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G protein-gated K⁺ channel ablation in forebrain pyramidal neurons selectively impairs fear learning

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Abstract

Background—Cognitive dysfunction occurs in many debilitating conditions including Alzheimer's disease, Down syndrome, schizophrenia, and mood disorders. The dorsal hippocampus is a critical locus of cognitive processes linked to spatial and contextual learning. G protein-gated inwardly rectifying K⁺ (GIRK/Kir3) channels, which mediate the postsynaptic inhibitory effect of many neurotransmitters, have been implicated in hippocampal-dependent cognition. Available evidence, however, derives primarily from constitutive gain-of-function models that lack cellular specificity.

Methods—We used constitutive and neuron-specific gene ablation models targeting an integral subunit of neuronal GIRK channels (GIRK2) to probe the impact of GIRK channels on associative learning and memory.

Results—Constitutive *Girk2*^{−/−} mice exhibited a striking deficit in hippocampal-dependent (contextual) and hippocampal-independent (cue) fear conditioning. Mice lacking GIRK2 in GABA neurons (GAD-Cre: *Girk2*^{flox/flox} mice) exhibited a clear deficit in GIRK-dependent signaling in dorsal hippocampal GABA neurons, but no evident behavioral phenotype. Mice lacking GIRK2 in forebrain pyramidal neurons (CaMKII-Cre(+): *Girk2*^{flox/flox} mice) exhibited diminished GIRK-dependent signaling in dorsal, but not ventral, hippocampal pyramidal neurons. CaMKII-Cre(+): *Girk2*^{flox/flox} mice also displayed a selective impairment in contextual fear conditioning, as both cue-fear and spatial learning were intact in these mice. Finally, loss of GIRK2 in forebrain

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pyramidal neurons correlated with enhanced long-term depression and blunted depotentiation of long-term potentiation at the Schaffer collateral/CA1 synapse in the dorsal hippocampus.

Conclusions—Our data suggest that GIRK channels in dorsal hippocampal pyramidal neurons are necessary for normal learning involving aversive stimuli, and support the contention that dysregulation of GIRK-dependent signaling may underlie cognitive dysfunction in some disorders.

Keywords

Kir3; learning; memory; anxiety; synaptic plasticity; hippocampus

INTRODUCTION

Cognitive dysfunction occurs in many debilitating conditions including Alzheimer's disease (AD), Down syndrome (DS), schizophrenia, and mood-related disorders (1–5). The dorsal hippocampus is a key anatomic substrate for cognition, as lesion, genetic, or pharmacologic disruption of this structure impairs the acquisition of contextual/spatial associations, and consolidation into long-term memory (6). Changes in CA1 pyramidal neuron activity and synaptic plasticity within the dorsal hippocampus are thought to be primary culprits for aberrations in associative learning and memory (7–12). To date, focus in this area has centered largely on excitatory neurotransmission.

Inhibitory G protein signaling plays an important role in hippocampal-dependent cognition. For example, acute pharmacologic activation of GABA_B and 5HT_{1A} receptors (GABA_BR and 5HT_{1A}R, respectively) in the dorsal hippocampus disrupts associative learning and impairs memory in spatial and contextual fear tasks (13–16). Constitutive ablation of GABA_BR or 5HT_{1A}R also yields associative learning and memory deficits (17, 18). Paradoxically, global over-expression of GABA_BR impairs contextual fear learning, an effect that is rescued by pharmacologic blockade of GABA_BR in the dorsal hippocampus (19). Collectively, these findings suggest that an optimal range of inhibitory G protein signaling is required for normal hippocampal-dependent cognition. The molecular and cellular mechanisms underlying this critical modulatory influence, however, are unclear.

G protein-gated inwardly rectifying K⁺ (GIRK/Kir3) channels mediate the postsynaptic inhibitory effect of many neurotransmitters acting via inhibitory G protein-coupled receptors, including GABA_BR and 5HT_{1A}R (20–22). Neuronal GIRK channels are homo- and heterotetrameric complexes formed primarily by assembly among GIRK1, GIRK2, and GIRK3 subunits (23). Studies of GIRK subunit expression patterns and knockout mice indicate that GIRK2 is an integral subunit of most, if not all, neuronal GIRK channels (21–23). For example, constitutive genetic ablation of *Girk2* in mice yields a dramatic reduction in GIRK1 protein levels and nearly-complete loss GABA_BR-dependent GIRK channel activity in CA1 pyramidal neurons (20, 24).

Several lines of evidence have implicated GIRK channel dysfunction in human psychiatric problems and cognitive dysfunction. Polymorphisms in human KCNJ genes have been associated with key cognitive abnormalities in depression and personality disorder (25), and schizophrenia (26). Post-mortem forebrain samples from individuals with schizophrenia or

bipolar disorder also show reduced GIRK mRNA levels (27). GIRK2 hypo-function is implicated in intellectual, cognitive and mental disabilities (28, 29), as well as elevated hippocampal network excitability in the early stages of AD (30). Mouse models of DS, including mice carrying an extra copy of the *Girk2* gene (GIRK2 trisomy mice), exhibit enhanced GIRK channel activity in hippocampal pyramidal neurons, as well as disrupted hippocampal-dependent synaptic plasticity and deficits in contextual fear learning and memory (31, 32). Similarly, constitutive loss of RGS7, a negative regulator of GIRK channel activity in CA1 pyramidal neurons, alters synaptic plasticity at the Schaffer collateral/CA1 synapse and impairs hippocampal-dependent learning and memory in multiple tasks (33).

While available evidence suggests that enhanced GIRK-dependent signaling is detrimental to cognition and normal synaptic plasticity (32, 34–37), less is known concerning the impact of diminished neuronal GIRK channel activity on these processes. Accordingly, we investigated the contribution of GIRK channels to associative learning and memory using three loss-of-function models. We demonstrate that diminished GIRK-dependent signaling in forebrain pyramidal neurons, but not inhibitory neurons, selectively impairs contextual fear learning.

METHODS AND MATERIALS

Animals

All animal experimentation was approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Constitutive *Girk2*^{-/-} and *Girk2*^{flx/flx} mice were generated as described (38, 39). *Girk2*^{flx/flx} mice were bred with B6.Cg-Tg(Camk2a-cre)T29-1Stl/J (CaMKII-Cre) and B6N.Cg-*Gad2tm2(cre)Zjh/J* (GAD-Cre) (The Jackson Laboratory; Bar Harbor, ME, USA) to generate CaMKII-Cre:*Girk2*^{flx/flx} and GAD-Cre:*Girk2*^{flx/flx} mice, respectively. B6.Cg-*Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J* mice (The Jackson Laboratory) were bred with B6.Cg-Tg(Camk2a-cre)T29-1Stl/J mice to generate CaMKII-Cre:Rosa-tdTomato mice. GAD67-eGFP mice (40) were generously provided by Dr. T. Kaneko and crossed with GAD-Cre:*Girk2*^{flx/flx} mice for electrophysiological studies. Mice were maintained on a 12 h light/dark cycle, with food and water available *ad libitum*.

Immunoblotting and immunohistochemistry

Procedures were performed as described to extract, process, and quantify target proteins in tissue micropunches (41), and to visualize GIRK2 immunoreactivity or tdTomato fluorescence in tissue sections (39). Polyclonal rabbit anti-GIRK2 (1:200 Alomone Labs; Jerusalem, Israel) and polyclonal mouse anti-β-actin (1:20,000; Abcam; Cambridge, MA, USA) antibodies were used in this study.

Slice electrophysiology

Somatodendritic GABA_BR-GIRK currents were measured in acutely-isolated mouse brain slices (4–6 wk), as described (41, 42). Neurons with a pyramidal-shaped soma located in dorsal and ventral CA1 *stratum pyramidale* were targeted for analysis of GIRK-dependent signaling in pyramidal neurons. GABA neurons were identified using GAD67-eGFP

reporter mice (40); eGFP-positive neurons with bipolar morphology, located near the *stratum pyramidale* in the *stratum oriens* and *stratum radiatum* were targeted for analysis of GIRK-dependent signaling in GABA neurons. All command potentials factored in junction potential of -15 mV.

Synaptic plasticity was measured in adult mice (8–20 wk), as described (33). In brief, field EPSPs (fEPSPs) were measured from the *stratum radiatum* of dorsal CA1 pyramidal neurons. LTP and long-term depression (LTD) were recorded for 1 h after theta-burst stimulation (TBS: 4 pulses at 100 Hz x 10, 200 ms intervals) or low-frequency stimulation (LFS: 2 Hz for 10 min, 1200 pulses). Depotentiation of LTP was achieved by applying high-frequency stimulation (HFS: 2 tetanized stimuli 100 Hz for 1 s each) to first induce LTP, followed shortly thereafter (1–2 min) by LFS.

Behavior

Adult mice (8–12 wk) were tested in open field activity, hot plate (55°C), and elevated plus maze (EPM) tests, as described (43, 44). Prior to the delay fear conditioning test, mice were acclimated to the testing room (2 h total), and handled within the testing room. On the first day of testing, floors of the test chamber (Med Associates, Inc.; St. Albans, VT, USA) were lined with corncob bedding, and walls were sprayed with 70% ethanol, prior to introducing the subject. During conditioning (7.5 min), percent time freezing was recorded (Video Freeze[®] 2.16, Med Associates, Inc.) across 3 pairings of an auditory tone (85 dB/3 kHz; 30 s presented in 1 min intervals) that co-terminated with an electric shock (0.70–0.72 mA, 2 s) delivered through metallic floor bars. Twenty-four hours later, percent time freezing was measured in response to chamber presentation alone (context test); during this 5 min test, the chamber floor was lined with bedding from the conditioning session, and no tones or shocks were delivered. The cue test occurred 1 h later, with freezing measured prior to and after tone presentation in a novel context (plastic context-changing inserts lined the floor and obscured the walls of the chamber, and were scented with vanilla). Cue testing lasted for 7 min, where a 3 min tone was delivered between intervals of silence (2 x 2 min).

The Barnes maze test was conducted using a round platform (100-cm diameter) with 20 holes equally spaced along the periphery (San Diego Instruments; San Diego, CA). The maze was elevated above the floor (100 cm), and visual cues were present on the walls of the testing room (within 1–2 m of the maze). One hole was connected to an escape box during training, and mice were trained to learn the escape box location over the course of 16 sessions (4/d x 4 d). The maximum length of each training session was 3 min, and the inter-trial interval was 15–20 min. Between each session, the maze was wiped with 70% EtOH and rotated 90° . Average escape latency was determined daily. A probe test was conducted on day 5; mice explored the maze for 90 s with escape box removed. Maze exploration during the probe test was analyzed by calculating time spent in each quadrant (target, +1, -1, and opposite). The average distance from, and latency to enter, the goal hole (a 5 cm circle circumscribing the escape hole location) were also calculated. All behaviors were recorded and analyzed using ANY-maze software (Stoelting Co.; Wood Dale, IL, USA).

Analysis

Data are presented throughout as mean $\pm$ SEM. Statistical analysis was performed using Prism 6.0 software (GraphPad Software, Inc.; La Jolla, CA, USA) or SigmaPlot (Systat Software, Inc; San Jose, CA, USA). With the exception of the slice electrophysiology and Barnes maze studies, which involved only male subjects, all studies involved balanced groups of male and female mice. In these cases, data were analyzed first for between-group effects of genotype and sex using two-way ANOVA; within-group cue and sex, genotype and sex, or cue and genotype effects were assessed using Repeated Measures ANOVA (Table S1). Significant between-group differences were assessed *post hoc* with Sidak's test. Where no sex differences were observed, data were pooled and assessed for genotypic effects with an unpaired Student's *t* test, or a Mann-Whitney *U* test when normality was violated, to increase statistical power of genotypic differences. In the absence of genotypic interactions or differences, sex differences were observed for a few parameters (e.g., freezing during the cue test for GAD-Cre: *Girk2*^{flox/flox} mice; Table S1). While these differences are noted in the appropriate figure legends, data are collapsed by genotype in figures to highlight the lack of genotypic effects. Pearson's Correlation Coefficient was used to determine the degree fear learning predicted fear memory in CaMKII-Cre: *Girk2*^{flox/flox} mice. Electrophysiological and synaptic plasticity data were analyzed for effects of genotype using an unpaired Student's *t* test, or a Mann-Whitney *U* test when normality was violated. Where applicable, values $\pm$ 2 standard deviations from the mean were eliminated as outliers. All comparisons were *a priori* specified. Differences were considered significant if $p < 0.05$.

RESULTS

Delay fear conditioning in constitutive *Girk2*^{-/-} mice

We began by testing constitutive *Girk2*^{-/-} mice in a delay fear conditioning paradigm that permits evaluation of both dorsal hippocampal-dependent (context) and independent (cue) associative learning and memory (6, 45, 46). Freezing triggered by the second and third conditioning tones was significantly reduced in *Girk2*^{-/-} mice relative to wild-type controls (Fig. 1A), as was total freezing measured during conditioning (Fig. 1B). On memory test day (24 h later), *Girk2*^{-/-} mice exhibited significantly decreased freezing in response to context presentation (Fig. 1C), and after cue presentation in a novel context (Fig. 1E). Freezing before tone presentation in the cue test was also reduced in *Girk2*^{-/-} mice relative to controls (Fig. 1D). Thus, constitutive ablation of *Girk2* in mice leads to an apparent disruption of both dorsal hippocampal-dependent and independent forms of associative learning and memory.

Characterization of GABA neuron-specific *Girk2*^{-/-} mice

In addition to exhibiting enhanced seizure susceptibility (44, 47), *Girk2*^{-/-} mice are hyperactive (Fig. S1A), show enhanced thermal nociception (Fig. S1B), and display decreased anxiety-related behavior (Fig. S1C). These neurological phenotypes, and potentially others, complicate the straightforward interpretation of their associative learning deficits. To gain more refined insight into the contribution of GIRK channels to learning and memory, we generated neuron-specific knockout lines by crossing a recently-derived conditional *Girk2* knockout (*Girk2*^{flox/flox}) line (39) with transgenic lines expressing Cre

recombinase in a neuron-specific manner. As GABA neuron dysfunction can disrupt cognition, including contextual fear learning and memory (48, 49), we first asked whether GIRK-dependent signaling in GABA neurons is required for normal fear learning. We crossed *Girk2^{flox/flox}* mice with GAD-Cre mice, which express Cre recombinase under the control of the *Gad2* promoter. Cre expression in these mice is found throughout the CNS, but is restricted to GABA neurons (50). GAD-Cre(+):*Girk2^{flox/flox}* mice were viable, and exhibited normal behavior the open-field, hot plate, and EPM tests (Fig. S1).

To facilitate the electrophysiological evaluation of GABA neurons in GAD-Cre:*Girk2^{flox/flox}* mice, we crossed this line with transgenic mice expressing eGFP under the control of the GAD67 promoter (GAD67-eGFP mice; (40)). Somatodendritic currents evoked by a saturating concentration of the GABA_BR agonist baclofen (200 μ M) were significantly smaller in dorsal hippocampal GABA neurons from GAD67eGFP(+)/GAD-Cre(+):*Girk2^{flox/flox}* mice than those measured in GAD67eGFP(+)/GAD-Cre(-):*Girk2^{flox/flox}* controls (Fig. 2A,B). Nevertheless, GAD-Cre(+):*Girk2^{flox/flox}* mice and GAD-Cre(-):*Girk2^{flox/flox}* mice exhibited comparable freezing in response to cue-shock pairing (Fig. 2C), and total freezing (Fig. 2D), on the first day of delay fear conditioning. Furthermore, no genotype-dependent differences in freezing were observed during context (Fig. 2E) or cue (Fig. 2F,G) memory tests.

Characterization of forebrain pyramidal neuron-specific *Girk2^{-/-}* mice

We next crossed *Girk2^{flox/flox}* mice with the CaMKII-Cre line, which drives Cre-dependent recombination in postnatal forebrain pyramidal neurons (7, 51). Using a conditional tdTomato reporter line, we observed that CaMKII-Cre-driven recombination was most prominent in the CA1 region of the dorsal hippocampus, and that the extent of recombination increased with age (Fig. S2). Consistent with these data, total GIRK2 protein levels were lower in the dorsal hippocampus of CaMKII-Cre(+):*Girk2^{flox/flox}* mice, but not the cerebellum (Fig. 3A,B). Immunohistochemical characterization revealed lower GIRK2 immunoreactivity in the dorsal hippocampus of CaMKII-Cre(+):*Girk2^{flox/flox}* mice, particularly in the *stratum radiatum*, which contains the CA1 dendritic field (Fig. 3C). In contrast, GIRK2 immunolabelling in the ventral hippocampus, thalamus, and midbrain was largely intact in CaMKII-Cre(+):*Girk2^{flox/flox}* mice.

Previous studies have shown that the baclofen-induced somatodendritic current in CA1 pyramidal neurons is mediated almost exclusively by activation of GIRK2-containing channels (20, 24). Indeed, reliable baclofen-induced outward currents were observed in both dorsal and ventral CA1 pyramidal neurons from CaMKII-Cre(-):*Girk2^{flox/flox}* mice (Fig. 3D,E). In contrast, baclofen-induced currents in dorsal CA1 pyramidal neurons from CaMKII-Cre(+):*Girk2^{flox/flox}* mice were significantly smaller than those recorded in controls. Consistent with our immunohistochemical data, which showed normal GIRK2 labeling in the ventral hippocampus, baclofen-induced currents in ventral CA1 pyramidal neurons from CaMKII-Cre(+):*Girk2^{flox/flox}* mice were normal. Thus, CaMKII-Cre(+):*Girk2^{flox/flox}* mice exhibit a deficit in GIRK-dependent signaling in dorsal, but not ventral, hippocampal CA1 pyramidal neurons.

In contrast to constitutive *Girk2*^{-/-} mice, CaMKII-Cre(+):*Girk2*^{flox/flox} mice exhibited normal open-field locomotor activity, thermal nociception, and anxiety-like behavior in the EPM (Fig. S1). In the fear conditioning test, freezing in response to the third cue-shock pairing (Fig. 4A), and total freezing during conditioning (Fig. 4B), were significantly lower in CaMKII-Cre(+):*Girk2*^{flox/flox} mice than in littermate controls. Reduced freezing in CaMKII-Cre(+):*Girk2*^{flox/flox} mice was not due to attributable to hyperactivity, as their motor activity during conditioning was comparable to that of CaMKII-Cre(-):*Girk2*^{flox/flox} control mice ($t_{14}=0.31$, $p=0.38$). During the memory test, CaMKII-Cre(+):*Girk2*^{flox/flox} mice showed significantly decreased freezing to context (Fig. 4C). In contrast to constitutive *Girk2*^{-/-} mice, however, baseline (Bsl) freezing and freezing during the cue test were normal in CaMKII-Cre(+):*Girk2*^{flox/flox} mice (Fig. 4D,E). Importantly, the deficit in contextual fear conditioning observed in CaMKII-Cre(+):*Girk2*^{flox/flox} mice is not attributable to altered sensitivity to the foot-shock, as the threshold to detect the shock was indistinguishable between CaMKII-Cre(+):*Girk2*^{flox/flox} and CaMKII-Cre(-):*Girk2*^{flox/flox} mice (Fig. S3). Furthermore, contextual freezing was significantly predicted by freezing to the last cue during conditioning for both CaMKII-Cre(+):*Girk2*^{flox/flox} ($R^2=0.47$, $p<0.005$) and CaMKII-Cre(-):*Girk2*^{flox/flox} ($R^2=0.40$, $p<0.01$) mice, indicating that a deficit in learning in the CaMKII-Cre(+):*Girk2*^{flox/flox} mice underlies the memory impairment.

To test whether the deficit in contextual fear conditioning displayed by CaMKII-Cre(+):*Girk2*^{flox/flox} mice extended to other types of hippocampal-dependent learning, we next evaluated these mice using a Barnes maze (Fig. 5). The Barnes maze evaluates spatial learning, and, unlike fear conditioning, does not employ a strong aversive stimulus (52). Over the 4-d training period, CaMKII-Cre(+):*Girk2*^{flox/flox} and CaMKII-Cre(-):*Girk2*^{flox/flox} mice learned to find the escape hole with similar latencies (Fig. 5A). During the probe test, both CaMKII-Cre(+):*Girk2*^{flox/flox} mice and littermate controls spent more time in the goal quadrant relative to other quadrants (Fig. 5B). No difference was observed in time spent in the goal zone or search strategy between CaMKII-Cre(+):*Girk2*^{flox/flox} and CaMKII-Cre(-):*Girk2*^{flox/flox} mice (Fig. 5B,C). Moreover, distance from the goal (Fig. 5D), and latency to enter the escape zone (Fig. 5E), during the probe test were comparable. Thus, hippocampal-dependent associative learning is not uniformly impaired in CaMKII-Cre(+):*Girk2*^{flox/flox} mice.

Synaptic plasticity in forebrain pyramidal neuron-specific *Girk2*^{-/-} mice

Lastly, we examined the impact of GIRK channel ablation in forebrain pyramidal neurons on LTP, LTD, and depotentiation of LTP, three forms of plasticity linked to hippocampal-dependent associative learning (8, 10, 53, 54). LTP at the Schaffer collateral/CA1 synapse in the dorsal hippocampus was induced by standard theta burst stimulation (TBS) in the *stratum radiatum* of dorsal CA1. We observed a trend toward enhanced fEPSP slope measured immediately after TBS in slices from CaMKII-Cre(+):*Girk2*^{flox/flox} mice (Fig. 6A), but the difference was not statistically significant ($t_4=1.89$, $p=0.13$). LTP expression, measured 55–60 min after TBS, did not differ between genotypes. Delivery of low-frequency stimulation (LFS), which had a transient inhibitory impact on fEPSP slope in slices from CaMKII-Cre(-):*Girk2*^{flox/flox} mice, evoked a persistent LTD in slices from CaMKII-Cre(+):*Girk2*^{flox/flox} mice (Fig. 6B). Finally, whereas consecutive application of

high-frequency stimulation (HFS) and LFS triggered a significant depotentiation in slices from CaMKII-Cre(-):*Girk2*^{flox/flox} mice, no depotentiation was observed in slices from CaMKII-Cre(+):*Girk2*^{flox/flox} mice (Fig. 6C). Thus, the loss of GIRK channels in forebrain pyramidal neurons impaired two distinct forms of inhibitory synaptic plasticity in the dorsal hippocampus.

DISCUSSION

Mouse models exhibiting enhanced GIRK-dependent signaling show altered learning and memory (19, 32–34). Neither the behavioral impact of GIRK channel ablation, nor the cell-specific contribution of GIRK channels to hippocampal-dependent cognition, however, has been examined. Here, we evaluated the impact of cell-specific ablation of GIRK channels on associative learning using a recently developed conditional *Girk2* knockout mouse. We found that the loss of GIRK channels in forebrain pyramidal (excitatory) neurons, but not GABA (inhibitory) neurons, impaired contextual fear learning, a task that relies primarily on the dorsal hippocampus (6, 45). The loss of GIRK channels in forebrain pyramidal neurons, however, did not impact spatial learning, another form of dorsal hippocampal-dependent cognition. Thus, the observed impact of GIRK channel ablation on cognition was task-specific. Further supporting the contention that GIRK channels make discrete contributions to certain forms of associative learning is the observation that despite the large deficits in fear learning measured in this study, constitutive *Girk2*^{-/-} mice can learn to perform an operant task with food as a reinforcer (44).

Cre recombinase-driven gene excision in CaMKII-Cre mice was initially reported to occur exclusively in CA1 pyramidal neurons (7). Available data suggest that the extent of Cre-dependent gene excision in pyramidal neurons observed with CaMKII-Cre mice is dependent on the reporter or “floxed” gene (*e.g.*, (51, 55–58)). In CaMKII-Cre(+):*Girk2*^{flox/flox} mice, loss of GIRK2 was most evident in the CA1 region of the dorsal hippocampus. Using a tdTomato reporter line, however, we observed evidence of Cre-dependent recombination in cortical and other forebrain regions. Thus, the loss of GIRK2 in pyramidal neurons outside of dorsal CA1 may contribute to the contextual fear learning deficit observed in CaMKII-Cre(+):*Girk2*^{flox/flox} mice.

Previously, we reported that constitutive *Girk2* ablation correlated with a 75% reduction in the magnitude of the baclofen-induced current in CA1 pyramidal neurons (24). While baclofen-induced currents were significantly diminished in CA1 pyramidal neurons from CaMKII-Cre(+):*Girk2*^{flox/flox} mice, the residual current was larger than expected (~50% of control). This could reflect study-specific differences involving animal age, or differential molecular adaptations occurring in the constitutive and cell-specific mutant lines. There may also have been significant residual GIRK2 protein in CA1 pyramidal neurons from CaMKII-Cre(+):*Girk2*^{flox/flox} mice at the time when slice recordings were obtained (30–35 d). Indeed, Cre expression does not begin until 2–3 weeks after birth in the CaMKII-Cre line (7), and we observed a notable increase in Cre-dependent recombination between P30 and P60. It is also important to consider that the complete elimination of GIRK2 protein may not occur until well after the Cre-dependent recombination of the *Girk2* gene.

Our data are consistent with observations that pyramidal neurons in the CA1 region are particularly important for cognitive function. fEPSPs at the Schaffer collateral/CA1 synapse increase significantly during associative classical conditioning (59), whereas genetic ablation of the NMDA receptor (NMDAR) in CA1 impairs spatial learning and memory (7). In the *stratum radiatum* of dorsal CA1, the number of mushroom spines and synapses on pyramidal neurons increases with learning and memory in the water maze (60). In addition, delay or trace eye-blink conditioning increases spine density at basal dendrites of pyramidal neurons exclusively in CA1, while leaving other hippocampal sub-regions and the cortex unchanged (61).

While normal performance in the Barnes maze and contextual fear conditioning tasks both rely on the dorsal hippocampus (6), contextual fear conditioning uses a strong aversive stimulus to induce learning. Thus, the stress associated with fear conditioning may explain the selective cognitive deficit observed in CaMKII-Cre(+):*Girk2*^{flx/flx} mice. In support of this contention, extremely high levels of glucocorticoid (*i.e.*, corticosterone in mice) disrupt the ability to learn and retrieve memories (62–66). Within minutes of stress, corticosterone promotes increased glutamate release in the hippocampus, while also enhancing the intrinsic excitability of CA1 pyramidal neurons (67–69). Collectively, these observations suggest the intriguing possibility that the stress induced by fear conditioning (70) exacerbated the deficit in GIRK-dependent neuronal inhibition in CaMKII-Cre(+):*Girk2*^{flx/flx} mice, leading to a selective impairment in contextual learning.

GABA interneurons of the dorsal hippocampus are thought to be important contributors to cognitive function. Indeed, a reduction in the number of inhibitory interneurons in the hippocampus has been linked to impaired contextual fear learning and memory (48). Similarly, mice lacking NMDAR in interneurons show impairments in working memory, and contextual and cue fear memories, while spatial cognition remains intact (71). In contrast, increasing the ratio of inhibitory interneurons to excitatory neurons throughout the dorsal hippocampus enhances working memory and reversal learning, while impairing LTP (72). In this study, we observed no change in learning or memory for mice lacking GIRK channels in GABA neurons. Together with findings in CaMKII-Cre(+):*Girk2*^{flx/flx} mice, these data indicate that the contribution of GIRK channels to hippocampal-dependent contextual fear learning stems primarily from their impact on excitatory (pyramidal) as opposed to inhibitory (GABA) neurons.

LTP, LTD, and depotentiation of LTP are forms of excitatory synaptic plasticity that have been implicated in the encoding of associative memories, and are variably influenced by genetic manipulations impacting GIRK channel function (10, 31, 35, 36, 53, 73–75). To date, clear relationships between gain- or loss-of-function manipulations of GIRK-dependent signaling and these measures of synaptic plasticity have been difficult to glean. While some studies have shown that hippocampal-dependent LTP is not impacted by either constitutive ablation or *Girk2* trisomy (32, 74), enhanced LTP has been reported in *Girk2*^{-/-} mice (31). Perhaps more surprisingly, CaMKII-Cre(+):*Girk2*^{flx/flx} (loss-of-function; this study) and GIRK2 trisomy (gain-of-function) mice (32) both exhibit blunted depotentiation of LTP and enhanced LTD. Furthermore, mice lacking *Rgs7*, which display enhanced hippocampal GABA_BR-GIRK signaling, exhibit both blunted depotentiation of LTP and

blunted LTD (33). These findings suggest that the contribution made by GIRK channels to synaptic plasticity is complex and perhaps model-specific, and that an optimal range of GIRK channel activity is required for normal synaptic plasticity. Interestingly, 5HT₄R antagonists also blunted depotentiation of LTP and enhanced LTD without affecting LTP in the hippocampus (76), findings similar to our observations in CaMKII-Cre(+):*Girk2*^{flox/flox} mice. As such, loss of GIRK2 in CA1 pyramidal neurons may alter the excitatory-inhibitory balance between 5HT_{1A}R and 5HT₄R (77), which is disrupted in psychiatric and cognitive conditions including schizophrenia and AD (18).

While LTD has been identified as an important contributor to hippocampal-dependent learning (*e.g.* contexts) (10), the behavioral relevance of depotentiation is less well-understood. Interestingly, both depotentiation of LTP and LTD require activation of NMDAR (10, 75), and NMDAR activation or inhibition can increase or decrease, respectively, GIRK-dependent signaling in hippocampal pyramidal neurons (78, 79). Moreover, genetic ablation of GluN1-containing NMDARs in forebrain pyramidal neurons correlates with decreased GIRK2 protein levels in cortical synaptic membranes (5). And, similar to our observations in CaMKII-Cre(+):*Girk2*^{flox/flox} mice, loss of GluN1-containing NMDAR channels in forebrain pyramidal neurons leaves spatial learning and memory intact, but impairs other forms of hippocampal-dependent cognition (80, 81). Altogether, these data suggest that the deficit in contextual fear learning seen in CaMKII-Cre(+):*Girk2*^{flox/flox} mice may reflect in part alterations in NMDAR levels and/or function.

Collectively, our work shows that GIRK channels in forebrain pyramidal neurons, but not GABA neurons, are necessary for normal hippocampal-dependent contextual fear learning and two forms of inhibitory synaptic plasticity implicated in associative learning. These data, along with work from others, support the emerging consensus that dysregulation of GIRK channel activity in excitatory neurons of the forebrain may contribute to cognitive dysfunction associated with aversive conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Delay fear conditioning in constitutive *Girk2*^{-/-} mice

A) Freezing in response to cue-shock presentation in *Girk2*^{-/-} and wild-type (WT) on day 1 of the delay fear conditioning test (n=15/genotype). Repeated Measures ANOVA revealed a significant interaction between cue and genotype across the 3 tone-shock presentations ($F_{2,56}=51.60$; $p<0.0001$). Significant main effects of cue ($F_{2,56}=80.48$; $p<0.0001$) and genotype ($F_{1,28}=54.20$; $p<0.0001$) were also observed. Symbols: **** $p<0.0001$ vs. WT (Sidak's *post hoc* test). **B)** Total freezing on day 1 of the fear conditioning test was also lower in *Girk2*^{-/-} mice than in WT controls ($t_{27}=5.58$, $p<0.0001$). Symbols: **** $p<0.0001$ vs. WT. **C)** *Girk2*^{-/-} mice exhibited decreased freezing in response to context presentation, measured 24 h after conditioning (Mann Whitney $U=5$, median_{1,2}=38.3, 4.1; $p<0.0001$). Symbols: **** $p<0.0001$ vs. WT. **D,E)** Baseline (Bsl) freezing prior to tone presentation (Mann Whitney $U=31$, median_{1,2}=4.63, 1.40; $p<0.001$), and cue-induced freezing ($t_{28}=6.78$; $p<0.0001$), were significantly reduced in *Girk2*^{-/-} mice during the cue memory test. Symbols: ***, **** $p<0.001$ and 0.0001 , respectively, vs. WT.

Figure 2. Characterization of GAD-Cre:*Girk2*^{lox/lox} mice

A) Representative currents ($V_{\text{hold}} = -60$ mV) evoked by baclofen (200 μM) in dorsal CA1 GABA neurons from male GAD67-eGFP(+)/GAD-Cre(-):*Girk2*^{lox/lox} (upper trace) and GAD67-eGFP(+)/GAD-Cre(+):*Girk2*^{lox/lox} (lower trace) mice (scale: 20 pA/100 s). Currents in slices from GAD67-eGFP(+)/GAD-Cre(-):*Girk2*^{lox/lox} mice were observed together with a decrease in input resistance (not shown), and were reversed by the GABA_BR antagonist CGP54626 (CGP, 2 μM). **B)** Summary of baclofen-induced currents in dorsal CA1 GABA neurons from male GAD67-eGFP(+)/GAD-Cre(+):*Girk2*^{lox/lox} and GAD67-eGFP(+)/GAD(-):*Girk2*^{lox/lox} mice ($t_8=5.63$; $p<0.001$; $n=5/\text{genotype}$). Symbols: *** $p<0.001$ vs. control. **C)** Freezing in response to cue-shock presentation in GAD-Cre(+):*Girk2*^{lox/lox} and GAD(-):*Girk2*^{lox/lox} on day 1 of the delay fear conditioning test ($n=20/\text{genotype}$). Repeated Measures ANOVA revealed a significant interaction between cue and sex ($F_{2,76}=6.26$; $p<0.005$) (Table S1), with females displaying significantly higher freezing than males to the 2nd ($p<0.01$) and 3rd ($p<0.001$) tones. No interaction between cue and genotype ($F_{2,76}=0.22$; $p=0.80$), however, was observed. A significant main effect of cue ($F_{2,76}=57.40$; $p<0.0001$) was revealed for freezing across 3 tone-shock pairings, but there was no main effect of genotype ($F_{1,38}=0.39$; $p=0.76$). **D)** Total freezing on day 1 of the fear conditioning test was comparable for GAD-Cre(+):*Girk2*^{lox/lox} and GAD(-):*Girk2*^{lox/lox} mice ($t_{38}=0.71$; $p=0.24$). No interaction was observed for genotype and sex (Table S1). While a significant main effect of sex was observed ($F_{1,36}=6.57$; $p<0.05$), *post hoc* analysis did not reveal any further difference between male and female mice ($p>0.05$). **E)** GAD-

Cre(+): *Girk2*^{flox/flox} and GAD(-): *Girk2*^{flox/flox} mice exhibited comparable freezing in response to context presentation, measured 24 h after conditioning ($t_{37}=0.53$; $p=0.30$). **F,G** Baseline (Bsl) freezing prior to tone presentation ($t_{38}=0.38$; $p=0.35$), and cue-induced freezing ($t_{38}=0.91$; $p=0.18$), were similar for GAD-Cre(+): *Girk2*^{flox/flox} and GAD(-): *Girk2*^{flox/flox} mice during the cue memory test. No interaction was observed for genotype and sex for cue memory (Table S1). While a significant main effect of sex was observed ($F_{1,36}=6.87$; $p<0.05$), *post hoc* analysis did not reveal any further difference between male and female mice ($p>0.05$).

Figure 3. Characterization of CaMKII-Cre(+):*Girk2*^{lox/lox} mice

A) Immunoblots showing GIRK2 (red) and β-actin (green) protein levels in micropunches of the dorsal hippocampus (dHPC) and cerebellum (CB) from adult (6–8 wk) CaMKII-Cre(+):*Girk2*^{lox/lox} and CaMKII-Cre(-):*Girk2*^{lox/lox} mice. Note that GIRK2 appears as a doublet, likely attributable to the presence of multiple GIRK2 splice isoforms (82). **B)** Summary of immunoblotting data. The GIRK2:β-actin ratio was calculated for each sample. GIRK2:β-actin ratio was significantly reduced in the dorsal hippocampus ($t_{11}=5.31$; $p=0.0001$) but not the cerebellum ($t_{11}=1.14$; $p=0.14$) for CaMKII-Cre(+):*Girk2*^{lox/lox} relative to CaMKII-Cre(-):*Girk2*^{lox/lox} mice. Symbol: **** $p<0.0001$ vs. control. **C)** GIRK2 immunoreactivity in sections containing the dorsal (dHPC; upper panels) and ventral (vHPC; lower panels) hippocampus in adult (8–12 wk) CaMKII-Cre(-):*Girk2*^{lox/lox} (left), CaMKII-Cre(+):*Girk2*^{lox/lox} (middle), and *Girk2*^{-/-} (right) mice. Abbreviations: *stratum oriens* (so), *stratum pyramidale* (sp), *stratum radiatum* (sr), *stratum lacunosum-moleculare*

(slm), *molecular layer* (ml), *granule cell* (gc), *hilus* (h) of the dentate gyrus (DG), substantia nigra (SN), ventral tegmental area (VTA). **D**) Currents ($V_{\text{hold}} = -60$ mV) evoked by baclofen (200 μM) in dorsal and ventral CA1 pyramidal neurons from male CaMKII-Cre(+): *Girk2*^{flox/flox} and CaMKII-Cre(-): *Girk2*^{flox/flox} mice (scale: 25 pA/2 min). Currents were observed together with a decrease in input resistance (not shown), and were reversed by the GABA_BR antagonist CGP54626 (CGP, 2 μM). **E**) Summary of baclofen-induced currents in dorsal and ventral CA1 pyramidal neurons from male CaMKII-Cre(+): *Girk2*^{flox/flox} and CaMKII-Cre(-): *Girk2*^{flox/flox} mice (n=4–5/genotype). CaMKII-Cre(+): *Girk2*^{flox/flox} mice showed reduced baclofen-induced currents in dorsal ($t_7=2.34$; $p=0.026$) but not the ventral hippocampus ($t_7=0.42$; $p=0.34$). Symbol: * $p<0.05$ vs. control.

Figure 4. Delay fear conditioning in CaMKII-Cre:Girk2^{lox/lox} mice

A) Freezing in response to cue-shock presentation in CaMKII-Cre(+):Girk2^{lox/lox} mice and CaMKII-Cre(-):Girk2^{lox/lox} mice on day 1 of the delay fear conditioning test (n=16–17/genotype). Repeated Measures ANOVA revealed a significant interaction between cue and genotype across the 3 tone-shock presentations ($F_{2,62}=13.07$; $p<0.0001$). Significant main effects of cue ($F_{2,62}=111.80$; $p<0.0001$) and genotype ($F_{1,31}=7.67$; $p<0.01$) were also observed. Symbol: **** $p<0.0001$ (Sidak's *post hoc* test). **B)** Total freezing on day 1 of the fear conditioning test was also lower in CaMKII-Cre(+):Girk2^{lox/lox} mice ($t_{31}=2.06$, $p<0.05$). Symbol: * $p<0.05$ vs. control. **C)** CaMKII-Cre(+):Girk2^{lox/lox} mice exhibited decreased freezing in response to context presentation, measured 24 h after conditioning ($t_{32}=2.83$; $p<0.01$). Symbol: ** $p<0.01$ vs. control. **D)** Baseline (Bsl) freezing prior to tone presentation ($t_{30}=1.13$; $p=0.27$) was not altered in CaMKII-Cre(+):Girk2^{lox/lox} mice relative to controls. Independent of genotype and in the absence of an interaction (Table S1), however, a significant effect of sex was observed ($F_{1,28}=7.51$; $p<0.05$). *Post hoc* analysis did not reveal any further difference between male and female mice ($p>0.05$). **E)** Cue-induced freezing ($t_{30}=1.21$; $p=0.12$), was not significantly reduced in CaMKII-Cre(+):Girk2^{lox/lox} mice during the cue memory test.

Figure 5. Barnes maze testing in CaMKII-Cre:*Girk2*^{flox/flox} mice

A) Latency to escape the Barnes maze during training for male CaMKII-Cre(+):*Girk2*^{flox/flox} and CaMKII-Cre(-):*Girk2*^{flox/flox} mice (n=14–15/group). Repeated Measures ANOVA revealed no significant interaction between genotype and training day ($F_{3,81}=0.87$; $p=0.46$). A main effect of training day ($F_{3,81}=44.6$; $p<0.0001$) was observed, but there was no main effect of genotype ($F_{1,27}=1$; $p=0.98$). **B)** Time spent during the probe test in the 4 zones of the maze: goal, opposite of goal (Opp), right of goal (+1), and left of goal (-1). Repeated Measures ANOVA revealed no significant interaction between zone and genotype ($F_{3,81}=0.71$; $p=0.55$). A main effect of zone ($F_{3,81}=23.22$; $p<0.0001$) was observed, but there was no main effect of genotype ($F_{1,27}=2.37$; $p=0.13$). **C)** No difference in search strategy, documented via heat maps of head location for each group, were observed between CaMKII-Cre(+):*Girk2*^{flox/flox} and CaMKII-Cre(-):*Girk2*^{flox/flox} mice during the probe test. **D)** No difference in distance from the escape hole was observed during the probe test ($t_{27}=0.98$; $p=0.17$) between CaMKII-Cre(+):*Girk2*^{flox/flox} and CaMKII-

Cre(-): *Girk2*^{flox/flox} mice. **E)** No difference was observed in the latency to enter the 5 cm circular perimeter around the sealed escape hole during the probe test for CaMKII-Cre(+): *Girk2*^{flox/flox} and CaMKII-Cre(-): *Girk2*^{flox/flox} mice (Mann Whitney $U=75$, median_{1,2}=11.90, 8.70; $p=0.10$).

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Figure 6. Synaptic plasticity in CaMKII-Cre:Girk2^{flox/flox} mice

A) LTP in hippocampal slices from CaMKII-Cre(+):Girk2^{flox/flox} and CaMKII-Cre(-):Girk2^{flox/flox} mice (left panel; n=3/genotype). Summary of the mean fEPSP slope measured 55–60 min following LTP induction, after normalization to pre-induction baseline ($t_4=2.39$; $p=0.075$; right panel). **B)** LTD was enhanced at the Schaffer collateral/CA1 synapse in slices from CaMKII-Cre(+):Girk2^{flox/flox} mice as compared with CaMKII-Cre(-):Girk2^{flox/flox} controls (left panel; n=3/genotype). Summary of the mean change in fEPSP slope, measured 55–60 min following LTD induction, after normalization to pre-induction baseline ($t_4=6.05$; $p<0.01$; right panel). Symbol: ** $p<0.01$ vs. control. **C)** Depotentialization (DP) of LTP was blunted in slices from CaMKII-Cre(+):Girk2^{flox/flox} mice, relative to controls (left panel; n=4–5/genotype). Summary of the mean fEPSP slope, measured 55–60 min following HFS/LFS stimulation, after normalization to pre-induction baseline ($t_7=3.51$; $p<0.01$; right panel). Symbol: ** $p<0.01$ vs. control.