Targeting light-gated chloride channels to neuronal somatodendritic domain reduces their excitatory effect in the axon

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Abstract

Light-gated chloride channels are emerging as promising optogenetic tools for inhibition of neural activity. However, their effects depend on the transmembrane chloride electrochemical gradient and may be complex due to the heterogeneity of this gradient in different developmental stages, neuronal types, and subcellular compartments. Here we characterized a light-gated chloride channel, GtACR2, in mouse cortical neurons. We found that GtACR2 activation inhibited the soma, but unexpectedly depolarized the presynaptic terminals resulting in neurotransmitter release. Other light-gated chloride channels had similar effects. Reducing the chloride concentrations in the axon and presynaptic terminals diminished the GtACR2-induced neurotransmitter release, indicating an excitatory effect of chloride channels in these compartments. A novel hybrid somatodendritic targeting motif reduced the GtACR2-induced neurotransmitter release while enhancing the somatic photocurrents. Our results highlight the

necessity of precisely determining the effects of light-gated chloride channels under specific experimental conditions and provide a much-improved light-gated chloride channel for optogenetic inhibition.

Introduction

Targeted manipulation of neural activity is a powerful approach in neuroscience that has provided fundamental insights into the roles of specific neurons in nervous system functions. Genetically encoded actuators such as light-gated ion channels or pumps enable control of neural activity with unprecedented spatiotemporal specificity and are transforming neuroscience research (Boyden et al., 2005; Han and Boyden, 2007; Li et al., 2005; Nagel et al., 2003; Zhang et al., 2007). Actuators that enable neuronal activation are frequently used, but inhibitory optogenetic tools are increasingly crucial because reversible and temporally precise suppression of neuronal activity is key to revealing the causal roles of specific neurons in network dynamics and behavior. The widely used light-driven inward chloride pumps and outward proton pumps, such as *Natronomonas pharaonis* halorhodopsin (NpHR) and *Halorubrum sodomense* archaerhodopsin (Arch), can hyperpolarize membrane potentials, independent of the electrochemical gradients, to inhibit action potentials with millisecond precision (Chow et al., 2010; Chuong et al., 2014; Han and Boyden, 2007; Han et al., 2011; Zhang et al., 2007). However, their efficacies are limited because only one ion is transported per absorbed photon, and their activation does not decrease membrane resistance. Light-gated chloride channels, such as *Guillardia theta* anion channelrhodopsin 1 and 2 (GtACR1 and GtACR2), iC++, and iChloC, overcome these limitations. They are highly sensitive to light, allow multiple ions to cross the membrane per photocycle, and reduce membrane resistance, thereby potently inhibiting action

47 potentials (Berndt et al., 2014; Govorunova et al., 2015; 2017b; Wietek et al., 2014). Thus, light-
48 gated chloride channels are emerging as promising optogenetic tools for suppressing neuronal
49 activity (Govorunova et al., 2017a).
50
51 Determining the precise effects of light-gated ion channels or pumps under defined conditions is
52 a prerequisite to use them for interrogating the functions of specific neurons and circuits. This is
53 due to the possibility that these channels or pumps not only modulate membrane potentials but
54 also may affect other processes such as ion homeostasis or neurotransmitter release. For
55 example, activation of a light-driven inward chloride pump, eNpHR3.0, can transiently change
56 the reversal potential of $GABA_A$ receptors and alter the inhibitory synaptic inputs (Raimondo et
57 al., 2012). Prolonged activation of a light-driven outward proton pump, eArch3.0, can increase
58 presynaptic calcium concentrations and spontaneous neurotransmitter release (Mahn et al.,
59 2016). Despite these potential confounds, these inhibitory optogenetic molecules are increasingly
60 essential tools for the targeted silencing of neuronal populations, as long as these confounds are
61 understood and controlled (Allen et al., 2015). Therefore, it is also crucial to thoroughly
62 characterize light-gated chloride channels, as their effect depends on the difference between the
63 membrane potential and the reversal potential for chloride, both of which can vary in different
64 neuronal types and subcellular compartments (Marty and Llano, 2005; Trigo et al., 2008).
65
66 To this end, we investigated the effects of activating GtACR2 in mouse cortical excitatory and
67 inhibitory neurons. Much to our surprise, wide-field light activation of GtACR2 not only
68 inhibited the soma, but also caused neurotransmitter release onto neighboring neurons. A similar
69 phenomenon was observed with GtACR1, iC++, and iChloC. We further showed that GtACR2

activation in the axon and presynaptic terminals directly depolarized the membrane to induce neurotransmitter release due to high chloride concentrations in these compartments. These data explain the recent observations that photostimulation of neurons expressing GtACR1 or GtACR2 can paradoxically release neurotransmitters or generate action potentials (Mahn et al., 2016; Malyshev et al., 2017). To reduce the excitatory effect of GtACR2, we screened a panel of somatodendritic targeting motifs to reduce the trafficking of GtACR2 to the axon and presynaptic terminals. We created a hybrid motif (Kv2.1C-linker-TlcnC) that is most effective in concentrating GtACR2 in the somatodendritic domain. Activation of somatodendritically targeted GtACR2 resulted in larger photocurrents at the soma and less neurotransmitter release than wild type GtACR2. These results demonstrate that restricting localization of light-gated chloride channels to the somatodendritic domain improves the inhibitory efficacy of these optogenetic tools.

Results

Light activation of GtACR2 in mouse cortical neurons causes neurotransmitter release

To examine the efficacy of GtACR2 in mouse cortical excitatory neurons, we expressed a GtACR2-EYFP fusion protein (referred to as GtACR2 below) together with a red fluorescent protein, tdTomato, in layer 2/3 pyramidal neurons of the mouse visual or somatosensory cortex by *in utero* electroporation of plasmids at embryonic day 14.5–15.5. We obtained acute coronal brain slices from 3–8-week-old mice and observed that GtACR2 was present in the soma, dendrites, and axon (***Figure 1A***). We performed whole-cell patch clamp recordings at the soma of neurons expressing GtACR2 (GtACR2^{+} neurons) with a K^{+}-based pipette solution (***Figure 1B***). As previously reported, activation of GtACR2 by wide-field blue light (455 nm) potently

inhibited current-induced spiking in these neurons (***Figure 1C***). However, when we voltage clamped the neurons to record GtACR2-mediated photocurrents, we unexpectedly found an inward current that was superimposed on the photocurrent. This inward current resembled an excitatory postsynaptic current (EPSC; ***Figure 1D***). To further investigate this phenomenon, we recorded layer 2/3 pyramidal neurons that did not express GtACR2 ($GtACR2^-$ neurons) with a Cs^+-based pipette solution (***Figure 1E***). GtACR2 activation generated inward currents in every recorded $GtACR2^-$ neuron that was voltage clamped at the reversal potential for GABAergic inhibition (-60 mV). The onsets of these inward currents followed the onset of the blue light by 3.19 ± 0.26 ms (mean ± s.e.m., n = 25). These currents were abolished by the glutamatergic receptor antagonists, NBQX and CPP (***Figure 1F***), or the voltage-gated sodium channel blocker, tetrodotoxin (TTX; ***Figure 1G***), indicating that they were indeed monosynaptic EPSCs caused by the glutamate transmitter released from $GtACR2^+$ neurons. Activation of GtACR2 also produced inhibitory postsynaptic currents (IPSCs) in $GtACR2^-$ neurons that were voltage clamped at the reversal potential for glutamatergic excitation (+10 mV). These IPSCs were disynaptic because they were abolished by NBQX and CPP (***Figure 1-supplement 1***), indicating that activating GtACR2 in pyramidal neurons can release sufficient glutamate to recruit inhibitory interneurons.

To determine if the phenomenon of GtACR2-induced neurotransmitter release is also present in inhibitory neurons, we expressed GtACR2 in parvalbumin-expressing (Pv) neurons by injecting a Flpo recombinase-dependent adeno-associated virus (AAV) into the visual cortex of *Pvalb-2A-Flpo* mice ($Pvalb^{Flpo/+}$) (Madisen et al., 2015) at postnatal day 1 (***Figure 1H***). Using acute brain slices from 3–6-week-old mice, we found that activation of GtACR2 in Pv neurons generated

IPSCs in every recorded GtACR2$^-$ layer 2/3 pyramidal neuron, and the IPSCs were abolished by Gabazine, a $GABA_A$ receptor antagonist, or TTX (***Figure 1I–K***). The onsets of the IPSCs followed the onset of the blue light by 2.47 ± 0.15 ms (mean ± s.e.m., n = 17), indicating that they were monosynaptic IPSCs caused by the GABA transmitter released from GtACR2$^+$ Pv neurons. Furthermore, repetitive activation of GtACR2 in layer 2/3 pyramidal neurons or Pv neurons with a high-frequency train of light pulses produced reliable EPSCs or IPSCs, respectively (***Figure 1-supplement 2A,B***).

These findings were unexpected, because the Nernst equilibrium potential of chloride becomes lower than the action potential threshold in rodent cortical neurons after the second postnatal week, and activation of chloride channels should not promote neurotransmitter release onto neighboring cells (Ben-Ari, 2002; Owens et al., 1996). We thus sought to identify the cause of this paradoxical neurotransmitter release and considered three possibilities. First, GtACR2 may conduct cations to depolarize neurons. Second, an increase in the intracellular chloride or strong hyperpolarization induced by GtACR2 activation may lead to rebound spikes. Third, GtACR2-mediated chloride currents may be excitatory.

A GtACR2-mediated excitatory chloride conductance causes neurotransmitter release

First, it was reported that GtACR2 did not conduct physiological cations (Govorunova et al., 2015), but some other light-gated chloride channels retained certain cation conductance (Berndt et al., 2014; Wietek et al., 2014). Thus, we sought to verify that in cortical neurons, GtACR2 has a similar reversal potential as a known chloride channel. To accomplish this, we determined the reversal potential of GtACR2-mediated photocurrents in comparison with that of IPSCs

mediated by the endogenous $GABA_A$ receptors. We used *in utero* electroporation to express GtACR2 in layer 2/3 pyramidal neurons and a Cre recombinase-dependent AAV to express a red light-gated cation channelrhodopsin, ReaChR, in Pv neurons of *Pvalb-2A-Cre* mice ($Pvalb^{Cre/+}$) (Madisen et al., 2010). We performed whole-cell voltage clamp recordings at the soma of a $GtACR2^+$ layer 2/3 pyramidal neuron and a nearby $GtACR2^-$ pyramidal neuron simultaneously (***Figure 2-supplement 1A***). In the $GtACR2^+$ neuron, we sequentially recorded the IPSCs induced by activating ReaChR in Pv neurons via 617-nm light and the GtACR2-mediated photocurrents activated by 455-nm light. Both IPSCs and photocurrents were recorded at different membrane potentials to determine their reversal potentials in the same neuron (***Figure 2-supplement 1B,C***). 617-nm light does not activate GtACR2 (Govorunova et al., 2015), whereas 455-nm light partially activates ReaChR (Lin et al., 2013). Thus, to avoid Pv neuron-mediated IPSCs contaminating GtACR2-mediated photocurrents, we monitored the IPSCs in the $GtACR2^-$ neuron at the membrane potential of +10 mV to ensure that the intensity of the 455-nm light was not sufficient to activate Pv neurons and generate IPSCs (***Figure 2-supplement 1B***). We found that the reversal potentials of GtACR2-mediated photocurrents and GABAergic IPSCs were similar for each neuron and well below the action potential threshold (***Figure 2-supplement 1D***), indicating that GtACR2 does not conduct cations to cause neurotransmitter release.

Second, we tested if GtACR2-induced neurotransmitter release could be due to rebound depolarization. An increase in the intracellular chloride caused by GtACR2 activation may trigger rapid efflux of chloride after the blue light illumination terminates. However, this possibility is unlikely because when GtACR2 was activated by a long pulse of blue light (e.g., 5 ms), neurotransmitter release occurred before the light illumination ended (see examples in

Figure 1F,G). Another possibility is that the strong hyperpolarization induced by GtACR2 activates hyperpolarization-activated I_h currents, which may depolarize the membrane potential above the action potential threshold. However, pharmacological inhibition of I_h currents slightly increased the amplitudes of GtACR2-induced EPSCs (***Figure 2-supplement 2A,B***), most likely because inhibiting I_h currents increases neuronal membrane resistances (Robinson and Siegelbaum, 2003). Thus, GtACR2-induced neurotransmitter release is not caused by rebound depolarization.

Third, although GtACR2-mediated photocurrents are inhibitory at the soma, it is possible that the chloride concentrations are higher in some other cellular compartments, such that the electrochemical gradient causes chloride to exit the cell upon GtACR2 channel opening, resulting in depolarization of the membrane potential. To test this hypothesis, we pharmacologically inhibited the activity of Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) with bumetanide (50 or 100 μM) to decease the intracellular chloride concentrations, as NKCC1 is responsible for transporting chloride into neurons (Ben-Ari, 2017). When we activated GtACR2 in layer 2/3 pyramidal neurons, the resulting EPSCs in $GtACR2^-$ pyramidal neurons were diminished by bath application of bumetanide (***Figure 2A,B***), indicating that GtACR2-induced neurotransmitter release requires high concentrations of intracellular chloride. An alternative interpretation of this result would be that bumetanide blocks GtACR2 itself. To test this possibility, we simultaneously recorded the photocurrents and EPSCs in $GtACR2^+$ and $GtACR2^-$ pyramidal neurons, respectively. While bumetanide diminished the EPSCs in $GtACR2^-$ neurons, it had no effect on the photocurrents in $GtACR2^+$ neurons (***Figure 2-supplement 3A,B***), thereby ruling out the possibility that bumetanide affects GtACR2 itself. Furthermore, when a cation

channel, channelrhodopsin-2 (ChR2) (Boyden et al., 2005; Li et al., 2005; Nagel et al., 2003), was expressed and activated in layer 2/3 pyramidal neurons, the resulting EPSCs in ChR2$^-$ neurons were not affected by bumetanide (***Figure 2C,D***), indicating that reducing the intracellular chloride concentration only affects chloride-mediated, and not cation-mediated, EPSCs. Finally, bumetanide also diminished the IPSCs resulting from GtACR2 activation in Pv neurons (***Figure 2E,F***). Together, these results demonstrate that light activation of GtACR2 generates an excitatory chloride conductance in certain neuronal compartments to trigger neurotransmitter release.

Expression and activation of GtACR2 in adult neurons causes neurotransmitter release

Since we expressed GtACR2 by *in utero* electroporation or neonatal AAV injection, we sought to determine if the excitatory effect of GtACR2 was caused by the long-term expression of GtACR2 throughout development that somehow altered the chloride homeostasis. To selectively express GtACR2 in adult neurons, we *in utero* electroporated a Flpo-dependent plasmid into layer 2/3 pyramidal neurons that will express GtACR2 only if Flpo is present (***Figure 2-supplement 2C,D***). A Flpo-expressing AAV was then injected into the electroporated mice at postnatal week 4 or 9 to turn on the GtACR2 expression. We obtained acute coronal brain slices 1–3 weeks after injecting Flpo-expressing AAV and found that light activation of GtACR2, again, produced bumetanide-sensitive EPSCs in GtACR2$^-$ neurons (***Figure 2-supplement 2E***). Thus, it is unlikely that GtACR2 expression during neuronal development alters the chloride homeostasis to render GtACR2 excitatory, as acute expression of GtACR2 in mature neurons had the same effect.

Activation of other light-gated chloride channels causes neurotransmitter release

To determine if activation of other light-gated chloride channels can trigger neurotransmitter release, we examined iC++ and iChloC, two engineered blue light-gated chloride channels that were converted from cation channelrhodopsins (Berndt et al., 2015; Wietek et al., 2015), and GtACR1, another natural anion channelrhodopsin from *Guillardia theta* (Govorunova et al., 2015). We *in utero* electroporated plasmids to express iC++ in layer 2/3 pyramidal neurons of the visual cortex and obtained acute coronal brain slices from 3–9-week-old mice. Similar to GtACR2, light activation of iC++ generated EPSCs in iC++$^{-}$ layer 2/3 pyramidal neurons that were abolished by NBQX and CPP (amplitude reduced by 97.3 ± 0.9%, mean ± s.e.m., n = 4). Bumetanide diminished the iC++-induced EPSCs (***Figure 2G,H***) without affecting the iC++-mediated photocurrents (***Figure 2-supplement 3C,D***). We also expressed iC++ in Pv neurons by injecting a Cre-dependent AAV into *Pvalb*$^{Cre/+}$ mice and found that light activation of iC++ caused bumetanide-sensitive IPSCs in iC++$^{-}$ layer 2/3 pyramidal neurons (***Figure 2I,J***). Similarly, when we expressed iChloC in Pv neurons, light activation of iChloC resulted in IPSCs in 10 out of 17 recorded iChloC^{-} layer 2/3 pyramidal neurons (183 ± 42 pA, mean ± s.e.m., n = 10), presumably because iChloC generated smaller photocurrents than iC++ (iChloC, 292 ± 62 pA, n = 7; iC++, 2182 ± 291 pA, n = 15; recorded at the membrane potential of +10 mV; mean ± s.e.m., $P < 0.0001$, t-test with Welch's correction). Finally, activation of GtACR1 in layer 2/3 pyramidal neurons produced EPSCs onto every recorded GtACR1^{-} pyramidal neuron (149 ± 47 pA, mean ± s.e.m., n = 11). Altogether, these results demonstrate that activation of different light-gated chloride channels in neurons can trigger neurotransmitter release.

GtACR2 activation directly depolarizes the presynaptic terminals

We hypothesized that the most likely neuronal compartments rendering GtACR2 excitatory were the distal axon and presynaptic terminals because of the following previous findings. First, activation of presynaptic $GABA_A$ or glycine receptors enhanced neurotransmitter release at several synapses of the hippocampus, cerebellum, and brainstem (Jang et al., 2006; Pugh and Jahr, 2011; Stell et al., 2007; Turecek and Trussell, 2001). Second, the chloride concentrations were 4–5 times higher in the presynaptic terminals of the Calyx of Held than the parent soma (Price and Trussell, 2006). Third, there appeared to be an axo-somato-dendritic gradient in which the reversal potentials of GABA from the axon to the soma and dendrites of cortical neurons become progressively more negative (Khirug et al., 2008). To test our hypothesis, we expressed GtACR2 in layer 2/3 pyramidal neurons of the visual cortex in one hemisphere as described above and obtained acute coronal slices from the contralateral hemisphere (***Figure 3A***). GtACR2 was present in the long-range callosal projections in the contralateral hemisphere (***Figure 3-supplement 1A***), which enabled us to activate GtACR2 in the axon and presynaptic terminals that were severed from their parent somas. Light activation of GtACR2 in the callosal projections generated EPSCs in layer 2/3 pyramidal neurons of the contralateral cortex, which were diminished by TTX (***Figure 3-supplement 1B,C***) or bumetanide (***Figure 3B***). These results demonstrate that activation of GtACR2 in the axon and presynaptic terminals is sufficient to trigger neurotransmitter release.

If GtACR2-mediated chloride currents are excitatory in the presynaptic terminals, then GtACR2 should be similar to ChR2, whose activation can directly depolarize the presynaptic membrane in the absence of action potentials to trigger neurotransmitter release (Petreanu et al., 2009). To test this prediction, we recorded EPSCs or IPSCs in $GtACR2^-$ neurons while activating GtACR2 in

layer 2/3 pyramidal neurons or Pv neurons, respectively (***Figure 3C,E***). As described above, bath application of TTX abolished the EPSCs and IPSCs. However, when we further blocked voltage-gated potassium channels by 4-aminopyridine (4-AP) and tetraethylammonium (TEA) to prolong membrane depolarization (Petreanu et al., 2009), the EPSCs and IPSCs were partially recovered (***Figure 3D,F***). These results indicate that in the absence of action potentials, light activation of GtACR2 is sufficient to depolarize the presynaptic membrane to open voltage-gated calcium channels and trigger neurotransmitter release.

We further tested if GtACR2-induced axonal depolarization could evoke antidromic action potentials by performing extracellular loose-patch or whole-cell current clamp recordings at the somas of GtACR2^{+} pyramidal neurons. We observed antidromic spikes in 9 out of 88 neurons recorded in loose-patch configuration and 21 out of 56 neurons recorded in whole-cell configuration in response to blue light stimulation (***Figure 3G,H***). In the whole-cell current clamp recordings, although the chloride concentration in the patch pipette solution sets the Nernst equilibrium potential of chloride around -85 mV (see Methods), blue light induced a depolarization following the initial hyperpolarization. This observation is consistent with the notion that the depolarization antidromically propagated from the distal axon to the soma (***Figure 3H***). The antidromic spikes were not affected by NBQX and CPP, but were abolished by TTX (***Figure 3H***), indicating that the spikes were generated within the GtACR2^{+} neurons, rather than by excitatory inputs from other neurons. Antidromic spikes were only observed in a subset of neurons, likely because the GtACR2 expression levels are heterogeneous in different neurons and the hyperpolarization initiated at the soma can orthodromically propagate to counteract the antidromic spikes. Similarly, TTX-sensitive antidromic spikes were observed in a subset of

277 GtACR2^{+} Pv neurons (3 out of 11 neurons in loose-patch configuration and 7 out of 17 neurons
278 in whole-cell configuration, ***Figure 3I,J***). These results show that GtACR2-induced axonal
279 depolarization can be sufficient to elicit antidromic action potentials.
280
281 **Targeting GtACR2 to the somatodendritic domain reduces light-induced neurotransmitter**
282 **release**
283 Activating GtACR2 and other light-gated chloride channels inhibits the soma but depolarizes the
284 presynaptic terminals to release neurotransmitters. This dichotomic effect can confound the
285 utilization of these channels as inhibitory optogenetic tools for suppressing neuronal activity. We
286 reasoned that reducing the trafficking of light-gated chloride channels into the axon and
287 presynaptic terminals should reduce or eliminate their depolarizing action. Thus, we sought to
288 restrict GtACR2 within the somatodendritic domain of neurons by fusing GtACR2 with a
289 number of reported somatodendritic targeting motifs including a 26-amino acid Myosin Va-
290 binding domain of Melanophilin (MBD) (Lewis et al., 2009), a 32-amino acid cytoplasmic C-
291 terminal motif of Neuroligin 1 (Nlgn1C) (Rosales et al., 2005), a 16-amino acid dileucine-
292 containing motif of potassium channel Kv4.2 (Kv4.2LL) (Rivera et al., 2003), the N-terminal
293 150 residues of kainate receptor subunit 2 (KA2N) (Shemesh et al., 2017), the C-terminal 17
294 residues of Telencephalin (TlcnC) (Mitsui et al., 2005), and a 65-amino acid cytoplasmic C-
295 terminal motif of potassium channel Kv2.1 (Kv2.1C) (Lim et al., 2000). Each of these GtACR2
296 variants (***Figure 4-supplement 1***), along with tdTomato, were expressed in layer 2/3 pyramidal
297 neurons of the visual cortex by *in utero* electroporation (***Figure 4A***). Since GtACR2 was also
298 tagged with EYFP or EGFP, we compared the EYFP or EGFP fluorescence in layer 5, which
299 only contains the axons of layer 2/3 pyramidal neurons, with the EYFP or EGFP fluorescence in

layer 2/3 to estimate the distribution of GtACR2 between the axon and somatodendritic domain. We normalized the EYFP or EGFP fluorescence ratio between layer 5 and layer 2/3 by the tdTomato fluorescence ratio between layer 5 and layer 2/3 to control for variations in the collateral axons. Among tested motifs, TlcnC and Kv2.1C were most effective in targeting GtACR2 to the soma and dendrites (***Figure 4B***). As these two motifs may engage different trafficking mechanisms, we combined them to create two hybrid motifs, Kv2.1C-TlcnC and Kv2.1C-linker-TlcnC. Kv2.1C-linker-TlcnC turned out to be the best in restricting GtACR2 within the somatodendritic domain (***Figure 4A,B,C***). Interestingly, GtACR2-EYFP-Kv2.1C and GtACR2-EYFP-Kv2.1C-linker-TlcnC showed less intracellular aggregation than wild type GtACR2 (***Figure 4C***), suggesting that the somatodendritic targeting motifs also enhance the surface expression of GtACR2. Finally, the EYFP fluorescence in the callosal projections in the contralateral hemisphere was also reduced for GtACR2-EYFP-Kv2.1C-linker-TlcnC as compared to GtACR2-EYFP (***Figure 4D,E***), demonstrating that Kv2.1C-linker-TlcnC decreased the trafficking of GtACR2 into the distal axon.

To determine how targeting GtACR2 to the somatodendritic domain affects its photocurrent and ability to trigger neurotransmitter release, we compared the somatodendritically targeted GtACR2 variants, GtACR2-EYFP-Kv2.1C or GtACR2-EYFP-Kv2.1C-linker-TlcnC, with wild type GtACR2 in the same litters of mice. We first recorded GtACR2^{+} layer 2/3 pyramidal neurons and found that the blue light-activated photocurrents of GtACR2-EYFP-Kv2.1C or GtACR2-EYFP-Kv2.1C-linker-TlcnC were 2.1- or 2.7-fold of GtACR2-EYFP photocurrents, respectively (***Figure 5A–C***). We then recorded the EPSCs in GtACR2^{-} layer 2/3 pyramidal neurons in response to different strengths of blue light stimulation. The EPSCs evoked by

activating GtACR2-EYFP-Kv2.1C or GtACR2-EYFP-Kv2.1C-linker-TlcnC were reduced by 52–60% or 65–77%, respectively, as compared to those evoked by activating GtACR2-EYFP (***Figure 5D–H***). Furthermore, the EPSCs in layer 2/3 pyramidal neurons of the contralateral cortex were reduced by 73% for GtACR2-EYFP-Kv2.1C-linker-TlcnC as compared to GtACR2-EYFP when photostimulating the callosal projections (***Figure 5I,J)***. These results show that the somatodendritic targeting motifs, especially Kv2.1C-linker-TlcnC, shift GtACR2 from the axon towards the soma and dendrites, thereby reducing the excitatory action in the axon and presynaptic terminals while enhancing the inhibitory currents at the soma and dendrites.

Discussion

Optogenetic suppression of neuronal activity and synaptic outputs is an essential approach for dissecting the roles of specific neurons in brain functions. Light-gated chloride channels, particularly GtACR1 and GtACR2, are increasingly used due to their large photocurrents and high sensitivity to light (Forli et al., 2018; Mardinly et al., 2018; Mauss et al., 2017; Mohamed et al., 2017; Mohammad et al., 2017). To use these tools to their full potentials, it is necessary that we understand their function and importantly, their limitations. The experiments here reveal that wide-field activation of these channels in visual cortical neurons suppresses action potentials at the soma but also triggers neurotransmitter release at the presynaptic terminals, therefore voiding inhibition of neuronal activity. As demonstrated, the excitatory action of chloride channels is due to the higher concentrations of chloride in the axon and presynaptic terminals relative to the somatodendritic domain. However, it is worth noting that the heterogeneity of chloride gradients across subcellular compartments may be cell-type dependent. For instance, gramicidin perforated patch recordings from the axonal blebs of layer 5 pyramidal neurons in the prefrontal cortex of

346 rats revealed that the reversal potential of $GABA_A$ receptors was more negative than the resting
347 membrane potential of the axon, which would indicate a hyperpolarizing effect of chloride
348 channels in this axon (Xia et al., 2014). Thus, it is critical to precisely determine the effect of
349 light-gated chloride channels, specific to each experimental design, when using them to
350 manipulate neuronal activity.
351
352 To create a better inhibitory optogenetic tool, we tested a number of somatodendritic targeting
353 motifs to confine GtACR2 in the soma and dendrites. We generated a hybrid motif, Kv2.1C-
354 linker-TlcnC, that was more effective than the widely used Kv2.1C (Baker et al., 2016; Mardinly
355 et al., 2018; Wu et al., 2013). The Kv2.1C motif was recently used in a bioRxiv preprint (Mahn
356 et al., 2017) to target GtACR2 to the somatodendritic domain. This GtACR2 variant achieved a
357 greater reduction of neurotransmitter release from the contralateral callosal projections of medial
358 prefrontal cortical neurons than what we observed with our GtACR2-EYFP-Kv2.1C and
359 GtACR2-EYFP-Kv2.1C-linker-TlcnC in visual cortical neurons. This quantitative difference is
360 likely due to different expression levels, light stimulation strengths, and neuronal types, which
361 reiterates the importance of characterizing the specific effect of light-gated chloride channels for
362 a given experiment.
363
364 Although the excitatory effect of light-gated chloride channels is undesired for neuronal
365 silencing, a potential utilization of their dual actions at the presynaptic terminals and soma is to
366 activate specific projections of neurons while minimizing the effect of antidromic spikes. For
367 example, long-range projection neurons often target multiple brain areas, and sometimes it is
368 desired to selectively excite the axonal terminals projecting to one particular area. If ChR2 is

used, local activation of ChR2-expressing axonal terminals may generate antidromic spikes, which will affect other projections (Kim et al., 2017). However, if GtACR2 is used, one can simultaneously activate GtACR2 in the soma and axonal terminals. Axonal depolarization will result in neurotransmitter release, but the antidromic spikes will be reduced or suppressed by the hyperpolarization originating from the somatodendritic domain, which reduces the likelihood of activating other projections.

While GtACR2-EYFP-Kv2.1C-linker-TlcnC can still traffic to the axon to cause neurotransmitter release, it is thus far the most improved light-gated chloride channel for optogenetic inhibition. Since it can generate much larger photocurrents in the somatodendritic domain than what is necessary to suppress action potentials, one approach to use this tool is to reduce the overall GtACR2 expression level to further decrease its presence in the axon while generating sufficient inhibitory photocurrents at the soma. Another approach is to selectively photostimulate GtACR2 at the soma by two-photon excitation (Forli et al., 2018; Mardinly et al., 2018). However, it is difficult to apply this photoactivation approach to freely moving animals or deep brain areas. Therefore, it is imperative that we further engineer the channels to eliminate their excitatory action in the axonal terminals. Future strategies include generating more effective somatodendritic targeting motifs, creating outwardly rectifying channels, and the combination of both strategies. The GtACR2-EYFP-Kv2.1C-linker-TlcnC reported here will serve as the foundation for future improvement, which will enhance the available toolkit for optogenetic inhibition.

Materials and methods

392 **Mice**

393 All procedures to maintain and use mice were approved by the Institutional Animal Care and
394 Use Committee at Baylor College of Medicine. Mice were maintained on 14-h:10-h light:dark
395 cycle with regular mouse chow and water ad libitum. Experiments were performed during the
396 light cycle. ICR female mice were purchased from Baylor College of Medicine Center for
397 Comparative Medicine or Charles River Laboratories. C57BL6/J, *Pvalb-2A-Cre*, and *Pvalb-2A-*
398 *Flpo* mice were obtained from Jackson Laboratory (stock numbers 000664, 012358, and 022730,
399 respectively). Both male and female mice were used in the experiments. The mice were used at
400 the age of 3–9 weeks for experiments, except for the conditional expression of GtACR2 in
401 adults, where mice were used at the age of 10–12 weeks.
402
403 **DNA constructs**
404 Plasmids pLenti-UbiC-GtACR2-EYFP (Addgene #67877) and pLenti-UbiC-GtACR1-EYFP
405 (Addgene #67795) were obtained from Dr. John Spudich, pAAV-CaMKIIα-iC++-TS-EYFP and
406 pAAV-EF1α-DIO-iC++-TS-EYFP from Dr. Karl Deisseroth, pAAV-EF1α-DIO-iChloC-T2A-
407 mCherry from Drs. Matthew Caudill and Massimo Scanziani, pCAG-tdTomato from Anirvan
408 Ghosh, and pCAG-Cre from Addgene (#13775). Plasmid pCAG-Flpo (Addgene #60662) was
409 previously described (Xue et al., 2014). pCAG-hChR2(H134R)-EYFP was created by replacing
410 the EGFP in pCAG-EGFP (Addgene #11150) with the hChR2(H134R)-EYFP from pAAV-
411 EF1α-DIO-hChR2(H134R)-EYFP (Addgene #20298). pAAV-EF1α-DIO-ReaChR-P2A-
412 dTomato was created by replacing the oChIEF(E163A T199C) in pAAV-EF1α-DIO-
413 oChIEF(E163A T199C)-P2A-dTomato (Addgene #51094) with the ReaChR from pAAV-hSyn-
414 FLEX-ReaChR-Citrine (Addgene #50955). pAAV-EF1α-FRT-FLEX-GtACR2-EYFP and

pAAV-EF1α-FRT-FLEX-GtACR1-EYFP were created by replacing the mNaChBac-T2A-tdTomato in pAAV-EF1α-FRT-FLEX-mNaChBac-T2A-tdTomato (Addgene #60658) with the GtACR2-EYFP and GtACR1-EYFP from pLenti-UbiC-GtACR2-EYFP and pLenti-UbiC-GtACR1-EYFP, respectively. Motifs MBD, Nlgn1C, Kv4.2LL, TlcnC, Kv2.1C, Kv2.1C-TlcnC, and Kv2.1C-linker-TlcnC (see ***Figure 4-supplement 1***) were generated by PCR and added to the C-terminus of the GtACR2-EYFP to create pAAV-EF1α-FRT-FLEX-GtACR2-EYFP-MBD, pAAV-EF1α-FRT-FLEX-GtACR2-EYFP-Nlgn1C, pAAV-EF1α-FRT-FLEX-GtACR2-EYFP-Kv4.2LL, pAAV-EF1α-FRT-FLEX-GtACR2-EYFP-TlcnC, pAAV-EF1α-FRT-FLEX-GtACR2-EYFP-Kv2.1C, pAAV-EF1α-FRT-FLEX-GtACR2-EYFP-Kv2.1C-TlcnC, and pAAV-EF1α-FRT-FLEX-GtACR2-EYFP-Kv2.1C-linker-TlcnC, respectively. pAAV-EF1α-FRT-FLEX-GtACR2-KA2N-EGFP was created by replacing the EYFP in pAAV-EF1α-FRT-FLEX-GtACR2-EYFP with the KA2N-EGFP from pAAV-hSyn-soCoChR-EGFP (Addgene #107708, obtained from Dr. Edward Boyden).

In utero electroporation

Female ICR mice were crossed with male C57BL6/J, *Pvalb-2A-Cre*, or *Pvalb-2A-Flpo* mice to obtain timed pregnancies. *In utero* electroporation was performed as previously described (Xue et al., 2014) with a square-wave pulse generator (Gemini X2, BTX Harvard Bioscience). To express GtACR2, GtACR1, iC++, or ChR2 in layer 2/3 pyramidal neurons, pLenti-UbiC-GtACR2-EYFP, pLenti-UbiC-GtACR1-EYFP, pAAV-CaMKIIα-iC++-TS-EYFP, or pCAG-hChR2(H134R)-EYFP (all 2μg/μl) was used, respectively. In a few experiments, pAAV-EF1α-FRT-FLEX-GtACR2-EYFP (2 μg/μl) with pCAG-Flpo (0.2 μg/μl), pAAV-EF1α-FRT-FLEX-GtACR1-EYFP (2 μg/μl) with pCAG-Flpo (0.2 μg/μl), or pAAV-EF1α-DIO-iC++-TS-EYFP (2

438 µg/µl) with pCAG-Cre (0.2 µg/µl) was used to express GtACR2, GtACR1, or iC++,
439 respectively. To express somatodendritically targeted GtACR2 variants in layer 2/3 pyramidal
440 neurons and compare them with wild type GtACR2, the pAAV-EF1α-FRT-FLEX constructs
441 described above were used (all 2 µg/µl) with pCAG-Flpo (0.2 µg/µl). pCAG-tdTomato
442 (0.1µg/µl) was included in all experiments. The plasmid concentrations stated above were final
443 concentrations in the plasmid mix. Transfected pups were identified by the transcranial
444 fluorescence of tdTomato with a MZ10F stereomicroscope (Leica) 1–2 days after birth.
445
446 **AAV production and injection**
447 All recombinant AAV serotype 9 vectors were produced by the Baylor College of Medicine
448 Gene Vector Core except AAV9-hSyn-Flpo (Addgene #60663), which was produced by the
449 Penn Vector Core and was previously described (Xue et al., 2014). To express GtACR2,
450 ReaChR, iC++, or iChloC in Pv neurons, 200–250 nl of the following recombinant AAV
451 serotype 9 vectors at their respective titer were injected into the visual cortex of *Pvalb*$^{Flpo/+}$ (for
452 GtACR2) or *Pvalb*$^{Cre/+}$ (for ReaChR, iC++, or iChloC) mice at postnatal day 1 as previously
453 described (Xue et al., 2014): AAV9-EF1α-FRT-FLEX-GtACR2-EYFP (3.8×10^{13} genome
454 copies/ml), AAV9-EF1α-DIO-ReaChR-P2A-dTomato (7.0×10^{13} genome copies/ml), AAV9-
455 EF1α-DIO-iC++-TS-EYFP (3.71×10^{13} genome copies/ml), and AAV9-EF1α-DIO-iChloC-2A-
456 mCherry (3.7×10^{14} genome copies/ml). To conditionally express GtACR2 in juvenile and adult
457 neurons, mice previously electroporated with plasmid pAAV-EF1α-FRT-FLEX-GtACR2-EYFP
458 into layer 2/3 pyramidal neurons were injected with 200 nl of AAV9-hSyn-Flpo (1.2×10^{12}
459 genome copies/ml) at postnatal day 23, 60, or 64. Injection was performed as previously
460 described (Xue et al., 2014) with an UltraMicroPump III and a Micro4 controller (World

461 Precision Instruments).

462

463 **Brain slice electrophysiology**

464 Mice were anesthetized by an intraperitoneal injection of a ketamine and xylazine mix (80 mg/kg
465 and 16 mg/kg, respectively) and transcardially perfused with cold (0–4°C) slice cutting solution
466 containing 80 mM NaCl, 2.5 mM KCl, 1.3 mM NaH_2PO_4, 26 mM $NaHCO_3$, 4 mM $MgCl_2$, 0.5
467 mM $CaCl_2$, 20 mM D-glucose, 75 mM sucrose and 0.5 mM sodium ascorbate (315 mosmol, pH
468 7.4, saturated with 95% O_2/5% CO_2). Brains were removed and sectioned in the cutting solution
469 with a VT1200S vibratome (Leica) to obtain 300-µm coronal slices. Slices were incubated in a
470 custom-made interface holding chamber saturated with 95% O_2/5% CO_2 at 34 °C for 30 min and
471 then at room temperature for 20 min to 10 h until they were transferred to the recording chamber.
472 Recordings were performed on submerged slices in artificial cerebrospinal fluid (ACSF)
473 containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 mM $MgCl_2$,
474 2.5 mM $CaCl_2$, 20 mM D-glucose and 0.5 mM sodium ascorbate (305 mosmol, pH 7.4, saturated
475 with 95% O_2/5% CO_2, perfused at 3 ml/min) at 30–32°C. For whole-cell recordings, we used a
476 K^+-based pipette solution containing 142 mM K^+-gluconate, 10 mM HEPES, 1 mM EGTA, 2.5
477 mM MgCl2, 4 mM ATP-Mg, 0.3 mM GTP-Na, 10 mM Na2-phosphocreatine (295 mosmol, pH
478 7.35) or a Cs^+-based pipette solution containing 121 mM Cs^+-methanesulfonate, 1.5 mM $MgCl_2$,
479 10 mM HEPES, 10 mM EGTA, 4 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM Na_2-Phosphocreatine,
480 and 2 mM QX314-Cl (295 mosmol, pH 7.35). Membrane potentials were not corrected for liquid
481 junction potential (experimentally measured as 12.5 mV for the K^+-based pipette solution and
482 9.5 mV for the Cs^+-based pipette solution).

483

Neurons were visualized with video-assisted infrared differential interference contrast imaging and fluorescent neurons were identified by epifluorescence imaging under a water immersion objective (40×, 0.8 numerical aperture) on an upright SliceScope Pro 1000 microscope (Scientifica) with an infrared IR-1000 CCD camera (DAGE-MTI). Data were low-pass filtered at 4 kHz and acquired at 10 kHz with an Axon Multiclamp 700B amplifier and an Axon Digidata 1550 Data Acquisition System under the control of Clampex 10.7 (Molecular Devices). Data were analyzed offline using AxoGraph X (AxoGraph Scientific). For the photostimulation of GtACR2-, iC++-, iChloC-, or ChR2-expressing neurons, blue light was emitted from a collimated light-emitting diode (LED) of 455 nm, whereas for the photostimulation of GtACR1- or ReaChR-expressing neurons, red light was emitted from a LED of 617 nm. The LEDs were driven by a LED driver (Mightex) under the control of an Axon Digidata 1550 Data Acquisition System and Clampex 10.7. Light was delivered through the reflected light fluorescence illuminator port and the 40× objective.

Synaptic currents and photocurrents were recorded in the whole-cell voltage clamp mode with the Cs^+-based patch pipette solution. Only recordings with series resistance below 20 MΩ were included. EPSCs and IPSCs were recorded at the reversal potential for IPSCs (-60 mV) and EPSCs (+10 mV), respectively, unless stated otherwise. Photocurrents were recorded at +10 mV unless stated otherwise. For light pulse stimulation, pulse duration (0.5–10 ms) and intensity (2.5–23.6 mW/mm^2) were adjusted for each recording to evoke small (to minimize voltage-clamp errors) but reliable monosynaptic EPSCs or IPSCs. Disynaptic IPSCs were evoked using the same light pulses that were used for evoking the corresponding monosynaptic EPSCs. Light pulses were delivered at 30-s interstimulus intervals. Antidromic spikes in $GtACR2^+$ neurons

were recorded with the K+-based patch pipette solution in whole-cell current clamp mode or with ACSF as the patch pipette solution in the loose-patch current clamp mode.

For pharmacology experiments, the baseline synaptic currents were recorded for at least 3 minutes in the absence of any drug. The drugs were then added to the ACSF at the following concentrations: TTX (1 μM), NBQX (10 μM), (*RS*)-CPP (10 μM), SR95531 (Gabazine, 10 μM), ZD7288 (20 μM), bumetanide (50 or 100 μM), TEA (1.5 mM), and 4-AP (1.5 mM). The synaptic currents were recorded for at least 3 minutes in the presence of drugs. For ZD7288, which did not inhibit GtACR2-induced neurotransmitter release, the efficacy of the drug was monitored by examining the I_h current of cortical layer 5 pyramidal neurons.

Fluorescent microscopy

Fluorescent images were taken from live brain slices, except for the conditional expression of GtACR2 in adults, where images were taken from fixed brain slices. Live brain slices were prepared as described for slice electrophysiology. For the fixed brain slices, mice were anesthetized by an intraperitoneal injection of a ketamine and xylazine mix (80 mg/kg and 16 mg/kg, respectively) and transcardially perfused with phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS (pH 7.4). Brains were further fixed overnight in 4% paraformaldehyde, cryoprotected with 30% sucrose in PBS, and frozen in optimum cutting-temperature medium until sectioning. A HM 450 Sliding Microtome (Thermo Scientific) was used to section the brains to obtain 30–50-mm coronal slices. Images were acquired on an Axio Zoom.V16 Fluorescence Stereo Zoom Microscope (Zeiss) and processed using National Institutes of Health ImageJ.

To determine the EYFP (or EGFP) fluorescence ratio between layer 5 and layer 2/3, one or two 350 μm-wide rectangular regions that were perpendicular to the pia and spanned all 6 cortical layers were selected in the most transfected regions of each slice. The mean EYFP (or EGFP) fluorescence was measured for layer 5 and layer 2/3 within the selected area. The mean tdTomato fluorescence was measured similarly for layer 5 and layer 2/3. The mean background fluorescence was measured from a nearby rectangular region (140.5 μm by 90.8 μm) where no cellular EYFP (or EGFP) and tdTomato fluorescence was present. The normalized EYFP (or EGFP) fluorescence ratio between layer 5 and layer 2/3 was calculated by

$$\frac{Layer\ 5_{EYFP}-Background_{EYFP}}{Layer\ 2/3_{EYFP}-Background_{EYFP}} \Big/ \frac{Layer\ 5_{tdTomato}-Background_{tdTomato}}{Layer\ 2/3_{tdTomato}-Background_{tdTomato}}.$$

To determine the ratio of EYFP fluorescence to tdTomato fluorescence in the callosal projections, one or two rectangular regions that contained the tdTomato-labeled axons were selected in each slice to measure the mean EYFP and tdTomato fluorescence. The mean background fluorescence was measured in a nearby cortical area spanning the same layers. The ratio of EYFP fluorescence to tdTomato fluorescence was calculated by

$$\frac{Fluorescence_{EYFP}-Background_{EYFP}}{Fluorescence_{tdTomato}-Background_{tdTomato}}.$$

Statistics

All reported sample numbers (*n*) represent biological replicates that are the numbers of recorded neurons for electrophysiology or the numbers of analyzed regions of interest (ROI) for fluorescent images. Statistical analyses were performed with Prism 7 (GraphPad Software). We first determined whether the data were normally distributed by performing the D'Agostino &

553 Pearson test, Shapiro-Wilk test, and KS test. If all data within one experiment passed all three
554 normality tests, we then performed the statistical test that assumes a Gaussian distribution.
555 Otherwise, we performed the statistical test that assumed a non-Gaussian distribution. All
556 statistical tests were two-tailed with an alpha of 0.05.
557
558 Wilcoxan matched-pairs signed rank test was used for Figure 1F,K; Figure 2B,D,F,J; Figure 2-
559 supplement 2B,E; Figure 2-supplement 3B (EPSCs); Figure 3B; and Figure 3-supplement 1C.
560 Paired *t* test was used for Figure 1G,J; Figure 2H; Figure 2-supplement 1D; and Figure 2-
561 supplement 3B (photocurrents),D. *t* test with Welch's correction was used for Figure 4E and
562 Figure 5C (WT vs. Kv2.1C). Mann-Whitney test was used for Figure 5C (WT vs. Kv2.1C-
563 linker-TlcnC),F,G,H,J. One-way ANOVA with Greenhouse-Geisser correction and Tukey
564 multiple comparisons was used for Figure 3D,F. Kruskal-Wallis test with Dunn's multiple
565 comparisons was used for Figure 4B.
566

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569 EYFP and pLenti-UbiC-GtACR1-EYFP plasmids, Anirvan Ghosh for the pCAG-tdTomato
570 plasmid, Karl Deisseroth for the pAAV-CaMKIIα-iC++-TS-EYFP and pAAV-EF1α-DIO-iC++-
571 TS-EYFP plasmids, Matthew Caudill and Massimo Scanziani for the pAAV-EF1α-DIO-iChloC-
572 T2A-mCherry plasmid, Zhuo-Hua Pan for the pAAV-EF1α-DIO-hChR2(H134R)-EYFP-
573 Kv2.1C plasmid, Edward Boyden for the pAAV-hSyn-soCoChR-EGFP plasmid, and Hsiao-
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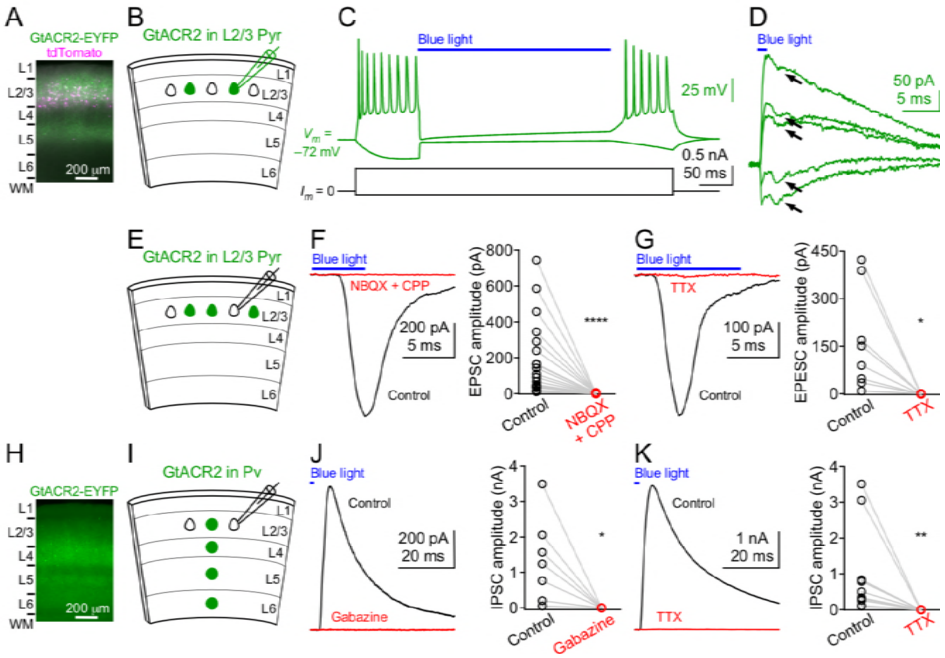
Competing interests

The authors declare no competing financial interests.

583 **Figures and legends**

Figure 1



584 **Figure 1. Light activation of GtACR2 causes excitatory and inhibitory neurons to release**

585 **neurotransmitter.**

586 (**A**) A representative fluorescent image of the visual cortex showing GtACR2-EYFP and

587 tdTomato expression in a subset of layer 2/3 pyramidal neurons. Note the strong EYFP

588 fluorescence in layer 5 that contains the axons of layer 2/3 pyramidal neurons. L, layer; WM,

589 white matter.

590 (**B**) Schematic of slice experiments in (C,D). GtACR2 in a subset of layer 2/3 pyramidal neurons.

591 (**C**) Membrane potentials (upper panel) in response to somatic current injections (lower panel)

592 from a GtACR2$^+$ neuron. Blue light activation of GtACR2 suppressed the action potentials

593 evoked by current injections (n = 5).

(**D**) Blue light-induced membrane currents recorded at membrane potentials of -75, -77, -78, -79, and -80 mV from the same GtACR2^{+} neuron in (C). Note the EPSC-like inward currents (arrows) superimposed on the GtACR2-mediated photocurrents (n = 2).

(**E**) Schematic of slice experiments in (F,G). GtACR2 in a subset of layer 2/3 pyramidal neurons.

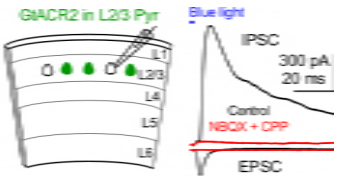
(**F**) Left, photoactivation of GtACR2 generated EPSCs in a GtACR2^{-} neuron, which were abolished by the glutamate receptor antagonists, NBQX and CPP. Right, summary graph of similar experiments (n = 17, $P < 0.0001$).

(**G**) Left, photoactivation of GtACR2 generated EPSCs in a GtACR2^{-} neuron, which were abolished by the voltage-gated sodium channel blocker, TTX. Right, summary graph of similar experiments (n = 8, P = 0.02).

(**H**) A representative fluorescent image of the visual cortex showing GtACR2-EYFP expression in Pv neurons.

(**I,J,K**) As in (E,F,G), but for GtACR2 in Pv neurons. GtACR2 activation-induced IPSCs were abolished by the $GABA_A$ receptor antagonist, Gabazine (J, n = 7, P = 0.02) or TTX (K, n = 10, P = 0.002).

Figure 1-supplement 1



610

611 **Figure 1-supplement 1. Recruitment of inhibitory neurons by activating GtACR2 in**

612 **excitatory neurons.**

613 Left, schematic of slice experiments. GtACR2 in a subset of layer 2/3 pyramidal neurons. Right,

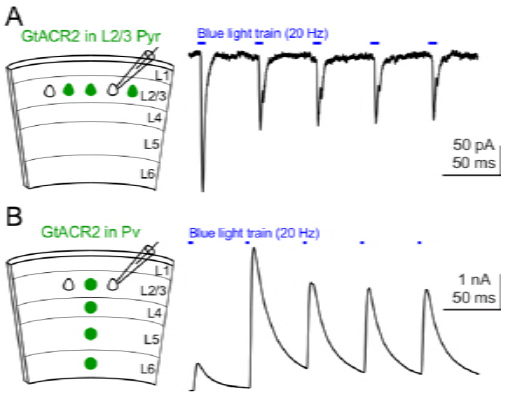
614 photoactivation of GtACR2 generated an EPSC (inward current) and IPSC (outward current) in a

615 GtACR2^{-} neuron. The IPSC was abolished by the glutamatergic receptor antagonists, NBQX and

616 CPP (red trace), indicating its disynaptic nature (n = 3).

617

Figure 1-supplement 2



618

619 **Figure 1-supplement 2. Repetitive activation of GtACR2 reliably causes neurotransmitter**

620 **release.**

621 (**A**) Left, schematic of slice experiments. GtACR2 in a subset of layer 2/3 pyramidal neurons.

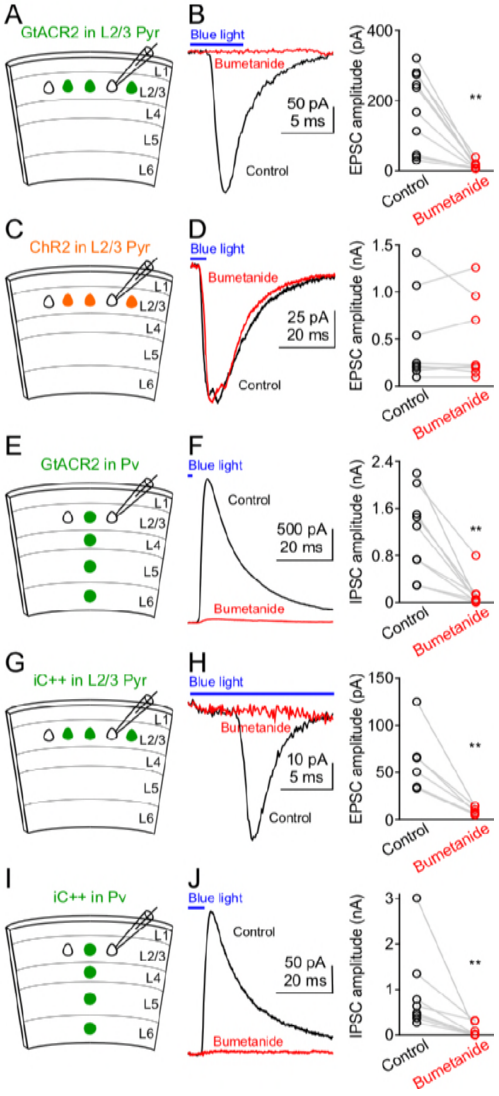
622 Right, a 20-Hz train of blue-light pulses activated GtACR2 to generate EPSCs in a GtACR2$^-$

623 neuron (n = 11).

624 (**B**) As in (A), but for GtACR2 in Pv neurons. A 20-Hz train of blue-light pulses activated

625 GtACR2 to generate IPSCs in a GtACR2$^-$ neuron (n = 14).

Figure 2



626

627 **Figure 2. Reducing intracellular chloride concentrations diminishes the neurotransmitter**

628 **release induced by activation GtACR2 or iC++.**

629 (**A**) Schematic of slice experiments in (B). GtACR2 in a subset of layer 2/3 pyramidal neurons.

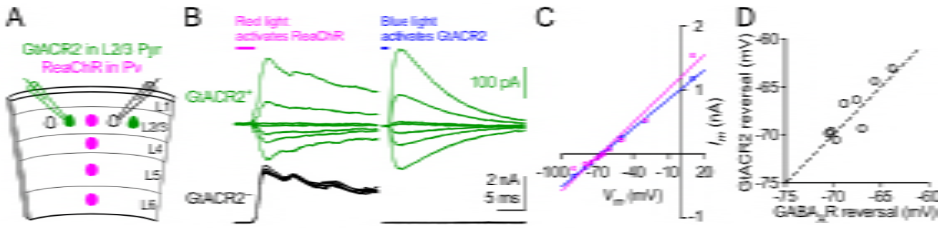
630 (**B**) Left, photoactivation of GtACR2 generated EPSCs in a GtACR2^{-} neuron, which were

631 diminished by decreasing intracellular chloride concentrations with an NKCC1 blocker,

632 bumetanide. Right, summary graph of similar experiments (n = 10, P = 0.002).

633 (**C,D**) As in (A,B), but for ChR2. ChR2 activation-induced EPSCs were not affected by
634 bumetanide (n = 8, P = 0.9).
635 (**E,F**) As in (A,B), but for GtACR2 in Pv neurons. GtACR2 activation-induced IPSCs were
636 diminished by bumetanide (n = 9, P = 0.004).
637 (**G,H**) As in (A,B), but for iC++. iC++-induced EPSCs were diminished by bumetanide (n = 6, P
638 = 0.009).
639 (**I,J**) As in (E,F), but for iC++. iC++-induced IPSCs were diminished by bumetanide (n = 8, P =
640 0.008).
641

Figure 2-supplement 1



642

643 **Figure 2-supplement 1. The reversal potential of GtACR2 at the soma is similar to that of**

644 **$GABA_A$ receptor.**

645 (**A**) Schematic of slice experiments in (B). GtACR2 in a subset of layer 2/3 pyramidal neurons

646 and ReaChR in Pv neurons.

647 (**B**) In a GtACR2$^+$ neuron, GABAergic IPSCs induced by activating ReaChR in Pv neurons (left

648 panel) and GtACR2-mediated photocurrents (right panel) were sequentially recorded at different

649 membrane potentials. The example traces show the currents at -50, -60, -65, -66, -68, -70, and -

650 75 mV. A GtACR2$^-$ neuron was simultaneously recorded at the membrane potential of +10 mV

651 to ensure that the blue light did not generate IPSCs (right panel).

652 (**C**) The amplitudes of IPSCs and GtACR2 photocurrents were plotted as a function of the

653 membrane potentials for the GtACR2$^+$ neuron in (B). Each set of data were fit with linear

654 regression and the reversal potentials were determined by the V_m-axis intercepts.

655 (**D**) Summary graph of similar experiments in (B,C) where the reversal potential of IPSCs was

656 plotted against that of GtACR2 photocurrents for each GtACR2$^+$ neuron. All data points fall

657 close to the dotted unity line and there is no significant difference between the reversal potentials

658 of IPSCs and photocurrents (n = 8, P = 0.4).

659

660

Figure 2-supplement 2

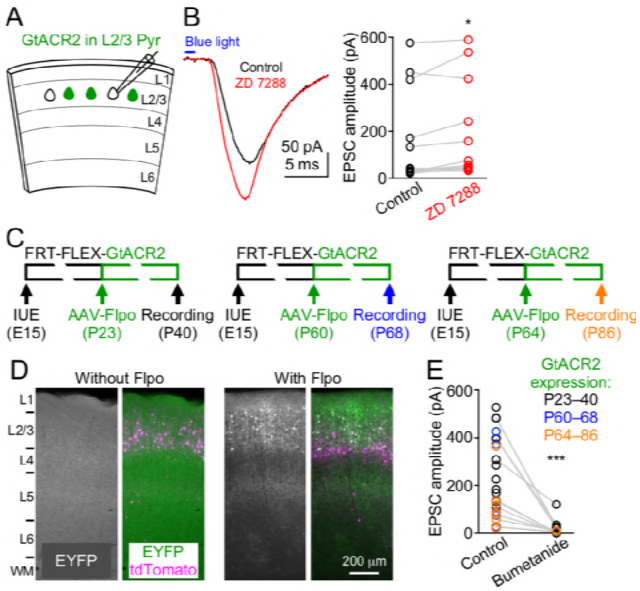


Figure 2-supplement 2. GtACR2-induced neurotransmitter release is not due to rebound depolarization or its long-term expression throughout development.

(**A**) Schematic of slice experiments in (B). GtACR2 in a subset of layer 2/3 pyramidal neurons.

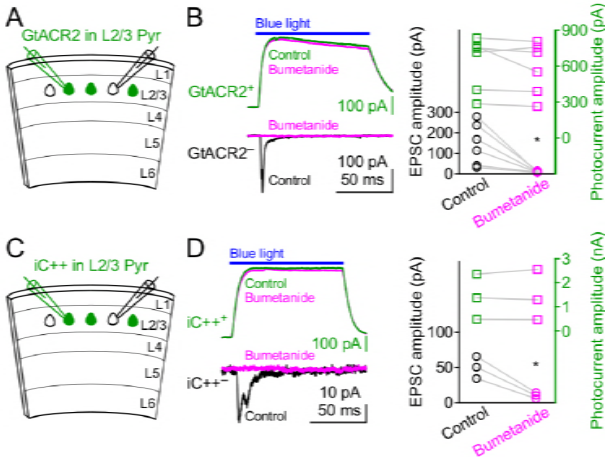
(**B**) Left, photoactivation of GtACR2 generated EPSCs in a GtACR2$^-$ neuron, which were not decreased by blocking the I_h current with ZD7288 (20 μM). Right, summary graph of similar experiments showing that the EPSC amplitudes were slightly increased by ZD7288 (n = 12, P = 0.03).

(**C**) Schematics of conditional expression of GtACR2 in a subset of layer 2/3 pyramidal neurons. A Flpo-dependent plasmid (FRT-FLEX-GtACR2) was electroporated at embryonic day 15 (E15) and AAV9-hSyn-Flpo was injected into the electroporated mice at different postnatal days (P23, P60, or P64). Green bars indicate the approximate GtACR2 expression time windows.

(**D**) Left, representative fluorescent images of the visual cortex obtained at postnatal day 73 showing that without injection of AAV9-hSyn-Flpo, transfected neurons (labeled by tdTomato)

675 did not express GtACR2-EYFP (left panel, n = 5 mice). Note that the image from the EYFP
676 channel was overexposed to show no $EYFP^+$ neurons. Right, representative fluorescent images
677 of the visual cortex obtained at postnatal day 40 showing that the expression of GtACR2-EYFP
678 in transfected neurons was turned on by the injection of AAV9-hSyn-Flpo at postnatal day 23
679 (right panel, n = 7 mice).
680 (**E**) Photoactivation of GtACR2 generated EPSCs in $GtACR2^-$ neurons when GtACR2 was
681 expressed during 3 different time windows as indicated in (C). Bumetanide was applied in a
682 subset of experiments and the EPSCs were diminished (n = 12, P = 0.0005).
683

Figure 2-supplement 3



684

685 **Figure 2-supplement 3. Bumetanide does not affect GtACR2 and iC++-mediated**

686 **photocurrents.**

687 (**A**) Schematic of slice experiments in (B). GtACR2 in a subset of layer 2/3 pyramidal neurons.

688 (**B**) Left, photoactivation of GtACR2 generated photocurrents in a GtACR2^{+} neuron (top panel,

689 recorded at the membrane potential of +10 mV) and EPSCs in a simultaneously recorded

690 GtACR2^{-} neuron (bottom panel, recorded at the membrane potential of -60 mV). Bumetanide

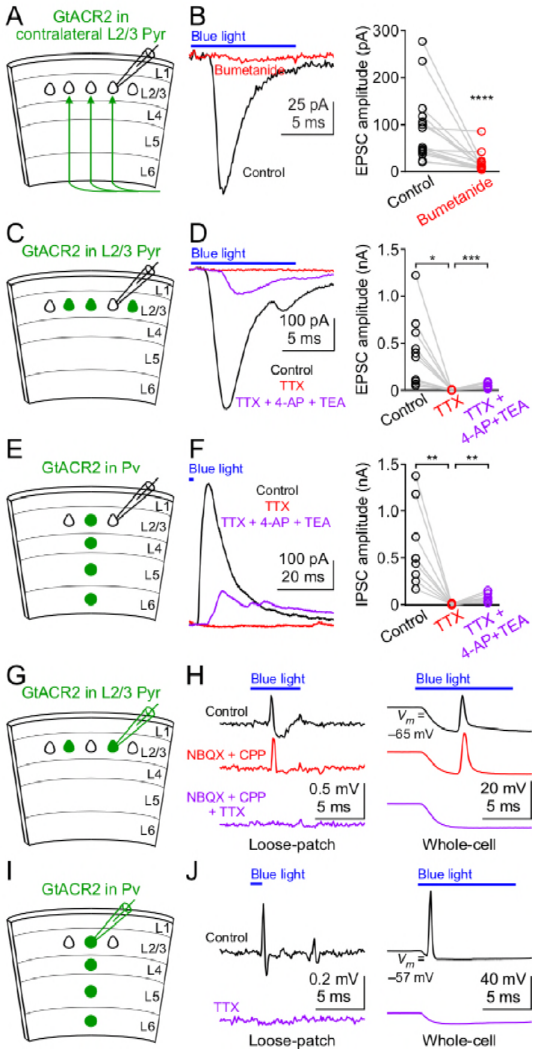
691 diminished the EPSCs (circle symbols) without affecting the photocurrents (square symbols).

692 Right, summary graph of similar experiments (n = 6; EPSCs, P = 0.03; photocurrents, P = 0.3).

693 (**C,D**) As in (A,B), but for iC++. Bumetanide diminished the EPSCs without affecting the

694 photocurrents (n = 3; EPSCs, P = 0.03; photocurrents, P = 0.7).

Figure 3



695

696 **Figure 3. GtACR2 activation directly depolarizes the distal axon and presynaptic terminals**

697 **and can result in antidromic spikes.**

698 (**A**) Schematic of slice experiments in (B). GtACR2 in a subset of layer 2/3 pyramidal neurons in

699 the contralateral hemisphere.

700 (**B**) Left, photoactivation of GtACR2 in the callosal axons, severed from their somas, generated

701 EPSCs in a GtACR2^{-} neuron, which were diminished by bumetanide. Right, summary graph of

702 similar experiments (n = 15, $P < 0.0001$).

(**C**) Schematic of slice experiments in (D). GtACR2 in a subset of layer 2/3 pyramidal neurons.

(**D**) Left, photoactivation of GtACR2 generated EPSCs in a GtACR2^{-} neuron, which were abolished by TTX, but partially recovered by the addition of voltage-gated potassium channel blockers, 4-AP (1.5 mM) and TEA (1.5 mM). Right, summary graph of similar experiments (n = 10; TTX vs. control, P = 0.02, average EPSC amplitude in TTX was 2% of control; TTX + 4-AP + TEA vs. TTX, P = 0.0004, average EPSC amplitude in TTX + 4-AP + TEA was 35% of control).

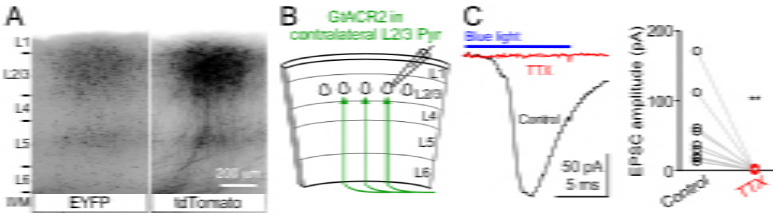
(**E,F**) As in (C,D), but for Pv neurons. GtACR2 activation-induced IPSCs were abolished by TTX, but partially recovered by 4-AP and TEA (n = 9; TTX vs. control, P = 0.006, average IPSC amplitude in TTX was 0.9% of control; TTX + 4-AP + TEA vs. TTX, P = 0.008, average IPSC amplitude in TTX + 4-AP + TEA was 23% of control).

(**G**) Schematic of slice experiments in (H). GtACR2 in a subset of layer 2/3 pyramidal neurons.

(**H**) Photoactivation of GtACR2 generated antidromic spikes in GtACR2^{+} pyramidal neurons, which were not affected by NBQX and CPP, but were abolished by TTX in both loose-patch (left panel) and whole-cell (right panel) recordings. In the whole-cell recordings, the resting membrane potentials of those neurons that generated antidromic spikes are -68.1 ± 1.7 mV (mean ± s.e.m., n = 21).

(**I,J**) As in (G,H), but for Pv neurons. The antidromic spikes in GtACR2^{+} Pv neurons were abolished by TTX. In the whole-cell recordings, the resting membrane potentials of those neurons that generated antidromic spikes are -60.0 ± 2.4 mV (mean ± s.e.m., n = 7).

Figure 3-supplement 1



724

725 **Figure 3-supplement 1. Activation of GtACR2 in the callosal axons causes**

726 **neurotransmitter release.**

727 (**A**) GtACR2-EYFP and tdTomato were expressed in a subset of layer 2/3 pyramidal neurons in

728 the visual cortex of one hemisphere. Representative fluorescent images of the contralateral

729 hemisphere showing GtACR2-EYFP (left panel) in the callosal projections labeled by tdTomato

730 (right panel). L, layer; WM, white matter.

731 (**B**) Schematic of slice experiments in (C). GtACR2-EYFP in a subset of layer 2/3 pyramidal

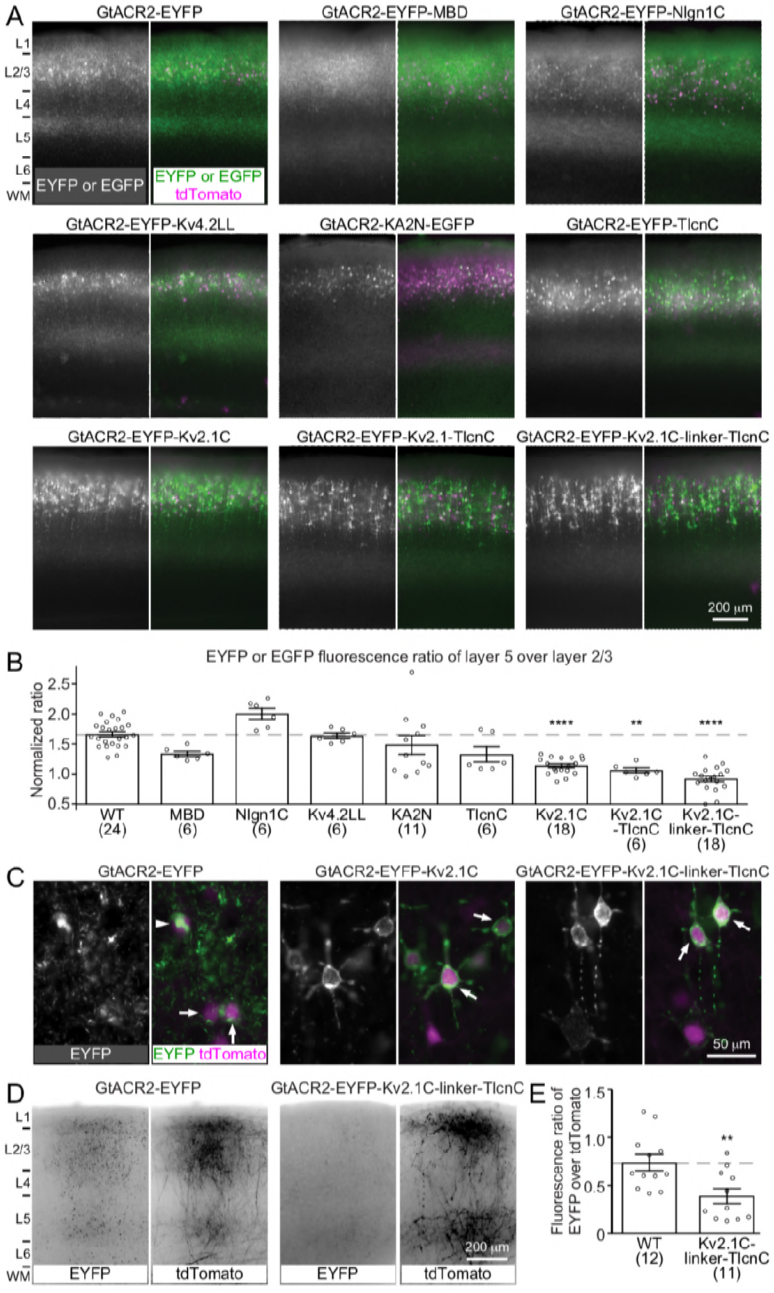
732 neurons in the contralateral hemisphere.

733 (**C**) Left, photoactivation of GtACR2 in the callosal axons generated EPSCs in a GtACR2^{-}

734 neuron, which were abolished by TTX. Right, summary graph of similar experiments (n = 9, P =

735 0.004).

Figure 4



736

737 **Figure 4. Targeting GtACR2 to neuronal somatodendritic domain.**

738 (**A**) Wild type (WT) GtACR2 and its variants tagged with EYFP or EGFP were expressed along

739 with tdTomato in a subset of layer 2/3 pyramidal neurons. Representative fluorescent images of

740 the cortices showing the distribution of GtACR2. Note the strong EYFP fluorescence in layer 5

741 for WT GtACR2 and weak EYFP fluorescence for some somatodendritically targeted GtACR2
742 variants (e.g., Kv2.1C-linker-TlcnC).
743 (**B**) The EYFP or EGFP fluorescence ratio between layer 5 and layer 2/3 was normalized by the
744 tdTomato fluorescence ratio between layer 5 and layer 2/3. A reduction in the normalized EYFP
745 or EGFP fluorescence ratio indicates a shift of the EYFP or EGFP distribution from the axon to
746 somatodendritic domain.
747 (**C**) Representative fluorescent images of electroporated neurons showing that GtACR2-EYFP-
748 Kv2.1C and GtACR2-EYFP-Kv2.1C-linker-TlcnC were more concentrated on the membranes at
749 the soma and proximal dendrites (arrows) than GtACR2-EYFP. Note the intracellular
750 aggregation of GtACR2-EYFP (arrow head).
751 (**D**) Representative fluorescent images of the contralateral hemisphere showing less GtACR2-
752 EYFP-Kv2.1C-linker-TlcnC in the callosal projections than GtACR2-EYFP. The callosal
753 projections were labeled by tdTomato.
754 (**E**) The ratio of EYFP fluorescence to tdTomato fluorescence in the callosal projections in the
755 contralateral hemisphere.
756 The numbers of analyzed slices were indicated in the panel. The columns and error bars are
757 mean ± s.e.m. ** $P < 0.01$, **** $P < 0.0001$.
758

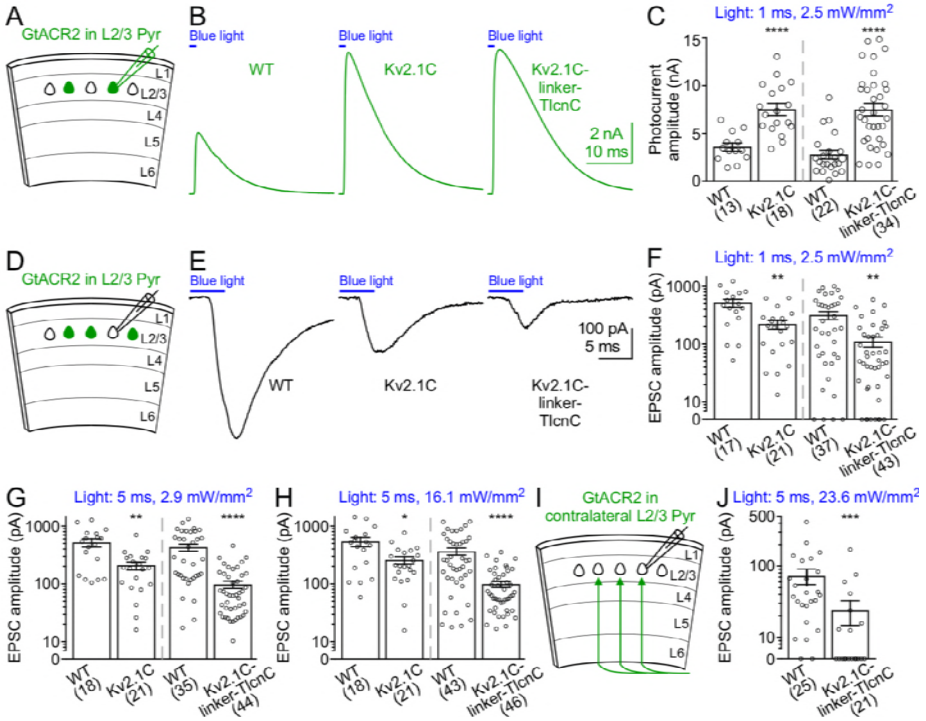
759 **Figure 4-supplement 1. Sequences of somatodendritic targeting motifs.**

760 Black, amino acid sequences of somatodendritic targeting motifs; blue, GtACR2; green, EYFP

761 or EGFP; magenta, linker.

Motif	Sequence
MBD	*GtACR2*-*EYFP*-GSGSGTRGSGS-RDQPLNSKKKKRLLSFRDVDFEEDSD
Nlgn1C	*GtACR2*-*EYFP*-VVLRTACPPDYTLAMRRSPDDIPLMTPNTITM
Kv4.2LL	*GtACR2*-*EYFP*-FETQHHHLLHCLEKTT
KA2N	*GtACR2*-GGSGGTGGSGGT-MPAELLLLLIVAFANPSCQVLSSLRMAAILDDQTVCGRGERLALALAREQINGIIEVPAKARVEVDIFELQRDSQYETTDTMCQILPKGVVSVLGPSSSPASASTVSHICGEKEIPHIKVGPEETPRLQYLRFASVSLYPSNEDVSLAVS-GASGGT-*EGFP*
TlcnC	*GtACR2*-*EYFP*-AESPADGEVFAIQLTSS
Kv2.1C	*GtACR2*-*EYFP*-QSQPILNTKEMAPQSKPPEELEMSSMPSPVAPLPARTEGVIDMRSMSSIDSFISCATDFPEATRF
Kv2.1C-TlcnC	*GtACR2*-*EYFP*-QSQPILNTKEMAPQSKPPEELEMSSMPSPVAPLPARTEGVIDMRSMSSIDSFISCATDFPEATRF-AESPADGEVFAIQLTSS
Kv2.1C-linker-TlcnC	*GtACR2*-*EYFP*-QSQPILNTKEMAPQSKPPEELEMSSMPSPVAPLPARTEGVIDMRSMSSIDSFISCATDFPEATRF-GSGSGSGSGS-AESPADGEVFAIQLTSS

Figure 5



762

763 **Figure 5. Activation of somatodendritically targeted GtACR2 variants generate larger**

764 **photocurrents but cause less neurotransmitter release than wild type GtACR2.**

765 (**A**) Schematic of slice experiments in (B,C). GtACR2-EYFP, GtACR2-EYFP-Kv2.1C, or

766 GtACR2-EYFP-Kv2.1C-linker-TlcnC in a subset of layer 2/3 pyramidal neurons.

767 (**B**) Somatic photocurrents recorded at the membrane potential of +10 mV from GtACR2-

768 EYFP-, GtACR2-EYFP-Kv2.1C-, or GtACR2-EYFP-Kv2.1C-linker-TlcnC-expressing neurons

769 in response to a blue light pulse of 1-ms and 2.5-mW/mm^2.

770 (**C**) Summary graph showing the photocurrent peak amplitudes of GtACR2-EYFP-Kv2.1C and

771 GtACR2-EYFP-Kv2.1C-linker-TlcnC were larger than those of GtACR2-EYFP.

772 (**D,E**) As in (A,B), but for recording EPSCs in GtACR2$^-$ layer 2/3 pyramidal neurons in response

773 to a blue light pulse of 5-ms and 2.9-mW/mm^2.

774 (**F,G,H**) Summary graphs showing the EPSC amplitudes in response to a blue light pulse of 1-
775 ms and 2.5 mW/mm^2 (F), 5-ms and 2.9 mW/mm^2 (G), or 5-ms and 16.1 mW/mm^2 (H).
776 (**I**) Schematic of slice experiments in (J). GtACR2-EYFP or GtACR2-EYFP-Kv2.1C-linker-
777 TlcnC in a subset of layer 2/3 pyramidal neurons in the contralateral hemisphere.
778 (**J**) Summary graphs showing the EPSC amplitudes in response to a blue light pulse of 5-ms and
779 23.6 mW/mm^2.
780 The numbers of recorded neurons were indicated in the panel. The columns and error bars are
781 mean ± s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.
782

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