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# Novel role of KCNQ2/3 channels in regulating neuronal cell viability

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**Overactivation of certain $K^+$ channels can mediate excessive $K^+$ efflux and intracellular $K^+$ depletion, which are early ionic events in apoptotic cascade. The present investigation examined a possible role of the KCNQ2/3 channel or M-channel (also named Kv7.2/7.3 channels) in the pro-apoptotic process. Whole-cell recordings detected much larger M-currents (212 ± 31 pA or 10.5 ± 1.5 pA/pF) in cultured hippocampal neurons than that in cultured cortical neurons (47 ± 21 pA or 2.4 ± 0.8 pA/pF). KCNQ2/3 channel openers *N*-ethylmaleimide (NEM) and flupirtine caused dose-dependent $K^+$ efflux, intracellular $K^+$ depletion, and cell death in hippocampal cultures, whereas little cell death was induced by NEM in cortical cultures. The NEM-induced cell death was antagonized by co-applied KCNQ channel inhibitor XE991 (10 μM), or by elevated extracellular $K^+$ concentration. Supporting a mediating role of KCNQ2/3 channels in apoptosis, expression of KCNQ2 or KCNQ2/3 channels in Chinese hamster ovary (CHO) cells initiated caspase-3 activation. Consistently, application of NEM (20 μM, 8 h) in hippocampal cultures similarly caused caspase-3 activation assessed by immunocytochemical staining and western blotting. NEM increased the expression of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), induced mitochondria membrane depolarization, cytochrome *c* release, formation of apoptosome complex, and apoptosis-inducing factor (AIF) translocation into nuclear. All these events were attenuated by blocking KCNQ2/3 channels. These findings provide novel evidence that KCNQ2/3 channels could be an important regulator in neuronal apoptosis.**
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Apoptosis is a programmed cell death that occurs during development or in pathological conditions such as after cerebral ischemia and trauma injury.[1] Hallmarks of apoptosis include cell body shrinkage, nuclear condensation, DNA fragmentation, mitochondrial depolarization, activation of apoptotic genes such as caspases, and mitochondrial release of apoptotic factors such as cytochrome *c* and/or apoptosis-inducing factor (AIF). Among them, cell volume decrease is a typical morphological phenotype of apoptotic cells.[2,3] Compelling evidence from our and others' investigations have revealed that excessive $K^+$ efflux and intracellular $K^+$ depletion are the main underlying ionic mechanism responsible for the apoptotic cell body shrinkage.[3–6]

$K^+$ is the predominant ion inside the cell. The intracellular environment contains high levels of $K^+$ (~150 mM), but low levels of $Na^+$ (~10 mM) and free $Ca^{2+}$ (~100 nM). Loss of ≥50% intracellular $K^+$ has been identified in many types of cells undergoing apoptosis, which is sufficient either to trigger or promote the activation of apoptotic cascade.[5,7] Increased $K^+$ efflux due to enhanced and/or prolonged activation of $K^+$ channels may serve as an initial step in cell volume shrinkage, and further cause caspase activation, mitochondrial depolarization, cytochorome *c* release, and DNA fragmentation. The $K^+$-mediated pro-apoptosis mechanism has been indicated in both the intrinsic (cytochrome *c* release from mitochondria) and extrinsic (death receptor mediated) pathways.[3,6] Blocking $K^+$ channel and $K^+$ efflux by $K^+$ channel blockers such as tetraethylammonium (TEA) or 4-aminopyridine (4-AP) antagonizes apoptotic cell death induced by a number of apoptotic insults. For example, TEA and/or 4-AP attenuated apoptosis induced by staurosporine in neurons[8,9] and pulmonary smooth muscle cells.[10] Some $K^+$ channels including outward/inward delayed rectifiers, transient A-type $K^+$ channel, $Ca^{2+}$-activated $K^+$ channels, ATP-sensitive $K^+$ channels, two-pore $K^+$ channels, and several others have been identified for their role in the $K^+$ mechanism of apoptosis.[1,3] So far, no study has indicated a role for M-channels in the pro-apoptotic ionic mechanism.

The M-type channels are unique voltage-gated and ligand-regulated $K^+$ channels with their distinct physiological and pharmacological characteristics.[11] They are activated at a voltage near the threshold for action potential initiation and regulate membrane excitability. M-channels were later categorized into the KCNQ or Kv7 channel family. Five genes of this family (KCNQ1–5) are all encoding $K^+$ channel subunits; among them homodimer and/or heterodimer of KCNQ2 and KCNQ3 are believed components of the M-channel.[12] KCNQ1 is restricted to the heart and smooth

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**Abbreviations:** NEM, *N*-ethylmaleimide; CHO, Chinese hamster ovary; AIF, apoptosis-inducing factor; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; DIV, day *in vitro*; TMRM, tetramethyrhodamine methyl ester; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor
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muscle cells, the other four KCNQ channels are expressed mainly in the nervous systems. Mutations of these genes have been reported to relate to many diseases. Interestingly, among 10 $K^+$ channel gene mutations related to human diseases, four come from the KCNQ gene family. Some compounds developed for treatment of Alzheimer's disease, epilepsy, and stroke were partly based on their effects on brain KCNQ channels.[13] In the present investigation, we identified and compared KCNQ2/3 channels in cultured hippocampal and cortical neurons. Overactivation or expression of these channels induced apoptosis. The KCNQ2/3 channel-induced neuronal cell death was likely mediated by the extracellular signal-regulated protein kinase (ERK) signaling pathway. Thus, M-channels may have broader physiological and pathological functions that were not recognized before.

## Results

**Identification of the KCNQ2/3 channels and M-current in hippocampal and cortical cultures.** In cultured hippocampal and cortical neurons, we first determined the expression levels of KCNQ2 and KCNQ3 subunits that are the molecular basis of the M-channel.[12] KCNQ2 and KCNQ3 proteins were detected in both cultured hippocampal and cortical neurons (14 day *in vitro* (DIV)) examined by immunostaining (Figure 1a and b) and western blotting (Figure 1c). In whole-cell recordings, the conventional protocol for activating/inactivating M-currents was applied.[14] The membrane potential was held at $-30$ mV in order to open the non-inactivating M-channels. Other voltage-gated channels were either not sensitive to this slight depolarization or inactivated at such a holding potential. Voltage pulses stepping from $-30$ mV to hyperpolarized potentials closed activated M-channels, and the channel closing relaxation current was recorded (Figure 2). Although KCNQ channel proteins were identified in both hippocampal and cortical neurons under the cultured condition, the electrophysiological assessment revealed a striking difference in the M-channel activity in these two types of neurons. M-currents carried by functional channels were much more prominent in hippocampal neurons ($212 \pm 31$ pA or $10.5 \pm 1.5$ pA/pF; $n = 24$) than that in cortical neurons ($47 \pm 21$ pA or $2.4 \pm 0.8$ pA/pF; $n = 20$) (Figure 2). The M-current was also identified by its $K^+$ dependence, slow kinetics, non-inactivation kinetic features, and sensitivity to block via its selective blockers such as muscarine and XE991 (Figure 2d).[14,15] Based on the large difference in the M-currents in hippocampal and cortical neurons, we next tested how functional KCNQ/M-channels might affect cell survival.

**KCNQ channel openers induced hippocampal neuronal death.** Activation of $K^+$ channels would promote $K^+$ efflux. Persistent opening of $K^+$ channels ultimately results in loss of intracellular $K^+$ and apoptosis.[5] *N*-ethylmaleimide (NEM), a cysteine-modifying reagent, was found to strongly augment M-currents by increasing their maximal open probability by alkylation of a cysteine residue in the channels' C terminus without affecting surface expression.[16] In hippocampal neurons, 30 $\mu$M NEM of bath application doubled the size of M-current in 20 min ($n = 13$), while it merely caused about 20% enhancement of the current in cortical neurons ($n = 5$) ($P < 0.05$ *versus* controls in both tests). To investigate whether persistent activation of KCNQ/M-channels might result in cell death in cultured neurons, we treated hippocampal neurons with different concentrations of NEM (5, 15, 20, 30, and 50 $\mu$M) for 24 h. TUNEL staining showed that NEM reduced viability of hippocampal neurons in a dose-dependent manner (Figure 3a and c). For instance, NEM at 30 and 50 $\mu$M induced 52 and 74% hippocampal cell death, respectively. Consistent with less functional M-channels in cortical neurons, the same concentrations of NEM induced only 14 and 19% death in these cells, respectively (Figure 3b and c).

To confirm that the NEM-induced neuronal death was indeed due to KCNQ channel activation but not some non-specific effects, hippocampal neurons were treated with NEM (30 $\mu$M, 24 h) combined with the KCNQ channel blocker XE991 (10 $\mu$M). The NEM-induced cell death was markedly reduced by inclusion of XE991 ($64 \pm 5$ and $20 \pm 9$% death in NEM alone and NEM plus XE991, respectively) (Figure 4a). Supporting that NEM-induced cell death was mediated by an excessive $K^+$ efflux, cell death was significantly attenuated by an external solution containing elevated $K^+$ (25 mM) that decreased the $K^+$ gradient crossing the plasma membrane (Figure 4a).

Subsequently, we tested a second KCNQ channel opener, flupirtine, for its ability to induce cell death in hippocampal cultures. Similar to NEM, exposure to flupirtine (100 $\mu$M, 48 h) caused significant cell death. The flupirtine-induced neuronal death was largely prevented by 10 $\mu$M XE991 ($61 \pm 3$ and $33 \pm 4$% death in flupirtine alone and flupirtine plus XE991, respectively) (Figure 4b).

Consistent with the idea that overactivation of $K^+$ channels and excessive $K^+$ efflux may lead to intracellular $K^+$ depletion, we detected substantial reduction of intracellular $K^+$ using the cell-permeable fluorescent $K^+$ dye potassium-binding benzofuran isophthalate (PBFI)-AM (Figure 4c and d). Under the control condition, intracellular $K^+$ was stable, whereas 80-min exposure to NEM caused about 40% of intracellular $K^+$ depletion, which was similar to the effect of the $K^+$ ionophore valinomycine in these cells (Figure 4e).

**Apoptosis induced by KCNQ2/3 expression in CHO cells.** We examined whether expression of KCNQ2/3 channels could directly cause apoptosis. We demonstrated before that Chinese hamster ovary (CHO) cells possess little endogenous $K^+$ currents, making these cells an ideal system for testing the role of an exogenously expressed specific $K^+$ channel.[17] CHO cells were transfected with KCNQ2 and KCNQ3 genes that were subcloned into pEGFP-C3 and pDsRed2-C1 vectors, respectively. The expression of these channel proteins could then be identified by the fluorescent green and red colors, respectively (Figure 5b and d). Expression of either homogenous KCNQ2 or heterogeneous KCNQ2/3 channels caused significant activation of caspase-3 after 24 h when compared with vector-transfected control CHO cells. The caspase-3 activation was completely blocked by the KCNQ channel blocker XE991 (Figure 5e). Expression of

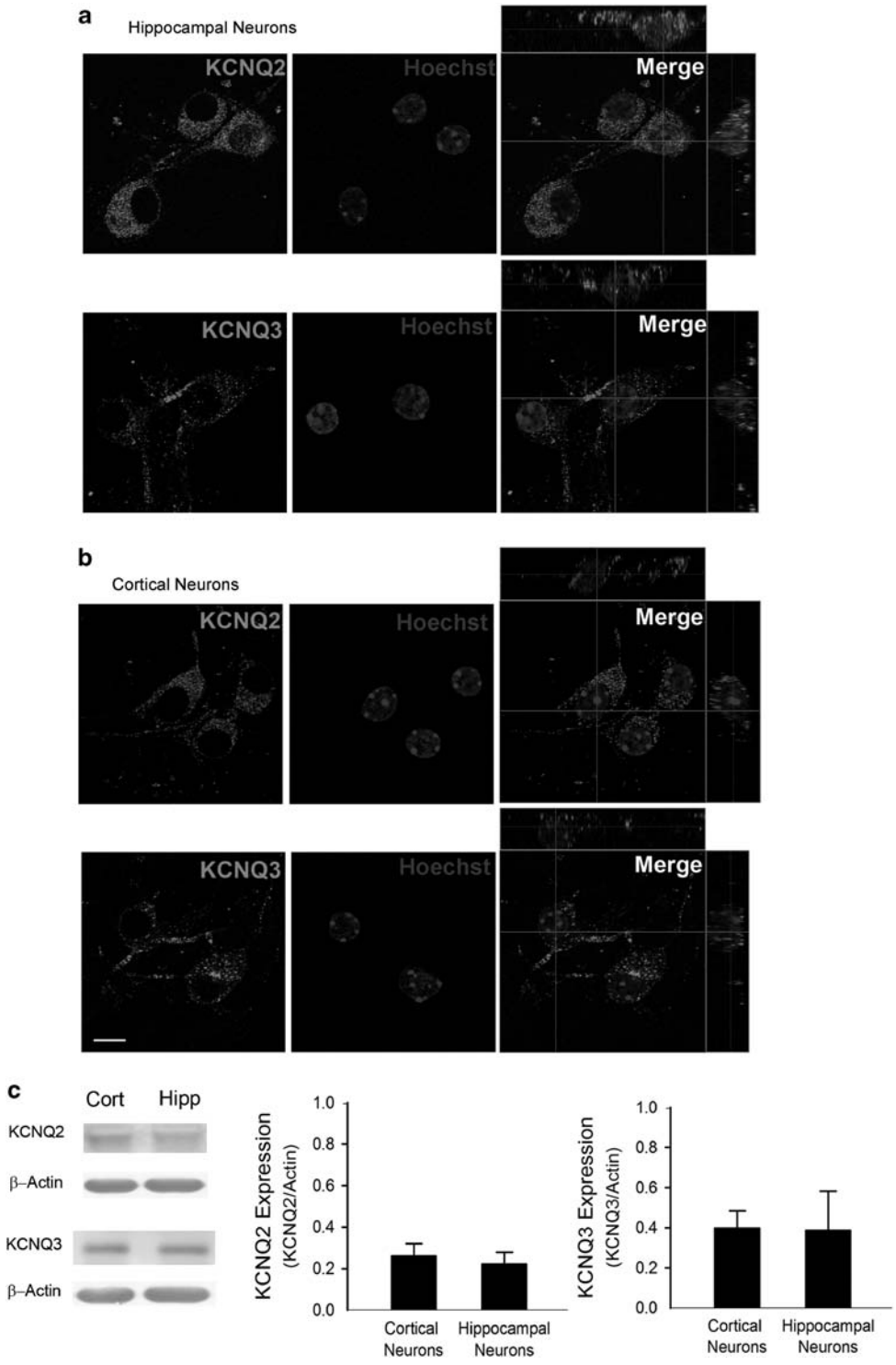


**Figure 1** KCNQ2 and KCNQ3 subunits in cultured hippocampal and cortical neurons. Expression and functional activity of KCNQ2 and KCNQ3 channels were tested in hippocampal and cortical neurons of 14 days *in vitro* (DIV). (**a**, **b**) Immunostaining for KCNQ2 (red), KCNQ3 (red), and Hoechst (blue) in hippocampal neurons (**a**) and cortical neurons (**b**). KCNQ2/3 was detected in both the cell body and dendrites of hippocampal and cortical neurons. Scale bar = 10 $\mu$m. (**c**) Western blotting showed that there is no difference in KCNQ2/3 expression level between cortical (Cort) and hippocampal (Hipp) neurons. $n = 3$ independent experiments. Error bars represent S.E.M.

homogenous KCNQ3 alone, on the other hand, showed much less efficacy in induction of apoptosis, which is consistent with the well-known fact that very little current is generated by expression of homogenous KCNQ3 subunit.[18] This observation additionally supports the importance of M-channel activities, but not just gene expression, in apoptosis.

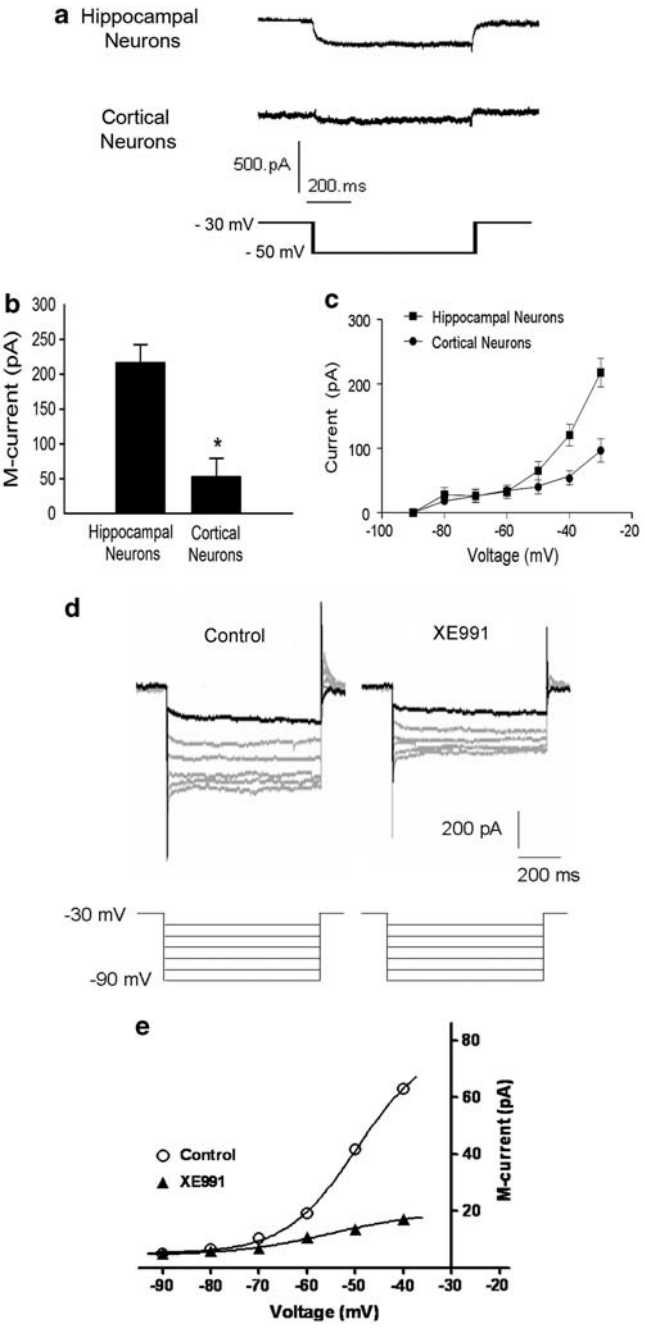


**Figure 2** M-currents in cultured hippocampal and cortical neurons. M-currents were recorded in 14–17 DIV hippocampal and cortical neurons using whole-cell recording. (**a**) Representative M-current traces recorded from hippocampal and cortical neurons, respectively. The recording of M-currents was started from a holding potential of $-30$ mV to allow the channels in activated state. A hyperpolarized pulse to $-50$ mV then close the M-channel, this hyperpolarization does not activate other voltage-gated channels that are sensitive only to depolarization. The M-current was also identified by its $K^+$ dependence, sensitivity to block by muscarine and XE991, its slow activation phase and non-inactivation feature.[14,15] (**b**) The bar graph shows mean values of M-currents in hippocampal neurons ($n=10$) and in cortical neurons ($n=7$). The current was enhanced by NEM in a dose-dependent manner, the enhanced current remained sensitive to block by XE991 (data not shown). (**c**) The I-V relationship of M-currents in hippocampal and cortical neurons. Although the amplitude of the currents was different in these two types of cells, both show typical voltage sensitivity and outward rectification features of the M-current. $N=5$ for each group. Error bars represent S.E.M. *$P<0.05$ *versus* hippocampal neurons. (**d**) M-currents (upper panel) in a hippocampal neuron triggered at different voltage steps (down panel) and their block by XE991 (5 $\mu$M). Current traces were from the same cell before and after 10 min application of XE991. (**e**) I-V curves of M-currents before and after 5 $\mu$M XE991. The I-V curves were constructed by plotting the amplitude of inward relaxation upon hyperpolarizing pulses. XE991 at 5 $\mu$M substantially (70–80%) blocked the current in 10 min. $N=3$ cells

**KCNQ channel-mediated activation of ERK and caspase-3.** In hippocampal neurons, 4 h application of NEM (20 $\mu$M) caused a fivefold increase in ERK1/2 phosphorylation (Figure 6a and b). NEM treatment for 8 h resulted in more than a twofold increase in caspase-3 activation measured by western blotting (Figure 6c and d)

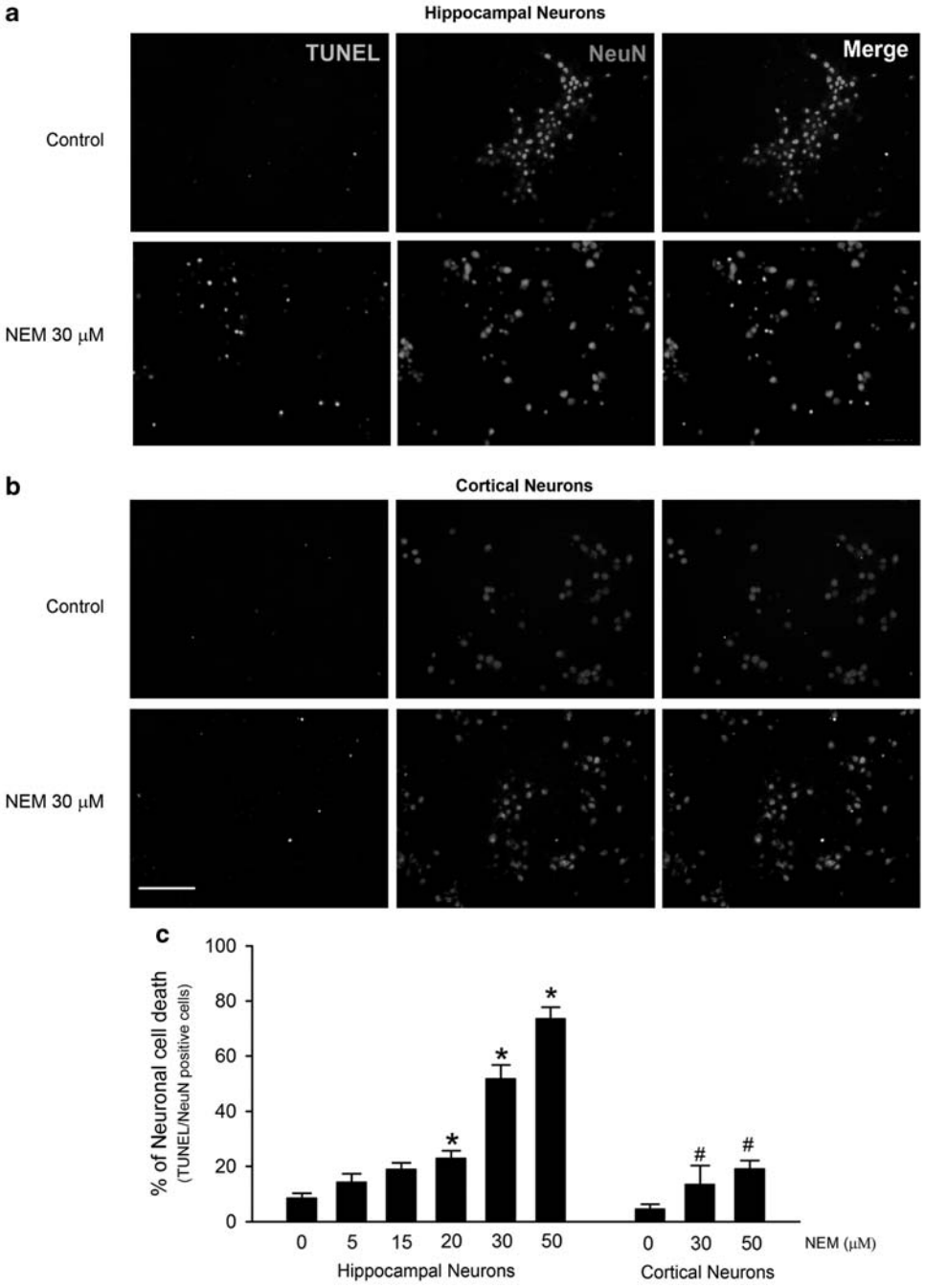


**Figure 3** KCNQ channel activation induced neuronal cell death in cultured hippocampal and cortical neurons. Cell viability in the presence of the KCNQ channel opener, NEM (30 or 50 $\mu$M, 24 h), was assessed by TUNEL staining. (**a**, **b**) NEM (30 $\mu$M) caused more TUNEL-positive cells in hippocampal neurons (**a**) than in cortical neurons (**b**). Scale bar = 100 $\mu$m. (**c**) Quantification of the percentage of TUNEL-positive neurons in hippocampal and cortical cultures. NEM caused dose-dependent significant cell death in hippocampal cultures, whereas much less cell death was detected in cortical neurons. Mean ± S.E.M. $N \geq 3$ independent experiments. $^{*}P < 0.05$ *versus* vehicle controls; $^{\#}P < 0.05$, *versus* NEM (30 or 50 $\mu$M) treatment in hippocampal cells

and immunostaining (Figure 6e and f). These effects were blocked by the KCNQ channel inhibitor XE991 (10 $\mu$M) (Figure 6).

Supporting that the ERK/mitogen-activated protein kinase (MAPK) pathway is a key factor involved in KCNQ channel-mediated cell death, NEM-induced neuronal death was significantly reduced by the ERK inhibitor UO126 (10 $\mu$M) (Figure 7a). XE991 and UO126 both dramatically reduced NEM-induced caspase-3 activation (Figure 7b).

**KCNQ channel activation-induced mitochondria membrane depolarization.** Tetramethyrhodamine methyl ester (TMRM) is a lipophilic potentiometric dye used as a fluorescent probe to monitor the membrane potential of

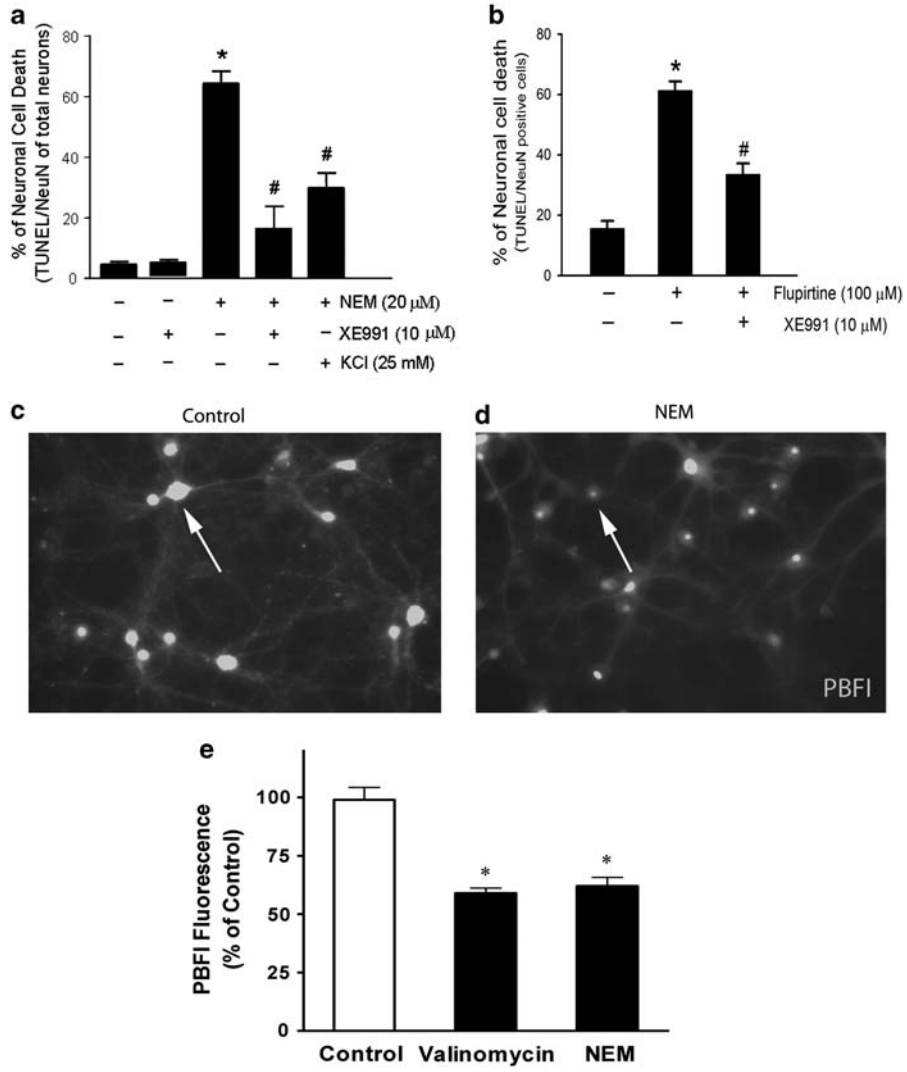


**Figure 4** Protective effects of KCNQ blocker or high external $K^+$ on NEM and flupirtine-induced hippocampal neuronal death. (**a**) KCNQ channel inhibitor XE991 (10 $\mu$M) or high $K^+$ external solution (25 mM KCl) showed protective effect on 30 $\mu$M NEM-induced cell death in hippocampal neurons, revealed using TUNEL assay. XE991 (10 $\mu$M) alone did not show significant effect on the basal level of cell viability. (**b**) KCNQ channel opener flupirtine (100 $\mu$M) cause more neuronal death compared with controls, and XE991 blocked flupirtine toxicity. Mean ± S.E. $N \geq 3$ independent experiments. (**c**, **d**) PBFI-AM imaging was applied to measure intracellular $K^+$ changes. There was no change in control cells (**c**) but significant decreases in PBFI fluorescence were detected after exposure to either NEM (30 $\mu$M, 80 min) (**d**) or the $K^+$ ionophore valinomycin (50 $\mu$M, 80 min) (**e**). Arrows point to PBFI imaging in representative cells under control and NEM conditions. (**e**) The bar graph summarizes experiments in (**c**) ($n = 3$ independent measurements in each group). $^*P < 0.05$ *versus* vehicle controls; $^\#P < 0.05$ *versus* NEM treatment

mitochondria. We used TMRM to determine mitochondria membrane potential before and after NEM application. NEM (20 $\mu$M) caused a drastic mitochondria membrane depolarization within 30 min; co-applied XE991 (10 $\mu$M) partially blocked this mitochondria dysfunction event (Figure 8).

**KCNQ channel activation-induced cytochrome *c* and AIF release and nuclear translocation.** Hippocampal neurons exposed to NEM (20 $\mu$M) for 4 h showed release of cytochrome *c* from mitochondria to cytosol, then translocation to nuclear (Figure 9a–d). Meanwhile, we observed similar nuclear translocation of the caspase-independent signaling protein AIF after KCNQ channel activation (Figure 9b and e). The nuclear translocations of cytochrome *c* and AIF were blocked by XE991 (10 $\mu$M) or ERK inhibitor UO126 (10 $\mu$M) (Figure 9a–e).

**KCNQ channel activation-promoted apoptosome formation.** Given that the KCNQ channel inhibitor XE991 only partially blocked mitochondria membrane depolarization and release of cytochrome *c* while it was much more effective in reversing the NEM-induced caspase-3 activation, we tested the hypothesis that blocking KCNQ channels might show greater effect on the formation of apoptosome, a protein complex of ATP, cytochrome *c*, Apaf-1 and caspase-9 that leads to caspase-3 activation. When the apoptosome formation in hippocampal neurons was examined using co-immunoprecipitation of Apaf-1 and caspase-9 antibodies

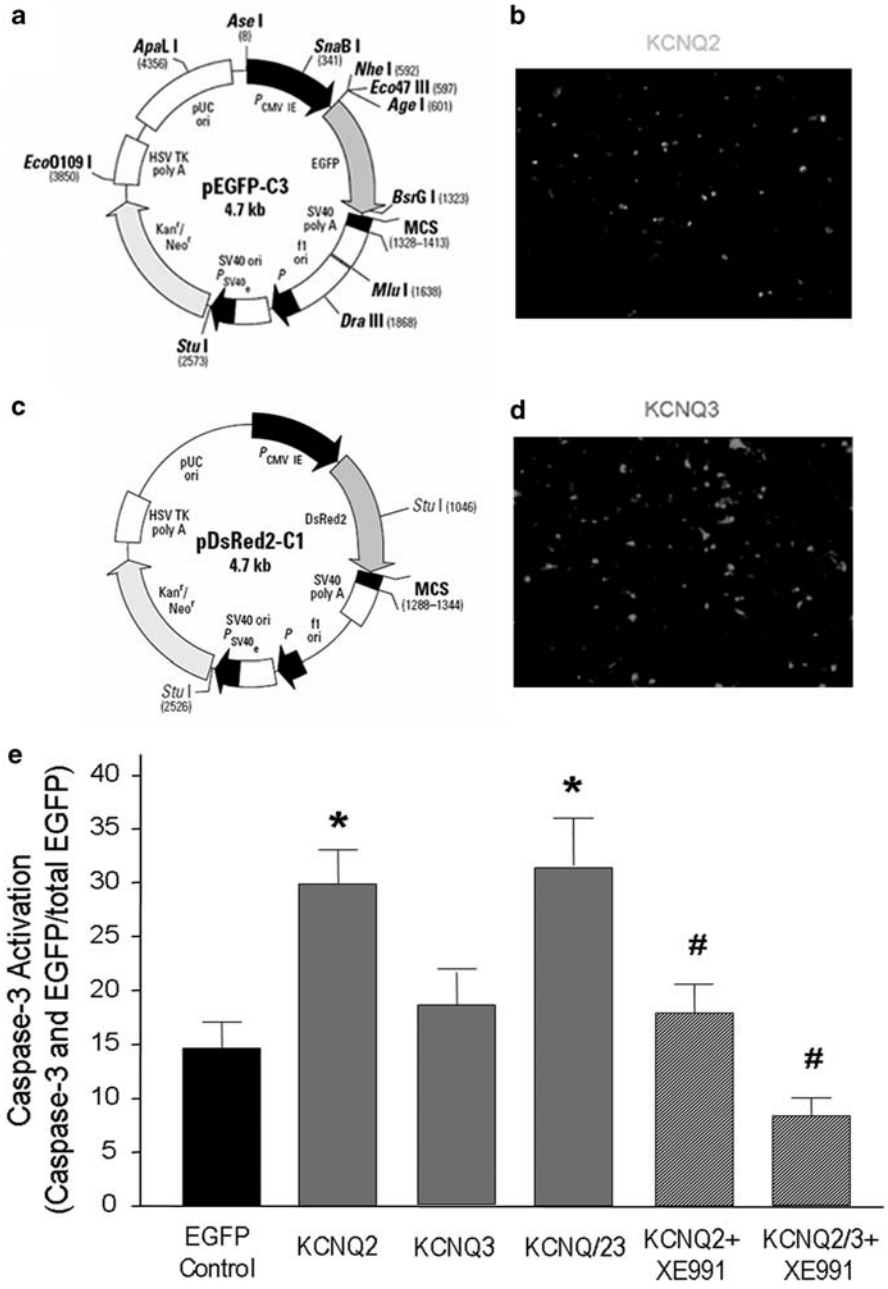


**Figure 5** Effects of expression of KCNQ2/KCNQ3 channels on caspase-3 activation. (**a**–**d**) KCNQ2 and KCNQ3 subunits were subcloned into pEGFP-C3 (**a**) and pDsRed2-C1 vectors (**c**) and transfected into CHO cells shown as green and red color in transfected cells (**b**, **d**). (**e**) The expression of KCNQ2 alone or KCNQ2/3 induced activation of caspase-3, and the KCNQ channel blocker XE991 suppressed caspase-3 activation in KCNQ-transfected CHO cells. Expression of homogenous KCNQ3 showed much less effect on caspase-3 activation, which is in line with previous reports that this subunit alone may not form functional M-channels (see text for discussion). $^{*}P<0.05$ *versus* control vector transfection; $^{\#}P<0.05$ *versus* KCNQ expression

4 h after NEM (20 $\mu$M) treatment, co-applied XE991 (10 $\mu$M) completely eliminated the NEM-induced apoptosome formation (Figure 10).

## Discussion

As a non-inactivating $K^+$ channel, the KCNQ/M-channel has significant roles in controlling neurotransmitter release, neuronal excitability, and generation of action potentials throughout the nervous system.[19] The present investigation provides the first evidence that, in addition to their well-defined physiological functions, the activity of KCNQ2/3 channels may affect viability of central neurons and perhaps other cells such as CHO cells tested in this investigation. This role of KCNQ/M-channels is consistent with the previous discovery that excessive $K^+$ efflux and intracellular $K^+$ depletion are key early steps in apoptosis, while inhibiting overactivated $K^+$ channels is neuroprotective.[3,5,7] Here, we specifically

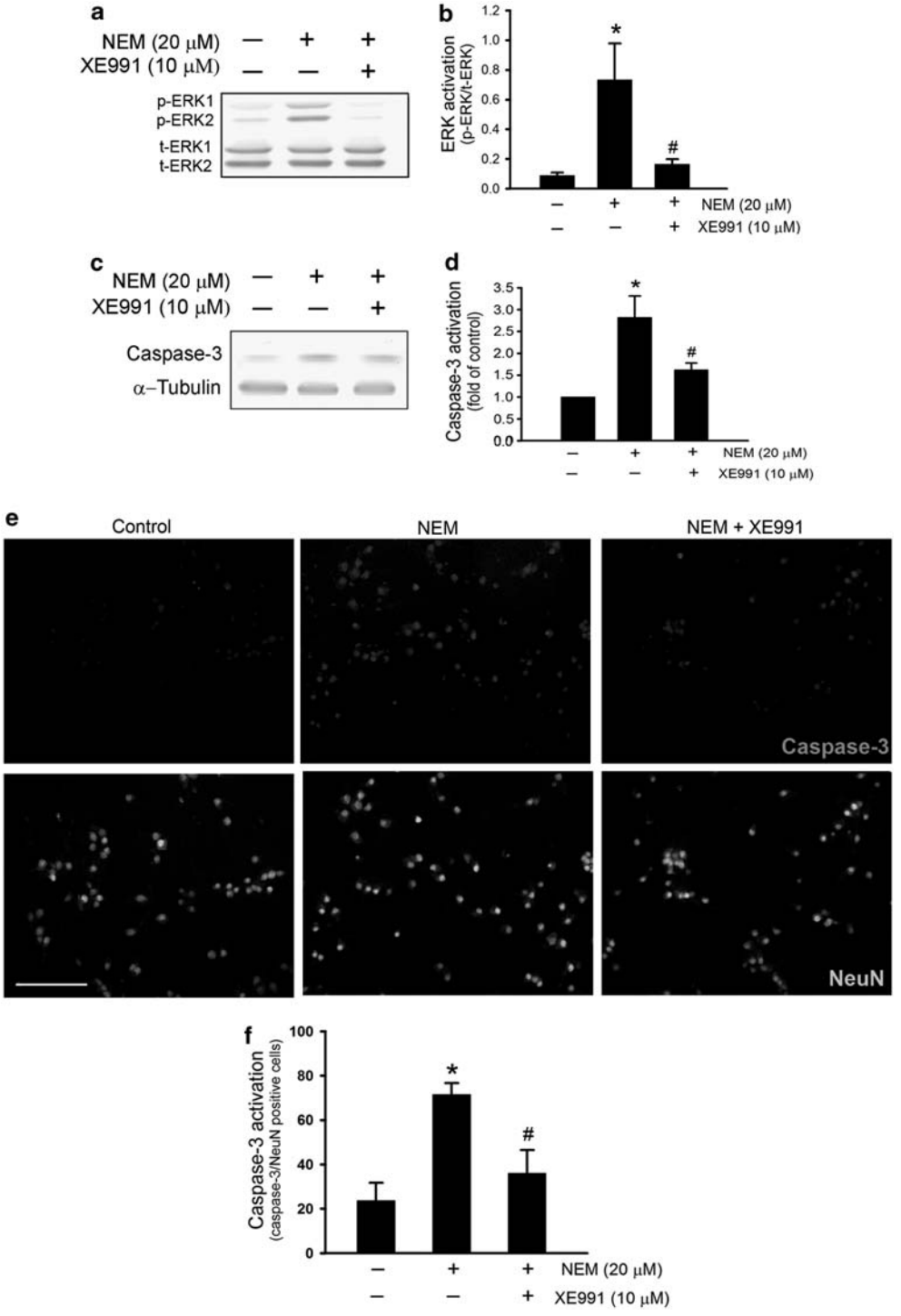


**Figure 6** KCNQ channel opener-induced increases in ERK phosphorylation and caspase-3 activation. The effects of NEM on apoptosis-related ERK phosphorylation and caspase-3 activation were detected in hippocampal cultures. (**a**–**d**) Representative western blotting showed expression levels of phosphor-ERK1/2 (44/42 KD) and total ERK1/2 (44/42KD) (**a**, **b**) or cleaved caspase-3 (**c**, **d**) in control, NEM (20 $\mu$M), or NEM (20 $\mu$M) plus XE991 (10 $\mu$M)-treated hippocampal neurons. XE991 drastically prevented NEM-increased ERK1/2 phosphorylation and caspase-3 activation. Mean measurement of ERK1/2 intensities was quantified. Mean ± S.E.M. $N \geq 3$ independent assays. (**e**) Immunostaining of caspase-3 (red) or NeuN (green) were shown in control, NEM (20 $\mu$M) or NEM plus XE991 (10 $\mu$M). (**f**) Quantification of capase-3-positive cells. XE991 significantly decreased NEM-induced caspase-3 activation. Scale bar = 100 $\mu$m. $^{*}P < 0.05$ *versus* vehicle controls; $^{\#}P < 0.05$ *versus* NEM

show that overactivation of KCNQ2/3 channels is associated with intracellular $K^+$ depletion, caspase-3 activation, mitochondrial depolarization, cytochrome *c*/AIF release from mitochondria and apoptotic death. Both caspase-dependent and caspase-independent pathways were activated during the process. Additional evidence suggests that KCNQ/M-channel overactivation

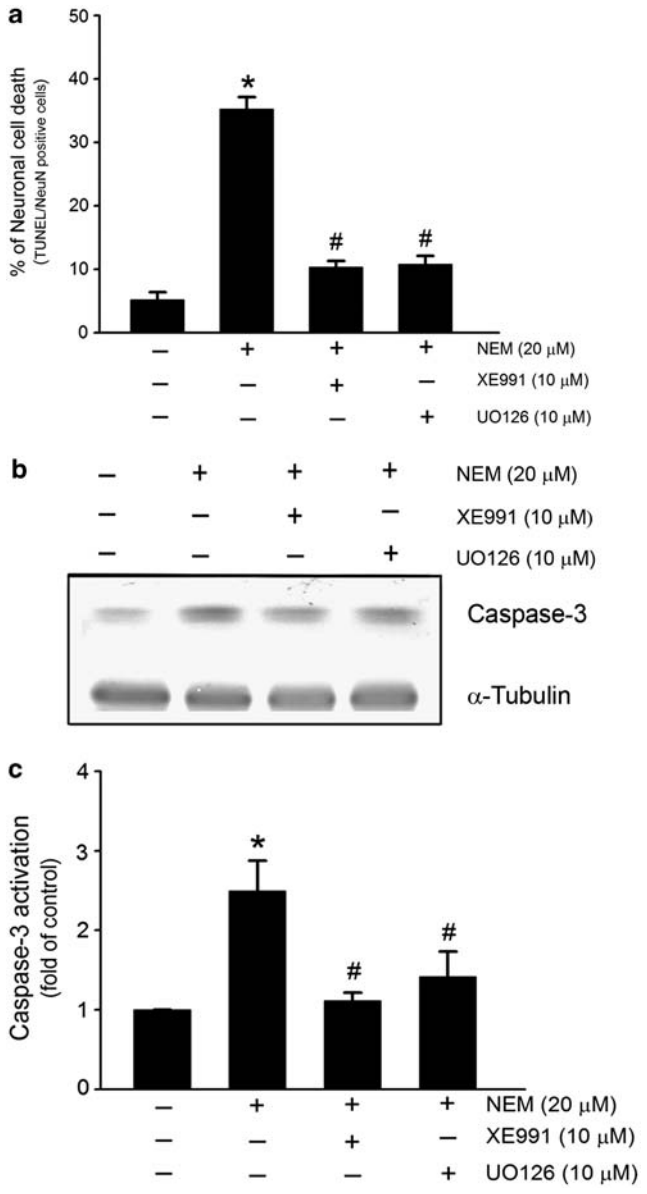


**Figure 7** Inhibition of ERK activity reduced neuronal death and caspase-3 activation in hippocampal neurons. Neuronal cell death and caspase activation were tested in hippocampal neurons using TUNEL staining and western blot analysis. (**a**) Exposure to NEM (20 $\mu$M) for 24 h caused about 35% cell death in hippocampal cultures measured by TUNEL assay. Both XE991 (10 $\mu$M) and UO126 (10 $\mu$M) showed protective effects against NEM toxicity. (**b**, **c**) Western blotting using the antibody at cleaved caspase-3 showed increased caspase-3 activation in hippocampal neurons after 8 h exposure to 20 $\mu$M NEM event was attenuated by XE991 (10 $\mu$M) or UO126 (10 $\mu$M). Mean ± S.E.M.; $N \geq 3$ independent assays. $^{*}P < 0.05$ *versus* vehicle controls; $^{\#}P < 0.05$ *versus* NEM treatment

may promote apoptosome formation, providing a new mechanism underlying $K^+$ depletion-induced apoptosis. We also present evidence that KCNQ/M-channel-mediated apoptosis is associated with activation of the ERK1/2 signaling pathway.

M-currents could be detected from cortex and hippocampus as shown in acutely isolated cells and *in vivo* studies.[11] On the other hand, none or very small M-currents are detected in cultured cortical neurons as shown in this study and in previous investigations.[20] The mechanism responsible for the difference in the M-channel activity in cultured cortical and hippocampal neurons is unknown. Nevertheless, this difference provides an advantage for the study of M-channel functions. The observation that KCNQ channel openers caused more cell death in hippocampal neurons than in cortical neurons endorses the idea that activation of functional KCNQ/M-channels is an important step in disruption of $K^+$ homeostasis and induction of apoptosis. Specifically, our study suggests that KCNQ/M-channel activation may have an important role for the intrinsic, mitochondria-dependent apoptotic pathway. Hippocampal neurons express death receptors such as FAS/CD95 and TNFR[21]; however, our preliminary study shows KCNQ channel openers do not increase caspase-8 activation (data not shown), suggesting that M-channel-induced cell death may not be mediated by the extrinsic signaling mechanism.

Our previous study demonstrated that M-channel blockers, XE991 and linopirdine, showed neuroprotective effects in rat sympathetic neurons deprived of nerve growth factor by blocking cytochrome *c* release and the development of 'competence-to-die.'[20] The present investigation further demonstrates that excessive activation of KCNQ2/3 channels induces a series of pro-apoptotic events in the apoptotic cascade. Supporting evidence came from the result that opening of KCNQ2/3 channels using the channel opener NEM led to neuronal apoptosis, which can be prevented by blocking KCNQ channels and attenuating $K^+$ efflux. In addition to NEM, another KCNQ channel opener, flupirtine showed similar effect. The effects on cell viability induced by two KCNQ channel openers and a specific channel blocker provide pharmacological evidence for a causal relationship between prolonged activation of KCNQ/M-channels and induction of apoptosis.

More specific molecular evidence came from the induction of cell death following expression of KCNQ2/3 channels in CHO cells. Wild-type CHO cells normally have undetectable endogenous $K^+$ currents and are relatively resistant to apoptotic insults.[17,22] Thus, we chose these cells to specifically test the ability of KNCQ channels in inducing apoptosis. Expression of KCNQ2 or KCNQ2/3 in CHO cells, without other manipulations, is capable of triggering caspase-3 activation, a landmark event in apoptosis. Moreover, XE991 prevented caspase-3 activation in these cells, indicating the causal role of KCNQ channels in induction of apoptosis. Transfection with KCNQ3 alone did not cause much increase in caspase-3 activation, likely due to the very little M-current carried by the KCNQ3 homodimer channel.[12,18] In the hippocampus, the KCNQ5 subunit may contribute to M-currents.[23] We cannot exclude the possibility that KCNQ5 subunit in hippocampal neurons might form M-channels with or without KCNQ2/3 and have a role in the pro-apoptotic activity. On the other hand, the channel expression experiments in CHO cells suggest that, in the absence of KCNQ5, KCNQ2/2, and KCNQ2/3 channels are capable to induce apoptosis in these cells.

The fluorescent indicator PBFI is broadly used to determine the intracellular concentrations of $K^+$. It contains two benzofuran isophthalate fluorophores linked to the nitrogens of diazacrown ether with a cavity size that has selectivity for

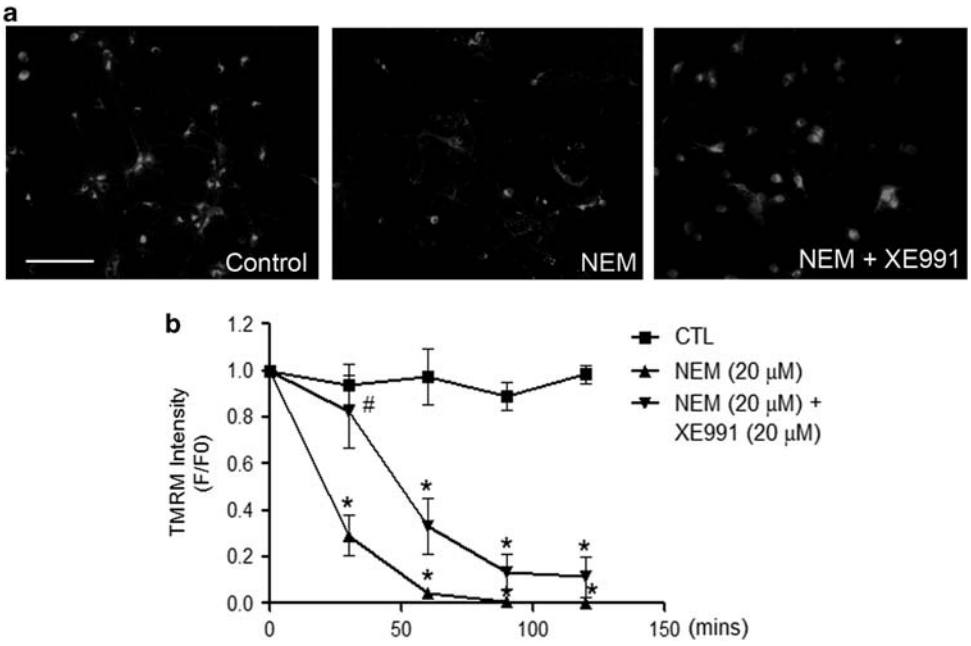


**Figure 8** Effects of KCNQ channel opener on mitochondria membrane potential. The mitochondrial membrane potential ($\Delta\Psi$) was monitored using TMRM staining. Hippocampal neurons were preloaded with TMRM (200 nM) 30 min before NEM (20 $\mu$M) treatment at indicated times. The intensity of TMRM fluorescence was detected using a fluorescent microscope. (**a**) NEM induced a dramatic collapse of the mitochondria membrane potential within 30 min. This mitochondria membrane depolarization was significantly blocked by XE991 (10 $\mu$M). (**b**) NEM-induced mitochondria membrane depolarization was partially inhibited by XE991. Mean ± S.E.M.; $N \geq 3$ separate experiments. $^{*}P < 0.05$ *versus* vehicle controls; $^{\#}P < 0.05$ *versus* NEM treatment

$K^+$.[24] Using this $K^+$ fluorescent dye, we showed that activation of KCNQ channels markedly decreased intracellular $K^+$, supporting the idea that KCNQ channels have the potential of influencing $K^+$ homeostasis. Activation of KCNQ channels is expected to cause the cell membrane potential moving toward hyperpolarized levels. It is also predictable that after prolonged channel openings, depolarization occurs when both intracellular $K^+$ and the $K^+$ gradient decrease. This depolarization due to prolonged $K^+$ channel activation and $K^+$ depletion has been demonstrated in previous investigations.[3,5]

The neuroprotective effect of elevated $K^+$ medium has been well known and was attributed to a membrane depolarization-induced $Ca^{2+}$ increase.[25] Although it is true that appropriate cellular $Ca^{2+}$ levels are critical for cell survival, it was noticed that the high $K^+$-mediated or 'depolarization-dependent' neuroprotection sometimes was not correlated with intracellular $Ca^{2+}$ changes.[3,4] In an elegant and comprehensive investigation, Franklin *et al*.[26] reported that at depolarized membrane potential levels achieved by increasing extracellular $K^+$ concentrations, 'there was no obvious concentration–response relationship between program cell death suppression and $[Ca^{2+}]_i$'. They concluded that while '$Ca^{2+}$ influx is required for depolarization-enhanced survival, the relationship between survival and $[Ca^{2+}]_i$ is more complex than previously suspected'.[26] Compelling evidence now supports that the high $K^+$-induced cytoprotection can be mainly a result from preventing excessive $K^+$ efflux as protective effect remains unchanged even when $Ca^{2+}$ influx and cellular $Ca^{2+}$ concentrations are constantly maintained at original levels.[3,5] It should be recognized that the approach of reducing $K^+$ efflux using a high $K^+$ solution does not distinguish between different $K^+$ channels that may mediate the pro-apoptotic $K^+$ efflux. Therefore, more specific methods are needed for delineating the mediator of $K^+$ efflux.

MAP kinases are involved in ion fluxes, cell volume regulation, and several apoptotic signaling pathways. Among three MAP subfamily members, ERK has been shown to be a key apoptotic factor in $K^+$ deprivation-induced neuronal cell death.[27] ERK is well characterized as a survival factor in the MAPK family, but recent studies also suggest that ERK is responsible for cell death in several cell types and organs under certain conditions.[27] In neuronal and renal epithelial cells, ERK is activated upon oxidative stress, depletion of growth factors and exposure to toxicants, whereas inhibition of ERK signaling reduced cell death.[28,29] ERK activation is also involved in various neurodegeneration models and renal ischemia; ERK inhibition attenuated tissue damage.[29,30] ERK acts as upstream signal of mitochondria membrane potential disruption, cytochrome *c* release, and caspase-3 activation. Our data support a role of ERK in the apoptotic response to $K^+$ channel activation and $K^+$ efflux. Activation of KCNQ channels leads to increases in phosphorylated-ERK, cytochrome *c* release from mitochondria, and caspase-3 activation, whereas ERK inhibition blocks these events. It remains to be tested whether activation of ERK stimulates KCNQ channel surface expression or affects biophysical properties of KCNQ channels during apoptosis.

During apoptosis, $K^+$ influx into mitochondria leads to mitochondria swelling and depolarization, which is one of the mechanisms causing the release of cytochrome *c* and AIF

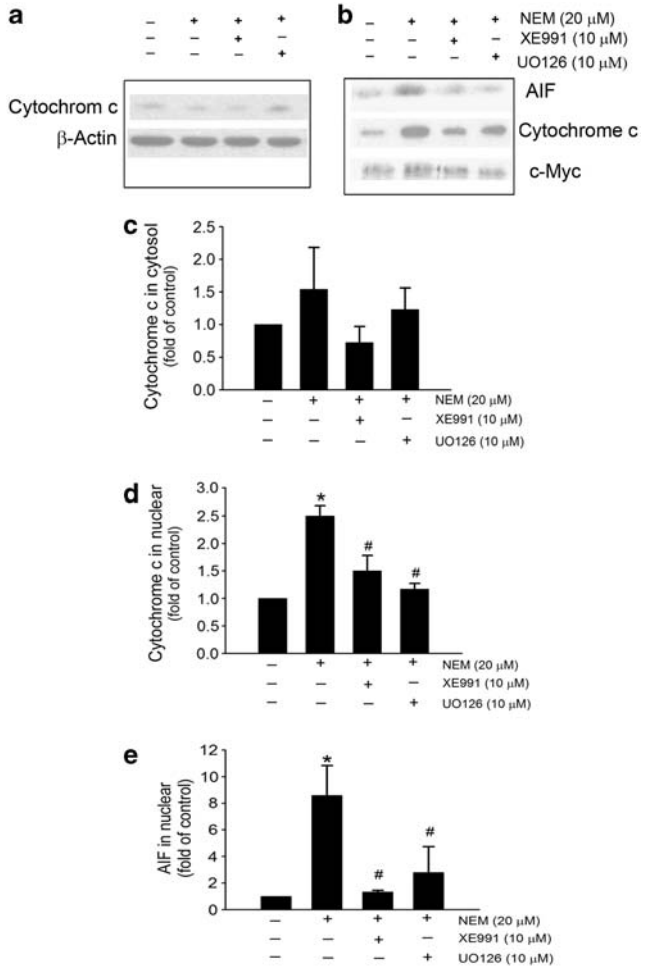


**Figure 9** Effects of KCNQ channel opener on cytosolic release and nuclear translocation of cytochrome *c* and AIF. Western blot analysis of cytochrome *c* and AIF in cytoslic and nuclear compartments after NEM exposure. (**a**, **b**) Hippocampal neurons were treated with NEM (20 $\mu$M) for 4 h. Cytosolic proteins (**a**) and nuclear proteins (**b**) were separated from mitochondria proteins and subjected to western blotting. (**c**–**e**) Summary of (**a**, **b**) NEM-induced cytochrome *c* release from mitochondria was blocked by XE991 (10 $\mu$M) or UO126 (10 $\mu$M) (**c**). XE991 and UO126 also significantly attenuated nuclear translocation of cytochrome *c* induced by NEM (**d**). The nuclear translocation of AIF was also largely prevented by co-applied XE991 or UO126 (**e**). Mean ± S.E.M.; $N \geq 3$ separate assays. $^{*}P < 0.05$ *versus* vehicle controls; $^{\#}P < 0.05$ *versus* NEM treatment

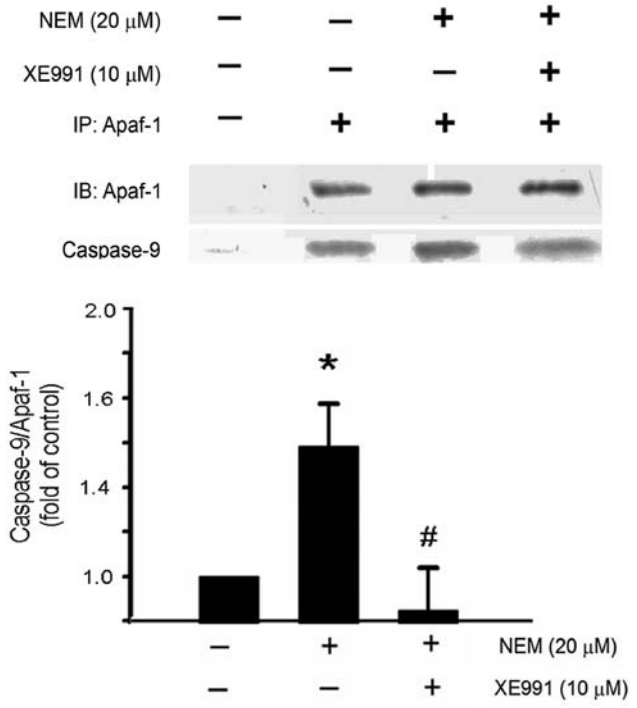


**Figure 10** KCNQ channel activation induced apoptosome formation in hippocampal neurons. Protein extractions from hippocampal neurons were collected and then used in immunoprecipitation assays against Apaf-1. Western blotting was then performed to detect the level of caspase-9 to indicate the formation of apoptosome complex. Note that the *y* axis value 1.0 is the basal level of apoptosome complex in control cells. Apoptosome formation was observed after NEM exposure, which was completely prevented by co-applied XE991 (10 $\mu$M). Mean ± S.E.M.; $N \geq 3$ separate experiments; $^{*}P < 0.05$ *versus* vehicle controls; $^{\#}P < 0.05$ *versus* NEM treatment

from mitochondria.[3] Cytochrome *c* and dATP/ATP binds to Apaf-1, mediates Apaf-1 oligomerization and recruitment of procaspase-9. The binding of procaspase-9 to the complex causes its activation and sequential activation of its downstream cascade, such as caspase-3 and endonuclease, eventually leads to apoptotic cell death.[3] The nuclear translocations of cytochrome *c* and AIF have been reported during caspase-independent cell death and associated with large-scale DNA fragmentation and chromatin condensation.[31] In the present investigation on neurons undergoing apoptosis, a KCNQ channel inhibitor partially attenuated mitochondrial membrane depolarization but more effectively inhibited apoptosome formation. Moreover, blocking the KCNQ channel prevents the translocation of cytochrome *c* and AIF to the nucleus. This agrees with the understanding that the $K^+$-mediated apoptosis involves caspase-dependent and -independent pathways. The stronger blocking effect of the KCNQ channel blocker on the formation of apoptosome is consisted with the previous report that the formation of apoptosome is regulated by the pro-apoptotic $K^+$ mechanism,[32] whereas mitochondrial depolarization can be induced by a number of non-specific pathways.

In addition to activating the KCNQ channel, NEM also could affect mitochondrial transition pore by thiol substitution or mediation of oxidative event.[33,34] This NEM action on mitochondria may explain why the KCNQ channel blocker XE991 could not fully inhibit NEM-induced mitochondrial depolarization. As a sulfhydryl reactive agent, NEM may stimulate intracellular zinc release.[35] However, the concentrations of NEM used in the present investigation are significantly lower than that for zinc release (e.g. 20 $\mu$M *versus* 100 $\mu$M). Moreover, another M-channel opener, flupirtine, tested in our study showed similar effects of inducing cellular $K^+$ depletion and apoptosis. As the molecular structure of flupirtine (ethyl [2-amino-6-[(4-fluorobenzyl)methylamino]pyridin-3-yl]carbamate) is much different from NEM, it is unlikely that they share the same non-specific effects in our experiments.

KCNQ/M-channel activity is essential for the normal hippocampus morphology development during the first postnatal weeks.[36] KCNQ channels are therapeutic drug targets

for anxiety, chronic and neuropathic pain, epilepsy, migraine, and other neuronal hyperexcitability disorders. Mutation of KCNQ2, KCNQ3, or KCNQ2/3 heteromultimeric channels results in benign familial neonatal convulsion in newborns.[37] KCNQ channel openers have been used clinically to treat epilepsy and neuropathic pain.[13] For example, retigabine is a widely used KCNQ channel opener, and has been tested to treat seizures in clinical trials.[38] On the other hand, evaluation of the long-term effects of KCNQ channel openers on neuronal apoptosis has not been reported. The present investigation provides new evidence that, like certain other $K^+$ channels, KCNQ channels contribute to pro-apoptotic mechanism of cellular $K^+$ depletion. As a $K^+$ channel opener, retigabine and other compounds are expected to cause hyperpolarization that is primarily accountable for neuro-protective and hypo-excitability effects. It has often been ignored, however, that prolonged applications of these compounds may ultimately result in membrane depolarization due to disruption of $K^+$ homeostasis. It is suggested that careful evaluation of the pro-apoptotic action of KCNQ2/3 openers and pre-caution of long-term treatment with these drugs will be necessary and important for the development of related drug therapies.

## Materials and Methods

**Materials.** Polyclonal antibodies to KCNQ2, KCNQ3, caspase-3, and AIF were obtained from Chemicon (Billerica, MA, USA). Monoclonal antibodies to cytochrome *c*, p-ERK, and t-ERK were purchased from Cell Signaling Technology (Danvers, MA, USA). Monoclonal antibodies to $\beta$-actin, NEM, and flupirtine were obtained from Sigma-Aldrich (St. Louis, MO, USA). XE991 was obtained from Tocris (Ellisville, MO, USA). Cell-permeable PBFI-AM was purchased from Sigma-Aldrich. Antibody to $\alpha$-tubulin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Primary neuronal cultures.** Primary cortical or hippocampal neurons were obtained from E15–E17 mice as described previously.[39] The mice were anesthetized with isoflurane. Dissociated hippocampal neurons using trypsin were plated on 35 mm poly-D-lysine and laminin-coated dishes (VWR, West Chester, PA, USA) at the density of $3 \times 10^5$ cells/ml in Neurobasal media (Invitrogen, Carlsbad, CA, USA) supplemented with 2% B-27 (Invitrogen) and, L-glutamine (0.5 mM). After DIV 3 days culture, 5 $\mu$M $\beta$-cytosine arabinoside (Sigma-Aldrich) was added to the medium to inhibit cell division. After 7 DIV, half of the medium was changed with fresh medium. Patch clamp recordings and other tests were performed 12–16 DIV.

**Expression of KCNQ2/3 channels in CHO cells.** Plasmids encoding rat KCNQ2 (GenBank: AF087453) and rat KCNQ3 (GenBank: AF-91247) were subcloned into pEGFP-C3 and pDsRed2-C1 (Clontech, Mountain View, CA, USA), respectively. CHO cells were obtained from ATCC (Mannassas, VA, USA). In order to express KCNQ2 or KCNQ3 in CHO cells, we amplified the coding region for each channel by PCR, using primers incorporating *Sal*I and *Bam*HI for KCNQ2 and *Eco*RI and *Bam*HI for KCNQ3 restriction sites. Then the amplified inserts were subcloned into the corresponding restriction sites of the pERFP-C3 or pDsRed2-C1 expression plasmids. The fidelity of all strands were confirmed by sequencing.

CHO cells were grown in 100-mm tissue culture dishes in DMEM (plus 10% fetal bovine serum plus 1% penicillin/streptomycin) in a humidified incubator at 37°C with 5% $CO_2$. Cells were seeded in 35-mm dishes and transfected when 70–80% confluenct. For tranfection, a mixture of 4 $\mu$g KCNQ, pEGFP-C3 cDNA, and 10 $\mu$l lipofectamine 2000 reagent (Invitrogen) was prepared in 1.0 ml of DMEM and incubated for 20 min according to the manufacturer's instruction. The mixture was then applied to the cell culture and incubated for 6 h. The cells were changed back to growth medium and tested 24 h after transfection.

**Electrophysiological recordings of M-currents.** A measure of 35-mm culture dishes containing cortical or hippocampal neurons were placed on the stage of an inverted microscope. Membrane currents were recorded by whole-cell configuration using an EPC-9 amplifier (HEKA Instruments Inc., Bellmore, NY, USA). Recording electrodes of 8–10 M$\Omega$ (fire polished) were pulled from Coming Kovar Sealing #7052 glass pipettes (PG52151-4; WPI, Sarasota, FL, USA) by a Flaming-Brown micropipette puller (P-80/PC; Sutter Instrument Co., Movat, CA, USA). A high-resistant gigaseal (1–3 G$\Omega$) was formed between the cell membrane and the electrode tip. Whole-cell configuration was then established by breaking through the cell membrane. Series resistance compensation was routinely applied during recording. Current and voltage signals were displayed on a computer monitor and collected by a data acquisition/analysis program PULSE (HEKA, Lambrect, Germany). Currents were digitally sampled at 50 $\mu$s (20 kHz) and were filtered at 3 kHz by a 3-pole Bessel filter. Pipettes were filled with an internal solution containing the following (in mM): KCl 120, $MgCl_2$ 1.5, $Na_2$-ATP 2, $CaCl_2$ 1.0, BAPTA 1.0, and HEPES 10. The external solution contains (in mM): NaCl 115, KCl 2.5, $MnCl_2$ 2.0, HEPES 10, BAPTA 0.1, Glucose 10, and 0.1 $\mu$M tetrodotoxin. The membrane potential was held at $-30$ mV for M-current activation. Hyperpolarizing steps were initiated by a digital pulse to deactivate M-current. All experiments were performed at room temperature (22–25°C) and pH 7.4.

**Western blotting and immunoprecipitation analysis.** Cell lysates were prepared by extracting proteins with lysis buffer (HEPES 50 mM, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 5 mM NaF, and 2.5 mM $NaVO_3$) supplemented with protease inhibitors. Lysates were centrifuged at $14\,000 \times g$ for 20 min at 4°C, and the supernatants were collected. Protein concentration in the supernatants was determined using bicinchoninic acid protein assay and adjusted to 1 mg/ml with lysis buffer. Samples containing 500 $\mu$g of proteins were incubated with 2 $\mu$g of anti-Apaf-1 or normal rat serum (Jackson ImmunoResearch, West Grove, PA, USA) overnight at 4°C. Immunoprecipitates were collected by the addition of 30 $\mu$l protein G and incubation for 2 h at 4°C. Complexes were collected by centrifugation and washed with lysis buffer. Finally, the pelleted beads were resuspended in $\times$ 2 SDS sample buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, and 5% $\beta$-mercaptoethanol). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked using 5% BSA in Tris-buffered saline, and then incubated with primary antibodies for 24 h at 4°C. Blots were developed using alkaline peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and proteins were visualized by addition of BCIP/NBT solution (Sigma-Aldrich). The membranes were scan analyzed by densitometry using the image analysis software Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) and ImageJ (NIH, Bethesda, MD, USA).

**Cytofluorometric determination of intracellular $K^+$ by PBFI-AM imaging.** Hippocampal neurons (12 DIV) were treated with 30 $\mu$M NEM for up to 3 h. Valinomycin (50 $\mu$M, 80 min) and drug-free medium were used as positive and sham controls. Intracellular $K^+$ content was assessed using the cell-permeable acetoxymethyl ester derivative of the fluorescent potassium-sensitive dye (PBFI-AM; Invitrogen). PBFI-AM stock solution was prepared by mixing 5 mM PBFI-AM in DMSO with 10% Pluronic-127 (Invitrogen). The stock solution was diluted upon use, to the final concentration using the medium (in mM): 120 NaCl, 25 Tris-HCl, 5.4 KCl, 1.8 $CaCl_2$, and adjusted to pH 7.4. Neurons were incubated in 1.5 ml medium containing 5 $\mu$M PBFI-AM for 40 min at 37°C. After loading with PBFI-AM, cells were washed twice with the medium. PBFI-AM fluorescence images in cell bodies were acquired using an inverted microscope (Leica DMIRB, Leica Microsystems, Bannockburn, IL, USA) with a $\times$ 40 objective lens; the dye was excited at 340 nm. The images of PBFI-AM fluorescence were analyzed offline using the software ImageJ (NIH).

**Measurement of mitochondrial membrane potential.** Mitochondrial membrane potential was determined by assaying TMRM (Invitrogen). TMRM (200 nM) was loaded for 30 min at 37°C in the culture media. The cells were washed with fresh medium containing 50 nM TMRM and were treated with NEM for 2 h with or without XE991 (10 $\mu$M). TMRM was excited at 543 nm, and the fluorescence was measured using a fluorescence microscope. Quantification of results was performed using ImageJ. Background fluorescent intensity was subtracted in data analysis.

**Preparation of cytosolic, mitochondria, or nuclear proteins fractions.** Cells were collected and mitochondria or nuclear proteins were separated from cytosolic protein using Mitochondria or Nuclear Isolation Kit (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations. The mitochondria-rich or nuclear-rich fractions were washed once with extraction buffer and resuspensed in lysis buffer containing protease inhibitors for western blotting analysis.

**Immunocytochemical staining.** Cells were fixed with 4% paraformaldyhyde for 5 min. A blocking solution of 1% fish gel in PBS was applied for 1 h, followed by overnight incubation in PBS containing primary antibody. The primary antibodies used were: mouse anti-NeuN (1:200; Chemicon), rabbit anti-caspase-3 (1:200; Chemicon), rabbit anti-KCNQ2 (1:100; Chemicon), and rabbit anti-KCNQ3 (1:100; Chemicon). Following several washes with $\times 1$ PBS, TRITC- or FITC-coupled secondary antibodies (1:600/1:200; Invitrogen) were applied for 1 h at room temperature. Cultures were washed and mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and were visualized with an Olympus IX61 fluorescent microscope.

**TUNEL staining.** DeadEnd Fluorometric TUNEL System (Promega Corporation, Madison, WI, USA) was used for TUNEL staining in primary hippocampal or cortical neurons to detect cell death. After incubation in control media or NEM for 24 h, cells were fixed, permeabilized, incubated in TUNEL reaction solution and counted to calculated mean percentage of TUNEL-positive cells of randomly selected six fields.

**Statistical analysis.** One-way ANOVA followed by Tukey's test was used for multiple comparisons; Student's two-tailed *t*-test was performed for comparison of two experimental groups. Changes were identified as significant if *P*-value was $<0.05$. Data were reported as mean $\pm$ S.E.M. (standard error of mean). The statistical analysis was performed using SigmaPlot (Systat Software Inc., Chicago, IL, USA) or GraphPad (GraphPad Software Inc., La Jolla, CA, USA).

## Conflict of interest

The authors declare no conflict of interest.

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