Cross-disorder risk gene CACNA1C differentially modulates susceptibility to psychiatric disorders during development and adulthood

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ORIGINIAL ARTICLE
Cross-disorder risk gene CACNA1C differentially modulates susceptibility to psychiatric disorders during development and adulthood

INTRODUCTION
Major psychiatric disorders including schizophrenia (SCZ), bipolar disorder (BPD), major depressive disorder (MDD), and autism are moderately to highly heritable, and increasing evidence is suggesting a shared genetic etiology among them.\(^1\)\(^-\)\(^6\) This might partially explain why many patients suffering from these illnesses display a substantial amount of overlapping symptoms.\(^7\) One of the most consistent and robust genetic findings in genome-wide association studies (GWAS) and meta-analyses of GWAS are associations of single-nucleotide polymorphisms (SNPs) in the α1C subunit (CACNA1C) of the voltage-gated L-type calcium channel Ca\(^{1,2}\)\(^+\) channel (LTCC) Ca\(^{1,2}\)\(^+\) with SCZ and BPD, and to a lesser extent with MDD and autism.\(^1\)\(^8\)\(^-\)\(^16\) In support, candidate analysis studies have clearly indicated a shared genetic risk for CACNA1C across these disorders.\(^1\)\(^5\)\(^-\)\(^17\) Further evidence comes from clinical studies, which have associated the primary disease-associated CACNA1C risk allele, rs1006737, with variations in human brain function and structure in patients, but also in healthy subjects.\(^18\)\(^-\)\(^20\) Hence, the available GWAS and clinical data establish CACNA1C as a possible shared susceptibility factor which influences disease vulnerability for BPD, SCZ and MDD across current diagnostic boundaries. This is of considerable interest, in view of the fact that LTCCs have a pivotal role in modulating neuronal excitability, synaptic plasticity and gene expression.\(^21\)\(^-\)\(^23\) However, the causality and mechanisms of how genetic alterations in CACNA1C affect the risk for an entire spectrum of psychiatric disorders remain largely unknown. Importantly, the associated CACNA1C variants by themselves only confer a marginal increase in disease risk. In addition to shared genetic risk factors, the environment represents an important contributor to the risk for many psychiatric disorders. In particular, adverse life events such as severe trauma and/or chronic stress represent strong risk factors not only for MDD but also for other psychiatric disorders including BPD and SCZ.\(^24\)\(^-\)\(^26\) However, no study to date has examined whether the established risk gene CACNA1C interacts with established environmental risk factors to shape disease outcome.

Functional LTCCs are hetero-oligomeric complexes consisting of multiple subunits: α\(^1\), β, α\(^2\), δ and/or γ. The voltage sensor,
selectivity filter, ion-conduction pore and binding site for all available calcium channel blockers is encoded by the α1 subunit. The LTCC family consists of four distinct members, Ca_{α1,1}–Ca_{α1,4}, with mainly Ca_{α1,2} and Ca_{α1,3} shown to have a prominent role in the brain, with Ca_{α1,2} and Ca_{α1,3} accounting for ~85% of the LTCCs. Pharmacological agents, such as the LTCC-targeting dihydropyridines, have frequently been applied to assess the function of LTCCs in the central nervous system (CNS). However, this approach is limited by the fact that all LTCC antagonists available to date are not completely selective for either Ca_{α1,2} or Ca_{α1,3}, and that most studies investigated acute rather than long-term effects. More sophisticated genetic approaches using transgenic mice have helped to address the selective functional roles of different LTCCs. Constitutive deletion of Ca_{α1c} was shown to result in embryonic lethality and conditional inactivation of Ca_{α1,2} in forebrain structures was repeatedly associated with impairments in cognitive function. More recently, haplo-insufficiency and forebrain-specific deletion of Ca_{α1c} were also associated with anxiety-like behavior and sleep disturbances, two core endophenotypes of MDD and BD. In addition, Ca_{α1c} was shown to mediate survival of young hippocampal neurons and brain-specific deletion of Ca_{α1c} impairs discrete forms of hippocampal-dependent memory and neurogenesis within the dentate gyrus. However, the underlying neuronal circuits that modulate the effects of CACNA1C on synaptic plasticity and behavior remain largely unknown.

In this study we aimed to investigate how CACNA1C modulates the risk to psychiatric disorders in dependence of environmental, developmental and circuit specific factors, using both genetic mouse models and human data.

**MATERIALS AND METHODS**

For a detailed description of the Materials and Methods, please refer to the Supplementary Information.

**Animals**

Male mice were used for all experiments. Inactivation of Ca_{α1c} from forebrain glutamatergic neurons during development was achieved by breeding Ca_{α1c}^{lox/lox} mice to Nex-Cre mice, to obtain Cav1.2-DevGlu-Het (Ca_{α1c}^{lox/+}:Nex-Cre). Homozygous and heterozygous deletion of Ca_{α1c} from forebrain excitatory projection neurons in adulthood was achieved by breeding Ca_{α1c}^{lox/+} mice to transgenic Camk2a-CreERT2 mice to obtain Cav1.2-AdGlu-CNS-CKO (Ca_{α1c}^{lox/+}:Camk2a-CreERT2). As Ca_{α1c} was indispensable in Cav1.2-AdGlu-CKO mice, Cav1.2-AdGlu-CNS-CKO mice (Ca_{α1c}^{lox/+}:Camk2a-CreERT2), respectively. Cav1.2 inactivation in Cav1.2-AdGlu-CNS-CKO mice was induced via 2 weeks of tamoxifen-containing food administration initiated during postnatal weeks 11–13. Both, control (Ctrl) and conditional knockout (CKO; CKO^{het}) mice received the identical tamoxifen diet. Behavioral experiments were started following an additional two-week washout period in which Cav1.2-AdGlu-CNS-CKO and Cav1.2-AdGlu^{het} mice received regular chow. Adult male CNS-specific Ca_{α1c} knockout mice and Ctrl littermates were obtained by initially breeding Ca_{α1c}^{lox/lox} CKO with Nestin-Cre mice. Subsequently Ca_{α1c}^{lox/+} mice were bred to Ca_{α1c}^{−/−}:Nestin-Cre mice to obtain Cav1.2-CNS-CKO (Ca_{α1c}^{lox/−}:Nestin-Cre) and Cav1.2-CNS^{het} (Ca_{α1c}^{−/−}:Nestin-Cre). Heterozygous Cav1.2 mice and their Ctrl littermates were obtained from the same breedings; Cav1.2^{+/−} (Ca_{α1c}^{lox/−} and Cav1.2^{het} (Ca_{α1c}^{−/−}). All animals were kept under standard laboratory conditions and were maintained on a 12 h light–dark cycle (lights on from 0700 to 1900 h), with food and water provided ad libitum. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

**Behavioral testing and study design**

Locomotion and sociability were investigated with the open field (OF) test and the three chamber apparatus respectively, as previously described. Anxiety-related behavior was assessed in the light/dark box test as previously described. Spatial memory learning and object recognition were investigated with the water cross maze (WCM) and spatial object recognition task. The forced swim test (FST) was used to assess passive vs active stress-coping behavior (behavioral despair) and corticosterone levels in response to an acute stressor as previously described. For details, refer to online Supplementary Information.

For the basal behavioral characterization of Cav1.2-DevGlu-CNS-CKO (Figure 2), Cav1.2-AdGlu-CNS-CKO (Figure 2) and Cav1.2-CNS^{het} mice (Supplementary Figure S7), one batch of animals was used for each line with the following order of tests (OF, sociability, dark/light box test, FST and corticosterone assessment). A second and third batch of animals was used for the WCM test and long-term potentiation (LTP) recordings, respectively. In addition, a fourth batch of Cav1.2-AdGlu-CNS^{het} mice (Figure 4) was used for the CSDS paradigm with the following test order (sociability, OF, object recognition test, dark/light box, FST and subsequent corticosterone, adrenal gland and thymus weight assessment). The same applies to Cav1.2-AdGlu^{het} mice (Supplementary Figure S9). For the CSDS experiment in Cav1.2^{het} mice (Figure 3), two separate batches of animals were used. For batch 1, the OF and dark/light box test were performed. For batch 2, the following tests were performed in this order: sociability, object recognition test, FST and subsequent corticosterone, adrenal gland and thymus weight assessment.

**Electrophysiology**

The influence of Ca_{α1c} deficiency on hippocampal LTP was conducted as previously described for details, please refer to online Supplementary Information.

**Single and double in situ hybridization**

Single and double in situ hybridization (ISH) was performed as previously described. The Cav1.2 probe was designed to target the loxP flanked exons 14 and 15 (nucleotides 2307–2427 of GenBank accession number NM_009781). In addition, the following riboprobes were used: Gad67: 984–1940 of NM_008077; Gad65: 753–1600 of NM_008078; Vglut2 (Slc17a6): 2427–3006 of NM_080853.3; and LacZ: 2649–3281 of X65335. Images were analyzed with ImageJ (http://rsweb.nih.gov/ij/). Three to four independent experiments were performed for all ISHs and double ISHs.

**Study design (gene × environment interaction in humans)**

Participants in this study belonged to a larger cohort (Grady Trauma Project) investigating the role of genetic and environmental factors in predicting outcomes to stressful life events. Phenotypes and genotypes were available for a total of 4,023 individuals, predominantly African Americans belonging to a highly traumatized, urban population of low social economic status. All procedures were approved by the institutional review boards of Emory University School of Medicine and Grady Memorial Hospital. Please see Supplementary Methods for further details and statistics.

**Statistical analysis**

Statistical analyses were performed using the commercially available software SPSS v16.0 (SPSS, Chicago, IL, USA) and GraphPad Prism v5.0 (GraphPad Software, La Jolla, CA, USA). The sample size was chosen such that with a type 1 error of 0.05 and a type 2 error of 0.2 the effect size should be at least 1.2-fold of the pooled s.d. All results are presented as mean ± s.e.m. Behavioral phenotypic differences between two genotypes were evaluated with Student’s t-test (two-tailed). If data were not normally distributed, the non-parametric Mann–Whitney test was used. Time-dependent measures were assessed with multi-factorial analysis of variance (ANOVA) with repeated measures. For CSDS experiments, the effects of genotype and condition on all other behavioral and neuroendocrine parameters were assessed by two factorial ANOVA (two-way ANOVA). Whenever significant main or interaction effects were found by the ANOVAs, Bonferroni post hoc tests were carried out to locate simple effects. Statistical significance was defined as P < 0.05. All data were tested for outliers using the Grubb’s test. Homogeneity of variance was tested using Bartlett’s test. Animals were allocated to the experimental groups in...
RESULTS

Forebrain Cacna1c is predominately expressed in glutamatergic neurons

Cacna1c is expressed throughout the mouse brain, including key limbic regions relevant for emotion and cognition such as the prefrontal cortex (Ctx), hippocampus (Hip) and amygdala (Figure 1a). Double ISH against Cacna1c and markers of excitatory glutamatergic (Vglut1 and Vglut2), as well as inhibitory GABAergic neurons (Gad65/Gad67), revealed a predominant expression of Cacna1c in glutamatergic Vglut1-positive neurons throughout the Ctx, Hip and basolateral amygdala. The prominent Cacna1c expression in the thalamus was largely restricted to glutamatergic Vglut2-positive neurons, although a number of Cacna1c-containing neurons within the latero-dorsal thalamus also co-expressed Vglut1 (Figures 1b and d and Supplementary Figure S1e,f). Minimal to no co-expression with Vglut1 was detected in the caudate putamen, central nucleus of the amygdala, bed nucleus of the stria terminals or granule cell layer of the olfactory bulb, confirming the abundance of GABAergic markers in these regions. Accordingly, Cacna1c expression in the olfactory bulb, CPU, bed nucleus of the stria terminals and central nucleus of the amygdala was mainly restricted to Gad65/67-positive GABAergic neurons. In addition, Cacna1c was also detected in scattered Gad65/67-positive neurons of the Hip and Ctx (Figures 1c and d and Supplementary Figure S1c,d).

Deletion of Cacna1c from forebrain glutamatergic neurons during development and adulthood induces differential effects on anxiety and opposing effects on cognition

In view of the results described above, we set out to genetically dissect the role of Cacna1c in glutamatergic neurons. For this, we crossed floxed Cacna1c mice to Nex-Cre mice, in which Cre-mediated recombination is initiated during embryonic development (E11.5) in forebrain glutamatergic neurons. As observed from the ISHs and double ISHs, Cacna1c ablation in forebrain glutamatergic neurons (Cav1.2-DevGlu-CKO) was largely restricted to Vglut1-expressing cells, including theCtx, CA1/2/3 of the Hip, and lateral and basolateral amygdala (Figures 2a and b, and Supplementary Figures S2). Next, we assessed behavioral endophenotypes known to be altered in animal models of MDD, BPD and SCZ, including locomotion/exploration in the OF test, anxiety in the dark/light box test, immobility in the FST, social behavior in the three-chamber test (sociability test), as well as spatial learning and memory in the WCM task. Similar to humans, deficits in cognition and social behavior are recapitulated by mouse models of numerous psychiatric disorders including SCZ, BPD and MDD. Hyperactivity in the OF and FST are often associated with mania in mouse models of BPD, and depressive phase of BPD in rodents. However, changes in locomotor activity are also commonly reported in animal models of SCZ and MDD. Compared with their respective littermate Ctrl mice, Cav1.2-DevGlu-CKO mice exhibited increased anxiety in the dark–light box test and decreased preference for the social counterpart in the sociability test (Figures 2c and d). In the FST, Cav1.2-DevGlu-CKO mice displayed an increased active stress-coping behavior (reduced behavioral despair), evident by decreased immobility (Figure 2e). In addition, Cav1.2-DevGlu-CKO mice displayed a pronounced hyperlocomotion in the OF test, which was not observed during the initial 5 min where novelty-induced anxiety is most prominent (Figure 2f). The OF was performed under low light conditions (15 lux) in order to minimize anxiety effects on locomotion, which likely explains the lack of effects on inner zone time (Figure 2f). Hip-dependent spatial learning and memory performance was investigated in the WCM. Compared with the regular Morris water-maze test, the simplicity of the WCM leads to short trial durations and therefore reduces the stress load compared to Morris water-maze training. In addition, using accuracy rather than speed as the main readout, allows for hippocampus-dependent strategies to be assessed from the first training day on. Cav1.2-DevGlu-CKO mice displayed accuracy levels that barely surpassed the chance level of 50%, both during learning and relearning (Figure 2g), suggesting drastically impaired cognitive performance. In view of this strong effect on cognition, we additionally assessed hippocampal LTP at the Schaffer collateral-CA1 synapses. One hour after a 100 Hz tetanus stimulation, Cav1.2-DevGlu-CKO showed a significant decrease in LTP compared with Ctrl mice (Figure 2h).

Next we addressed whether the underlying neurobiological changes responsible for the behavioral alterations in Cav1.2-DevGlu-CKO mice occur during development or adulthood. Floxed Cacna1c mice were bred to inducible Camk2a-CreERT2 animals with the aim of deleting Cacna1c in forebrain glutamatergic neurons (Cav1.2-AdGlu-CKO) during adulthood (postnatal weeks 11–13). We deliberately chose the Camk2a-CreERT2 line because of the strongly overlapping expression pattern with the Nex-Cre line (Figures 2a and i, and Supplementary Figures S2 and S5). Ca1.2 was inactivated upon tamoxifen administration in forebrain excitatory projection neurons, which predominantly include glutamatergic pyramidal neurons of the Ctx, Hip and basolateral amygdala. As expected, the mRNA deletion pattern strongly resembled that of Cav1.2-DevGlu-CKO mice, with loss of Cacna1c expression in Vglut1-positive neurons of the lateral and basolateral amygdala, as well as the entire cerebral Ctx and Hip (Figures 2i and j, and Supplementary Figures S3 and S4). Along these lines, remaining hippocampal protein levels did not significantly differ between Cav1.2-DevGlu-CKO and Cav1.2-AdGlu-CKO (Supplementary Figure S6a,b). In addition, deletion of Cacna1c mRNA expression in Cav1.2-DevGlu-CKO mice was also observed throughout the DG and within a few neurons of the latero-dorsal thalamus and medial parts of the thalamus, central nucleus of the amygdala and the geniculate nucleus (Supplementary Figures S2). In the OF, Cav1.2-AdGlu-CKO mice displayed enhanced locomotion (Figure 2i), although this was not as strongly pronounced as in Cav1.2-DevGlu-CKO mice. However, immobility in the FST and sociability were not significantly altered in Cav1.2-DevGlu-CKO mice (Figures 2i and m). Surprisingly, Cav1.2-AdGlu-CKO mice displayed a marginal increase in time spent in the lit zone and number of entries in the dark/light box test (Figure 2k), and even demonstrated enhanced cognitive flexibility during the relearning trial of the WCM (Figure 2o). Considering that deletion of forebrain Cacna1c was reported to impair long-term memory, we re-exposed the animals to the WCM 30 days after relearning, but did not observe any differences between Cav1.2-AdGlu-CKO and Ctrl mice (Supplementary Figure S6g). In view of our results that developmental inactivation of Cacna1c from glutamatergic neurons decreases NMDA receptor (NMDAR)-dependent synaptic plasticity and impairs spatial memory, we additionally assessed hippocampal LTP in Cav1.2-AdGlu-CKO mice. Intriguingly, 1 h after a 100 Hz tetanus stimulation, Cav1.2-AdGlu-CKO showed increased LTP compared with Ctrl mice (Figure 2p). Importantly, we observed that CNS-specific Cacna1c deletion (Cav1.2-NS-CKO) mice, which is initiated at E8, results in similar behavioral alterations compared with Cav1.2-DevGlu-CKO mice (Supplementary Figure S7). This further supports partially opposing roles for developmental and adult Cacna1c in emotion and cognition, and argues against the possibility that differences between Cav1.2-DevGlu-CKO and Cav1.2-AdGlu-CKO mice are due to the slight discrepancies in recombination patterns between the Nex-Cre and Camk2a-CreERT2. In addition, the observed behavioral changes were not caused by...
Figure 2. Absence of Cacna1c in forebrain glutamatergic neurons during development, but not adulthood, promotes aversive behavioral and cognitive deficits. (a) Cacna1c expression pattern determined by in situ hybridization (ISH) following developmental deletion of Cacna1c (E11.5 in forebrain glutamatergic neurons (Cav1.2-DevGlu-CKO). (b) Double ISH (DISH) in Cav1.2-DevGlu-CKO mice demonstrates absence of Cacna1c mRNA expression (silver grains) in Vglut1-positive neurons (red staining) in the hippocampal CA3 region (conditional knockout (CKO)). (c) Distance travelled in an inner zone time in the open field (OF) test (distance segments: repeated measures (RM)-ANOVA: genotype, F(1,130) = 365.4, P < 0.0001; n = 16 Ctrl, 12 CKO). (d) Sociability test (t(26) = 2.7, P < 0.05; n = 16 Ctrl, 12 CKO). (e) Forced swim test (FST; t(26) = 3.6, P < 0.005; n = 16 Ctrl, 12 CKO). (f) Distance travelled an inner zone time in the open field (OF) test (distance segments: repeated measures (RM)-ANOVA: genotype, F(1,130) = 365.4, P < 0.0001; n = 16 Ctrl, 12 CKO). (g) Water cross-maze test (RM-ANOVA: genotype, F(1,130) = 7.5, P < 0.05; time × genotype, F(2,130) = 5.7, P < 0.0001; genotype, F(1,130) = 45.9, P < 0.0001; total distance: t = 6.8, P < 0.0001; n = 16 Ctrl, 12 CKO). (h) Schaffer collateral/CA1-long-term potentiation (LTP) in Cav1.2-DevGlu-CKO mice (fEPSP last 10 min: t(26) = 2.36, P < 0.05; n = 14 slices from 4 Ctrl mice and 12 slices from 4 CKO mice) (i) Cacna1c expression pattern determined by ISH following adult-specific deletion (postnatal week 11–13) of Cacna1c in forebrain glutamatergic neurons (Cav1.2-AdGlu-CKO). (j) DISHs in Cav1.2-AdGlu-CKO mice demonstrated the absence of Cacna1c mRNA expression in Vglut1-positive neurons in the hippocampal CA3 region (CKO: 4.5 ± 0.7% double-positive neurons normalized to Ctrl; genotype, F(1,11) = 365.4, P < 0.0001; n = 4, 1–2 sections per mouse). (k)–(m) Dark/light box, sociability and FST (t = trend, P = 0.09, n = 17 Ctrl, 11 CKO) in Cav1.2-AdGlu-CKO mice. (n) OF test (distance segments: RM-ANOVA: genotype, F(1,130) = 9.06, P < 0.05; total distance: t(26) = 3.01, P < 0.05; n = 17 Ctrl, 11 CKO) in Cav1.2-AdGlu-CKO mice. (o) Water cross-maze test (RM-ANOVA: genotype, F(1,130) = 4.36, P < 0.05; n = 11 Ctrl, 10 CKO) in Cav1.2-AdGlu-CKO mice. (p) Schaffer collateral/CA1-LTP in Cav1.2-AdGlu-CKO mice (fEPSP last 10 min: t(26) = 2.06, P < 0.05; n = 13 slices from 6 Ctrl mice and 16 slices from 7 CKO mice). Student’s t-test for simple comparisons, *P < 0.05. Data are means ± s.e.m.

Figure 1. Cacna1c is predominantly expressed in forebrain glutamatergic neurons. (a) Cacna1c mRNA expression in the mouse brain (C57BL/6J mouse strain), determined by in situ hybridization (ISH). (b) and (c) Co-localization of Cacna1c with glutamatergic (Vglut1) and GABAergic (Gad65/67) markers with double ISH (DISH). Black arrowheads indicate cells only expressing Cacna1c (silver grains). Gray arrowheads indicate cells co-expressing Cacna1c and the respective neurotransmitter marker (red staining). (d) Quantifications of Cacna1c co-expression with Vglut1 and Gad65/67 (n = 3, 2–3 sections per mouse). Scale bar = ISH, 1 mm; DISH, 50 μm.
Cacna1c differentially modulates susceptibility to CSDS during development and adulthood

Chronic stress and/or trauma represent strong risk factors for a number of psychiatric disorders, including MDD, BPD, and anxiety disorders such as post-traumatic stress disorder. In view of the phenotype in Cav1.2-DevGlucKO mice, we wondered whether developmental deletion of Cacna1c would increase the susceptibility to CSDS. We choose to investigate heterozygous Cacna1c animals (Cav1.2Het, Figure 3a), considering that Cav1.2-DevGlucKO mice already display strong behavioral deficits under baseline conditions, which might not be further aggravated by CSDS. Importantly, no gross behavioral abnormalities were previously reported for Cav1.2Het mice. We were able to detect robust physiological and neuroendocrine changes evoked by CSDS, independent of genotype, demonstrating the efficacy of the paradigm (Supplementary Figures S8a–d). Chronically stressed Cav1.2Het mice displayed drastically reduced locomotion throughout the entire test duration compared to both, non-stressed Cav1.2Het and stressed Cav1.2Ctrl mice (Figure 3b). In addition, only chronically stressed Cav1.2Het mice showed a significant reduction in the number of inner zone entries of the OF, indicating increased anxiety-related behavior even under low-light test conditions (Figure 3c). Moreover, CSDS increased the latency to enter the light compartment and decreased the time spent in the light zone and number of entries to a much greater extent in Cav1.2Het than in Cav1.2Ctrl mice (Figures 3d–f), further supporting increased stress-induced anxiety in heterozygous Cacna1c mice. Sociability, immobility in the FST and spatial object recognition memory were not differentially affected by CSDS (Figures 3g–i), suggesting a specific impact of the interaction between Cacna1c and chronic stress on anxiety.

In view of the partially opposing effects of Cacna1c on anxiety and cognition during development and adulthood, we wondered whether stress vulnerability would also be differentially affected upon deletion of the calcium channel in adulthood. Physiological and neuroendocrine parameters were similarly altered by CSDS in Ctrl and Cav1.2-AdGlucKO mice (Supplementary Figures 8e–h). In contrast to Cav1.2Het mice, locomotion/exploration in the OF was only decreased in stressed Ctrl but not stressed Cav1.2-AdGlucKO mice, with a similar trend in the number of inner zone entries (Figures 4a and b). In the dark/light box test, only Ctrl mice responded to CSDS with a significant reduction in time spent in the lit zone and number of entries, and a trend toward delayed latencies (Figure 4c), which implies enhanced resilience to CSDS in Cav1.2-AdGlucKO mice. FST behavior, sociability and spatial object were similarly affected by CSDS in Ctrl and Cav1.2-AdGlucKO mice (Figures 4d–f). Considering that we observed susceptibility to CSDS in heterozygous Ca1.2 mice, we additionally assessed whether a heterozygous deletion in glutamatergic neurons during adulthood would be sufficient to induce stress resilience (Supplementary Figure S9). However, compared with Cav1.2-AdGlucKO, Cav1.2-AdHet mice only exhibited partial resilience to CSDS-induced anxiety in the dark/light box test (Supplementary Figure S9f). This points to a gene-dosage effect and suggests that complete absence of Cacna1c during adulthood, in forebrain glutamatergic neurons, is required to induce the extent of resilience to CSDS observed in Cav1.2-AdGlucKO mice.

CACNA1C interacts with adult trauma to predict depression symptoms in humans

A number of CACNA1C SNPs associated with BPD, SCZ and MDD were also shown to affect measures of anxiety, depression,
psychosocial functioning and cognitive aspects in healthy
Ctrls, which might represent increased susceptibility factors for
psychiatric disorders upon exposure to adverse environ-
ments. This is especially interesting in the context of MDD, in
which disease susceptibility is strongly influenced by previous
exposures to chronic and/or severe stress. Considering that most
GWAS do not Ctrl for life-time adversity, we wondered whether
certain CACNA1C SNPs might primarily influence the risk for MDD
in combination with stressful life events. Consequently, we
analyzed the effects of all CACNA1C variants and adult trauma
on current depression symptoms, determined by the Beck
Depression Inventory in a large African American cohort of
non-psychiatric clinical patients (n = 4808) from the Grady trauma
project. Importantly, symptoms of depression are not only
classic for MDD and BPD but can also occur as part of
affective dysregulation in SCZ. After linkage disequilibrium-
pruning based on $r^2$-values of 0.2, 32 of the 465 tested SNPs in the
CACNA1C locus showed nominal signifi-
cant interactions with
adult trauma exposure on Beck Depression Inventory scores. Of
these, two remained significant after Bonferroni correction for
multiple testing; rs73248708, nominal $P = 1.38 \times 10^{-5}$, Bonferroni
corrected $P = 0.004$; rs116625684, nominal $P = 5.2 \times 10^{-5}$, Bonfer-
roni corrected $P = 0.0016$ (Figures 5a and b, and Supplementary
Table S1 and S2). Both SNPs also showed nominally signifi-
cant main effects (rs73248708: nominal $P = 1.88 \times 10^{-4}$, rs116625684: nomi-
inal $P = 0.02$). Interestingly, in both cases, individuals carrying at
least one minor allele (CT/TT or AG/AA) and without trauma
history displayed significantly higher Beck Depression Inventory

![Figure 4. Deletion of Cacna1c from forebrain glutamatergic neurons during adulthood promotes resilience to chronic social defeat stress (CSDS). (a) Locomotion (distance segments: time x stress, $F_{2,45} = 10.7$, $P < 0.0001$; total distance: genotype, $F_{1,45} = 19.6$, $P < 0.0001$; stress, $F_{1,45} = 4.3$, $P = 0.04$) and (b) number of inner zone entries in the open field (OF). (c) Dark/light box test (Lit zone time: genotype, $F_{1,45} = 9.3$, $P = 0.004$; Lit zone entries: stress, $F_{1,45} = 5.8$, $P = 0.02$; genotype, $F_{1,45} = 12.3$, $P < 0.001$). (d) Sociability test (stress, $F_{1,45} = 52.52$, $P < 0.0001$) and (e) forced swim test (FST; genotype, $F_{1,45} = 7.5$, $P = 0.009$; stress, $F_{1,45} = 8.3$, $P = 0.006$). (f) Spatial object recognition test (stress, $F_{1,42} = 7.1$, $P = 0.01$). Two-way analysis of variance (ANOVA)+Bonferroni post hoc test and repeated measures (RM)-ANOVA+Bonferroni post hoc test; *significantly different from the control (Ctrl) group of the same condition, #significantly different from the basal condition of the same
genotype. OF, dark/light box test, sociability and FST (n = 13 Ctrl basal, 12 Ctrl stress, 12 conditional knockout (CKO) basal, 12 Het stress). Spatial object recognition test (n = 12 Ctrl basal, 10 Ctrl stress, 12 CKO basal, 12 CKO stress). Data are means ± s.e.m.
scores compared with the major allele group, whereas the opposite was observed for minor allele carriers with the highest degree of lifetime trauma exposure.

**DISCUSSION**

Our results demonstrate that spatial memory, hippocampal plasticity and anxiety-related behavior are differentially affected by the loss of Cacna1c from excitatory circuits depending on whether it is deleted during embryonic development or adulthood. Increased anxiety-related behavior as well as reduced social and cognitive performance, observed in Cav1.2-DevGlu-CKO mice, are considered core endophenotypes of MDD and/or the depressive phase of BPD, whereas increased locomotion and activity in the FST are often associated with mania in animal models of BPD. Decreased immobility in the FST was previously reported for heterozygous mice by Dao et al., which is in line with our results and earlier work demonstrating antidepressant-like effects for LTCC blockers. However, studies using LTCC blockers have to be interpreted with caution, as these have been reported to induce nonspecific, aversive and stress-like behavioral effects possibly due to excessive blood pressure lowering caused by inhibition of Cav1.2 in the cardiovascular system. Importantly, hyperlocomotion, altered social behavior, and learning and memory impairments, are also analogous to negative and cognitive symptoms of SCZ, a disorder believed to have neurodevelopmental origins. Additional evidence also suggests that two missense mutations in CACNA1C predispose to autism, another neurodevelopmental disorder characterized by impaired social interactions and altered cognition. In accordance with the observed cognitive impairments, Cav1.2-DevGlu-CKO mice also displayed deficits in hippocampal LTP, which is considered the cellular correlate of learning and memory. Moreover, we show that Cacna1c strongly interacts with the environment to shape anxiety-related behavior. Heterozygous deletion of Cacna1c, which is present throughout life starting at the earliest point of development (one-cell stage), significantly increased the susceptibility to CSDS and is likely to be mediated by Cacna1c absence in forebrain glutamatergic neurons. Although CACNA1C SNPs have not been consistently associated with anxiety in humans, altered anxiety-related behavior is considered a core endophenotype of MDD and BPD in animal models. Different to our observations, a recent study showed that chronic unpredictable stress induces similar behavioral deficits in heterozygous Cav1.2 mice and their wild-type littermates when assessed 1–2 days post stress. Surprisingly, the stress effects persisted in wild-type mice when tested 5–7 days later but not in heterozygous knockout mice. These discrepancies might be related to the different chronic stress paradigms.

In contrast to a developmental inactivation, deletion of Cacna1c from forebrain glutamatergic neurons during adulthood blocked the adverse effects of CSDS on anxiety, enhanced LTP and improved hippocampus-dependent cognitive flexibility during the WCM, without affecting long-term memory. Our findings in Cav1.2-DevGlu-CKO mice are supported by previous studies, showing that deletion of Cacna1c from pan-neuronal or glutamatergic neurons during development impairs synaptic plasticity and/or spatial learning, some of which might be attributed to deficits in adult neurogenesis. However, it should be kept in mind that our LTP results cannot be entirely correlated with cognitive performance considering that we stimulated CA3-CA1 Schaffer collaterals, which leads to the potentiation of most CA3-CA1 synapses rather than those that would selectively be recruited during a learning and memory task.
pharmacological data have indicated that LTP induction is mainly initiated through NMDAR-activity but can also include NMDAR-independent and VGCC-dependent mechanisms.\textsuperscript{69,70} Interestingly, the decline of some memory processes is attributed to the shift from NMDAR-dependent to VGCC-intracellular calcium store-dependent regulation of synaptic plasticity, especially during aging.\textsuperscript{71–73} Along these lines, a recent study provides evidence for an association between increased hippocampal Cacna1c expression and age-related cognitive decline.\textsuperscript{74} Although we did not assess aged animals, it can be speculated that ablation of Ca\textsubscript{a,1,2} in adulthood partially blocks an evolving shift towards VGCC- and/or intracellular calcium store-dependent regulation of synaptic plasticity, hence facilitating NMDAR-dependent LTP. This potentially suggests that Hip-dependent spatial memory formation requires Ca\textsubscript{a,1,2} during prenatal development, but not in adulthood where activation of Ca\textsubscript{a,1,2} channels is even detrimental for LTP. Notably, calcium channel antagonists were shown to ameliorate age-related deficits in hippocampus-dependent memory, possibly mediated through facilitation of NMDAR-LTP or inhibition of long-term depression.\textsuperscript{71,73,75–78} On the other hand, specific inactivation of Cacna1c in the anterior cingulate between postnatal weeks 8–10, impaired observational fear learning without altering novel object recognition memory and classical fear conditioning,\textsuperscript{70} highlighting the requirement for Ca\textsubscript{a,1,2} during adulthood for specific cognitive functions. The fact that we observed no significant changes in spatial object memory performance under basal or stress conditions further implies that adult-specific Cacna1c deletion in excitatory circuits does not impact all cognitive domains.\textsuperscript{79}

As observed in Cav1.2-Dev\textsubscript{Glu\textsuperscript{−/−}}-Cre\textsubscript{−} mice, locomotion was also enhanced in Cav1.2-Ad\textsubscript{α\textsubscript{1}}-Cre\textsubscript{−} animals; however, social and active stress-coping behavior were only mildly affected, suggesting that these endophenotypes are partly regulated by forebrain-Ca\textsubscript{a,1,2} during development and adulthood. In contrast, an earlier study demonstrated that forebrain-specific elimination of Cacna1c, using a non-inducible Camk2a-Cre, also results in increased anxiety-like behavior.\textsuperscript{80} The discrepancy could potentially be attributed to different Cacna1c deletion time windows (p18\textsuperscript{79} vs p91–97 in our study).

Although the Nex-Cre and Camk2a-Cre\textsubscript{ER2} drivers primarily target glutamatergic forebrain neurons, they do slightly differ in their recombination patterns (for example, the Camk2a-driven Cre\textsubscript{ER2} is also expressed in parts of the thalamus, DG and central nucleus of the amygdala). However, the fact that developmental, CNS-specific Cacna1c knockout mice showed similar behavioral deficits as Cav1.2-Dev\textsubscript{Glu\textsuperscript{−/−}}-Cre\textsubscript{−} mice, further supports opposing roles for Ca\textsubscript{a,1,2} during development and adulthood. Nevertheless, we cannot rule out an important contribution of Ca\textsubscript{a,1,2}-expressing, GABAergic neurons in many of the disease-associated pheno-types. In fact, Cacna1c was reported to modulate processes related to drug addiction via GABAergic medium spiny neurons in the nucleus accumbens\textsuperscript{80,81} and promote susceptibility to social stress when deleted from this specific brain region.\textsuperscript{82} It is important to point out that we only assessed a subset of behavioral tests performance under basal or stress conditions further implies that fear conditioning,\textsuperscript{30} highlighting the requirement for Ca\textsubscript{v1.2} without altering novel object recognition memory and classical memory, possibly mediated through facilitation of NMDAR-LTP. Notably, calcium channel antagonists were shown to

In the clinical context this could imply that, individuals with genetic variants promoting reduced CACNA1C expression may either be at higher or lower risk to develop psychopathologies, depending on whether they experienced severe stress/trauma during development or adulthood. The pronounced effect of a prenatal Cacna1c depletion on emotional behavior and cognitive performance might account for its association with disorders such as SCZ and autism, which are increasingly believed to result from synaptic dysfunction during development. The fact that CACNA1C is also able to interact with the environment during adulthood, by modulating stress susceptibility, could explain its linkage to stress-related disorder such as MDD and BPD. At the same time, Ca\textsubscript{a,1,2} and Ca\textsubscript{a,1,3} are expressed in midbrain dopaminergic neurons and several studies suggest a neuroprotective effects of L-TCC blockers...
in Parkinson’s disease, which further emphasizes the possibility that inhibition of Ca1.2 may be of therapeutic value in brain disorders that manifest at later stages in life. Concluding, we propose that the association of CACNA1C with multiple psychiatric disorders is related to its broad expression within key limbic regions and neuronal circuits relevant to emotion, motivation and cognition, and that alterations in CACNA1C gene expression during development and adulthood can result in diverging behavioral outcomes and differentially impact stress susceptibility.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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