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1 GABA_B receptor/HCN channel complexes in VTA dopamine neurons limit synaptic
2 inhibition and prevent anxiety-like behavior

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Abstract

Aversive stimuli inhibiting dopamine neurons in the ventral tegmental area (DA^{VT}_A neurons) induce anxiety-like behaviors. The inhibition of DA^{VT}_A neurons is prolonged by GABA_B receptor (GBR)-activated K⁺-currents, which exhibit a rapid desensitization of unknown physiological relevance. We now report that GBRs associate via auxiliary KCTD16 subunits with HCN channels, which facilitates activation of hyperpolarization-activated currents (*I_h*) by GBR-activated K⁺ currents. Activation of *I_h* underlies rapid K⁺ current desensitization in DA^{VT}_A neurons and limits GBR-mediated inhibition. Disruption of the GBR/HCN complex in *KCTD16*^{-/-} mice or blockade of *I_h* prolongs optogenetically driven inhibition of DA^{VT}_A neuron firing. *KCTD16*^{-/-} mice exhibit an increased anxiety-like behavior in response to stressful stimuli, which is reproduced by *in vivo* CRISPR/Cas9-mediated *KCTD16* ablation in DA^{VT}_A neurons or intra-VTA infusion of HCN antagonist to wild-type mice. Our data reveal that GBR-induced *I_h* protect DA^{VT}_A neurons from prolonged GBR-mediated inhibition in response to stressors, which moderates anxiety-like behaviors.

Introduction

Dopamine neurons of the ventral tegmental area (DA^{VT}_A) are implicated in the processing of reward and emotions (Chaudhury et al., 2013; Coque et al., 2011; Tovote, Fadok, & Luthi, 2015; Tye et al., 2013; Zweifel et al., 2011). Aberrant DA^{VT}_A neuron activity is associated with psychiatric disorders, including addiction, depression and anxiety (Bellone, Loureiro, & Luscher, 2021; Jennings et al., 2013; Nestler & Carlezon, 2006). In the latter case, environmental stimuli are perceived as threatening or overly aversive. Aversive stimuli that attenuate DA^{VT}_A neuron activation induce avoidance and cause a persistent, generalized anxiety-like phenotype in mice (Tan et al., 2012; Ungless, Magill,

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& Bolam, 2004; Zweifel et al., 2011). Conversely, disinhibition of DA^{VT}A neurons produces rewarding and anxiolytic phenotypes, which are reproduced by direct inhibition of GABA^{VT}A neurons synapsing onto DA^{VT}A neurons (Jennings et al., 2013). Synaptic activation of GABA_B receptors (GBRs) triggers Kir3-type K⁺ currents (I_{Kir3}) generating late inhibitory postsynaptic potentials (IPSP) that prolong neuronal inhibition (Edwards et al., 2017; Gassmann & Bettler, 2012; Lecca et al., 2016; Luscher & Slesinger, 2010; Schwenk et al., 2016). In DA^{VT}A neurons, the current activated by the GBR agonist baclofen (I_{bac}) exhibits an atypically rapid and pronounced attenuation compared to neighboring GABA^{VT}A neurons, which was attributed to fast I_{Kir3} desensitization (Arora et al., 2011; Cruz et al., 2004; Labouebe et al., 2007; Lalive et al., 2014; Padgett et al., 2012). Rapid and pronounced I_{bac} attenuation in the continuous presence of baclofen suggests that prolonged GBR-mediated inhibition of DA^{VT}A neurons is harmful. The mechanism underlying rapid I_{bac} attenuation and a causal link to behavior, however, remain elusive.

DA^{VT}A neurons exhibit a tonic firing (Overton & Clark, 1997) that is supported by a mixed inward Na⁺/K⁺ current, I_h , gated by hyperpolarization-activated cyclic nucleotide-gated HCN channels (Biel, Wahl-Schott, Michalakakis, & Zong, 2009; Seutin, Massotte, Renette, & Dresse, 2001). The depolarizing I_h sets the membrane potential (V_m) to more positive voltages, near the threshold of L-type Ca²⁺ channel activation, to maintain tonic DA^{VT}A neuron firing (Biel et al., 2009; Chan et al., 2007; Nedergaard, Flatman, & Engberg, 1993). Proteomic work surprisingly revealed a physical interaction of GBRs with HCN channels (Schwenk et al., 2016). GBRs additionally assemble with a family of auxiliary KCTD subunits, which regulate signaling kinetics and act as scaffolds for ion channels (Bhandari et al., 2021; Schwenk et al., 2010; Schwenk et al., 2016). However, it is presently unknown how GBRs and HCN channels work together and whether this interaction is behaviorally relevant.

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Here we show that GBRs associate via auxiliary KCTD16 subunits with HCN channels, which facilitates I_h activation in DA^{VT}A neurons through I_{Kir3} -mediated hyperpolarization. I_h activation counteracts the I_{Kir3} , thus mimicking a strong I_{Kir3} desensitization. Functional interaction of GBR-induced I_{Kir3} and I_h shortens IPSPs and reduces inhibition of DA^{VT}A neuron firing *in vivo*. I_h activation in GBR/HCN complexes represents a hitherto unknown feedback mechanism for limiting GBR-mediated inhibition, which in DA^{VT}A neurons serves to moderate the expression of anxiety-like behaviors in response to stressful stimuli.

Results

KCTD16 scaffolds HCN2 and HCN3 subunits to GBRs

GB1a, GB1b and GB2 subunits assemble heterodimeric GB1a/2 and GB1b/2 receptors that interact via GB2 with pentamers of auxiliary KCTD8, -12 and -16 subunits (Hannan, Wilkins, & Smart, 2012; Pin & Bettler, 2016; Schwenk et al., 2010; Schwenk et al., 2016; Shaye, Stauch, Gati, & Cherezov, 2021; Zheng, Abreu, Levitz, & Kruse, 2019; Zuo et al., 2019). KCTD subunits additionally bind to the G protein $\beta\gamma$ subunits, which accelerates activation and deactivation kinetics of GBR-activated I_{Kir3} (Fritzius et al., 2017; Turecek et al., 2014; Zheng et al., 2019). Proteomic work also indicated that KCTD subunits recruit HCN channels to GBRs (Schwenk et al., 2016). To study the functional consequences of GBR/HCN interactions we first identified the KCTD and HCN channel subunits involved in complex formation. Confirming earlier proteomic data (Schwenk et al., 2016), KCTD16 efficiently co-purified the HCN2 subunit from wild-type (WT) brain membranes (Figure 1A). GBRs co-purified HCN2 in the presence of KCTD16 but none of the other KCTDs from transfected HEK293 cells (Figure 1B). KCTD16 also co-purified HCN2 in the absence of

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GBRs, indicating that HCN2 binds via KCTD16 to GBRs (Figure 1 – figure supplement 1A). Indeed, GBRs did not co-purify HCN2 when mutation of Y902A in GB2 (Schwenk et al., 2016; Sereikaite et al., 2019) prevented binding of KCTD16 to the receptor (Figure 1C). Four different subunits assemble homo- and hetero-tetrameric HCN channels (Biel et al., 2009). KCTD16 selectively binds to HCN2 and HCN3 subunits (Figure 1 – figure supplement 1B). We determined that the H2 domain of KCTD16 (Gassmann & Bettler, 2012) binds to the N-terminal intracellular domain of HCN2 (aa1-215, Figure 1D-F). In summary, biochemical data show that KCTD16 recruits HCN channels containing HCN2 or HCN3 to GBRs.

Superimposed I_h mimics I_{Kir3} desensitization

DA^{VT}A neurons, which express GBRs, HCN channels and KCTD16 constitute a suitable cellular system to study putative functional interactions between GBRs and HCN channels (Cruz et al., 2004; Metz, Gassmann, Fakler, Schaeren-Wiemers, & Bettler, 2011; Ungless & Grace, 2012) (Figure 1 – figure supplement 1C). For electrophysiological recordings, we typically identified DA^{VT}A neurons in horizontal midbrain slices by their spontaneous firing activity, the presence of I_h and, occasionally, by staining for tyrosine hydroxylase (TH) (Figure 2A and C) (Cruz et al., 2004; Ungless & Grace, 2012). When clamped at -60 mV, DA^{VT}A neurons exhibited a pronounced I_{bac} desensitization ($46.0 \pm 4.0\%$, mean $\pm$ s.e.m. $n = 9$ neurons; Fig. 2b, d), as reported earlier (Arora et al., 2011; Cruz et al., 2004; Labouebe et al., 2007; Lalive et al., 2014; Padgett et al., 2012). During I_{bac} desensitization, the input resistance R_i surprisingly only recovered by $9.4 \pm 3.1\%$, while the R_i strongly increased after GBR inhibition with the antagonist CGP54626 (Figure 2B and D). The small decrease in I_{bac} conductivity during continuous baclofen application is at odds with the pronounced I_{bac} desensitization. Because GABA^{VT}A neurons, which do not express

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HCN channels, lack the pronounced I_{bac} desensitization observed in DA^{VT}A neurons (Cruz et al., 2004), we tested whether pharmacological blockade of I_h influences the time course of I_{bac} in DA^{VT}A neurons. Indeed, the I_h blocker zatebradine (Bucchi, Baruscotti, & DiFrancesco, 2002) significantly attenuated I_{bac} desensitization in WT DA^{VT}A neurons ($p < 0.001$, Figure 2B and D), showing that I_h contribute to I_{bac} desensitization and that I_{Kir3} exhibit *per se* little desensitization. $KCTD16^{-/-}$ DA^{VT}A neurons also exhibited a significantly reduced I_{bac} desensitization compared to WT neurons ($p < 0.01$, Figure 2B and D), suggesting that disruption of the GBR/HCN complex reduces desensitization. $KCTD12^{-/-}$ neurons exhibited similar I_{bac} desensitization as WT neurons, showing that KCTD12 does not contribute to I_{bac} desensitization in DA^{VT}A neurons. These findings support that activation of I_h counteracts I_{Kir3} and that superimposed I_h and I_{Kir3} underlie the apparent I_{bac} desensitization. The results additionally indicate that GBRs are more efficient in triggering I_h in WT than in $KCTD16^{-/-}$ neurons, showing that HCN/GBR complex formation promotes I_h activation.

GBR-induced hyperpolarization activates dendritic I_h

For I_{bac} recordings, DA^{VT}A neurons were clamped at -60 mV, which normally should prevent I_h activation by hyperpolarization. We therefore tested whether incomplete space clamp is responsible for hyperpolarization of DA^{VT}A neurons by GBR-activated I_{Kir3} and for activation of I_h (Williams & Mitchell, 2008). Indeed, somatic voltage clamp did not prevent baclofen-induced hyperpolarization of proximal dendrites in dual patch clamp experiments (Figure 2 – figure supplement 1A and B). Dendritic hyperpolarization increased linearly with the distance from the soma (Figure 2 – figure supplement 1C), in line with a decreasing influence of the somatic voltage clamp. The I_{bac} exhibited a strong attenuation without a corresponding increase in Ri (Figure 2 – figure supplement 1B), indicating that

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dendritic hyperpolarization due to incomplete voltage clamp activates dendritic I_h , which opposes the I_{Kir3} . Of note, due to the influence of the somatic voltage clamp, I_{bac} -induced hyperpolarization in the proximal dendrites was slow with maximal hyperpolarization only observed after approximately a minute (Figure 2 – figure supplement 1B).

Blocking I_h with zatebradine in somatic current-clamp recordings resulted in a significantly larger baclofen-induced maximal hyperpolarization (control: -75.2 ± 0.9 mV, $n = 10$ neurons; zatebradine: -85.6 ± 1.4 mV, $n = 6$ neurons; mean $\pm$ s.e.m. $p < 0.001$, unpaired Welch's t-test) (Figure 3A-C). The conductance at maximal hyperpolarization (g_{max}) was significantly reduced in the presence of zatebradine, revealing that g_{HCN} contributes to the conductance induced by GBR activation (control: 15.6 ± 1.7 nS, zatebradine: 5.2 ± 1.6 nS; $p < 0.001$, unpaired Welch's t-test). Zatebradine also significantly reduced the conductance at steady state (g_{steady}) (Figure 3D), showing that HCN channels largely remain open during GBR activation by baclofen.

The activation kinetics of HCN channels reported in the literature are approximately 100 ms (Biel et al., 2009; Kaupp & Seifert, 2001). Therefore, voltage-dependent activation of I_h appears to be too fast to account for the attenuation of the I_{bac} in DA^{VTa} neuron ($\tau = 156.4 \pm 19.2$ sec, mean $\pm$ s.e.m. $n = 9$ neurons, calculated for the 5 minutes following I_{max} ; Figure 2B). We therefore determined I_h activation kinetics in DA^{VTa} neurons by injecting rectangular voltage commands from -60 (the holding potential in voltage-clamp experiments) to -75 mV (the maximal hyperpolarization induced by baclofen in DA^{VTa} neuron) (Figure 3E). Fitting a double exponential function to the I_h yielded time constants in the order of tens of seconds ($\tau_1 = 9.5 \pm 2.1$ and $\tau_2 = 58.0 \pm 7.6$ sec, mean $\pm$ s.e.m. $n = 8$ neurons, Figure 3F). The non-instantaneous hyperpolarization induced by diffusion of baclofen into the brain slice and the somatic voltage-clamp opposing hyperpolarization will further slow I_h activation kinetics. Therefore, the kinetics of I_h

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1 activation in DA^{VTa} neurons under our experimental conditions are compatible with the
2 kinetics of I_{bac} attenuation.

3 We further addressed whether GBRs allosterically modulate HCN channels in
4 DA^{VTa} neurons in the absence of Kir3 channel activation. In the presence of the Kir
5 channel-blocker Ba²⁺ (Alagem, Dvir, & Reuveny, 2001), baclofen did not significantly alter
6 the voltage-sensitivity of I_h currents (Figure 3 – figure supplement 1A). In addition,
7 activation of GBRs with baclofen did not induce any conformational changes between
8 GBRs and HCN2 in bioluminescence resonance energy transfer (BRET) experiments in
9 transfected HEK293 cells (Figure 3 – figure supplement 1B). In line with a recruitment of
10 HCN channels to GBRs by KCTD16, we observed saturating BRET between Rluc-GB1b
11 and YFP-HCN2 in the presence but not in the absence of KCTD16 (Figure 3 – figure
12 supplement 1B). Altogether, these data indicate that GBRs do not allosterically influence
13 HCN channel activity. Therefore, exclusively hyperpolarization-dependent activation of
14 HCN channels accounts for the observed attenuation of GBR-mediated inhibition.
15 Dissociation of HCN channels from GBR-complexes in the absence of KCTD16 impairs
16 this regulatory mechanism, indicating that it relies on the GBR-dependent subcellular
17 localization of HCN channels in the dendrites.

18

19 **Increased inhibition of *KCTD16*^{-/-} DA^{VTa} neurons after optogenetic activation of** 20 **GABA^{VTa} neurons**

21 Inhibitory inputs onto DA^{VTa} neurons can be optogenetically activated in mice expressing
22 ChR2 under the VGAT promoter (ChR2^{VGAT}). Illumination of ChR2^{VGAT} horizontal midbrain
23 slices with blue light via the microscope objective induced intense firing activity in GABA^{VTa}
24 neurons (Figure 4A) and evoked IPSPs in DA^{VTa} neurons (Figure 4B). In WT::ChR2^{VGAT}

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mice, these IPSPs were followed by a rebound depolarization (Figure 4B) that has been attributed to I_h activation (Biel et al., 2009). Pharmacological blockade of HCN channels with zatebradine prevented rebound depolarization and prolonged the duration of IPSPs ($p < 0.01$, Figure 4B and C). Similarly, DA^{VTa} neurons from *KCTD16*^{-/-}::ChR2^{VGAT} mice exhibited longer IPSPs compared to DA^{VTa} neurons from WT::ChR2^{VGAT} mice ($p < 0.05$, Figure 4B and C) and rebound depolarization was observed less frequently (in 2 out of 8 neurons). IPSP amplitudes were similar in all groups (Figure 4B and C).

To study whether KCTD16 regulates DA^{VTa} neuron firing *in vivo*, we performed optogenetic experiments in anesthetized ChR2^{VGAT} mice (Figure 5A). As previously observed (Tan et al., 2012), optogenetic activation of GABA^{VTa} neurons (Figure 5B) resulted in a time-locked decrease of DA^{VTa} neuron firing (Figure 5C). The inhibition of DA^{VTa} neurons was more pronounced in *KCTD16*^{-/-}::ChR2^{VGAT} mice compared to WT::ChR2^{VGAT} mice, as indicated by a reduced firing rate ($p < 0.05$, Figure 5D) and a longer latency to fire an action potential after the onset of inhibition ($p < 0.05$, Figure 5E). GABA^{VTa} and DA^{VTa} neurons were identified based on validated *in vivo* electrophysiological properties, such as action potential width and the basal firing frequency (Ungless & Grace, 2012), which were similar between genotypes (Figure 5F and G). *In vivo* recordings thus corroborate that GBR/HCN complexes serve to limit synaptic inhibition of DA^{VTa} neurons.

***KCTD16*^{-/-} mice exhibit increased anxiety-like behavior**

A decrease in DA^{VTa} neuron excitability was shown to result in an anxiety-like behavior in mice (Zweifel et al., 2011). The increased inhibition of DA^{VTa} neuron firing observed in *KCTD16*^{-/-} mice in response to GABA^{VTa} activation may therefore increase anxiety-like

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behaviors in stressful situations. We used the light/dark chamber test to analyze whether *KCTD16*^{-/-} mice exposed to a mild naturalistic stressor exhibit an exacerbated anxiety-like behavior. This test relies on the innate aversion of mice to brightly illuminated areas. However, when presented in a novel environment, mice also have a tendency to explore. This conflicting situation leads to anxiety-like behaviors (Takao & Miyakawa, 2006). In the light/dark chamber test, *KCTD16*^{-/-} mice showed increased anxiety-like behavior, as indicated by fewer entries ($p < 0.05$) and less time spent in the light chamber ($p < 0.01$) compared to WT littermate mice (Figure 6A and C). *KCTD16*^{-/-} mice also covered less distance than WT mice ($p < 0.01$, Figure 6B). This was not due to a motor impairment, as both genotypes exhibited similar locomotor behavior in an open arena, when spontaneous locomotion was decomposed into bouts (number, length, speed and duration) to test for possible motor differences (Figure 6 – figure supplement 1).

To confirm exaggerated anxiety in response to stressors, we tested mice in a mild version of the classical fear-conditioning paradigm. The test consisted of one training session with five auditory stimuli (tones) paired with an aversive foot shock. 24 hours later, in a novel context (different from the training context), freezing behavior in response to the novel context and to the tones in the absence of foot shock was assessed (Figure 6D). At baseline, both genotypes showed a similar low level of freezing in the test chamber (Figure 6E). However, after the first tone and throughout the entire session, *KCTD16*^{-/-} mice displayed significantly more freezing episodes ($p < 0.01$) and spent more time freezing ($p < 0.05$) than WT littermate mice (Figure 6F). These data indicate an increased anxiety-like behavior in response to the novel context and the predictive tone.

In further experiments we used the elevated zero and plus mazes as non-invasive tests for anxiety. *KCTD16*^{-/-} and WT littermate mice spent significantly more time ($p < 0.001$, Figure 6G, H, K and L) and travelled larger distances (Figure 6 – figure supplement

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2A and C) in the closed arms compared to the open arms of the mazes. *KCTD16*^{-/-} mice explored the open arms significantly less than WT mice, as shown by a decreased dwell time ($p < 0.05$, Figure 6I and M), decreased number of entries ($p < 0.05$, Figure 6J and N) and lower travelling speed in the open arms (Figure 6 – figure supplement 2B and D). These data corroborate that *KCTD16*^{-/-} mice exhibit increased anxiety-like behaviors in stress situations.

CRISPR/Cas9-mediated *KCTD16* ablation in DA^{VTA} neurons promotes anxiety-like behaviors

AAV-mediated CRISPR/Cas9 technology has recently been established for cell-type specific gene editing in the VTA (DeBaker, Marron Fernandez de Velasco, McCall, Lee, & Wickman, 2021). We applied this approach to ablate *KCTD16* specifically in DA^{VTA} neurons of adult mice (Figure 7A and B). For efficient targeting we designed two single guide RNAs (sgRNAs) binding in the *KCTD16* open reading frame upstream of the H2 domain mediating interaction with HCN channels. Following *in vitro* validation (Figure 7 – figure supplement 1A), the sgRNAs were cloned individually into AAV vectors expressing mCherry as a fluorescent reporter and subsequently packed into AAV serotype 8 capsids (AAV8-sg*KCTD16*-mCherry). A 1:1 mixture of both AAV8-sg*KCTD16*-mCherry was then injected into the VTA of Cre-dependent LSL-Cas9/EGFP knock-in mice heterozygous for the DA neuron-directed driver DAT-Cre (LSL-Cas9/EGFP::DAT-Cre) to produce mice that are deficient for *KCTD16* specifically in DA^{VTA} neurons (DA^{VTA}-*KCTD16*^{-/-} mice). As a control, the same AAV8 preparation was injected into LSL-Cas9/EGFP knock-in mice lacking the DAT-Cre driver (Figure 7A). 6 weeks after AAV8 injection, we tested mice for their anxiety levels in the elevated zero and elevated plus mazes (Figure 7C-H). DA^{VTA}-*KCTD16*^{-/-} mice spent significantly less time in the open arms of the mazes compared to

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control mice ($p < 0.01$ and $p < 0.05$, Figure 7D and G), similar to pan *KCTD16*^{-/-} mice. Post hoc analysis revealed that 55.7% of TH positive neurons in the VTA of DA^{VTA}-*KCTD16*^{-/-} mice expressed Cas9/EGFP and where transduced with AAV8-sg*KCTD16*-mCherry (Figure 7 – figure supplement 1C and D). Amplicon deep sequencing of genomic DNA extracted from the VTA confirmed *in vivo* editing of *KCTD16* in DA^{VTA}-*KCTD16*^{-/-} mice, revealing insertion and deletions (indel mutations) near the predicted Cas9 cleavage sites (Figure 7I). Importantly, more than 80% of the editing events resulted in out-of-frame mutations. These data confirm that selective ablation of *KCTD16* in DA^{VTA} cells is sufficient to exacerbate anxiety-like behaviors in stressful conditions.

Intra-VTA inhibition of HCN channels induces an anxiety-like phenotype

Our data support that the increased anxiety-like behavior of *KCTD16*^{-/-} mice in stress situations relates to a reduced activation of HCN channels in DA^{VTA} neurons. We therefore tested whether zatebradine exhibits anxiogenic properties when infused into the VTA. One group of WT mice was first infused with saline and 24 hrs later with zatebradine. The mice were tested 30 min after each infusion for their anxiety levels in the elevated zero maze and subsequently in the elevated plus maze (Figure 8A). A second group of mice first received zatebradine and 24 hrs later saline before testing in the mazes (Figure 8A). Because the behavioral data in the presence of saline or zatebradine of the two groups of mice were similar, we pooled the data for analysis. After infusion with zatebradine, all mice spent less time in the open arms of the mazes ($p < 0.05$, Figure 8C-F), thus mimicking the anxiety-like behavior of *KCTD16*^{-/-} mice in the mazes. The infusion site was validated by infusion of green fluorescent beads and staining for TH in brain slices (Figure 8B). Pharmacological data therefore corroborate that lack of HCN channel activity in DA^{VTA} neurons increases anxiety-like behavior in the elevated mazes.

1

2 Discussion

3 A well-known postsynaptic function of GBRs is the activation of Kir3 channels, which
 4 underlie the late phase of IPSPs and prolong the time window of synaptic inhibition
 5 (Gassmann & Bettler, 2012; Luscher & Slesinger, 2010). To prevent excessive inhibitory
 6 influences on neuronal activity, the gating of Kir3 channels by GBRs must be under
 7 stringent temporal control. Endocytosis of GBRs, which typically terminates second
 8 messenger signaling, is too slow to terminate Kir3 activity in a timely manner (Gassmann
 9 & Bettler, 2012). GBRs therefore use RGS and KCTD12 proteins, which directly interfere
 10 with G protein activity, to rapidly terminate signaling to Kir3 channels (Mutneja, Berton,
 11 Suen, Luscher, & Slesinger, 2005; Raveh, Turecek, & Bettler, 2015; Schwenk et al., 2010;
 12 Turecek et al., 2014; Xie et al., 2010; Zheng et al., 2019). Here, we describe a novel
 13 mechanism that allows for rapid inactivation of GBR-activated I_{Kir3} . This mechanism relies
 14 on association of HCN channels with GBRs, which facilitates activation of HCN channels
 15 through hyperpolarization by I_{Kir3} . In contrast to rapid termination of I_{Kir3} by RGS and
 16 KCTD12 acting at the G protein, this novel mode of I_{Kir3} inactivation relies on activation of
 17 I_h that counteract I_{Kir3} . GBR-dependent I_h activation provides a straightforward explanation
 18 for the more pronounced I_{bac} desensitization observed in DA^{VTa} neurons compared to
 19 neighboring GABA^{VTa} neurons, as the latter lack I_h (Cruz et al., 2004).

20 Pharmacological blockade of HCN channels or disruption of the GBR/HCN
 21 complex by genetic removal of KCTD16 prolongs optogenetically induced IPSPs in DA^{VTa}
 22 neurons of acute slices, showing that I_h impede the I_{Kir3} -dependent late phase of IPSPs.
 23 Likewise, optogenetic activation of GABA^{VTa} neurons *in vivo* produces a more pronounced
 24 and prolonged inhibition of DA^{VTa} neuron firing in *KCTD16*^{-/-} mice than in WT mice, further
 25 supporting that GBR/HCN complex formation is physiologically relevant and serves to limit

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inhibition. Our data are consistent with GBRs capturing HCN channels at postsynaptic sites where they are gated by hyperpolarizing IPSPs. The activation of HCN channels is not dependent on a direct modulation or allosteric regulation by GBRs and simply relies on the spatial co-localization with hyperpolarizing conductances. HCN channels have a propensity to traffic to the distal dendrites (Lorincz, Notomi, Tamas, Shigemoto, & Nusser, 2002). Translocation of HCN channels to distal dendrites therefore likely explains why HCN channels fail to regulate GBR-mediated inhibition in *KCTD16*^{-/-} mice. *I_h* activation explains the small contribution of GBR-activated *I_{Kir3}* to IPSPs in DA^{VTa} neurons during inhibitory activity, for example during reward omission or the presentation of aversive stimuli (Bromberg-Martin, Matsumoto, & Hikosaka, 2010; Cohen, Haesler, Vong, Lowell, & Uchida, 2012; Schultz, 2013; Tan et al., 2012). GBR-dependent *I_h* activation may not be limited to DA^{VTa} neurons and is expected to play a role in shortening the duration of IPSPs in other neurons as well. It is assumed that GBR-mediated slow IPSPs are mostly generated during periods of intense GABAergic activity, when GABA concentrations are high enough to reach extrasynaptic GBRs (Gassmann & Bettler, 2012). Our data imply that postsynaptic GBRs may also be activated during less intense GABAergic activity, but that *I_h* occlude GBR-mediated IPSPs.

Our experiments show that lack of *I_h* activation in DA^{VTa} neurons of *KCTD16*^{-/-} mice correlates with an increased anxiety-like behavior in response to stressful situations and aversive conditioning. This finding is in line with the reported hyper-reactivity of *KCTD16*^{-/-} mice in response to aversive stimuli (Cathomas et al., 2017). Spontaneous firing frequencies and action potential waveforms were similar in *KCTD16*^{-/-} and WT DA^{VTa} neurons, while optogenetic inhibition of DA^{VTa} neurons by GABA^{VTa} neurons was increased in *KCTD16*^{-/-} mice. Since a reduced excitability of DA^{VTa} neurons is anxiogenic (Zweifel et al., 2011), we expected that increased DA^{VTa} neuron inhibition in response to

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stressors underlies the increased anxiety-like behavior of *KCTD16*^{-/-} mice. Indeed, cell-type specific ablation of *KCTD16* in DA^{VTA} neurons is sufficient to promote anxiety phenotypes, supporting that the GBR/HCN interaction in DA^{VTA} neurons is behaviorally relevant. This is corroborated by intra-VTA blockade of *I_h* in WT mice, which is expected to increase DA^{VTA} neuron inhibition in response to stressors and replicates the anxiety phenotype of *KCTD16*^{-/-} mice.

DA^{VTA} neurons can be separated into anatomically and functionally distinct subpopulations that interact with specific neural circuits associated with anxiety and reward-seeking behaviors (Morales & Margolis, 2017). The GBR/HCN regulatory mechanism described here will be operational in “conventional” DA^{VTA} neurons characterized by large *I_h* current densities but not in DA^{VTA} neurons lacking *I_h* currents (Lammel, Lim, & Malenka, 2014). *I_h* expressing DA^{VTA} neurons project primarily to the nucleus accumbens (NAc), while DA^{VTA} neurons lacking *I_h* additionally project to the medial prefrontal cortex and amygdala (Lammel et al., 2008). Recent data show that aversive stimuli inhibit a specific subpopulation of NAc-projecting DA^{VTA} neurons (de Jong et al., 2019). Thus, we speculate that primarily these neurons use the GBR/HCN complex to limit GBR-mediated inhibition and to prevent anxiety-like behaviors. In this context, it is important to note that these DA^{VTA} neurons are directly inhibited by GABAergic projections from the nucleus NAc via the activation of GBRs (Edwards et al., 2017; Yang et al., 2018). However, DA^{VTA} neurons are also under inhibition from GABA^{VTA} neurons that receive inputs from a great number of brain areas associated with anxiety (Jennings et al., 2013; Morales & Margolis, 2017; Tovote et al., 2015). Thus, it is possible that several projections activate GABA^{VTA} neurons in our behavioral experiments.

Increasing *I_h* activity or stabilizing GBR/HCN interaction in DA^{VTA} neurons may therefore represent a novel concept for the treatment of phobia, panic attacks, post-

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1 traumatic stress disorders or other mental health diseases characterized by pathological
 2 responses to negative stimuli. It is interesting to note that lamotrigine, a clinically used
 3 mood-stabilizer, enhances I_h in DA^{VTA} neurons, leading to a resilient phenotype in a mouse
 4 model of depression (Friedman et al., 2014).

5

1 **Materials and Methods**

2 ***Mice***

3 Experiments with mice (*KCTD12*^{-/-} (Turecek et al., 2014), *KCTD16*^{-/-} (Turecek et al., 2014),
4 VGAT-ChR2-YFP (JAX#014548 (Zhao et al., 2011)), LSL-Cas9/EGFP (JAX#024857
5 (Platt et al., 2014)), DAT-Cre (Turiault et al., 2007) were approved by the veterinary office
6 of the canton of Basel-Stadt, Switzerland. No blinding was done.

8 ***Biochemistry***

9 Brains were washed in ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8
10 mM KH₂PO₄) and placed at 100 mg/ml in ice-cold homogenization media containing:
11 320 mM sucrose, 4 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA and protease
12 inhibitors (Roche Diagnostics). For homogenization, we used a glass-teflon homogenizer
13 (mouse brain) or a microcentrifuge tube pestle grinder (VTA brain slice) with 30 passes
14 on ice. The homogenized material was cleared by centrifugation at 10³ x g (4°C, 15 min),
15 the membrane-enriched fraction isolated by ultracentrifugation at 4.8 x 10⁴ x g (4°C,
16 45 min) and solubilized at 2 mg protein/ml for 3 hrs at 4°C in NETN-based solubilization
17 buffer containing: 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris/HCl (pH
18 7.4) and protease inhibitors. The solubilized fraction was cleared by ultracentrifugation at
19 10⁵ x g (4°C, 45 min) and directly used for Western blots (input 100 µg of total membrane
20 protein) or precleared for 1 hr using 30 µl dry volume of a 1:1 mixture of protein-A and
21 protein-G agarose (Roche Diagnostics). Solubilized membranes (3 mg of total membrane
22 protein) of WT and *KCTD16*^{-/-} mouse brains were incubated overnight at 4°C with 2 µl of
23 affinity-purified guinea pig anti-KCTD16 antibody, followed by 40 min of incubation with 10

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1 μ l of a 1:1 mixture of protein-A/-G agarose. After repeated washing, bound proteins were
2 eluted with Laemmli buffer and resolved using standard SDS-PAGE.

3 HEK293 cells were transfected at 80–90% confluency using PEI transfection reagent
4 (Sigma) with 2 mg/ml PEI per mg of DNA. The total amount of DNA in the transfections
5 was kept constant by supplementing with empty pCI plasmid (Promega). Plasmids
6 encodings HCN1-4, Flag-, Myc- and HA-tagged GB1 and GB2 as well as Flag- and Myc-
7 tagged KCTDs were as described (Schwenk et al., 2010; Seddik et al., 2012; Zolles et al.,
8 2009). All mutant constructs were generated using overlap extension PCR. To generate
9 KCTD16 Δ H2 a stop codon was introduced after E²⁷⁹. To generate KCTD16T1 a stop
10 codon was introduced after L¹¹⁸. To generate KCTD16 Δ T1 the region between M¹ and V¹¹⁹
11 was excised. To generate KCTD16H1 a stop codon was introduced in KCTD16 Δ T1 after
12 E²⁷⁹; in KCTD16H2 the region between M¹ and P²⁸⁰ was excised. To generate HCN2 aa1
13 – 215 a stop codon was introduced after D²¹⁵. HEK293 cells were harvested 48 hrs after
14 transfection in NETN lysis buffer (with protease inhibitors added) by rotating them for 10
15 min at 4°C. Lysates were cleared by centrifugation at 10³ x g for 20 min at 4°C and directly
16 used for Western blot analysis (input lanes) or precleared as described above and
17 immunoprecipitated for 3 hrs at 4°C with the following antibodies: mouse anti-Myc (9E10,
18 Santa Cruz Biotechnology, 1 μ g) or mouse anti-Flag (M2, Sigma, 1 μ g), followed by 40
19 min of incubation with 10 μ l of a 1:1 mixture of protein-A/-G agarose. Lysates and
20 immunoprecipitates were resolved using SDS-PAGE and probed with the primary
21 antibodies rabbit anti-KCTD16 (RRID: AB_2631050, 1:2500), rabbit anti-GB1 (AB26, rat
22 aa 857–960, 1:3500), rabbit anti-GB2 (C44A4, #4819, Cell Signaling Technology, RRID:
23 AB_2108339, 1:1500), rabbit anti-Myc (C3956, Sigma, RRID: AB_439680, 1:2500), rabbit
24 anti-Flag (F7425, Sigma, RRID: AB_439687, 1:2500), rabbit anti-HA (RHGT-45A, ICL
25 Lab, RRID: AB_2861135, 1:100), guinea pig anti-HCN1 (AGP-203, Alomone labs,

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1 1:1000), rabbit anti-HCN2 (AGC-030, Alomone labs, 1:1000), rabbit anti-HCN3 (AGC-057,
2 Alomone labs, 1:1000), guinea pig anti-HCN4 (AGP-004, Alomone labs, 1:1000), mouse
3 anti- β -tubulin III (T8660, Sigma, 1:1000), rabbit anti-TH (ab112, Abcam, 1:1000) and
4 peroxidase-coupled secondary antibodies donkey anti-guinea pig (A7289, Sigma,
5 1:10000), donkey anti-rabbit (NA934, GE Healthcare, 1:10000) and sheep anti-mouse
6 (NA931, GE Healthcare, 1:10000). The guinea-pig anti-KCTD16 antibody was raised
7 against a synthetic peptide derived from mouse KCTD16 (aa7-23) (Metz et al, 2011). The
8 Bradford assay was used to ensure equal protein loading. Antibody incubation was in 5%
9 nonfat dry milk in PBS containing 0.1% Tween-20. The chemiluminescence detection kit
10 SuperSignal West (Thermo Scientific) was used for visualization of proteins on Western
11 blots.

12 13 ***In vitro electrophysiology***

14 Mice (P18-P22) were deeply anesthetized with isoflurane prior decapitation and brain
15 isolation. Acute horizontal brain slices (250 μ m thick) from the midbrain were cut with a
16 vibrating microslicer (Leica VT1200S) in ice-cold oxygenated artificial cerebral spinal fluid
17 (ACSF) containing: NaCl (119 mM), 26.2 NaHCO₃ (26.2 mM), KCl (2.5 mM), NaH₂PO₄ (1
18 mM), MgCl₂ (1.3 mM), CaCl₂ (2.5 mM) and glucose (11 mM), pH 7.4, constantly bubbled
19 with 95% O₂ and 5% CO₂. Slices were maintained at 37°C in ACSF for 15–120 min and
20 continuously perfused with ACSF at 35.0 $\pm$ 2.0°C throughout the experiments. Whole-cell
21 recordings from somata and/or from dendrites were obtained using oblique illumination on
22 a Olympus BX51WI equipped with a 60x objective (Olympus LUMPplanFI, NA 0.9).
23 Somatic (4-6 M Ω) and dendritic (10-20 M Ω) recording pipettes were filled with an
24 intracellular solution containing: K-gluconate (140 mM), NaCl (4 mM), MgCl₂ (2 mM),
25 EGTA (1.1 mM), HEPES (5 mM), Na₂ATP (2 mM), Na₂-phosphocreatine (5 mM), Na₃GTP

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(0.6 mM), 0.2% biocytin, pH 7.2 (with KOH); 312.3 mOsm. For double patch-clamp recordings, Alexa 594 (10 μ M) was added to the intracellular solution. Drugs (TOCRIS Bioscience) were freshly prepared the day of the experiment. Bipolar electrodes (theta glass with a tip diameter of 4-8 μ m, located at ~50 μ m laterally from distal dendrites). Electrophysiological recordings were obtained using a Multiclamp 700B amplifier and digitized at 10 kHz. For voltage-clamp recordings the series resistance was 15-20 M Ω and uncompensated. In experiments performed with series resistance compensation (40-50%, band width 3.5 kHz) and at holding potentials more depolarized (-45 mV in the presence of tetrodotoxine) than the threshold for I_h voltage activation, the I_{bac} desensitized less ($18.0 \pm 3.5\%$ vs $46.0 \pm 4.0\%$ at V_{hold} -60 mV with uncompensated series resistance and -10 mV test pulses), in keeping with a space-clamp deficit being responsible for I_h activation. I_{bac} desensitization and Ri recovery values were calculated as the percentage of $\Delta I_{steady}/\Delta I_{max}$ and $\Delta Ri_{steady}/\Delta Ri_{max}$, Δ denotes the amplitudes (max and steady) relative to baseline (hold): $\Delta I_{max} = I_{max} - I_{hold}$, $\Delta I_{steady} = I_{steady} - I_{hold}$, $\Delta Ri_{max} = Ri_{max} - Ri_{hold}$, $\Delta Ri_{steady} = Ri_{steady} - Ri_{hold}$. Optogenetic stimulation of brain slices was induced with blue light applied via the microscope objective. Blue light was generated by means of an excitation filter (460 - 495 nm; Olympus filter cube U-MWIBA3) mounted in the light path originating from the mercury lamp-house.

In vivo electrophysiology

Extracellular single unit recordings were performed as described (Tan et al., 2012). In brief, mice were anesthetized with isoflurane (induction, 5%; maintenance, 0.5 to 1.5%) and placed into a stereotaxic frame (World Precision Instruments). The body temperature was maintained at 36° C using a heating pad. A cranial window was opened above the VTA (AP -3.4 to 3.8, ML 0.3 to 1.0) and the dura retracted. Glass recording pipets (GC120F-10

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Harvard Apparatus) were back filled with oxygenated ACSF (in mM: NaCl 119, NaHCO₃ 26.2, KCl 2.5, MgCl₂ (6H₂O) 1.3, NaHPO₄ 1, CaCl₂ 2.5, Glucose 11, pH 7.3) and contained an optic fiber coupled to a blue laser (10-20 mW at the tip). The spontaneous firing rate was continuously recorded while a blue light pulse of 40 Hz was applied for 1 sec every 10 sec. DA^{VTa} neurons have been reported to display larger action potential width (>1.1 msec from the start to the through) and a lower spontaneous average firing rate (<10 Hz) than neighboring GABA^{VTa} neurons(Ungless & Grace, 2012). Recordings were analyzed using MATLAB (Mathworks).

AAV-mediated CRISPR/Cas9 editing in vivo

CRISPR/Cas9 target sites in the Mus musculus (mm10/GRCm3) *KCTD16* were identified using the ChopChop online tool (<http://chopchop.cbu.uib.no/>). Two target sequences upstream of the KCTD16 H2 domain with an on-target efficacy score >0.6 were pre-validated with *in vitro* assembled ribonucleoprotein complexes using Alt-RTM sgRNAs (integrated DNA Technologies), *Streptococcus pyogenes* Cas9 nuclease V3 (Provider?) and PCR-amplified genomic *KCTD16* DNA as a double-stranded target. The two target sequences sg-*KCTD16*-1 (5'-GCTTTGCTTGACATCCTCGG-3') and sg-*KCTD16*-2 (5'-TTCCGCAAACAAAATCCGG-3') were then cloned into the AAV-U6-sgRNA-hSyn-mCherry plasmid (RRID:Addgene_87916) using the SapI site 5' of the gRNA scaffold. For optimal RNA expression from the U6 promoter a guanine nucleotide was added to the 5' end of the sg*KCTD16*-2 sequence. AAV8 particles were produced in HEK293T cells using the triple transfection strategy and purified using IDX gradient ultracentrifugation to remove impurities and empty capsids (Vigene Biosciences large-scale AAV packaging service). 500 nl of a 1:1 mixture of AAV8-sg*KCTD16*-1 (5.6x10¹³ genome copies (GC)/ml) and AAV8-sg*KCTD16*-2 (3.7x10¹³ GC/ml) were injected into the VTA of LSL-

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Cas9/EGFP::DAT-Cre (Fig. S6b) and control Cas9/EGFP mice using a stereotaxic apparatus (from bregma, ML:0.8, AP:3.4, DV:4.4, Paxinos). AAV8-transduction of VTA^{DA} neurons was assessed by confocal imaging of the AAV-reporter mCherry (expressed under the hSyn promoter) in VTA slices immunostained for TH as described previously (Rizzi, Li, Hogrefe, & Tan, 2021). Cre-mediated activation of Cas9/EGFP in DA neurons was assessed by imaging EGFP. CRISPR/Cas9 editing *in vivo* was validated by next-generation sequencing of a 375 bp PCR amplicon containing the two sgRNA target sites. The first-step PCR was performed on genomic DNA extracted from the VTA with the locus-specific forward (5'-TCACTTTCCAGAAAGAGGAAGG-3') and reverse (5'-GTGTATCTTTCTGGAGCTCGGT-3') primers containing the following 5' tails (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG -3') and (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'), respectively, required for binding of the second-step PCR primers containing barcode and Illumina adapter sequences. PCR amplifications were performed with Phusion High-Fidelity DNA Polymerase (Thermo Scientific) according to the manufacturers protocol. 2 x 250 bp reads were generated on the Illumina MiSeq/NextSeq Platform (Microsynth). Analysis of genome editing outcomes from deep sequencing data was performed using CRISPResso2 (Clement et al., 2019).

Behavioral tests

To assess spontaneous locomotion in an open field, mice were free to explore a rectangular (30 x 45 cm) arena for a total of 6 min. Automated software (ANY-maze, Stoelting Co) was used to track movements. Data were post hoc analyzed using MATLAB, as described (Ruder, Takeoka, & Arber, 2016). In the light/dark chamber test the apparatus (20 x 60 cm) was divided into a dark and a brightly illuminated chamber. Mice

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were allowed to explore and move freely between the chambers for a total of 15 min. The apparatus was elevated 60 cm from the table and mice video tracked using ANY-Maze software. The dark chamber was lit with red LEDs to allow video tracking. The reported parameters were automatically calculated by the software. In the elevated zero maze mice were free to explore for a total of 6 min an O-shaped maze (5 cm width, 25 cm radius) divided into two open arms and two closed arms (15 cm height). The maze was elevated 60 cm from the table. The bottom of the floor of each arm was coated with a semi-transparent paint that allowed video tracking of the mice. In the elevated plus maze mice were placed for 6 min in a plus-shaped maze consisting of two open arms (7.5 width x 30 cm length) and two enclosed arms (7.5 cm width x 30 cm length x 30 cm height) extending from a center platform (7.5 cm width x 7.5 cm length). The maze was elevated 60 cm from the table. ANY-Maze software was used to track movements. For pharmacological inhibition of HCN channels *in vivo*, four WT mice were infused with 500 µl of saline and 24 hrs later with zatebradine (10 µM) via a cannula implanted above the VTA (Plastics One, 5 mm length guide with a 26 gauge, implanted from bregma at AP-3.3, ML-0.8 with a 10° angle, DV-4.1). 30 min after saline or zatebradine infusion mice were tested in the mazes. To avoid that the sequence of the infusions impacts behavioral results, we reversed the order of the infusions such that four additional mice first received zatebradine and 24 hrs later saline. The behavioral results of the eight mice obtained in the presence of zatebradine and saline were pooled. Infusion sites were confirmed on VTA slices after infusion of fluorescent beads. For fear conditioning we used a standard mouse-conditioning chamber (16 x 14 x 12 cm; Med-Associates, Vermont, USA) with the stainless-steel rod floor coupled to a stand-alone aversive stimulator (Med-Associates). The experiment was a simplified version of the classical fear-conditioning task and was divided in two steps with a conditioning session followed by a test session 24 hrs later (10 min each). The conditioning context (17 cm x 26 cm x 17 cm) was patterned with checks

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and diagonal stripes whereas the test context (17 cm x 26 cm x 17 cm) exhibited large black dots. During the conditioning session, mice received 5 auditory stimuli (25 sec, 1 Hz pure tone), each paired with a foot shock (2 s, 0.6 mA). On the test session freezing was measured in response to the predictive auditory stimuli in the absence of foot shocks. Freezing was continuously assessed with ANY-Maze.

BRET studies

BRET experiments reporting allosteric changes between Rluc-GB1b and YFP-HCN2 in transfected CHO cells were conducted and analyzed as described (Turecek et al., 2014).

Statistical analysis

All statistical tests and graph preparations were done using Prism (GraphPad, San Diego, CA), and figure assembly was done in Photoshop (Adobe, San Jose, CA). Sample size in all experiments was based on those of similar experiments in previous studies. Samples were randomly allocated to the experimental groups unless they were predefined by the genotype. Masking was not performed for any experiment. Data are presented as mean $\pm$ standard error of the mean (s.e.m.) unless indicated otherwise. To determine whether to use parametric or non-parametric statistical tests, Shapiro-Wilk test for normality of residuals was applied. The statistical tests adopted are described in the figure legends. All p values reported are two-tailed, and a p value of < 0.05 was considered statistically significant.

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1 **Data availability**

2 All data needed to evaluate the conclusions in this paper are present in the main
3 manuscript and/or the Supplementary Materials. Numerical data that are represented in
4 graphs are also provided as source data excel files.

5

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Figures legends

Figure 1. KCTD16 mediates GBR/HCN channel complex formation. (A) KCTD16 co-immunoprecipitated HCN2 from brain membranes of WT mice. Immunoprecipitation with *KCTD16*^{-/-} brain membranes confirmed specificity of the anti-KCTD16 antibody. (B) Immunoblot showing that GB1a/b and GB2 subunits co-immunoprecipitated HCN2 in the presence of KCTD16, but not in the presence of KCTD8 and -12, from membrane preparations of transfected HEK293 cells. (C) GBRs did not co-immunoprecipitate HCN2 when mutation of Y902 in GB2 prevented binding of KCTD16 to GB2. (D) The H2 domain of KCTD16 co-immunoprecipitated the HCN2 subunit. Mutations: KCTD16ΔH2 lacks the H2 domain; KCTD16ΔT1 lacks the T1 domain; KCTD16T1, KCTD16H1, KCTD16H2 express the T1, H1 or H2 domains in isolation. (E) KCTD16 co-immunoprecipitated the N-terminal peptide containing amino acid residues 1-215 of HCN2. (F) KCTD16 links the N-terminal domain of HCN2 to the C-terminal domain of GB2. GB2 binds to the T1 domain of KCTD16 (Gassmann & Bettler, 2012). HCN2 binds to the H2 domain of KCTD16. Source data for all Western blots are provided in Figure 1 – source data 1.

Figure 2. Pharmacological blockade of *I_h* or lack of KCTD16 reduces *I_{bac}* desensitization in DA^{VT}A neurons. (A) Hyperpolarizing steps from -50 to -120 mV applied via the somatic patch pipette evoked *I_h* in DA^{VT}A neurons. Digital subtraction of currents recorded in the absence (control) and presence of the *I_h* blocker zatebradine (zb, 30 μM) revealed the *I_h*. **B** Top: *I_{bac}* traces in DA^{VT}A neurons induced by bath application of baclofen (300 μM). The GBR antagonist CGP54626 (CGP, 4 μM) fully blocked *I_{bac}*. From the holding current (*I_{hold}*), the *I_{bac}* reached an initial peak (*I_{max}*) and desensitized to a steady-state (*I_{steady}*). Recordings are from WT neurons (n = 9, black), WT neurons in the presence

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1 zatebradine (30 μ M, n = 6, blue), *KCTD16*^{-/-} neurons (n = 8, red) and *KCTD12*^{-/-} neurons
2 (n = 8, orange). Traces with similar I_{max} values were selected for comparison. Bottom: Ri
3 time courses of the I_{bac} traces shown above. (C) Micrograph of the WT DA^{VT}A neuron
4 (arrowhead) analyzed in the presence of zatebradine shown in (a), labeled with biocytin
5 (green), stained for TH (red) and a cell nuclei marker (DAPI, blue). The merged image
6 confirms that the recorded neuron is a DA^{VT}A neuron. (D) Summary bar graph of I_{bac}
7 desensitization and Ri recovery during desensitization. WT neurons incubated with
8 zatebradine and *KCTD16*^{-/-} neurons exhibited reduced I_{bac} desensitization. The Ri
9 recovery during desensitization was similar for all groups. **p < 0.01; ***p < 0.001; ns, not
10 significant; Kruskal-Wallis test followed by Dunn's multiple comparison test (compared to
11 WT). Data are presented as mean $\pm$ s.e.m., n indicates the number of neurons analyzed.
12 Source data for all plots are provided in Figure 2 – source data 1.

13
14 **Figure 3. I_h decreases baclofen-induced hyperpolarization of DA^{VT}A neurons. (A)**
15 Representative traces of the baclofen (300 μ M) induced-changes in Vm and Ri in the
16 presence of tetrodotoxin (TTX, 1 μ M) in WT DA^{VT}A neurons. Vm values sampled every 5
17 seconds during baclofen application. The Vm hyperpolarized from Vm_{rest} to Vm_{max} and
18 gradually repolarized to Vm_{steady}. Application of CGP54626 (4 μ M) returned the Vm to
19 Vm_{rest}. For each recorded sweep (1 second duration), a hyperpolarizing current step of 10
20 pA and 200 ms duration was injected via the recording pipette to measure the Ri. (B)
21 Recordings from WT DA^{VT}A neurons in the presence of the I_h blocker zatebradine (zb, 30
22 μ M). (C) Baclofen-induced hyperpolarization was significantly increased in the presence
23 of zatebradine (n = 6 neurons) compared to control (n = 10 neurons). (D) Conductance
24 values for individual neurons before ($g_{rest} = 1/Ri_{rest}$) and at steady-state of GBR activity
25 ($g_{steady} = 1/Ri_{steady}$) for each experimental group. Averages of the baclofen-induced

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conductance increase for each group are shown in the bar graph on the right. *** $p < 0.001$; unpaired Welch's t-test. Data are represented as mean $\pm$ s.e.m. (E) Example trace of I_h evoked in DA^{VTA} neurons by a voltage command from -60 to -75 mV during 5 minutes. Fitting the activation phase to a double exponential function is shown in red. The dashed line indicates the base line. (F) Summary box plots (interquartile range and median) of the time constants obtained for I_h activation. Whiskers denote the minimum and maximum values ($n = 8$ neurons). Source data for all plots are provided in Figure 3 – source data 1.

Figure 4. Pharmacological blockade of I_h or lack of KCTD16 prolongs IPSP duration in DA^{VTA} neurons. (A) Exposure of VTA slices from WT::ChR2^{VGAT} mice to blue light induced firing activity in GABA^{VTA} neurons. (B) Top: Examples of averaged IPSPs (5 consecutive sweeps) of WT::ChR2^{VGAT} (black), zatebradine treated (zb, 30 μ M) WT::ChR2^{VGAT} (blue) and *KCTD16*^{-/-}::ChR2^{VGAT} (red) DA^{VTA} neurons evoked by optogenetic stimulation for 1 sec. Spontaneous firing activity in DA^{VTA} neurons was prevented by current injection via the recording pipette to a $V_{m_{rest}}$ of ~ -55 mV. Nonetheless, action potentials were sometimes triggered. The arrow depicts the depolarizing rebound in WT neurons caused by I_h activation. Bottom: Same traces as above scaled to the initial IPSP amplitude. (C) Quantification of IPSP duration and amplitude for each experimental group. Data are represented as mean $\pm$ s.e.m. Neurons analyzed per group: WT, $n = 9$; *KCTD16*^{-/-}, $n = 8$; WT+zb; $n = 6$. * $p < 0.05$, ** $p < 0.01$ (Welch's ANOVA, $F_{2, 7.84} = 32.53$). Source data for all plots are provided in Figure 4 – source data 1.

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Figure 5. *KCTD16*^{-/-} mice show increased inhibition of DA^{VT}A neuron firing in response to optogenetic stimulation of GABA^{VT}A neurons. (A) Single-unit recordings were performed in anesthetized *KCTD16*^{-/-}::ChR2^{VGAT} and WT::ChR2^{VGAT} mice. Optogenetic stimulation was for 1 sec at 40Hz. (B) Example trace, raster plot and action potential waveform of the *in vivo* activity of a WT GABA^{VT}A neuron before, during and after optogenetic stimulation. (C) Optogenetic stimulation of GABAergic inputs to DA^{VT}A neurons induced a stronger inhibition of DA^{VT}A neuron firing in *KCTD16*^{-/-} (red) than in WT (black) mice. (D) The firing rate ratio (firing frequency during the 1 sec light stimulation divided by firing frequency 1 sec before) was significantly lower in DA^{VT}A neurons of *KCTD16*^{-/-} than WT mice (*p < 0.05, unpaired t-test). (E) The latency to fire an action potential (AP) after light-induced inhibition was increased in *KCTD16*^{-/-} mice (*p < 0.05, unpaired t-test). (F,G) The full width at half maximum (FWHM) of action potential (F) and the spontaneous firing rate (G) were not significantly different between *KCTD16*^{-/-} and WT DA^{VT}A neurons but differed from those of GABA^{VT}A neurons. DA^{VT}A neurons: WT, n = 16; *KCTD16*^{-/-}, n = 12. GABA^{VT}A neurons: WT, n = 11; *KCTD16*^{-/-}, n = 12. FWHM: ***p < 0.001 (ANOVA, F_{3,47} = 15.90); Firing rate: *p < 0.05 (ANOVA, F_{3,47} = 2.92). Data are presented as mean ± s.e.m., n indicates the number of neurons analyzed. Source data for all plots are provided in Figure 5 – source data 1.

Figure 6. *KCTD16*^{-/-} mice exhibit an anxiety-like phenotype in response to mild aversive stimuli. (A) Representative tracking traces of a WT (black) and a *KCTD16*^{-/-} (red) mouse in the light/dark chamber test. (B) *KCTD16*^{-/-} mice covered significantly less distance than WT mice (n = 6, **p < 0.01, unpaired t-test). (C) *KCTD16*^{-/-} mice spent significantly less time in the light chamber resulting in a increased Δtime (time spent in dark – time spent in light chamber) compared to WT (**p < 0.01, unpaired t-test). *KCTD16*^{-/-}

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^{-/-} mice also made less entries to the light chamber than WT mice (*p < 0.05, unpaired t-test). **(D)** Experimental design of the mild fear-conditioning paradigm. The training session consisted of pairing a 1 Hz tone for 25 sec with a 2 sec foot shock. Five pairings were randomly presented during a 10 min session. During the test session 24 hrs later, the freezing behavior was assessed in a novel environment, in which five tones were presented randomly in the absence of foot shock. **(E)** *KCTD16*^{-/-} and WT mice showed no significant difference in baseline freezing in the new environment (n = 6, p = 0.214, unpaired t-test). **(F)** *KCTD16*^{-/-} mice exhibited more freezing episodes and froze significantly more than WT mice in the new environment throughout the session after presentation of the first conditioned auditory stimulus (*p < 0.05, **p < 0.01, unpaired t-test). **(G)** Representative tracking traces of a WT (left) and a *KCTD16*^{-/-} (right) mouse in the elevated zero maze. **(H)** WT and *KCTD16*^{-/-} mice spent significant more time in the closed compared to the open arms, showing that both genotypes exhibit anxiety-like behavior in the maze (n = 6, ***p < 0.001, 2-way RM ANOVA F_{1, 10} = 372.3, followed by Bonferroni's multiple comparisons test). **(I)** *KCTD16*^{-/-} mice spent significantly less time in the open arms of the maze than WT mice, indicating that *KCTD16*^{-/-} mice exhibit increased anxiety-like behavior (*p < 0.05 unpaired t-test). **(J)** *KCTD16*^{-/-} mice made fewer entries to both the open and closed arms of the maze compared to WT mice (*p < 0.05, 2-way RM ANOVA F_{1, 10} = 19.29, followed by a Bonferroni's multiple comparisons test). **(K)** Representative tracking traces of a WT (left) and a *KCTD16*^{-/-} (right) mouse in the elevated plus maze. **(L)** WT and *KCTD16*^{-/-} mice spent significantly more time in the closed compared to the open arms of the maze (n = 6, ***p < 0.001, 2-way RM ANOVA F_{1, 10} = 400.8, followed by a Bonferroni's multiple comparisons test). **(M)** *KCTD16*^{-/-} mice spent significantly less time in the open arms than WT mice (*p < 0.05 unpaired t-test). **(N)** *KCTD16*^{-/-} mice made fewer entries to both the open and closed arms of the maze compared to WT mice, which however did not reach statistical significance. (2-way RM

1 ANOVA $F_{1,10} = 2.701$). Data are represented as mean $\pm$ s.e.m., n indicates the number of
2 mice tested. Source data for all plots are provided in Figure 6 – source data 1.

3

4 **Figure 7. DA^{VTA} neuron-specific *KCTD16* ablation increased anxiety-like behavior.**

5 (A) Experimental design for Cre-dependent *KCTD16* gene editing in the VTA using
6 CRISPR/Cas9. DA^{VTA} neuron-specific *KCTD16*^{-/-} (DA^{VTA}-*KCTD16*^{-/-}) mice were generated
7 by intra-VTA injection of AAV-sg*KCTD16*-mCherry into LSL-Cas9/EGFP::DAT-Cre mice.
8 Control mice (Ctl) were generated by AAV-sg*KCTD16*-mCherry injection into LSL-
9 Cas9/EGFP mice lacking the DAT-Cre driver. (B) Viral spread (mCherry fluorescence in
10 magenta) was assessed by confocal imaging of ventral midbrain slices counterstained for
11 TH (green). SNc, substantia nigra pars compacta; SNr substantia nigra pars reticulata;
12 vertical line marks the midline. (C) Representative tracking traces of a control (Ctl) and a
13 DA^{VTA} neuron-specific *KCTD16*^{-/-} (DA^{VTA}-*KCTD16*^{-/-}) mouse in the elevated zero maze. (D)
14 DA^{VTA}-*KCTD16*^{-/-} mice (n = 8) spent significant less time in the open arms of the maze
15 than Ctl mice (n = 7), indicating an increased anxiety-like behavior in DA^{VTA}-*KCTD16*^{-/-}
16 mice (**p < 0.01, unpaired t-test). (E) The number of entries to the open and closed arms
17 of the maze was not significantly different between DA^{VTA}-*KCTD16*^{-/-} and Ctl mice (ns, 2-
18 way RM ANOVA $F_{1,26} = 0.042$, followed by a Bonferroni's multiple comparisons test). (F)
19 Representative tracking traces of a Ctl and a DA^{VTA}-*KCTD16*^{-/-} mouse in the elevated plus
20 maze. (G) DA^{VTA}-*KCTD16*^{-/-} mice (n = 8) spent significantly less time in the open arms of
21 the elevated plus maze than Ctl mice (n = 7) (*p < 0.05 unpaired t-test). (H) DA^{VTA}-
22 *KCTD16*^{-/-} mice made significantly fewer entries to the open arms compared to the closed
23 arm of the maze (2-way RM ANOVA $F_{1,26} = 12.36$, followed by a Bonferroni's multiple
24 comparisons test, ***p < 0.001). Data are represented as mean $\pm$ s.e.m., n indicates the
25 number of mice tested. (I) *In vivo* editing of *KCTD16* in DA^{VTA}-*KCTD16*^{-/-} mice. Amplicon

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deep sequencing of genomic DNA extracted from the VTA of DA^{VTA}-*KCTD16*^{-/-} mice identified insertions and deletions (indels) near the predicted Cas9 cleavage sites. The most prevalent reads from two DA^{VTA}-*KCTD16*^{-/-} mice are shown below the sgRNA sequences (5' to 3') with corresponding insertions (bold letter) and deletions (dashes). Arrowheads mark the predicted Cas9 cleavage sites. The percentage of frameshift (fs) and non-frameshift (nfs) indel mutations is indicated at the bottom. PAM, protospacer adjacent motif. Source data for all plots are provided in Figure 7 – source data 1.

Figure 8. Intra-VTA infusion of zatebradine in WT mice recapitulates the anxiety phenotype of *KCTD16*^{-/-} mice. (A) Scheme depicting behavioral testing of WT mice after saline and zatebradine (10 μM) infusion via a cannula implanted above the VTA. Four WT mice received first saline and then zatebradine. The sequence of the infusions was reversed in four additional mice. The behavioral results of the eight mice were pooled. The infusion sites were confirmed on VTA slices after infusion of fluorescent beads at the end of the experiments. (B) Example of post-hoc confocal image showing green fluorescent beads at the injection site in the VTA, counterstained for TH (red). SN, substantia nigra. Scale bar 500 μm. (C) Example track plots of mice infused with saline (left) or zatebradine (right) in the elevated zero maze. (D) Intra-VTA infusion of zatebradine significantly decreased the time spent in the open arms of the elevated zero maze (n = 8 mice, *p < 0.05, paired t-test). (E) Example track plots of mice infused with saline (left) or zatebradine (right) in the elevated plus maze. (F) Intra-VTA infusion of zatebradine significantly decreased the time spent in the open arms of the elevated plus maze (n = 8 mice, *p < 0.05 paired t-test). Data are represented as mean ± s.e.m., n indicates the number of mice tested. Source data for all plots are provided in Figure 8 – source data 1.

1 **Legends for figure supplements**

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3 **Figure 1 – figure supplement 1. Specificity of the KCTD16/HCN channel**

4 **interaction.** (A) KCTD16 co-immunoprecipitated HCN2 in the presence and absence of
5 GBRs from membrane preparations of transfected HEK293 cells. (B) KCTD16 co-
6 immunoprecipitated HCN2 and HCN3 but not HCN1 or HCN4. KCTD12 did not co-
7 immunoprecipitate HCN subunits. (C) Left: Immunoblot of membrane preparations of the
8 VTA, cortex, hippocampus and cerebellum demonstrating HCN2, KCTD16 (lower band),
9 TH, GB1a and GB1b expression in the VTA. β -tubulin III served as a loading control.
10 Right: All HCN subunits are expressed in the VTA. (D) Immunoblot of similar amounts of
11 HCN subunits (same amounts of transfected cDNA) expressed in HEK293 cells showed
12 subunit-specificity of the HCN antibodies. Source data for all Western blots are provided
13 in Figure 1 – source data 1.

14

15 **Figure 2 – figure supplement 1. Incomplete space-clamp of DA^{VTA} neurons. (A)**

16 Reconstruction of a biocytin-filled DA^{VTA} neuron showing the positioning of the somatic
17 voltage-clamp (VC) and dendritic current-clamp recording pipettes (CC). (B) Top: VC
18 recording of I_{bac} at the soma in response to baclofen application (300 μ M). The I_{bac} was
19 inhibited by CGP54626 (CGP, 4 μ M). Middle: Ri measured at the soma. Bottom: CC
20 recording showing membrane hyperpolarization in the dendrite. (C) Hyperpolarization for
21 each dendritic recording ($n = 7$ neurons) as a function of the distance between VC and CC
22 pipettes (linear regression fitted to the data; $p < 0.05$; f-test of overall significance). The
23 red circle depicts the experiment shown in (B). Source data for all plots are provided in
24 Figure 2 – source data 1.

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Figure 3 – figure supplement 1. GBR activity does not allosterically alter biophysical properties of receptor-associated HCN channels. (A) I_h recorded from DA^{VT}A neurons in the presence of 300 μ M Ba²⁺ to block Kir3 channels before (black traces) and during baclofen application (300 μ M; red traces). Baclofen did not significant change the I_h voltage-dependency (leak subtracted) ($p = 0.33$, ESS F-test $F_{4, 72} = 1.17$, $n = 5$ neurons), (B) BRET experiments in transfected CHO. cells. BRET titration curves using a fixed amount of Rluc-GB1b and increasing amounts of YFP-HCN2 were performed in the absence (black) or in the presence (red) of baclofen (100 μ M). No specific BRET was observed in control cells that were not transfected with KCTD16. Bar graph shows the Bmax BRET values in the presence and absence of KCTD16 (two-way ANOVA $F_{1, 16} = 79.55$ followed by Tukey's multiple comparison; *** $p < 0.001$; ns, not significant; $n = 5$ independent experiments). Data are represented as mean $\pm$ s.e.m. Source data for all plots are provided in Figure 3 – source data 1.

Figure 6 – figure supplement 1. KCTD16^{-/-} mice exhibit normal locomotor activity. (A) Example track plots of spontaneous locomotion in an open rectangular arena of a WT (left) and a KCTD16^{-/-} (right) mouse, color-coded for speed. (B) Example traces of decomposed locomotion bouts for WT (left) and KCTD16^{-/-} (right) mice, color-coded for single bouts, aligned to the start. (C-G) No differences in the total distance traveled (c), the number of bouts (d), bout length (e), the speed of travel (f) or the duration of each bout (g) were found between WT and KCTD16^{-/-} mice ($n = 6$ mice per group). Data are represented as mean $\pm$ s.e.m., n indicates the number of mice tested. Source data for all plots are provided in Figure 6 – source data 1.

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Figure 6 – figure supplement 2. *KCTD16*^{-/-} mice exhibit an anxious phenotype in the elevated zero and plus mazes. (A) WT and *KCTD16*^{-/-} mice travelled significantly larger distances in the closed sections of the elevated zero maze (ANOVA, $F_{1,10} = 80.11$). (B) WT mice travelled at higher speed in the open sections of the elevated zero maze than *KCTD16*^{-/-} mice (ANOVA, $F_{1,10} = 8.539$). (C) WT and *KCTD16*^{-/-} mice travelled larger distances in the closed sections of the elevated plus maze ($F_{1,10} = 29.6$). (D) WT mice travelled at higher speed in the open sections of the elevated plus maze than *KCTD16*^{-/-} mice ($F_{1,10} = 13.59$). $n = 6$ mice per group. Bonferroni's multiple comparison test was used for post-hoc analysis: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are represented as mean $\pm$ s.e.m., n indicates the number of mice tested. Source data for all plots are provided in Figure 6 – source data 1.

Figure 7 – figure supplement 1. DA^{VT}A neuron-specific *KCTD16* ablation by AAV-mediated CRISPR/Cas9 editing. (A) *In vitro* validation of the *KCTD16*-targeting sgRNAs. In the presence of the sgRNAs, incubation with recombinant Cas9 results in the specific cleavage of PCR products (amplicons) containing the *KCTD16* target sites. Uncut amplicons and cleavage products were resolved on 2.5% agarose gels (left). The sequence of the target sites and the PCR primers used to generate the amplicons are shown to the right. (B) Cre-dependent expression of Cas9 in DA neurons in LSL-Cas9/EGFP::DAT-Cre mice. PCR of genomic DNA extracted from midbrain slices reveals deletion of the STOP-cassette, flanked by two loxP sites, in the presence of the DAT-Cre driver (right). The positions of the PCR primers (P1, P2) and the loxP sites (open triangles) are indicated in the scheme to the left. (C) Representative immunofluorescence images of the VTA of DA^{VT}A-*KCTD16*^{-/-} mice. Cre-mediated activation of Cas9 in TH-positive neurons (cyan) is revealed by the EGFP reporter (green). AAV8-mediated transduction of the

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1 *KCTD16*-targeting sgRNAs is visualized by mCherry fluorescence (magenta). (**D**)
 2 Representative immunofluorescence images of the VTA of Ctl mice. Note the absence of
 3 Cas9/EGFP expression due to the presence of the STOP cassette in the LSL-Cas9/EGFP
 4 locus. mCherry fluorescence reveals AAV8 transduction of TH-positive neurons. Scale
 5 bars, 25 μ m.

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