

# Altered chloride homeostasis removes synaptic inhibitory constraint of the stress axis

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In mammals, stress elicits a stereotyped endocrine response that requires an increase in the activity of hypothalamic parvocellular neuroendocrine neurons. The output of these cells is normally constrained by powerful GABA-mediated synaptic inhibition. We found that acute restraint stress in rats released the system from inhibitory synaptic drive *in vivo* by down-regulating the transmembrane anion transporter KCC2. This manifested as a depolarizing shift in the reversal potential of GABA<sub>A</sub>-mediated synaptic currents that rendered GABA inputs largely ineffective. Notably, repetitive activation of GABA synapses after stress resulted in a more rapid collapse of the anion gradient and was sufficient to increase the activity of neuroendocrine cells. Our data indicate that hypothalamic neurons integrate psychological cues to mount the endocrine response to stress by regulating anion gradients.

The brain responds to perceived stress by invoking neural and hormonal changes that have widespread effects throughout the body. In mammals, stress activates corticotrophin-releasing hormone neurons in the paraventricular nucleus of the hypothalamus (PVN), which in turn increase circulating levels of corticosteroids. The activity of these neurons is tightly regulated by GABAergic synaptic input<sup>1,2</sup> that originates from interneuron populations located adjacent to the nucleus<sup>1-3</sup>. This inhibition is critical, as manipulations that decrease GABA drive to these cells increase neural activity and circulating corticosteroids. Given that synaptic inhibition actively restrains the output of this system, it has been proposed that release from inhibition is necessary for the initiation of the neuroendocrine response to stress<sup>4</sup>. However, the cellular mechanisms through which this inhibitory release may be accomplished have not been resolved. In the CNS, fast GABA transmission relies on the influx of Cl<sup>-</sup> through the GABA<sub>A</sub> receptor. Influx of Cl<sup>-</sup> is exquisitely sensitive to the ionic electrochemical gradient<sup>5,6</sup>. Anionic Cl<sup>-</sup> homeostasis in adults is primarily maintained by KCC2 and previous work has demonstrated that disruption of transporter activity via pharmacological or physiological manipulations strongly affects the efficacy of synaptic inhibition<sup>7-11</sup>.

We hypothesized that alterations in postsynaptic Cl<sup>-</sup> homeostasis, which ultimately reduce synaptic inhibition, may contribute to the disinhibition and increased excitability of neuroendocrine neurons following acute stress. Using an *in vivo* restraint stress protocol combined with *in vivo* microinjections, hormone measurements and patch clamp recordings from brain slices prepared from control and stressed animals, we found that a single bout of acute stress neutralized inhibitory signaling via a loss of KCC2 function and a resulting decrease in Cl<sup>-</sup> extrusion capacity.

## RESULTS

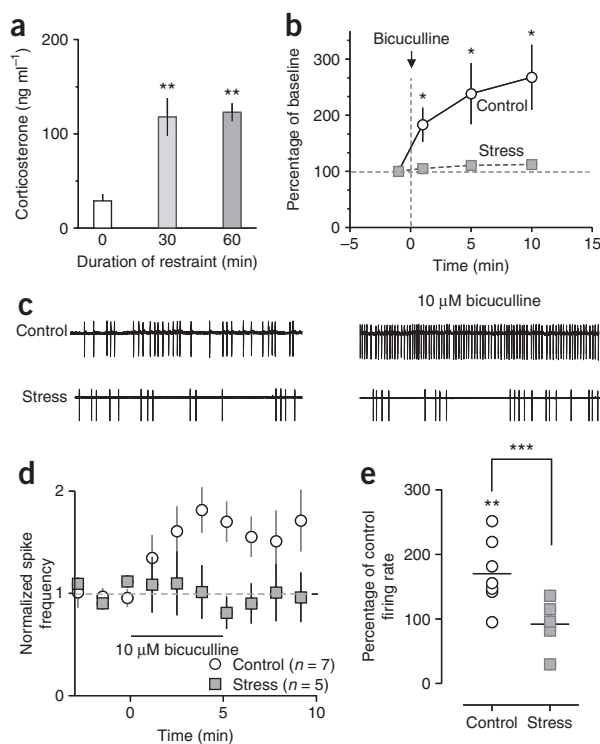
### Acute stress decreases synaptic GABA<sub>A</sub>-mediated inhibition

We first established that a 30-min restraint stress protocol was sufficient to increase circulating corticosteroids. Immediately following the protocol, we measured a robust and reliable increase in circulating corticosteroids (control = 28.9 ± 7.3 ng ml<sup>-1</sup>, *n* = 6; 30-min protocol = 117.9 ± 20.1 ng ml<sup>-1</sup>, *n* = 6; **Fig. 1a**). Because a 60-min restraint elicited a similar elevation of corticosteroids (122.9 ± 9.7, *n* = 6, *P* < 0.01 compared with control), we used a 30-min restraint stress for all subsequent experiments.

We then conducted *in vivo* experiments to examine the effect of synaptic GABAergic inhibition after stress. Pharmacological blockade of GABA<sub>A</sub> receptors in PVN elicits a robust increase in circulating corticosteroids<sup>4</sup> in control conditions. Because an effective stress response requires a disinhibition of neuroendocrine neurons, we hypothesized that synaptic GABA<sub>A</sub>-mediated inhibition may be compromised following stress. To test this possibility, we injected the GABA<sub>A</sub> antagonist bicuculline (2 μl, 0.1 nM) directly into PVN and measured the corticosteroid response at different times postinjection (**Fig. 1b**). Consistent with a previous report<sup>4</sup>, this manipulation elicited a robust increase in circulating corticosteroids in control animals (1 min = 183.3 ± 30.6%, 5 min = 238.5 ± 54.4%, 10 min = 267.6 ± 58.1%; all values reported as a percentage of control). In contrast, bicuculline microinjection had no effect on circulating corticosteroids in stressed animals (1 min = 105.0 ± 8.2%, 5 min = 110.5 ± 6.3%, 10 min = 112.4 ± 6.8%; *P* < 0.05 for each time point compared with identical time points in control animals). This observation is consistent with our hypothesis that, following stress, the ability of inhibitory synaptic GABA<sub>A</sub> to dampen the activity of neuroendocrine cells and curb corticosteroid release is markedly reduced.

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**Figure 1** Acute stress reduces the strength of synaptic GABA<sub>A</sub>-mediated inhibition. **(a)** Acute restraint stress (30 or 60 min) significantly increased circulating corticosteroids ( $n = 6$  for each group,  $P < 0.01$  compared with control). **(b)** Summary of the percentage change in circulating corticosteroids following microinjection of the GABA<sub>A</sub> antagonist bicuculline into PVN. Corticosteroids levels in naive, unstressed rats ( $n = 10$ ) were significantly increased compared with those in stressed rats ( $n = 7$ ) ( $P < 0.05$  compared with vehicle injection). **(c)** Cell-attached recordings showed effects of bath application of bicuculline (10  $\mu$ M) in controls (left) and after stress (right). **(d)** Summary data showing that blockade of GABA<sub>A</sub> receptors caused an increase in neuronal activity in control, but had no effect following stress. **(e)** Effect of bicuculline on spike rate in each cell tested under control and stress conditions. The firing rate was calculated 5 min after onset of bicuculline (control,  $170 \pm 20\%$  of baseline; stress,  $92 \pm 17\%$  of baseline;  $* P < 0.05$ ;  $** P < 0.01$  versus control baseline,  $*** P < 0.01$  versus bicuculline in stress). All values are mean  $\pm$  s.e.m.

extrusion capacity through a downregulation in the activity of KCC2. To directly test for changes in Cl<sup>-</sup> homeostasis following stress, we measured  $E_{\text{GABA}}$  in slices obtained from an acutely stressed animal. Using gramicidin (40  $\mu$ M) perforated patch recordings<sup>25</sup>, we first examined the effect of postsynaptic membrane voltage on evoked inhibitory postsynaptic currents (eIPSCs) in control slices (**Fig. 2a**). When this experiment was repeated following stress, we observed a depolarizing shift in  $E_{\text{GABA}}$  (control =  $-70 \pm 3.4$  mV,  $n = 11$ ; stress =  $-55 \pm 2.3$  mV,  $n = 7$ ;  $P < 0.01$ ; **Fig. 2a**). A depolarizing shift in  $E_{\text{GABA}}$  was also observed when the whole-cell recording configuration was used (control =  $-54.6 \pm 1.6$  mV,  $n = 17$ ; stress =  $-47.9 \pm 1.8$  mV,  $n = 21$ ;  $P < 0.01$ ; **Supplementary Fig. 1** online). This is consistent with previous reports showing that  $E_{\text{GABA}}$  is a reliable reporter for changes in Cl<sup>-</sup> homeostasis, even in whole-cell conditions<sup>12</sup>. Consequently, unless otherwise specified, we used the whole-cell recording configuration for the remainder of the experiments in which we assessed  $E_{\text{GABA}}$ . Both chronic stress and prolonged application of corticosteroids are reported to decrease the release probability at GABA synapses in PVN<sup>26</sup> and may also decrease the expression of GABA<sub>A</sub> receptors<sup>27</sup>. We found no changes in release probability (as assessed by calculating the paired pulse ratio, PPR) following acute stress ( $\text{PPR}_{\text{control}} = 0.84 \pm 0.1$ ,  $n = 21$ ;  $\text{PPR}_{\text{stress}} = 0.93 \pm 0.1$ ,  $n = 20$ ;  $P > 0.05$ ). Our observations indicate that stress causes a depolarizing shift in  $E_{\text{GABA}}$  and suggest that mechanisms that control the transmembrane anion gradient, such as KCC2, may be important in promoting synaptic disinhibition *in vivo*.

### $E_{\text{GABA}}$ is determined by KCC2

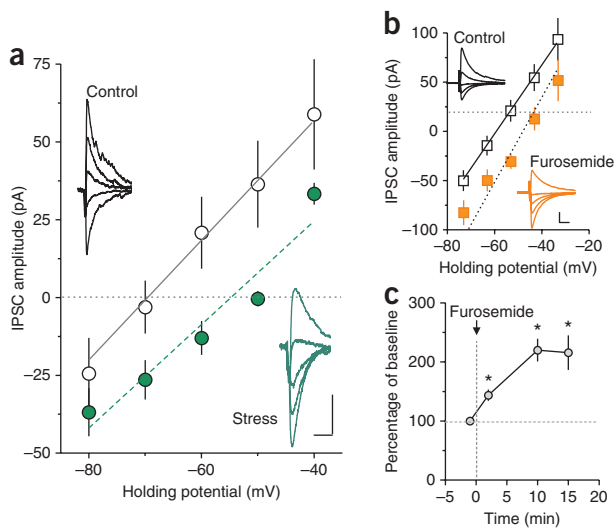
To determine the contributions of KCC2 to setting  $E_{\text{GABA}}$  under these conditions, we first used a pharmacological inhibitor of KCC2, furosemide (200  $\mu$ M), and tested its effects on  $E_{\text{GABA}}$ . Consistent with previous reports<sup>12</sup>, this manipulation caused a depolarizing shift in  $E_{\text{GABA}}$  (whole-cell recording: control =  $-59.1 \pm 1.4$  mV,  $n = 21$ ; furosemide =  $-48.0 \pm 2.2$  mV,  $n = 16$ ,  $P < 0.05$  compared with control; **Fig. 2b**). To demonstrate that KCC2 inhibition is responsible for the stress-induced shift in  $E_{\text{GABA}}$ , we examined the effects of furosemide on slices obtained from stressed animals. In these conditions, furosemide elicited no further changes in  $E_{\text{GABA}}$  (whole-cell recording: paired data, stress =  $-50.0 \pm 3.0$  mV, stress plus furosemide =  $-49.1 \pm 2.3$  mV,  $n = 6$ ,  $P > 0.05$ ; **Supplementary Fig. 2** online). Because furosemide can also partially inhibit NKCC1 (refs. 5,14,19,28), we conducted additional experiments using the specific NKCC1 inhibitor bumetanide (10  $\mu$ M). Bumetanide had no effect on  $E_{\text{GABA}}$  (whole-cell recording: control =  $-54.6 \pm 1.6$  mV,  $n = 17$ ; bumetanide =  $-56.6 \pm 2.5$ ,  $n = 4$ ;  $P > 0.05$ ), thereby ruling out any involvement for NKCC1. These data demonstrate that KCC2 is an important determinant of the intracellular

To directly test whether this was the result of a loss of inhibitory drive to parvocellular neuroendocrine cells (PNCs), we carried out tight-seal cell-attached recordings, which do not perturb intracellular Cl<sup>-</sup>, and assessed the effects of bicuculline (10  $\mu$ M) on spike activity. Bicuculline increased the activity of neurons in controls ( $170 \pm 20\%$ ,  $n = 7$ ; **Fig. 1c–e**) but had no effect on neurons recorded from slices obtained from stressed animals ( $91.8 \pm 17\%$ ,  $n = 5$ ;  $P = 0.019$  versus effects of bicuculline on control cells; **Fig. 1c–e**). Collectively, these observations indicate that GABA<sub>A</sub>-mediated inhibition is absent following stress.

### Acute stress causes a depolarizing shift in $E_{\text{GABA}}$

We next tested for a possible mechanism that would explain this loss of synaptic inhibition following stress. A number of observations demonstrate that the strength of GABA drive can be modified by changes in transmembrane ionic gradients<sup>9,12–15</sup>. Signaling through the GABA<sub>A</sub> receptor depends on the electrochemical gradient for Cl<sup>-</sup>, which is determined in turn by the activity of transmembrane transporters, specifically the Na/K/2Cl co-transporter NKCC1 and the K/Cl co-transporter KCC2<sup>13,14,16–18</sup>. Developmental studies indicate that there is a decline in NKCC1 expression following birth and a concomitant increase in KCC2 expression, which leaves KCC2 as the predominant determinant of Cl<sup>-</sup> homeostasis in the mature nervous system<sup>19–21</sup>. By harnessing the potassium electrochemical gradient to extrude Cl<sup>-</sup> ions, KCC2 ensures an inward driving force for Cl<sup>-</sup> at resting membrane potentials<sup>5,12,17,18,22</sup>. Acute depolarizing shifts in  $E_{\text{GABA}}$  have previously been observed following injury<sup>7,23</sup>, cellular oxidative stress<sup>11</sup> and seizure activity<sup>8–11,24</sup>. In contrast, immature neurons undergo a slower shift from a depolarizing to a hyperpolarizing Cl<sup>-</sup> flux, reflecting a change from high to low intracellular Cl<sup>-</sup> concentrations, during the course of development<sup>19–21</sup>.

In each case, these shifts are coupled to changes in the Cl<sup>-</sup> extrusion capacity of KCC2. Therefore, we hypothesized that the apparent disinhibition of neuroendocrine cells after stress mirrors a loss of Cl<sup>-</sup>



**Figure 2** KCC2 regulates  $\text{Cl}^-$  homeostasis. **(a)** Gramicidin recordings show IPSCs from a cell in a control slice (black) and a cell in a slice obtained from a stressed animal (green).  $I$ - $V$  plot showed a reversal of IPSCs in control (black,  $n = 11$ ) and after stress (green,  $n = 7$ ). Holding potential for traces corresponds with each point in the  $I$ - $V$  plot. **(b)** Whole-cell recordings showed that bath application of furosemide ( $200 \mu\text{M}$ ) caused a depolarizing shift in the  $I$ - $V$  relationship ( $E_{\text{GABA}}$  control =  $-59.1 \pm 1.4 \text{ mV}$ ,  $n = 21$ ;  $E_{\text{GABA}}$  furosemide =  $-48.0 \pm 2.2 \text{ mV}$ ,  $n = 16$ ;  $P < 0.05$ ; control shown in black, furosemide shown in orange). Inset traces are from holding potentials corresponding to the  $I$ - $V$  plot. **(c)** Summary of the percentage change in circulating corticosteroids following microinjection of furosemide ( $200 \mu\text{M}$ ) into PVN ( $n = 7$ – $10$ ). Scale bars represent  $50 \text{ pA}$  and  $10 \text{ ms}$ . All values are mean  $\pm$  s.e.m. \*  $P < 0.05$ .

$\text{Cl}^-$  concentration in PNCs and ensures that  $\text{Cl}^-$  extrusion is adequate to maintain an inward driving force for the anion at rest.

Finally, we hypothesized that if corticosteroid release during stress results from a loss of synaptic inhibition due to compromised KCC2 activity, then inhibition of KCC2 *in vivo* should also increase circulating corticosteroids. Furosemide ( $200 \mu\text{M}$ ) was microinjected directly into PVN of control rats ( $n = 7$ – $10$ ; **Fig. 2c**) and blood corticosteroid levels were sampled at different time points. This elicited a robust increase in circulating corticosteroids (1 min =  $144 \pm 9.4\%$ , 10 min =  $220 \pm 19.2\%$ , 15 min =  $216 \pm 29\%$ ;  $P < 0.05$  for each time point compared with identical time points in control animals). Taken together, these observations indicate that stress decreases KCC2 activity and that KCC2 activity is necessary to maintain synaptic inhibition of the hypothalamic-pituitary-adrenal (HPA) axis *in vivo*.

### Mechanisms for KCC2 downregulation

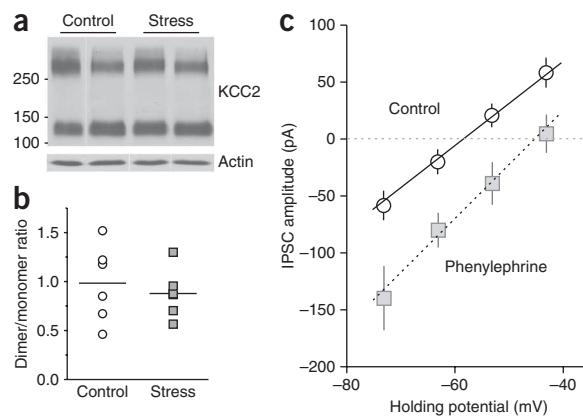
Changes in  $\text{Cl}^-$  homeostasis can be the result of either changes in the expression of KCC2 (refs. 7,29) or changes in transporter activity<sup>30,31</sup>. To determine whether the expression of KCC2 was decreased following stress, we prepared whole-cell extracts from dissected hypothalamic tissue and assessed KCC2 expression levels by immunoblotting with an antibody to KCC2. In extracts from both control and stressed animals, this revealed two prominent bands at  $\sim 140 \text{ kDa}$  and  $\sim 270 \text{ kDa}$ , which is consistent with the previously reported presence of monomeric and dimeric KCC2 protein<sup>16,32</sup> (**Fig. 3a**). There was no difference in the expression of total protein between control and stress samples ( $n = 6$ ; **Fig. 3a**). Because the oligomerization of KCC2 appears to be necessary for conferring activity<sup>32</sup>, we tested whether there may be a change in the relative ratio of the dimeric (active) band to the monomeric (inactive) band between control and stress samples. Densitometric analysis revealed no difference in the dimer/monomer ratio between the two conditions (control =  $0.98 \pm 0.16$ , stress =  $0.88 \pm 0.25$ ,  $n = 6$ ,  $P = 0.6$ ; **Fig. 3b**).

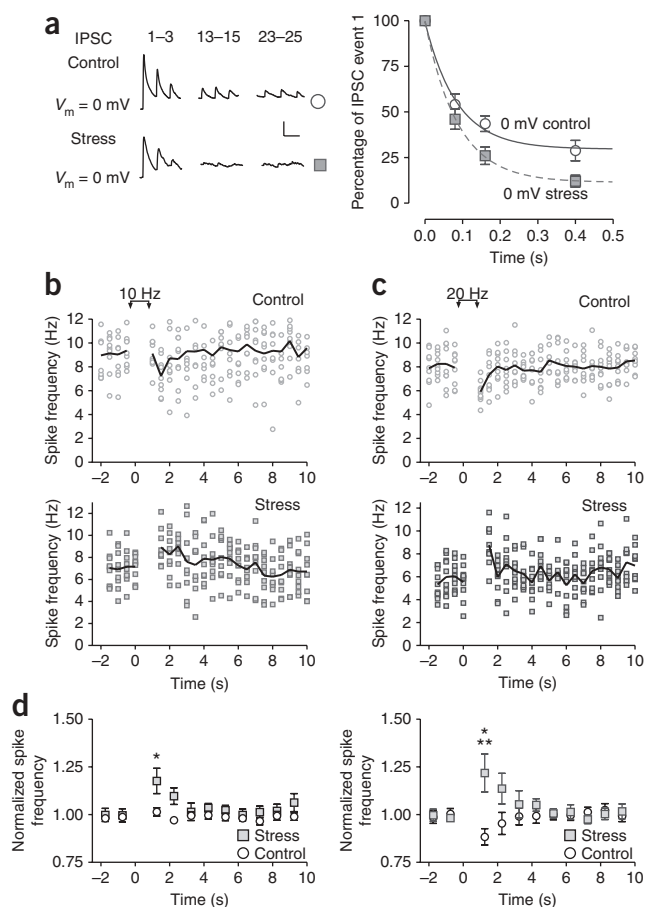
**Figure 3** Stress-induced shift in  $E_{\text{GABA}}$  is not associated with a change in protein expression and can be mimicked by  $\alpha_1$  adrenoceptor activation. **(a)** Immunoblots of KCC2 from PVN extracts in control and stress. Bands are  $\sim 140 \text{ kDa}$  and  $\sim 270 \text{ kDa}$ . **(b)** Quantification of optical density measurements of ratio of dimer/monomer bands showed no change in relative ratio of oligomers after stress ( $P > 0.05$ ). **(c)**  $I$ - $V$  plot, obtained using whole-cell recordings, showing that incubation of control slices with phenylephrine ( $100 \mu\text{M}$  for 30 min) resulted in a depolarizing shift in  $E_{\text{GABA}}$  ( $n = 8$ ,  $P < 0.01$  versus control). All values are mean  $\pm$  s.e.m.

The activation of the HPA axis and the release of corticosteroids in response to stress both require the release of noradrenaline into PVN<sup>33</sup>. Furthermore, changes in KCC2 activity have been reported to be dependent on the activation of second messengers, such as protein kinase C, and an increase in intracellular  $\text{Ca}^{2+}$  (ref. 30). Because PNCs express functional  $\alpha_1$  adrenoceptors<sup>34</sup> that are coupled to protein kinase C and can increase the release of  $\text{Ca}^{2+}$  from intracellular stores, we hypothesized that their stimulation by the exogenous  $\alpha_1$  ligand phenylephrine may be sufficient to decrease KCC2 activity. To test this idea, we incubated slices with phenylephrine ( $100 \mu\text{M}$ , 30 min) and evoked IPSCs in whole-cell configuration to determine  $E_{\text{GABA}}$ . In the phenylephrine-treated slices,  $E_{\text{GABA}}$  was  $-46 \pm 2.5 \text{ mV}$  ( $n = 8$ , whole cell recording; **Fig. 3c**), which was significantly more depolarized than controls ( $P < 0.01$  versus control; **Supplementary Fig. 1**). Together, these results indicate that KCC2 protein expression is unaffected by stress, but the activity of the transporter can be attenuated by activation of  $\alpha_1$  adrenoceptors.

### $\text{Cl}^-$ gradient collapse during repetitive synaptic activity

Computational analyses of changes in  $\text{Cl}^-$  extrusion capacity indicate that shifts in  $E_{\text{GABA}}$  strongly affect GABA inhibition<sup>6</sup>. Furthermore, sustained  $\text{Cl}^-$  extrusion is necessary to prevent the collapse of the  $\text{Cl}^-$  gradient during repetitive synaptic activity<sup>15,30,35,36</sup>. To examine the contribution of KCC2-mediated  $\text{Cl}^-$  extrusion during repetitive activity in control slices, we conducted experiments in conditions that either favored  $\text{Cl}^-$  influx (cell membrane voltage clamped to  $0 \text{ mV}$ ) or efflux (cell membrane voltage clamped to  $-80 \text{ mV}$ ) through the  $\text{GABA}_A$  receptor (**Supplementary Fig. 3** online). This allowed us to isolate the relative contribution of KCC2-mediated  $\text{Cl}^-$  extrusion to the development of activity-dependent synaptic depression. To maintain





**Figure 4** Effects of repetitive synaptic stimulation on GABA IPSCs and postsynaptic activity. **(a)** Left, IPSCs recorded at 0 mV during 500-ms, 50-Hz pulse trains under control and stress conditions. Sample IPSCs are shown for events 1–3, 13–15 and 23–25. Right, summary of synaptic depression under stress (filled squares) and control (open circles) conditions. Scale bars represent 200 pA (top trace) or 50 pA (bottom trace) and 20 ms. **(b)** Top, effect of 10-Hz stimulation on firing in a single control cell (cell-attached recording). Each of seven trials is shown. The solid black line represents the average of all trials in this cell. Bottom, 10-Hz stimulation in a cell from a stressed rat. The solid black line represents the average of all eight trials. **(c)** Top, effect of 20-Hz stimulation on firing in a single control cell as in **b**. The solid black line represents the average of all eight trials. Bottom, 20-Hz stimulation in a cell from a stressed rat. **(d)** Left, summary of six control cells and nine stressed cells in response to a 10-Hz synaptic stimulation. The increase in firing after synaptic stimulation in stress was significantly different from the firing rate before stimulation ( $P < 0.05$ ). Right, summary of six control cells and nine stressed cells in response to a 20-Hz stimulation. The increase in firing after synaptic stimulation in stress was significantly different from the firing rate before stimulation ( $P < 0.05$ ) and from the firing rate at the same time point following stimulation in control slices ( $P < 0.05$ ). All values are mean  $\pm$  s.e.m. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

### GABA signaling is conditionally excitatory after stress

Previous reports indicate that epileptiform activity<sup>8–11</sup> and injury-induced decreases in spinal KCC2 (ref. 7) result in GABA-mediated depolarizations and an increase in neuronal excitability. To examine the consequences of impaired  $\text{Cl}^-$  extrusion at high rates of activity, we carried out experiments using the cell-attached recording mode and stimulated synaptic GABA inputs at 10 or 20 Hz for 1 s. Seven to nine trials were conducted at each frequency in each cell and the data from all trials at each frequency were averaged for a given cell (**Fig. 4b–d**). Under control conditions, stimulation of GABA inputs (10 Hz, 1 s) had no effect on firing (prestimulation,  $0.99 \pm 0.01$ ; poststimulation,  $1.00 \pm 0.02$ ;  $n = 6$  cells,  $P > 0.05$ ; **Fig. 4b,d**). Under stress conditions, the same stimulation protocol elicited a transient, but significant, increase in spike activity (prestimulation,  $1.01 \pm 0.02$ ; poststimulation,  $1.2 \pm 0.07$ ;  $n = 9$ ,  $P < 0.05$ ; **Fig. 4b,d**). In response to 20-Hz stimulation, control cells showed a decrease in spiking immediately after the synaptic stimulation (prestimulation,  $1.00 \pm 0.02$ ; poststimulation,  $0.88 \pm 0.04$ ;  $n = 6$ ,  $P < 0.05$ ; **Fig. 4c,d**). Under stress conditions, 20-Hz stimulation caused an increase in spike activity (prestimulation,  $1.00 \pm 0.01$ ; poststimulation,  $1.22 \pm 0.09$ ;  $n = 9$ ,  $P < 0.05$ ; **Fig. 4c,d**). Increasing the rate of synaptic activation to 50 Hz elicited a more robust inhibition of firing (prestimulation,  $1.01 \pm 0.02$ ; poststimulation,  $0.25 \pm 0.05$ ;  $n = 7$ ,  $P < 0.01$ ). Following stress, however, the same stimulation protocol failed to modify firing rate (prestimulation,  $0.99 \pm 0.01$ ; poststimulation,  $1.06 \pm 0.04$ ;  $n = 7$ ,  $P > 0.05$ ).

These observations demonstrate that there is a loss of synaptic inhibition at 50 Hz, but not an accompanying GABA-mediated excitation, as was observed at 10- and 20-Hz stimulation, indicating that additional mechanisms, probably resulting from the spillover of GABA and the activation of extrasynaptic GABA<sub>B</sub> receptors, contribute to the prolonged inhibition following 50-Hz stimulation. Collectively, these experiments indicate that repetitive activation of GABA inputs following stress causes a collapse of the  $\text{Cl}^-$  gradient that, under specific conditions, is sufficient to promote GABA-mediated excitations.

### DISCUSSION

Our data demonstrate that neural pathways activated during stress target KCC2 in neuroendocrine command cells in the PVN that control the output of the HPA axis. By compromising KCC2 activity, stress increases intracellular  $\text{Cl}^-$ , which is reflected as a depolarizing shift in the reversal potential for GABA<sub>A</sub>-mediated synaptic events, and

synaptic strength during a burst of presynaptic activity under conditions of high  $\text{Cl}^-$  influx, KCC2 must rapidly extrude  $\text{Cl}^-$ . At 0 mV, a brief train (six pulses) resulted in the development of frequency-dependent synaptic depression (**Supplementary Fig. 3**). In contrast, the activation of GABA<sub>A</sub> receptors at  $-80$  mV results in  $\text{Cl}^-$  efflux and imposes no load on KCC2. Consequently, the collapse of the  $\text{Cl}^-$  gradient should not contribute to the development of depression. Under these conditions, depression was less robust and was only partly frequency dependent (**Supplementary Fig. 3**). Thus,  $\text{Cl}^-$  extrusion is a critical determinant of synaptic depression and the activity of the cell would be highly affected by alterations in its ability to extrude  $\text{Cl}^-$ .

Because stress downregulates KCC2 function and compromises  $\text{Cl}^-$  extrusion capacity, one prediction is that depression during high-frequency synaptic stimulation should be more pronounced following stress. We tested this idea by using a longer, 500-ms 50-Hz train to elicit robust depression at holding potentials favoring the influx of  $\text{Cl}^-$  ( $E_{\text{hold}} = 0$  mV) in slices obtained from control and stressed animals. Sample traces from eIPSC 1–3, 12–15 and 23–25 during the train were plotted, which revealed that activity-dependent depression was more robust following stress (**Fig. 4a**). In contrast, no difference in synaptic depression was observed between control and stress when the  $\text{Cl}^-$  driving force was outward ( $E_{\text{hold}} = -80$  mV, data not shown), suggesting that the effects of acute stress on short-term depression are derived from changes in the efficacy of  $\text{Cl}^-$  transport. These observations indicate that the decrease in KCC2-mediated  $\text{Cl}^-$  efflux following stress compromises the ability of GABA synapses to sustain inhibition when activated repetitively.

profoundly weakens synaptic inhibition. *In vivo*, this translates into a complete absence of GABA inhibitory control of the HPA axis following stress. Decreased KCC2 activity allows for the buildup of intracellular  $\text{Cl}^-$  during repetitive synaptic activation, which reverses the ionic flux through the receptor and promotes the depolarization and resultant excitation of neuroendocrine cells. Our findings provide, to the best of our knowledge, the first demonstration of ionic plasticity in the mature nervous system in response to acute physiological stimuli. This and other reports<sup>7,37</sup> demonstrate that subtle changes in the function of transmembrane ion transport mechanisms profoundly affect the output of neural networks in the adult CNS.

Although reciprocal changes in the expression of the  $\text{Cl}^-$  transporters NKCC1 (ref. 24) and KCC2 (ref. 19–21) are important for the hyperpolarizing shift in  $E_{\text{GABA}}$  during development, pathological conditions in the adult CNS that are associated with depolarizing shifts in  $E_{\text{GABA}}$  all point to an essential role for KCC2 (refs. 7–11,24). Using both cell-attached recordings that do not perturb intracellular  $\text{Cl}^-$  and *in vivo* microinjections and hormone measurements, we found that inhibition mediated by the activation of GABA<sub>A</sub> receptors was absent following stress. When GABA synapses were repetitively activated, however, GABA conditionally became excitatory. This observation is supported by computational modeling work indicating that the depolarizing shift in the reversal potential of the anion ( $E_{\text{anion}}$ ) markedly reduces the ability to inhibit firing until some threshold shift is reached, after which point hyperexcitability results<sup>6</sup>.

We provide evidence that the change in  $E_{\text{GABA}}$  is a result of altered KCC2-mediated  $\text{Cl}^-$  extrusion capacity. Collapse of the  $\text{Cl}^-$  gradient as a result of downregulation of KCC2 occurs in response to sustained neuronal activity<sup>15,30,35,36</sup>. Here we directly examined the activity-dependent effect of a stress-induced decrease in KCC2 activity using high-frequency stimulation procedures in situations designed to favor either the influx or efflux of  $\text{Cl}^-$ . This experimental design allowed us to examine the contribution of  $\text{Cl}^-$  extrusion independently from the development of synaptic depression. Adequate  $\text{Cl}^-$  extrusion is a requirement for the maintenance of a sustained  $\text{Cl}^-$  gradient favoring the influx of  $\text{Cl}^-$ . We found that, even under control conditions, the extrusion capacity of KCC2 was saturated at high rates of activity and was important in the depression throughout the pulse train. Under stress conditions, however, this contribution was greatly enhanced. The lack of parallel changes in activity-dependent synaptic depression is consistent with a downregulation of KCC2 activity<sup>31</sup>.

Our data demonstrating that the activation of  $\alpha_1$  adrenoreceptors causes a depolarizing shift in  $E_{\text{GABA}}$  provide a cellular mechanism by which ascending noradrenergic fibers<sup>33</sup> can cause a sustained release of pituitary hormones. This probably occurs through a mechanism that is not dissimilar to one in which bouts of enhanced neuronal activity downregulate KCC2 through the recruitment of protein kinase C and intracellular  $\text{Ca}^{2+}$  (ref. 30). Whether this is the only means by which KCC2 can be affected in this system is not known. Additional mechanisms, such as coincident and repetitive activation of pre- and postsynaptic elements<sup>38</sup> or the actions of different chemical messengers<sup>23,29</sup>, certainly cannot be ruled out. On the basis of our evidence that neither the expression nor the oligomerization of KCC2 is altered after stress, we favor a scenario whereby activity is compromised independent of change in the expression of the KCC2 protein<sup>31</sup>.

In both physiological and pathological states, inhibitory plasticity appears to be extremely diverse. Our results support the hypothesis that changes in membrane anion transporter activity provide a powerful mechanism for altering the output of neural networks in response to behavioral stimuli.

## METHODS

**Slice preparation.** PVN hypothalamic slices (300  $\mu\text{m}$  thick) were prepared from postnatal day 21 (P21) to P28 male Sprague Dawley rats as described previously<sup>39</sup>. Our animal experiments were approved by the University of Calgary Animal Care and Use Committee. The control group was comprised of rats that were anaesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg per kg of body weight) prior to decapitation. The stress group was comprised of animals placed in a plexiglass restrainer for 30 min, and then quickly anaesthetized and decapitated as described above. Slices (300  $\mu\text{m}$ ) were superfused with artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 2.5 mM KCl, 26 mM  $\text{NaHCO}_3$ , 2.5 mM  $\text{CaCl}_2$ , 1.5 mM  $\text{MgCl}_2$ , 1.25 mM  $\text{NaH}_2\text{PO}_4$  and 10 mM glucose (bubbled with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ ). We added 10  $\mu\text{M}$  6,7-dinitroquinoxaline-2,3-dione and 100  $\mu\text{M}$  DL-2-amino-5-phosphonovaleric acid to specifically measure GABA<sub>A</sub> currents.

**Recordings.** Whole-cell recordings were obtained from PNCs that were visually identified using an upright microscope (Olympus Optical) that was fitted with infrared differential interference contrast optics. PNC cell type was verified on the basis of the electrical fingerprint in current-clamp configuration<sup>40</sup>. All recordings were obtained at 30–32 °C using borosilicate glass microelectrodes (tip resistance of 3–7 M $\Omega$ ) that were filled with intracellular solution containing 123 mM potassium gluconate, 2 mM  $\text{MgCl}_2$ , 8 mM NaCl, 1 mM EGTA, 4 mM ATP and 0.3 mM GTP. For some experiments, gramicidin (40  $\mu\text{M}$ ) was added to the intracellular solution. In these experiments, a high  $\text{Cl}^-$  intracellular solution was used (150 mM KCl and 10 mM HEPES, buffered to pH 7.2 with KOH) to detect rupture of the patch. In other whole-cell experiments, intracellular  $\text{Cl}^-$  was altered from 12 mM to 4, 8 or 24 mM. Because varying intracellular  $\text{Cl}^-$  concentration resulted in predictable shifts in  $E_{\text{GABA}}$ , we subsequently used whole-cell recordings (12 mM  $\text{Cl}^-$ ) to measure  $E_{\text{GABA}}$ . Cell-attached recordings used 12 mM  $\text{Cl}^-$  intracellular solution and whole-cell configuration was achieved following the experiment to determine cell type and to ensure that synaptic stimulation activated GABA synapses. Data acquisition and analysis of postsynaptic currents were carried out as previously described<sup>39</sup>. Recordings were accepted when access resistance changes were <15%.

**Data analysis.** Whole-cell and gramicidin-perforated patch data were analyzed using pCLAMP 10 (Molecular Devices). Cell-attached data were analyzed using MatLab 7.1 (Mathworks). In **Figure 4a**, points were fit with a mono-exponential decay curve. When comparing two datasets, statistical significance was tested using Student's *t* test. For more than two datasets, statistical significance was determined with a one-way ANOVA with a Newman-Keuls *post hoc* test. All measurements are given as mean  $\pm$  s.e.m.

**Corticosterone assay.** Blood was collected into heparinized syringes, centrifuged (15 min at 9,500g) and plasma was stored at –80 °C until assayed. Plasma corticosterone was determined using an enzyme immunoassay kit (Correlate-EIA, Assay Designs) and expressed as ng  $\text{ml}^{-1}$  of plasma. The intra-assay coefficient of variation was 6.6% and the sensitivity was 27.0 pg  $\text{ml}^{-1}$  according to the manufacturer's protocol.

**PVN microinjection.** Rats (P21–28) were anesthetized with ketamine (1 mg per kg) and the femoral artery was cannulated for blood collection. A control blood sample was collected (250–300  $\mu\text{l}$ ) approximately 1 min before microinjection. The animal was then placed into a stereotaxic unit and secured. The coordinates used for PVN microinjection were 0.8 mm posterior to bregma and 0.3 mm lateral to midline. A small hole was drilled and the cannula was lowered into position (8 mm ventral from dura). We injected bicuculline (2  $\mu\text{l}$ , 0.1 nM), furosemide (2  $\mu\text{l}$ , 200  $\mu\text{M}$ ) or vehicle into PVN with 2% (wt/vol) Evans Blue dye. Subsequent blood samples (250–300  $\mu\text{l}$ ) were collected at 1, 5 and 10 min after microinjection. At the termination of the experiment, the animal was killed by transcardial perfusion with formalin and the brain was collected for *post hoc* analysis of cannula placement.

**Protein extraction and immunoblotting.** Rats were anesthetized and decapitated, and the hypothalamus rapidly excised and homogenized in lysis buffer (20 mM MOPS, 150 mM KCl, 4.5 mM magnesium acetate and 1% (vol/vol) Triton X-100) containing protease inhibitors (2  $\mu\text{g}$   $\text{ml}^{-1}$  aprotinin (Roche Applied Science), 2  $\mu\text{g}$   $\text{ml}^{-1}$  leupeptin (Roche Applied Science), 2 mM

phenylmethylsulfonyl fluoride (Sigma) and 1  $\mu\text{g ml}^{-1}$  pepstatin A (Roche Applied Science)). Protein concentrations of cleared lysates were determined using a detergent-compatible protein assay (Bio-Rad) with bovine serum albumin as a standard. Samples (50  $\mu\text{g}$  of protein) were resolved on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose and blocked for 1 h in 25% nonfat dry milk in blocking buffer (50 mM Tris, 150 mM NaCl, 0.1% (vol/vol) Tween 20) before overnight incubation at 4 °C in rabbit antibody to KCC2 (1:3,000 in blocking buffer, Millipore). After washing in blocking buffer, blots were incubated for 1 h in horseradish peroxidase-conjugated goat antibody to rabbit IgG (1:3,000 in blocking buffer, Bio-Rad). Antibody binding was determined by chemiluminescent detection (ECL, GE Healthcare-Life Sciences) and exposure to Fuji SuperRX film. Densitometric analysis was performed using ImageQuant software (GE Healthcare-Life Sciences).

Note: Supplementary information is available on the Nature Neuroscience website.

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#### AUTHOR CONTRIBUTIONS

S.A.H. conducted the *in vivo* stress and corticosteroids measurements, the  $E_{\text{GABA}}$ , PPR and repetitive synaptic activation experiments, analyzed the data and wrote the manuscript. J.I.W. conducted the cell-attached experiments in Figure 1, as well as performing some gramicidin recordings. E.U.K. designed and performed immunoblot experiments and analyses. J.S.B. designed the experiments, analyzed the data, prepared the manuscript and supervised the project.

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