Regulation of intrinsic excitability in hippocampal neurons by activity-dependent modulation of the Kv2.1 potassium channel

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Kv2.1 is the prominent somatodendritic sustained or delayed rectifier voltage-gated potassium (Kv) channel in mammalian central neurons, and is a target for activity-dependent modulation via calcineurin-dependent dephosphorylation. Using hanatoxin-mediated block of Kv2.1 we show that, in cultured rat hippocampal neurons, glutamate stimulation leads to significant hyperpolarizing shifts in the voltage-dependent activation and inactivation gating properties of the Kv2.1-component of delayed rectifier K+ (IK) currents. In computer models of hippocampal neurons, these glutamate-stimulated shifts in the gating of the Kv2.1-component of IK lead to a dramatic suppression of action potential firing frequency. Current-clamp experiments in cultured rat hippocampal neurons showed glutamate stimulation induced a similar suppression of neuronal firing frequency. Membrane depolarization also resulted in similar hyperpolarizing shifts in the voltage-dependent gating properties of neuronal IK currents, and suppression of neuronal firing. The glutamate-induced effects on neuronal firing were eliminated by hanatoxin, but not by dendrotoxin-K, a blocker of Kv1.1-containing channels. These studies together demonstrate a specific contribution of modulation of Kv2.1 channels in the activity-dependent regulation of intrinsic neuronal excitability.

Introduction

Homeostatic processes that operate at both synaptic and cellular levels regulate overall activity in neuronal networks. Without these mechanisms, changes in synapse number and strength could threaten the stability of neuronal networks.1 One mechanism for achieving homeostasis is to link synaptic activity to intrinsic excitability through activity-dependent modulation of voltage-gated ion channels. Voltage-gated potassium (Kv) channels play a crucial role in regulating neuronal excitability.2 Different Kv channels are localized to presynaptic terminals, axons, somata and dendrites,3,4 where they perform specific functions in maintaining and dynamically regulating membrane excitability.5,6 Two major classes of Kv currents in central neurons are the sustained delayed rectifier current IK and the transient A-type current IA.7 Kv2.1 channels constitute the major component of the somatodendritic sustained or delayed rectifier current IK and play a major role in regulating membrane excitability and Ca2+ influx in hippocampal and cortical neurons during high-frequency repetitive firing.8,12-14 The Kv2.1 channel is abundantly expressed in mammalian brain neurons,4 and specifically localized in high-density clusters in somata and proximal dendrites15,16 as well as the axon initial segment.17

Recently we found that the phosphorylation state and clustered localization of Kv2.1 in rat brain and in cultured hippocampal neurons are dynamically regulated in response to altered neuronal activity,18 ischemia19 and muscarinic modulation.20 These stimuli also lead to calcineurin-dependent changes in the voltage-dependence of activation of hippocampal neuronal IK.18-20 Similar modulation of voltage-dependent channel gating properties is induced by Ca2+/calcineurin-dependent dephosphorylation of recombinant Kv2.1 in heterologous cells,20 at a large number of cytoplasmic serine phosphorylation sites.21

Here, we address the role of modulation of the Kv2.1-based neuronal IK in regulating firing of hippocampal neurons. We first define the signaling pathway whereby glutamate stimulation leads to modulation of neuronal IK and then use a realistic model of neuronal firing to determine the potential effects of such modulation. We use current-clamp experiments and specific neurotoxin blockade to determine the contribution of Kv2.1 modulation to the changes
in neuronal firing observed upon glutamate treatment. Together these studies demonstrate a critical role for calcineurin-mediated modulation of KV2.1 in the suppression of neuronal firing following high levels of neuronal activity.

**Results**

Glutamate stimulation of hippocampal neurons has diverse effects on voltage-dependent activation and inactivation of $I_K$ via Ca$^{2+}$/calcineurin-dependent intracellular signaling. We previously found that seizures in rats in vivo, or glutamate stimulation of cultured rat hippocampal pyramidal neurons, led to calcineurin-dependent dephosphorylation of KV2.1, and dispersion of KV2.1 clusters. Initial experiments showed that glutamate stimulation of cultured neurons also led to significant hyperpolarizing shifts in voltage-dependent activation of neuronal $I_K$ \(^{18}\) However, the potential impact of these changes on neuronal function could not be accurately deduced without a more comprehensive analysis of the effects of glutamate stimulation on the gating properties of neuronal KV2.1 channels. To electrophysiologically isolate $I_K$ from $I_A$, neurons held at -80 mV were subjected to a conditioning step to -10 mV (30 ms) to eliminate the majority of fast-activating and inactivating component of $I_A$, briefly repolarized, and test-pulses delivered to evoke $I_K$ currents. Under control conditions, $I_K$ in cultured hippocampal neurons has half-maximal activation ($G_{1/2}$) and inactivation ($V_{1/2}$) voltages of +16.8 ± 0.3 mV and -28.5 ± 0.5 mV (n = 5), respectively (Fig. 1C and Table 1). These parameters were stable and not significantly different over an experimental time course of up to 20 min after the completion of the protocol for steady-state inactivation, in the absence of further experimental manipulation. However, treatment of neurons with 10 μM glutamate for 10 minutes after the completion of the protocol for steady-state inactivation, and subsequent wash-out for 2 min, significantly increased $I_K$ amplitude (Fig. 1A and B), and yielded ≈25 mV hyperpolarizing shifts in the voltage-dependence of both activation and inactivation gating ($G_{1/2} = -8.6 ± 0.9$ mV; $V_{1/2} = -54.7 ± 0.5$ mV, n = 5, Fig. 1C and Table 1). Glutamate treatment also led to the suppression of most of the $I_A$ component, and an increase in the amplitude of $I_K$ component, as evident from the current traces at -10 mV conditioning pulse before each test pulse (Fig. 1A). Recovery from inactivation at -80 mV was also slowed. In control neurons the recovery from inactivation was quite fast and was complete within 1 sec (half-maximal recovery ($\tau_{rec}$) = 0.2 ± 0.001 sec, n = 5), whereas in glutamate-treated neurons, recovery was incomplete even at 10 sec ($\tau_{rec}$ = 4.1 ± 0.3 sec, n = 5; p < 0.001) (Fig. 1D).

We previously showed that glutamate treatment of cultured rat hippocampal neurons led to dephosphorylation and lateral translocation of KV2.1.\(^{18}\) This involved activation of ionotropic glutamate receptors, leading to increased [Ca$^{2+}$] and activation of the protein phosphatase calcineurin, resulting in KV2.1 dephosphorylation.\(^{18}\) Here, we investigated whether the same signaling pathway underlies the glutamate-induced modification in the functional properties of $I_K$ in cultured hippocampal neurons. Glutamate-induced hyperpolarizing shifts in the voltage-dependent gating parameters of $I_K$ were abolished when the neurons were treated with glutamate in Ca$^{2+}$-free extracellular buffer, and also almost completely blocked by pre-treatment of neurons with APV, a specific antagonist of NMDA receptors (Fig. 2). Glutamate-induced shifts in $I_K$ gating were also blocked by intracellular dialysis with a complex of cyclosporine A (CsA) and cyclophilin A (CypA), which specifically antagonizes the activity of calcineurin, but were not blocked by a low concentration of extra-cellular okadaic acid, which antagonizes the activity of the protein phosphatases 1A and 2A (Fig. 2). Extracellular perfusion of neurons with the Ca$^{2+}$-ionophore ionomycin also led to hyperpolarizing shifts in $I_K$ gating (Fig. 2). These results suggest that the glutamate-induced hyperpolarizing shifts in $I_K$ gating are mediated by a pathway identical to that which regulates KV2.1 channel dephosphorylation and localization,\(^{18}\) namely activation of ionotropic glutamate receptors, increased [Ca$^{2+}$], and activation of calcineurin.

Glutamate-induced modulation of $I_K$ in hippocampal neurons is achieved primarily through effects on KV2.1 channels. To identify the component of neuronal $I_K$ sensitive to glutamate-induced gating modulation, we tested the effects of externally applied hanatoxin (HaTx), a KV2.1 blocker/gating-modifier neurotoxin isolated from the venom of the Chilean tarantula.\(^{22,23}\) We first tested the blocking efficiency of bath applied HaTx on KV2.1 channels expressed stably in HEK293 cells. Extracellular perfusion of 100 nM HaTx onto these cells for 10 min led to an ≈93% decrease in the KV2.1 current amplitude (from 3.31 ± 0.49 nA to 0.21 ± 0.04 nA) at 0 mV, and an ≈62% decrease (from 11.7 ± 1.9 nA to 4.2 ± 0.4 nA) at +50 mV (n = 4, p < 0.05; Fig. 3A and B). That HaTx inhibition...
of $I_K$ activation and inactivation gating (from $\approx 25$ mV without HaTx to $\approx 5$ mV with HaTx; Fig. 3D and E and Table 1). That the glutamate-induced effects on $I_K$ gating were lost upon blockade of $K_V2.1$ with HaTx indicates that the glutamate-induced shifts in the voltage-dependent activation and inactivation properties of the $I_K$ in hippocampal neurons are primarily contributed by the $K_V2.1$ component of this current.

Modeling the effects of $K_V2.1$ dephosphorylation on hippocampal neuronal firing. Because $K_V2.1$ is the major component of neuronal $I_K$ in hippocampal neurons, altered activation and inactivation of $K_V2.1$ upon glutamate stimulation might change neuronal action potential firing. To test this we first developed detailed models of the effect of glutamate-mediated $K_V2.1$ current modulation on hippocampal neuron firing using NEURON 5.6 software.\textsuperscript{24} We incorporated the data on the biophysical properties of the $K_V2.1$ component of $I_K$ as we recorded from control and glutamate-treated neurons (see previous section), and kept all else constant in this hippocampal neuron model that resembles previously used models.\textsuperscript{25,26} Compared to control neurons, decreased action potential firing frequencies were observed in the glutamate-treated neurons at a number of different levels of current injections (Fig. 4A and C). Glutamate treatment also resulted in a fraction of $K_V2.1$ currents being active at resting membrane potentials, and during interspike intervals (Fig. 4B), which might contribute to increase in the duration of interspike intervals (Fig. 4A). As such the glutamate-stimulated modulation of $K_V2.1$ would be predicted to suppress hippocampal neuronal firing.

Glutamate-induced modification of the voltage-dependent gating of $K_V2.1$ contributes to suppression of action potential firing in cultured hippocampal neurons. To test the predictions obtained from these computer models of hippocampal neuron firing, we performed current-clamp experiments on cultured hippocampal neurons under control and glutamate-stimulated conditions. Under control conditions, neurons showed frequent spontaneous action potential firings presumably due to the high spontaneous activity of cultured neurons.\textsuperscript{27} Firing was consistent over time periods of 15–17 min with continuous perfusion of ACSF. Glutamate treatment (10 μM, 10 min), led to a brief period of increased spontaneous firing, followed by a membrane depolarization to approximately -20 mV, at which point firing ceased (Fig. 5A). After glutamate washout, the neurons re-established a normal resting membrane potential within 2 min, but sustained the decreased spontaneous firing (Fig. 5A and B).

### Table 1 Voltage-dependent gating properties of $I_K$ currents in cultured rat hippocampal neurons without or with treatment with different drugs

| Drug treatments | $G_{1/2}$ (mV) | $k$ | $V_{1/2}$ (mV) | $k$ | $n$ |
|-----------------|----------------|-----|----------------|-----|-----|
| Control         | 16.6 ± 0.3     | 16.6 ± 0.3 | -28.8 ± 0.5 | 11.4 ± 0.6 | 5    |
| 10 μM Glutamate-10 min | -8.6 ± 0.9a       | 14.2 ± 1.4 | -54.7 ± 0.5a | 11.3 ± 0.4 | 5    |
| 5 μM Glutamate-10 min     | -4.1 ± 0.6a       | 15.2 ± 0.7 | -52.2 ± 0.6a  | 10.7 ± 0.5 | 4    |
| 100 nM HaTx-0 min       | +15.4 ± 0.4        | 14.5 ± 0.2  | -25.7 ± 0.5   | 12.6 ± 0.6 | 4    |
| 100 nM HaTx-10 min      | +16.2 ± 0.5        | 17.9 ± 0.3  | -25.8 ± 0.8   | 12.7 ± 0.4 | 4    |
| 100 nM HaTx-10 min + 10 μM Glutamate-10 min | +10.1 ± 0.4a       | 15.1 ± 0.2  | -32.7 ± 0.3a  | 11.2 ± 0.2 | 4    |

*Significantly different from the control values. Abbreviation: HaTx, hanatoxin.

Figure 2. Ca$^{2+}$/calcineurin-dependent modulation of the half-maximal voltage-dependent activation ($G_{1/2}$) and steady-state inactivation ($V_{1/2}$) potentials of $I_K$ currents from cultured rat hippocampal neurons with different drug treatments. Glutamate (10 μM), AP-V (5 μM), Ionomycin (1 μM), Okadaic acid (OA, 0.1 μM) and cadmium chloride (CdCl$_2$, 1 μM) were applied extracellularly, whereas cyclosporin A (CsA; 1 μM) and cyclophilin A (CypA; 1 μM) were applied intracellularly by dissolving in the pipette solution. Data are presented as mean ± SEM ($n = 5, 5, 5, 6, 5, 6, 4, 4$ and $4$ for Control, Glu, Glu + AP-V, Glu[Ca$^{2+}$-free], Ionomycin, Glu + CsA + CypA, Glu + OA, Glu + CsA + CypA + OA, Depol.[-20 mV], Depol.[20 mV] + CdCl$_2$, and Depol.[-20 mV] + CsA + CypA respectively). Asterisks indicate significant difference in the $G_{1/2}$ potentials (*) and $V_{1/2}$ potentials (**) as compared to respective values under control conditions ($p < 0.05$).
Figure 3. Block of Kv2.1 currents by hanatoxin (HaTx) eliminates the glutamate-induced shifts in the voltage-dependent gating properties of $I_K$. (A) Representative whole-cell current recordings from HEK293 cells stably expressing recombinant rat Kv2.1 channels, from a holding potential of -100 mV with 10 mV incremental depolarizing potentials to +80 mV before and after extracellular application of 100 nM HaTx for 10 min. (B and C) Current density plot (B) and steady-state activation curves (C) of recordings obtained from experiments as shown in (A). Data are presented as mean ± SEM. (D and E) Glutamate-induced shifts in $I_K$ are specific to currents carried by Kv2.1 channels. (D) Representative whole-cell current recordings obtained from a cultured rat hippocampal neuron following the pulse protocol depicted in Figure 1A, before and after 10 min superfusion of 100 nM hanatoxin (HaTx), followed by superfusion of 10 M glutamate along with 100 nM HaTx. (E) Steady-state activation and inactivation curves for $I_K$ recorded from neurons as shown in (A). The voltage-dependent biophysical parameters are detailed in Table 1.

Figure 4. Glutamate-induced alterations in the activation and inactivation properties of Kv2.1/$I_K$ lead to decreased action potential firing frequency in model hippocampal neurons. (A) Action potential firing patterns in an idealized hippocampal pyramidal neuron. Firing patterns were obtained by injecting 75 pA (upper) and 150 pA (lower) current from a membrane potential of -70 mV into model hippocampal pyramidal neurons using Kv2.1 functional parameters obtained from control or glutamate-treated neurons. Parameters of all other ion channels were kept constant. (B) Kv2.1 current in control [thin black line] and glutamate-stimulated [thick grey line] model neurons invoked by action potential waveforms obtained from a 150 pA current injection. (C) Frequency-intensity plot of action potential spikes generated by the hippocampal pyramidal neuron model upon step-wise current injections.
Injection of different amplitudes of currents into neurons led to a consistent frequency of action potential firing that was stable for more than 15 minutes (not shown). However, treatment with 10 μM glutamate for 10 min led to a significant decrease in action potential firing frequency (Fig. 6A and C), similar to that predicted from computer modeling (Fig. 4A and C). Similar experiments using 5 μM glutamate treatment for 10 min also led to significant suppression of firing (Fig. 6D and F). Note that treatment of cultured hippocampal neurons with 5 μM glutamate for 10 min had also resulted in large (20–25 mV) hyperpolarizing shifts in the voltage-dependent gating properties of $I_{Kv2.1}$ (Table 1). No significant differences in the membrane potential ($V_m$), input resistance ($R_{input}$), action potential amplitude ($AP_{amplitude}$), and action potential duration ($AP_{duration}$) were observed before and after glutamate treatment (Table 2). However, a decrease in the latency to the first spike was observed in glutamate-treated neurons (77.4 ± 4.2 ms and 34.9 ± 3.9 ms for 5 μM and 10 μM glutamate-treated neurons versus 87.2 ± 4.5 ms for control neurons), as measured with 20 pA.

Figure 5. Glutamate treatment of cultured rat hippocampal neurons leads to suppression of spontaneous firing. (A) Representative traces of spontaneous firing patterns in cultured hippocampal neurons for a period of 17 min with continuous bath perfusion of ACSF (upper), and with 10 μM glutamate application in ACSF for 10 min following an initial ACSF perfusion for 2.5 min and post-glutamate ACSF wash of 4.5 min (bottom). (B) Quantitation of spontaneous firing before and after glutamate treatment as shown in (A). Data are presented as mean ± SEM of the number of spontaneous spikes per minute, before and after glutamate treatment ($n = 4$). * indicates significant difference in the number of spontaneous spikes ($p < 0.05$).

Figure 6. Decreased action potential firing in cultured rat hippocampal neurons upon glutamate treatment is dependent on $K_{V2.1}$. (A and D) Representative traces of action potential firing in cultured hippocampal neurons upon injection of 20 pA (upper) and 100 pA (lower) currents, respectively, over a period of 1 sec, and before and after the treatment of 10 μM glutamate (A) or 5 μM glutamate (D) for 10 min, followed by wash-out for 2 min. (B and E) Representative traces of action potential firing patterns in cultured hippocampal neurons upon superfusion of 100 nM HaTx for 10 min [before clamping] with injection of 20 pA (upper) and 100 pA (lower) currents, respectively, over a period of 1 sec, and before and after the treatment with 10 μM (B) or 5 μM (E) glutamate for 10 min, followed by wash-out for 2 min. (C and F) Frequency-intensity plots of action potential spikes generated upon step-wise current injections from experiments in (C) panels (A and B), and (F) panels (D and E). Data are presented as mean ± SEM ($n = 5$ and 3 for experiments from (A and D) and (B and E) respectively).
The voltage-dependent gating parameters of KV2.1 channels by using suppression of neuronal firing was dependent on the modified 20 pA current injections.

Treated neurons versus 15.0 ± 0.6 ms for control, measured with current injections. The action potential rise-time was also decreased external HaTx treatment to block KV2.1 currents in cultured hippocampal slices on pyramidal cell firing. Interestingly, glutamate suppression of firing observed upon glutamate treatment of neurons increase in firing frequency (Fig. 6B, C, E and F), consistent with previous reports on DTX treatment had no effect on the glutamate-induced hyperpolarizing shifts in the voltage-dependent IK gating properties (not shown). Glutamate stimulation (5 μM for 10 min) of DTXκ-pretreated neurons still yielded a significant decrease in firing frequency (Fig. 7A and B). These data together demonstrate the important role of the HaTx-sensitive KV2.1 currents in mediating the glutamate-induced suppression of neuronal firing.

The close correlation between predictions derived from model hippocampal neurons, and experimental data from cultured hippocampal neurons, combined with the use of HaTx to block KV2.1, suggest that dephosphorylation-induced changes specific to KV2.1 gating contribute to the suppression of neuronal excitability observed upon glutamate treatment.

Sustained membrane depolarization also leads to modulation of the voltage-dependent gating of KV2.1-based IK and suppression of neuronal firing. We next studied whether membrane depolarization in itself was sufficient to modulate KV2.1-based IK and suppress firing in cultured hippocampal neurons. After achieving the whole-cell patch-clamp mode and recording IK, the membrane potential of the neurons was depolarized to -20 mV for 10 min in the patch-clamp amplifier, followed by a return to the holding potential (-80 mV). This protocol resulted in ≈20–25 mV hyperpolarizing shifts in the voltage-dependent gating properties of the IK, which were antagonized by including 1 mM CdCl2 in the extracellular buffer to block Ca2+ entry, or by pre-treatment with intracellular CsA and CypA complex to block calcineurin activity (Fig. 2). Under current-clamp conditions, similar membrane depolarization (-20 mV for 10 min) led to significant suppression of spontaneous firing (Fig. 8A and B), as well as suppression of action potential firing upon injection of different amplitudes of currents into cultured hippocampal neurons (Fig. 8C and D). No significant changes in the Vm and Rinput of neurons were observed during the resting period after 10 min of membrane depolarization to -20 mV (Table 2). We also subjected the neurons to short (≈30 sec) episodes of membrane depolarization (to -20 mV) interspersed with 1 min resting periods and recorded the spontaneous activity throughout the experiments. This type of episodic depolarizing stimulus led to a significant graded suppression of spontanous spiking frequencies after subsequent stimul

### Table 2  Electrophysiological properties of cultured rat hippocampal neurons under current-clamp conditions

| Drug treatment | Vm (mV) | Rinput (mΩ) | AP amplitude (mV) | AP duration (ms) | n |
|----------------|--------|------------|-------------------|-----------------|---|
| Control—2 min  | -63.8 ± 1.6 | 139.8 ± 11.3 | 79.4 ± 4.8 | 8.1 ± 0.6 | 4 |
| —15 min        | -62.9 ± 1.3 | 147.4 ± 10.5 | 76.2 ± 3.6 | 7.9 ± 0.9 | 4 |
| Before 10 μM Glutamate | -63.3 ± 2.1 | 146.2 ± 15.1 | 73.9 ± 5.8 | 7.5 ± 0.6 | 5 |
| After 10 μM Glutamate | -59.7 ± 1.0 | 153.2 ± 20.7 | 64.8 ± 3.4 | 6.4 ± 0.5 | 5 |
| Before 5 μM Glutamate | -64.9 ± 2.1 | 140.5 ± 10.8 | 73.5 ± 4.1 | 7.7 ± 0.8 | 4 |
| After 5 μM Glutamate | -61.8 ± 2.8 | 155.0 ± 17.1 | 78.0 ± 5.0 | 6.9 ± 0.7 | 4 |
| Before Membrane Depolarization (-20 mV) | -62.9 ± 3.5 | 139.3 ± 13.6 | 78.3 ± 8.6 | 7.4 ± 0.8 | 3 |
| After Membrane Depolarization (-20 mV) | -58.2 ± 2.3 | 176.3 ± 23.6 | 85.6 ± 7.2 | 5.9 ± 0.7 | 3 |

*Calculated from the first action potential generated in response to a 20 pA current injection.*

![Figure 7](https://www.landesbioscience.com/Channels/51/figures/7.jpg)
show that multiple stimuli resulting in an increase in the \(\text{Ca}^{2+}/\text{calcineurin} \) activity in hippocampal neurons can affect \(\text{K}_{\text{V}2.1} \) gating and lead to changes in neuronal firing.

**Discussion**

The studies presented here show that glutamate treatment of hippocampal neurons leads to pronounced changes in voltage-dependent gating of the \(\text{K}_{\text{V}2.1} \) channels that constitute the majority of \(I_K \) currents. That altered gating properties of \(I_K \) are due to dephosphorylation of \(\text{K}_{\text{V}2.1} \) is supported by a number of experimental observations. \(\text{K}_{\text{V}2.1} \) undergoes calcineurin-dependent dephosphorylation in response to altered neuronal activity, membrane depolarization, and neuromodulation, with parallel changes in the voltage-dependent gating of \(I_K \) (Figs. 1 and 2). Blocking \(\text{K}_{\text{V}2.1} \) currents with HaTx prevented the glutamate-induced changes in \(I_K \) gating (Fig. 3), showing that the \(\text{K}_{\text{V}} \) channels that comprise the minority non-\(\text{K}_{\text{V}2.1} \) component of \(I_K \) are refractory to glutamate-dependent modulation (Fig. 3D and E).

We showed here that glutamate treatment of hippocampal neurons, through activation of \(\text{Ca}^{2+} \) permeable ionotropic glutamate receptors, leads to increased \([\text{Ca}^{2+}]_i \) that is both necessary and sufficient for the calcineurin-dependent modulation of \(I_K \) gating properties. This conclusion is drawn from our results showing that under \(\text{Ca}^{2+} \)-free extracellular conditions, or upon treatment with the specific NMDA receptor antagonist APV, effects of glutamate on voltage-dependent gating of \(I_K \) currents in neurons were eliminated (Fig. 2). Treatment of neurons with the \(\text{Ca}^{2+} \) ionophore ionomycin or increasing \([\text{Ca}^{2+}]_i \) through sustained membrane depolarization mimicked the glutamate-induced effects on \(I_K \) (Fig. 2), showing that, in the absence of glutamate stimulation, increased \([\text{Ca}^{2+}]_i \) is sufficient to induce modulation of neuronal of \(I_K \). A central role for calcineurin in mediating the effects of increased \([\text{Ca}^{2+}]_i \) is evident from our experiments showing that dephosphorylation of \(\text{K}_{\text{V}2.1} \) and, as shown here, modulation of \(I_K \) gating properties, in response to diverse stimuli that raise \([\text{Ca}^{2+}]_i \) is in all cases blocked by pretreatment of neurons with specific calcineurin inhibitors. A recent study revealed that endogenous glutamate released in response to ischemia is also sufficient to induce \(\text{K}_{\text{V}2.1} \) dephosphorylation and

**Figure 8.** Membrane depolarization in cultured rat hippocampal neurons leads to suppression of action potential firing frequencies. (A) Representative trace of spontaneous firing in a cultured hippocampal neuron for a period of 18 min with membrane depolarization to -20 mV for 10 min following an initial holding period of 3 min, and post-depolarization period of 5 min, with continuous perfusion of ACSF. (B) Quantitation of spontaneous spikes before and after membrane depolarization to -20 mV as shown in [A]. Data are presented as mean ± SEM of the number of spontaneous spikes per minute, before and after membrane depolarization (n = 3). *indicates significant difference in the number of spontaneous spikes (p < 0.05). (C and D) Decreased action potential firing in cultured hippocampal neurons upon membrane depolarization (-20 mV). (C) Representative traces of action potential firing patterns in cultured hippocampal neurons upon injection of 20 pA (upper) and 100 pA (lower) currents, respectively, over a period of 1 sec, and before and after membrane depolarization (-20 mV) for 10 min. (D) Frequency-intensity plot of action potential spikes generated upon step-wise current injections from experiments in (C). Data are presented as mean ± SEM (n = 3). (E) Representative trace of spontaneous firing patterns in a cultured hippocampal neuron for a period of 8 min with episodic membrane depolarization to -20 mV for 30 sec interspersed with 1 min intervals of resting potential (∼63 mV), with continuous perfusion of ACSF. (F) Quantitation of spontaneous spikes under resting conditions before and after episodic membrane depolarization to -20 mV as shown in (E). Data are presented as mean ± SEM of the number of spontaneous spikes per minute, from every 1 min resting interval (n = 2).
phosphorylated on Kv2.1 purified from rat brain and in HEK293 and potentiation.39 Indeed our results also show that glutamate treatment of Kv4 channels and inhibition of Kv2.1, as occurs upon induction of apoptosis, 33 or prolonged mediated Ca2+ entry in hippocampal neurons leads to increased here. Treatment of cultured rat hippocampal neurons with high Ca2+ levels yields time-dependent increase in the density of I{sub K} and decreased cell viability,35 through an as yet unknown mechanism. We previously found that reversible calcineurin-dependent phosphorylation of Kv2.1 purified from rat brain and in HEK293 and COS cells.21,34 This site is not regulated by calcineurin-dependent dephosphorylation of Kv2.1 in HEK293 cells, and mutation of S800 to Ala or Asp does not affect Kv2.1 gating.21 As such the events that are associated with Kv2.1 activation during apoptosis appear to occur independently of those induced by glutamate stimulation as applied here. Treatment of cultured rat hippocampal neurons with high levels of glutamate (200 μM) for long periods (20 min) yields time-dependent increase in the density of I{sub K} and decreased cell viability,35 through an as yet unknown mechanism. We previously found that reversible calcineurin-dependent Kv2.1 phosphorylation as induced by ischemic insult is neuroprotective.19 However, sustained activation of Kv2.1, as occurs upon induction of apoptosis,33 or prolonged treatment with high levels of glutamate,35 might lead to excessive K+ efflux, and subsequent induction of apoptotic or excitotoxic cell death, as we suggested previously.19

The potential impact of modulating Kv2.1 channel gating in suppressing excitability of hippocampal neurons was proposed initially based on preliminary computer simulations.36 Our more detailed modeling analyses presented here show that, using biophysical parameters of Kv2.1 activation and inactivation derived from control and glutamate-treated hippocampal neurons, the hyperpolarizing shift in the voltage-dependent gating of Kv2.1 channels alone was sufficient to dramatically reduce hippocampal neuronal firing in response to a broad range of excitatory current injections (Fig. 4). The computer simulations were predictive of experiments in cultured hippocampal neurons, which show drastic suppression of firing frequency upon glutamate treatment (Fig. 6). Similar suppression of action potential firing was previously reported for kainate treatment of retinal ganglion neurons,37 which also express Kv2.1.38 That glutamate-induced suppression of firing in cultured hippocampal neurons was reversed upon functional blockade of Kv2.1 currents with HaTx (Fig. 6), supports that modulation of Kv2.1 channels largely mediates the suppression of neuronal excitability. We should note that HaTx has some blocking activity against Kv4 channels,23 which underlie the neuronal I{sub A} that prevent back propagation of action potentials into dendrites.39 Activation of synaptic NMDA receptor-mediated Ca2+ entry in hippocampal neurons leads to increased phosphorylation of Kv4 channels and inhibition of I{sub A},40 presumably via phosphorylation-induced endocytosis of Kv4 channels,41 which results in increased dendritic excitability and induction of long-term potentiation.39 Indeed our results also show that glutamate treatment led to the suppression of I{sub A} currents (Fig. 1A). Increased firing of cultured rat hippocampal neurons is observed upon blockade of Kv4 channels with Heteropodatoxin-3.42 However, our results here show a drastic reduction of neuronal firing frequency upon glutamate treatment (Fig. 6A, C, D and F), which suppresses I{sub A} currents (Fig. 1A), but increases the amplitude and voltage-dependent gating properties of Kv2.1 (Fig. 1A–C). We did observe an increased firing frequency in HaTx-treated neurons (Fig. 6B, C, E and F), presumably due to blocking effects of HaTx on both Kv2.1,8 and Kv4,42 channels. Given these results and a recent report showing activity-dependent suppression of Kv4 channel function and increased neuronal excitability,6 it seems unlikely that Kv4 channels mediate the glutamate-induced, calcineurin-dependent suppression of neuronal excitability documented here. Although glutamate stimulation could affect the activity of other ion channels, the sufficiency of Kv2.1 modulation in model neurons in mimicking the experimentally observed suppression of neuronal excitability, and the loss of such suppression in HaTx-treated neurons, taken together argue that Kv2.1 contributes to the glutamate-induced suppression of firing.

While the model neurons accurately predicted the effects of glutamate on firing frequency, we should note that the model predicted an opposite effect of glutamate with regards to the latency to the first action potential following current injections (model: increased latency; cultured neurons: decreased latency). The differences between model and cultured neurons are presumably due to inhibitory effects of glutamate on Kv4 channels, which has been previously shown to yield decreased latency to the first spike.42 That HaTx did not inhibit the glutamate-induced effects on the decreased latency to the first spike mediated by Kv4 channels, but eliminated the suppression of firing, supports that the effects of HaTx on the latter are mediated through Kv2.1 channels. Note however that glutamate induced an increase in the latency to the first spike in DTXx-treated neurons (Fig. 7) suggesting that Kv1.1-containing channels also contribute to determining the latency to first spike.

Such activity-dependent effects on neuronal firing represent a form of homeostatic plasticity, whereby enhanced synaptic or intrinsic activity subsequently depresses intrinsic neuronal excitability.53 That glutamate treatment and membrane depolarization lead to similar modulation of Kv2.1/I{sub K} gating and suppresses neuronal firing is consistent with a general contribution of Kv2.1 modulation to homeostatic suppression of neuronal firing under conditions of hyperexcitability. The graded suppression of neuronal firing properties observed upon episodic membrane depolarization may correlate with a graded regulation of Kv2.1 gating properties upon dephosphorylation of individual phosphorylation sites on the channel protein.21,32 Several classes of ion channels, including Ca2+-dependent K+ channels,44 voltage-dependent Na+ channels45,46 and HCN channels47 have also been implicated in mediating changes in excitability associated with homeostatic plasticity. Our studies, together with those previously performed on cortical pyramidal neurons,48 argue that activity-dependent modulation of Kv2.1/I{sub K} is also an important determinant of this type of plasticity. Modulation of Kv2.1 with subsequent effects on neuronal excitability may also contribute to changes in excitability seen in spreading depression49 and ischemia/hypoxia, when excess glutamate may accumulate.50 Furthermore, the broad expression of Kv2.1 in mammalian brain suggests that this Kv2.1-dependent form of homeostatic plasticity may be found throughout the nervous system.
**Materials and Methods**

**Materials.** Glutamate, aminophosphonovaleric acid (APV), ionomycin, okadaic acid (OA), dendrotoxin-kappa (DTXκ), 4-aminopyridine (4-AP), cyclosporin A (CsA) and cyclophilin A (CypA), were obtained from Sigma Chemical (St. Louis, MO). HPLC-purified hanatoxin (HaTx) was a generous gift from Dr. Kenton J. Swartz, NINDS, Porter Neuroscience Institute, NIH, Bethesda, MD. All the drugs were diluted in the extracellular buffer, except for CsA and CypA, which were dissolved in the pipette solution to achieve the final desired concentrations.

**Culture of rat embryonic hippocampal neurons.** Hippocampal neurons isolated from E18 or E19 rat embryos were cultured for 12–15 days on poly-L-lysine coated glass cover-slips or plastic tissue culture dishes as described previously.18

**Culture of HEK293 cells stably expressing recombinant rat Kv2.1.** Generation of the Human Embryonic Kidney (HEK) 293 cells (ATCC, Manassas, VA) stably expressing recombinant rat Kv2.1 was described previously.21 Cells were maintained in Dulbecco’s Modified Eagle's Media (DMEM) with 10% FBS and 1% penicillin and streptomycin, Glutamax (Invitrogen), and 10% Fetal Bovine Serum (FBS; HyClone), along with 500 μg/ml of active G418 (Invitrogen), and plated 24 hours before use in electrophysiological experiments.

**Electrophysiological recordings and data analyses.** **Voltage-clamp.** Outward potassium currents were recorded from cultured rat hippocampal neurons in whole-cell mode using an EPC10 patch-clamp amplifier (HEKA Electronik, Lambrecht/Pfalz, Germany), at room temperature (23–25°C) as described.20 Patch pipettes were pulled from borosilicate glass tubes (Sutter Instrument, Novato, CA) to give a tip resistance of 1.5–3.0 MΩ when filled with the pipette solution. The extracellular buffer contained (in mM) 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose, pH 7.3. The pipette solution contained (in mM) 140 KCl, 5 NaCl, 2 MgCl2, 1 CaCl2, 5 EGTA and 10 HEPES, pH 7.3. The free Ca2+, Mg2+ and EGTA concentrations in the pipette solution were calculated using the MaxChelator software (Stanford, CA), and are 0.027 mM, 1.6 mM and 2.6 mM respectively. Glutamate and HaTx were dissolved in the extracellular buffer to achieve the final desired concentration. Control and drug-added extracellular buffer were applied to the bath for 10 min immediately after recording the currents for activation, steady-state inactivation, and recovery from inactivation using a polytetrafluoroethylene glass multiple-barrel perfusion system. In voltage-clamp experiments using HaTx, the first set of currents (for activation and steady-state inactivation) were recorded soon after obtaining the whole-cell configuration in voltage-clamp mode and compensating capacitance, membrane voltages were recorded in current-clamp mode by holding the neurons at their inherent membrane potentials (-57 mV to -68 mV). Voltage-dependent current activation and inactivation curves were generated using established protocols2122 and the voltage-dependent parameters are given in Table 1. To determine the recovery time period from steady-state inactivation of IK, the cells were held at -80 mV and depolarized to +50 mV for 500 ms, followed by subsequent pulses again at +50 mV with different interval durations in 200 ms increments with an intermediate holding at -80 mV. 2 mM 4-AP was used in the extracellular buffer to block IK. Peak IK amplitudes from the second pulse were normalized to the current obtained in the respective first pulse and plotted against the respective interval time.

**PULSE 8.65 software (HEKA Elektronik) was used for acquisition and analysis of currents. IGOR Pro4 (WaveMetrax, Lake Oswego, OR), and Origin7 software (OriginLab, Northampton, MA) were used to perform least-squares fitting and to create figures. Data are presented as mean ± SEM or fitted value ± SE of the Boltzmann equation fit. Paired or unpaired Student’s t-tests (Origin; OriginLab) were used to evaluate significance of changes in mean values. p values <0.05 were considered statistically significant.

**Current-clamp.** Action potential firing patterns in cultured rat hippocampal neurons were recorded in current-clamp mode of standard patch-clamp technique at room temperature (23–25°C). Patch pipettes were pulled from borosilicate glass tubes to give a tip resistance of 1.5–4.5 MΩ when filled with pipette solution. Membrane voltages were recorded either with an Axopatch 1D patch-clamp amplifier and monitored with a PC running pClamp6 (Axon Instruments), or using PULSE 8.65 and Patchmaster 2.10 software in an EPC10 patch-clamp amplifier (HEKA Electronik). After achieving whole-cell configuration in voltage-clamp mode and compensating capacitance, membrane voltages were recorded in current-clamp mode by holding the neurons at their inherent membrane potentials (~57 mV to ~68 mV). The extracellular buffer (ACSF) contained (in mM) 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 25 NaHCO3, 1.25 NaH2PO4 and 10 glucose, pH 7.3, and was continuously bubbled with CO2 during the experiment. The pipette solution contained (in mM) 140 KCl, 5 NaCl, 2 MgCl2, 1 CaCl2, 3 Mg-ATP, 0.2 Na-GTP, 5 EGTA and 10 HEPES, pH 7.3. Glutamate, HaTx, and DTXx were diluted in the ACSF to achieve the final desired concentration. Control and drug-containing ACSF were applied to the bath using a polytetrafluoroethylene glass multiple barrel perfusion system. Glutamate was applied to the bath immediately after recording the firing by injecting different amplitude of currents, and after 10 min next set of current-injected firings were recorded. In experiments using HaTx, the first set of firings was recorded soon after 10 min treatment of neurons with HaTx in extracellular buffer. Further, HaTx and glutamate were applied together in the extracellular buffer for 10 min and the next set of firings was recorded. In experiments using glutamate and drug (or drug-containing ACSF), the first set of firings was recorded soon after 10 min treatment of neurons with glutamate in extracellular buffer. Further, glutamate and drug were applied together in the extracellular buffer for 10 min and the next set of firings was recorded. Origin7 software (OriginLab) was used to perform least squares fitting and to create figures. Data are presented as mean ± SEM. Paired or unpaired Student’s t-tests were used to evaluate significance of changes. p values < 0.05 were considered statistically significant.

**Generation of current-clamp data by computer simulation.** Simulations were performed using NEURON 5.7.24 Because the
employed electrophysiological protocols were similar to those used by Hodgkin and Huxley,\(^{51,52}\) our simulations used a mathematical model with similar features. First, \(K_{v,2.1}\) channel gating was assumed to conform to a Hodgkin-Huxley-like formalism \(I = G_{\text{max}} x^2(t,V) [(a)h(t,V) + (1 - a)](V - E_K),\) where \(I\) is the current, \(G_{\text{max}}\) is the maximum conductance, \(V\) is the transmembrane voltage, \(E_K\) is the \(K^+\) equilibrium potential, and \(a\) and \(h\) are the gating particles that control activation and inactivation of the \(K_{v,2.1}\) channel, respectively. Both \(a\) and \(h\) are functions of time and transmembrane voltage that satisfied the partial differential equations:

\[
\frac{\partial a}{\partial t} = \frac{n(x,V) - a(t,V)}{\tau_a(V)}
\]

\[
\frac{\partial h}{\partial t} = \frac{h(x,V) - h(t,V)}{\tau_h(V)}
\]

where

\[
n(x,V) = 1/[1 + \exp\{(V - V_{nh})/V_{nc}\}]
\]

\[
\tau_a(V) = C_n/[\exp\{(V - V_{tn})/\tau_{tc1}\} + \exp\{-(V - V_{tn2})/\tau_{tc2}\} + \tau_{a\phi}]
\]

\[
h(x,V) = (1 - P)/\{1 + \exp\{-(V - V_{hh})/V_{hc}\}\} + P
\]

\[
\tau_h(V) = C_n/[\exp\{(V - V_{thh})/\tau_{tc1}\} + \exp\{-(V - V_{th2})/\tau_{tc2}\} + \tau_{h\phi}]
\]

and where \(V_{nh}\) is the half-maximal activation of the steady-state activation curve; \(V_{nc}\) is the slope of the steady-state activation curve; \(C_n\) is assumed to be the maximal activation time constant taking into account that \(\tau_{a\phi}\) is close to zero; \(\tau_{a\phi}\) is the activation time constant at maximal positive or negative potentials; \(V_{tn2}\) is the voltage at which the time constant for activation is maximum; \(\tau_{tc1}\) is the slope of the activation curve for the negative side of the \(V_{nh}; \tau_{tc2}\) is the slope of the activation curve for the positive side of the \(V_{nh}; \tau_{hh}\) is the half-maximal steady-state inactivation voltage; \(V_{hc}\) is the slope of the steady-state inactivation curve; \(\tau_{h\phi}\) is assumed to be the maximal inactivation time constant; \(\tau_{h\phi}\) is the inactivation time constant at maximal positive or negative potentials; \(V_{th2}\) is the voltage at which the time constant for inactivation is maximum; \(P\) is the fraction of non-inactivating current.

This formalism was coded in a NEURON mod file and then virtual channels were distributed in the membrane of cylindrical soma (35 \(\mu\)m length, 25 \(\mu\)m diameter) of a model hippocampal pyramidal neuron with a single bifurcating apical dendrite and two basal dendrites. To model currents generated from somatic point clamp recordings, two different Hodgkin-Huxley-style \(K_{v,2.1}\) channel models were created to represent control and glutamate-modulated conditions. The \(K_{v,2.1}\) density was assumed to be zero in the all compartments except the soma. Manually adjusting values of various parameters in steady-state activation, steady-state inactivation, activation time constant and inactivation time constant formulae led to a reasonably accurate simulation of experimental data for both control (with \(G_{1/2} = +16\) mV and \(V_{1/2} = -25\) mV) and glutamate-modulated conditions (with \(G_{1/2} = -10\) mV and \(V_{1/2} = -55\) mV). A spiking model of a hippocampal pyramidal neuron was constructed by adding \(Na_{v,1}, Ca_{v,1}, Ca_{v,2}, K_{v,2}, K_{v,3}, K_{v,4}, K_{v,7}\) (KCNQ), SK, BK, KIR2 and HCN channel models that were based upon standard experimental measurements reported in the literature. These mod files and the details of this model are available upon request. Action potential firing patterns from this model neuron were predicted upon somatic injection of different current amplitudes ranging from 0 to 500 pA over a period of 500 ms from a holding potential of -65 mV using the NEURON utilities in current-clamp mode. The control and glutamate-induced altered biophysical properties of \(I_K\) were simulated separately to obtain the action potential firing patterns upon altered \(K_{v,2.1}\) channel activity. Upon publication, this model will be available on the Neuron Model Database website (http://senselab.med.yale.edu/NeuronDB/).

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