-GAL4) that also drives expression throughout the MB (Joiner et al., 2006; Tong et al., 2016). Specifically, c309 > cac-RNAi (0.08 ± 0.07) showed significantly less STM than c309/+ (0.52 ± 0.06) and cac-RNAi KK/+ (0.54 ± 0.07) control lines. ITM was also affected when knocking down cac in the mushroom bodies (F(4,37) = 6.296, p = 0.0006; Fig. 5B). Both, OK107 > cac-RNAi KK (0.18 ± 0.06) and c309 > cac-RNAi (−0.03 ± 0.13) displayed reduced ITM compared to control lines OK107/+ (0.42 ± 0.04), c309/+ (0.53 ± 0.09) and cac-RNAi KK/+ (0.38 ± 0.05). To confirm these findings, we performed the same experiments with a second validated RNAi line (cac-RNAi 27,244) with consistent similar results indicating that loss of cac in the MB causes STM and ITM disruption (Fig. 1S).
Given the role of cac in mediating calcium influx at the active zone, we analyzed the effect of cac knock-down on evoked calcium responses of the MB lobes, the region that contains the presynaptic terminals of the MB neurons (Eichler et al., 2017; Guven-Ozkan and Davis, 2014). The genetically-encoded calcium sensor, GCaMP6f, was expressed in the MBs and the calcium transients evoked by high [KCl] were assessed in the MB lobes (Fig. 5C). Bath application of KCl evoked a robust increase in the normalized fluorescence, that decayed when KCl was washed out (Fig. 5D). Quantification of the peak normalized fluorescence induced by this stimulation in GCaMP6f; OK107 > cac-RNAi KK flies (1.18 ± 0.09) showed a reduction (t(6) = 3.067, p = 0.0220; Fig. 5E) by about 40% compared to response detected in control GCaMP6f; OK107/+ flies (1.96 ± 0.24).

3.4. cacophony loss of function mutants reduce mushroom body neuron calcium transients

Given the role of cac in mediating calcium influx at the active zone, we analyzed the effect of cac knock-down on evoked calcium responses of the MB lobes, the region that contains the presynaptic terminals of the MB neurons (Eichler et al., 2017; Guven-Ozkan and Davis, 2014). The genetically-encoded calcium sensor, GCaMP6f, was expressed in the MBs and the calcium transients evoked by high [KCl] were assessed in the MB lobes (Fig. 5C). Bath application of KCl evoked a robust increase in the normalized fluorescence, that decayed when KCl was washed out (Fig. 5D). Quantification of the peak normalized fluorescence induced by this stimulation in GCaMP6f; OK107 > cac-RNAi KK flies (1.18 ± 0.09) showed a reduction (t(6) = 3.067, p = 0.0220; Fig. 5E) by about 40% compared to response detected in control GCaMP6f; OK107/+ flies (1.96 ± 0.24).

4. Discussion

In this study we show that loss of function mutants of the fly ortholog of schizophrenia-associated CACNA1A and CACNA1B genes, cacophony, shows a range of behavioural and cognitive deficits. These include a reduction in night-time sleep mediated by clock neurons accompanied by a reduction in rhythmic circadian behaviour and lengthening of 24 h period. The mutants were also impaired in a cognitive task, showing reduction in short and intermediate memory underlined by a reduction in MB neuron calcium transients. Our data help establish Drosophila as a model to understand how Ca_{2.1} channel mutants contribute to the pathophysiology underlying the behavioural deficits associated with the disease (Beuckmann et al., 2003; Murakami et al., 2007).

Damaging mutations (Gulsuner et al., 2020) and deletions (Glessner et al., 2010) affecting CACNA1A and CACNA1B, respectively have been observed in schizophrenia cases suggesting a loss of function of CACNA1A–CACNA1B. Here we use the cac^{H18} allele, a nonsense mutation resulting in a truncated transcript and reduced cac expression; Smith et al., 1998; Tong et al., 2016) and a specific RNAi knock-down of cac (Brusich et al., 2013; Limpitikul et al., 2018; Weiss et al., 2019) to mimic the effect of such given mutations in CACNA1A/CACNA1B expression.

Locomotor activity was shown to be impaired in the cac^{H18} mutant (Fig. 1A-B) resulting from an increase in the total activity observed in these flies caused by night-time hyperactivity. Interestingly, these results are in line with observations in Cac^{H18}+/− mice mutants that displayed increased night-time activity while day activity remained unchanged (Beuckmann et al., 2003). The changes in locomotor activity under LD, were accompanied by an overall decrease in the total sleep time. However, this was accounted for by a reduction in both day and night sleep (Fig. 2B). Insomnia has been reported in schizophrenia patients, as well as disrupted activity patterns throughout the day and night with hyperactivity under night-time being particularly common (Cosgrave et al., 2018; Wulff et al., 2012).

As cac^{H18} mutants have defects in visual processing (Smith et al., 1998), we targeted knockdown of cac to the central clock, and showed cac regulated day/night locomotion. Interestingly, cac regulated when opposed to how much- total sleep time was changed, with animals sleeping less at night but more in the day. The reduction in nocturnal sleep was seen in both cac^{H18} and Tim > cac-RNAi mutants. Furthermore a recent study also showed that knock-down of cac in the MBs decreased sleep time during the night with no apparent effect in daytime sleep (Tong et al., 2016). Although the activity phenotypes were similar for cac^{H18} and Tim > cac-RNAi KK flies during the night when compared with their respective controls, the changes in daytime were not.
possible explanation for this finding could arise from the nature of the genetic manipulations used here. cacH18 mutation is a constitutive hypomorphic chromosomal mutation that results in a truncated Ca\textsubscript{v}2 channel being expressed when and where ever the endogenous channel is expressed (Smith et al., 1998). These include both the neurons that regulate circadian rhythms and sleep but also peripheral neurons such as motoneurons and neuromuscular junctions, where cac has important functions (Astorga et al., 2016; Gaviño et al., 2015; Gratz et al., 2019; Müller and Davis, 2012; Ryglewski et al., 2012), required to bring about locomotion by which circadian rhythms and sleep are measured. Thus, phenotypes observed here are likely the sum of the contributions of both clock and non-clock structures. Therefore, it is possible that the effects observed at night in the mutant might be driven principally by the clock network, whereas the effects observed during the day are not.

A recent study also confirmed that knocking-down cac in the clock caused arrhythmicity, an effect that was mimicked by expressing cac RNAi in the LNvs (Palacios-Munoz and Ewer, 2018). Similar results were obtained here, using both the cacH18 mutant and clock wide knockdown of cac, two different genetic manipulations resulting in reduced rhythmicity (Fig. 3-4). It is likely that cac knockdown reduces N/P/Q/R-type high voltage activation voltage-gated calcium currents as reported in larval motoneurons (Ryglewski et al., 2012). Although Ca\textsubscript{v} channels are expressed in the clock, it has not been possible to record Ca\textsubscript{v} currents from adult clock neurons and Ca\textsubscript{v} channel blockade reversibly suppresses action potential firing of clock neurons (Nahm et al., 2005; Sheeba et al., 2008b, 2008a). Our GCaMP data showed a reduction in Ca\textsuperscript{2+} transients with neuronal cac RNAi expression, consistent with a reduction in neuronal activity. In the mammalian clock, the suprachiasmatic nucleus (SCN) clock neurons express Ca\textsubscript{v}2 channels and Ca\textsubscript{v} currents are larger in the day than at night contributing to the higher firing rate during the day (McNally et al., 2019). Blockade of SCN Ca\textsubscript{v}2 suppresses rhythmic expression of clock genes such as PERIOD and

**Fig. 4.** cac knock-down throughout the clock reduced rhythmicity and increased period length. Rhythmicity Statistic was used as a measure of rhythm strength after five days of continuous darkness, which was also the point at which period length of the circadian rhythm was measured. cacH18 mutants showed a reduction in RS (A, purple bar) without a change in the period (B, purple bar) compared to control flies (open black bars) (Mann-Whitney test). (C) In order to map this phenotype, cac was knocked down throughout the clock again leading to a reduction in rhythmicity (D) while increasing the period length compared to Tim/- and cac-RNAi KK/+ control flies (E). Data was analysed using a Kruskal-Wallis test with Dunn’s post hoc test. n (CSw+) = 62, n (cacH18) = 26, n (Tim/-) = 46, n (Tim > cac-RNAi KK) = 112 and n (cac-RNAi KK/+) = 64 flies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
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Fig. 5. cac knockdown in the mushroom bodies impaired aversive olfactory memory and reduced evoked calcium transients of mushroom body lobes. Olfactory aversive conditioning was used to test memory in flies with reduced cac expression in the mushroom body (MB). Knocking-down cac using the OK107-Gal4 driver impaired two-minute memory (short-term memory; STM) (A) and 1-h memory (intermediate-term memory; ITM; B). A reduction in performance index was also found using the c509-Gal4 driver in both STM (C) and ITM (D). Data in A-D were analysed using one-way ANOVA and Holm-Sidak’s post hoc test. n = 3 replications (each n consisted of ~100 flies) for each genotype and condition. (E) The genetically encoded calcium sensor, GCaMP6f, was expressed in the MB neurons and its fluorescence was measured in the lobes, which contain the presynaptic terminals of the MB neurons. Example images showing the (E left panel) basal fluorescence and (right panel) fluorescence after the addition of depolarizing high concentration of potassium chloride (KCl). Calibration bar showing the changes in fluorescence can be observed to the right. Scale bar is 20 μm. (F) Transients were observed in controls (black) and in the cac knock-down condition (red), however, the changes in the peak was lower in the GCaMP; OK107 > cac-RNAI KK flies (mean fluorescence as solid lines ± SEM in grey) (G) A reduction in the amplitude of the peak fluorescence was found upon cac knock-down (Unpaired t-test). n (GCaMP; OK107/+)= 4 and n (GCaMP; OK107 > cac-RNAI KK)= 4 brains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A recent study showed that MB knockdown of the Drosophila orthologue of the schizophrenia-related gene CACNA1C, called Ca1d, which encodes a L-type Ca channel, also reduced STM and ITM (Higham et al., 2019a). Interestingly no differences in MB lobe Ca²⁺ transients were reported when knocking down Ca1d, suggesting that cac (N/P/Q/R-type) and Ca1d (L-type) differentially contribute to calcium handling in the MBs (Higham et al., 2019a) with cac working via a presynaptic mechanism as described at the NMJ terminals (Gaviño et al., 2015; Gratz et al., 2019; Müller and Davis, 2012). On the other hand, Ca1d may localise to axons and dendrites modulating the propagation of action potential and intrinsic excitability in segments in the neuron (Kadas et al., 2017).

In summary, we showed that loss of function of the fly ortholog of the schizophrenia-associated genes CACNA1A and CACNA1B, cac, displayed impaired day/night locomotor activity and reduced night-time sleep. Loss of function cac mutants also showed a reduction in rhythmic circadian behaviour. We showed that the majority of the locomotor, sleep and circadian phenotypes described in the cacH18 mutant resulted from loss of function of cac in the clock. Cac mutants also showed cognitive impairment with cac knockdown in the fly memory centre resulting in reduced short- and intermediate-term memory accompanied by a reduction in MB lobe Ca²⁺ transients. Because analogous phenotypes are seen with pharmacological or genetic blockade of Ca2.2 in rodents and similar behavioural and cognitive deficits are seen in schizophrenia. This work establishes Drosophila as a high throughput genetically tractable in vivo model to elucidate CACNA1A and CACNA1B contribution to behavioural mechanisms and pathophysiology related to schizophrenia, with the potential to perform genetic and drug screens for new therapies for this common, poorly understood and treated disorder.

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BMAL2, which are required for the generation of circadian rhythms (Lundkvist et al., 2005; Nahm et al., 2005). Therefore the loss of rhythmic expression of circadian behaviour in flies with clock neuron Ca2.2 knockdown is a result of suppression of the molecular clock mediated by rhythmic expression of clock genes like per. In addition, Ca2.2 channels are also likely to regulate clock output: reduction in Ca2.2 would suppress the previously reported increase in firing of action potentials in the day as opposed to night, resulting in dampened rhythmic behaviour (Buhl et al., 2019; Curran et al., 2019; Sheeba et al., 2008b).

Consistent with this and our results, physiological blockade of Ca2.2 channels decreased rhythmic circadian behaviour in rats (Harvey et al., 2020), and two studies have shown Ca2.2 knockout mice are hyperactive at night (Beuckmann et al., 2003; Nakagawasai et al., 2010), the same phenotype we report in flies (Fig. 1B and D). Like the cac loss of function flies, the Ca2.2 knockout mice also showed disrupted sleep (Beuckmann et al., 2003).

The role of cac in memory has not been previously reported, although a recent transcriptomic study showed that cac expression changes in the MBs after a form of memory training called courtship conditioning (Jones et al., 2018). Interestingly, Ca2.2.2 knockout mice displayed impaired long term potentiation (LTP) and disrupted memory (Jean et al., 2007). Therefore, we evaluated the role of cac in MB mediated aversive olfactory memory (Fig. 5), showing that MB cac knockdown reduced both STM and ITM. These results suggest that, like circadian rhythms and sleep, the role of Cav2.2 in memory appears to be conserved between flies and mammals. Furthermore, several studies have reported deficits in different types of memory in individuals with schizophrenia. It is important to understand the mechanisms underlying these cognitive deficits in order to find new targets for the development of novel drugs for the poorly treated negative symptoms of the disorder (Dollfus and Lyne, 2017; Topolov and Getova, 2016; Yang and Tsai, 2017).

Ca2.2 currents have not been recorded in the MB; however Ca2.2 blockers such as Plectreurys toxin (PLTX), which block Cav2.2, decrease MB spontaneous calcium transients and MB loss of cac decreases spontaneous activity in these neurons (Gu et al., 2009; Jiang et al., 2005). Consistent with these studies we show that MB cac knock-down caused a 40% reduction in calcium transients recorded in MB lobes, a fly brain region which contains the presynaptic terminals of these neurons (Fig. 5E). This suggests that cac might be important for evoked calcium influx into presynaptic terminals required for neurotransmitter release (Simms and Zamponi, 2014). Such increases in presynaptic terminal calcium influx can act as a substrate for LTP (Kaeser and Südhof, 2005; Kavalali, 2015; Leresche and Lambert, 2017) with Cav2.2/Cac2.2 mutants showing reduced LTP (Jean et al., 2007). In Drosophila, cac mediates a similar form of synaptic plasticity called presynaptic homeostatic potentiation (PHP) at the larval NMJ (Akkergenova et al., 2018; Gratz et al., 2019; Müller and Davis, 2012). Other presynaptic proteins and cact interacting molecules such as unc-13 and Dlg also impairs STM (Böhme et al., 2019; Davis and Müller, 2014; Lee et al., 2014; Ortega et al., 2018; Peng and Wu, 2007). Therefore, it is possible that cac mediated deficits in STM and ITM could arise from its role in PHP or other presynaptic plasticity mechanism compromised by the reduction in MB Ca²⁺ transients, as we demonstrated here.

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Appendix A. Supplementary data

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