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The neurotoxic MEC-4(d) DEG/ENaC sodium channel conducts calcium: implications for necrosis initiation

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Hyperactivation of the *Caenorhabditis elegans* MEC-4 Na^+ channel of the DEG/ENaC superfamily (MEC-4(d)) induces neuronal necrosis through an increase in intracellular Ca^{2+} and calpain activation. How exacerbated Na^+ channel activity elicits a toxic rise in cytoplasmic Ca^{2+}, however, has remained unclear. We tested the hypothesis that MEC-4(d)-induced membrane depolarization activates voltage-gated Ca^{2+} channels (VGCCs) to initiate a toxic Ca^{2+} influx, and ruled out a critical requirement for VGCCs. Instead, we found that MEC-4(d) itself conducts Ca^{2+} both when heterologously expressed in *Xenopus* oocytes and *in vivo* in *C. elegans* touch neurons. Data generated using the Ca^{2+} sensor cameleon suggest that an induced release of endoplasmic reticulum (ER) Ca^{2+} is crucial for progression through necrosis. We propose a refined molecular model of necrosis initiation in which Ca^{2+} influx through the MEC-4(d) channel activates Ca^{2+}-induced Ca^{2+} release from the ER to promote neuronal death, a mechanism that may apply to neurotoxicity associated with activation of the ASIC1a channel in mammalian ischemia.

Rises in intracellular Ca^{2+} have crucial roles in promoting apoptosis[1] and necrosis[2], contributing to the debilitating neuronal damage that accompanies ischemia, injury and neurodegenerative disease[3]. The ER can be the source of a life-threatening increase in Ca^{2+} in these conditions[3–5]. Despite considerable therapeutic relevance, however, the molecular mechanisms by which the neurotoxic release of Ca^{2+} from ER stores is provoked are not well understood.

Hyperactivation of ion channels initiates excitotoxicity and underlies several inherited neurodegenerative disorders across species[6–8]. One of the best-studied genetic initiators of necrosis is *C. elegans mec-4(d)*, which encodes a mutant subunit of the DEG/ENaC Na^+ channel superfamily[6,9]. Wild-type MEC-4 functions as the core subunit of a multimeric mechanically gated Na^+ channel complex[10–12] that is normally activated in response to gentle touch stimuli[13]. The neurotoxic change in MEC-4(d) is a large side chain amino-acid substitution adjacent to, or part of, the channel pore (A713V or A713T[6,9]). Analogous substitutions in heterologously expressed MEC-4(d) and other neuronal DEG/ENaC channels markedly enhance channel activity[10,14–17]. The mammalian acid-sensing ion channel (ASIC) subfamily can influence cutaneous mechanoreceptor function[18] and contribute to pain sensation, synaptic plasticity, fear conditioning[19,20] and cell loss in tissue acidosis[21,22]. Similar to other members of the DEG/ENaC family, the MEC-4(d) channel is inhibited by amiloride[10].

In vivo, *mec-4(d)*-induced neuronal death requires the function of ER Ca^{2+}-storing proteins calreticulin and calnexin as well as ER Ca^{2+} release channels[5], suggesting that the ER regulates an essential cytoplasmic concentration of Ca^{2+} required for progression through necrosis. When the intracellular Ca^{2+} concentration rises to a neurotoxic threshold, specific Ca^{2+}-activated calpain proteases function to dismantle the neuron[23]. Ion channel hyperactivation, the requirement for a rise in intracellular Ca^{2+}, and calpain activation are common features of mammalian necrosis, indicating that, similar to apoptosis[24], necrotic neuronal death occurs by an evolutionarily conserved mechanism[8].

A key issue in the nematode necrosis model, which is relevant to problems in mammalian traumatic neuronal injury and channel-induced death, is how an increase in Na^+ influx might provoke release of Ca^{2+} from the ER, the paradox being that Ca^{2+} release from the ER is usually induced by signals that are not dependent on Na^+, namely, inositol (1,4,5)-trisphosphate (IP3) or Ca^{2+} itself[25,26]. Here we report that the MEC-4(d) Na^+ channel conducts Ca^{2+}, suggesting that a Ca^{2+} influx via the MEC-4(d) channel directly signals the release of Ca^{2+} from the ER. Our data require a rethinking of the mechanism by which DEG/ENaC channels can be neurotoxic and implicate Ca^{2+}-induced Ca^{2+} release in necrosis initiation.

RESULTS

VGCCs are not required for *mec-4(d)*-induced necrosis

We tested the hypothesis that hyperactivation of the MEC-4(d) channel induces secondary Ca^{2+} influx through plasma membrane sources. In response to gentle touch, *mec-4(+)* touch receptor neurons generate Ca^{2+} transients that depend on MEC-4 as well as on the EGL-19 α and UNC-36 α-2/δ L-type VGCC subunits[13]. To test whether Ca^{2+} influx through these or other VGCC subunits are needed for necrosis (**Fig. 1a**), we constructed double mutants of *mec-4(d)* and

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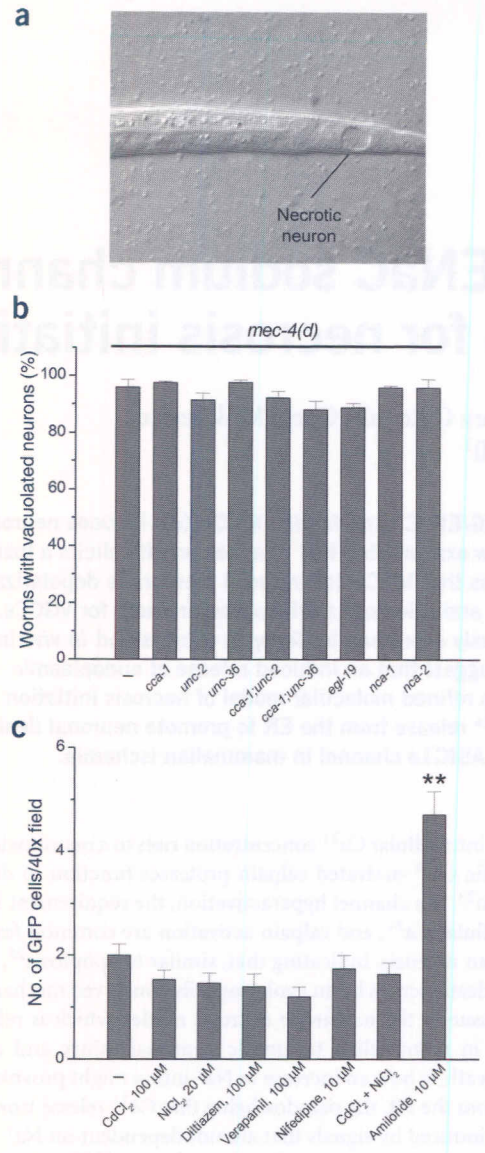

Figure 1 VGCCs do not influence *mec-4(d)*-induced cell death. (**a**) Necrotic swollen touch neurons are evident in the L1 stage of *mec-4(d)* nematodes. (**b**) Degeneration in PLM touch neurons was assayed in the *mec-4(d)* strain (–) and in the indicated *mec-4(d)* and VGCC double and triple mutants (alleles: T-type *cca-1(gk30)*, N-P/Q-type *unc-2(e55)*, L-type *egl-19(ad1006)* and *unc-36(e251)*, novel *nca-1(gk9)* and *nca-2(gk5)*) by determining the percentage of L1 worms with one or two degenerating PLM neurons up to 5 h after hatching. Data are the mean ± s.e.m. (n = 2–4 repetitions; sample size ≥ 192 larvae). (**c**) *mec-4(d)*-expressing touch neurons were cultured for 24 h under standard conditions in the absence (–) or presence of the indicated VGCC blockers. Numbers of touch neurons per field were scored (at ×40 magnification) after cell fixation in 4% paraformaldehyde. Data are the mean ± s.e.m. (n = 5–30 fields scored). $^{**}P < 0.01$ versus untreated cells by t-test.

each of the four VGC channels[27] and two distantly related *nca-1* and *nca-2* channels[28]. Quantification of cell death did not, however, detect significant effects of any VGCC mutation on either the time of onset or the extent of necrosis (**Fig. 1b**).

In complementary studies, we tested whether cultured *mec-4(d)* neurons (which differentiate and die similarly in culture as *in vivo*; **Supplementary Fig. 1** online) would be protected from necrosis by treatment with nifedipine, verapamil or diltiazem (L-type blockers that are effective on nematode channels; C.F.-J. and W.R.S., unpublished data), Ni^{2+} (a T-type channel blocker) or Cd^{2+} (a more general Ca^{2+} channel blocker that blocks all high-voltage-gated Ca^{2+} channels at 100 μM), either alone or in combination with Ni^{2+}. Although amiloride was found to be neuroprotective in the culture assay, none of the VGCCs blockers reduced cell death (**Fig. 1c**). Thus, VGCCs do not contribute a source of Ca^{2+} that is essential for necrosis activation.

We tested several alternative sources of Ca^{2+} influx by using genetic and RNA interference disruption approaches, but none of these sources was implicated in necrosis (**Supplementary Table 1** online). In considering alternative models of how *mec-4(d)* might elicit Ca^{2+} release from the ER, we recognized that the potential Ca^{2+} permeability of the MEC-4(d) channel itself had not been addressed. We therefore tested whether the MEC-4(d) channel might directly conduct Ca^{2+}.

MEC-4(d) conducts Ca^{2+} in *Xenopus* oocytes

The touch-transducing MEC Na^+ channel is thought to include the DEG/ENaC subunits MEC-4 and MEC-10, MEC-2 (a stomatin-related protein)[10,16] and MEC-6 (homologous to paraoxonases)[11]. Consistent with previous data[10,11], coexpression of MEC-4(d), MEC-10(d), MEC-2 and MEC-6 (hereafter referred to as the MEC-4(d) channel complex) in *Xenopus* oocytes induced a large Na^+ current (**Fig. 2a**). When we exposed these oocytes to carrier ion Ca^{2+} rather than Na^+, we detected a large inward Cl^- current on membrane hyperpolarization (**Fig. 2b**) that was not evident in non-injected oocytes (**Fig. 2c,d**). This current showed the same characteristics as the slow component of the *Xenopus* oocyte endogenous Ca^{2+}-activated Cl^- current[29]: namely, slow activation kinetics (τ = 311 ± 37.7 ms; n = 34), slow inactivation kinetics (τ = 1,451 ± 152 ms; n = 30), a reversal potential of –53.6 ± 2.2 (n = 37) and sensitivity to 200 μM 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), a specific Cl^- channel blocker that inhibits the endogenous Ca^{2+}-activated Cl^- current[30] (data not shown). The amplitude of the MEC-4(d) Na^+ current in a given injected oocyte strongly paralleled the amplitude of the Ca^{2+}-activated Cl^- current (**Fig. 2e,f**).

Because the endogenous Ca^{2+}-activated Cl^- current in *Xenopus* oocytes is known to be activated by an intracellular increase in Ca^{2+}, we tested whether we could block activation of the MEC-4(d)-dependent current by buffering intracellular Ca^{2+} with EGTA. We found that the Ca^{2+}-activated Cl^- current was markedly suppressed by EGTA injection both before (n = 8; data not shown) and during (n = 33; **Fig. 3a,b**) electrophysiological recordings. Taken together, these observations suggest that the MEC-4(d) channel complex directly conducts Ca^{2+} into the oocyte, thereby activating an endogenous Ca^{2+}-sensitive current.

Amiloride sensitivity of MEC-4(d) Ca^{2+} currents

Our successful blocking of the Ca^{2+}-activated Cl^- current by EGTA[29] (**Fig. 3a,b**) enabled us to identify a MEC-4(d)-dependent Ca^{2+} influx. We therefore tested whether the isolated current would be inhibited by amiloride; this inhibition is well documented for the MEC-4(d) channel complex reconstituted in oocytes[10]. Indeed, we found that application of amiloride blocked a portion of the residual current (**Fig. 3c**). (The remaining amiloride-insensitive current is probably carried by endogenous Ca^{2+}-insensitive Cl^- channels that are present in *Xenopus* oocytes[31].)

We found that the amiloride-sensitive Ca^{2+} current (**Fig. 3d**) had the following properties: first, it was not present in oocytes not injected with *mec* RNA (**Fig. 3e**), indicating that it depends on the MEC-4(d) channel complex; second, it reversed at –49.3 ± 0.5 mV (n = 9) in 73 mM $CaCl_2$, typical of a channel with low Ca^{2+} permeability; third,

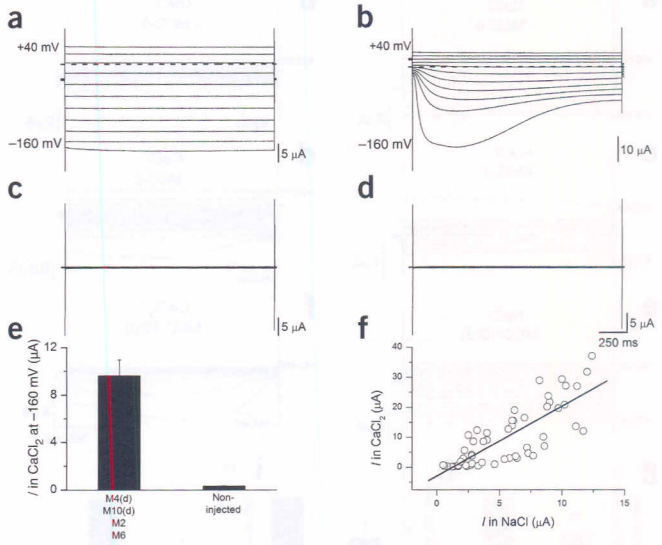

Figure 2 Endogenous Ca^{2+}-activated Cl^- currents are activated in *Xenopus* oocytes injected with *mec-4(d)* when exposed to $CaCl_2$ solutions. (**a**) Example of Na^+ currents elicited by voltage steps from −160 to +40 mV from a holding potential of −30 mV in an oocyte injected with *mec-4(d)*, *mec-10(d)*, *mec-2* and *mec-6* and exposed to NaCl solution ($n = 332$). The reversal potential of the current is 0 mV. (**b**) The same oocyte was exposed to a solution in which NaCl was replaced with $CaCl_2$ and the voltage protocol in **a** was applied (holding potential 0 mV). The reversal potential is −40 mV ($n = 271$). Both Na^+- and Ca^{2+}-activated Cl^- currents can be simultaneously detected in oocytes perfused with a solution containing 50 mM NaCl and 33 mM $CaCl_2$ (not shown). (**c**) Exposure to NaCl solution does not activate endogenous currents in a non-injected oocyte (voltage protocol as in **a**; $n = 30$). (**d**) The endogenous Ca^{2+}-activated Cl^- current is not activated in non-injected oocytes on exposure to $CaCl_2$ solutions (voltage protocol as in **b**; $n = 30$). (**e**) Average Cl^- current at −160 mV recorded from oocytes injected with *mec-4(d)*, *mec-10(d)*, *mec-2* and *mec-6* ($n = 56$) and from non-injected ($n = 16$) oocytes. Data are the mean ± s.e.m. (**f**) Amplitude of the Na^+ current carried by the MEC-4(d) channel complex plotted against amplitude of the Ca^{2+}-activated Cl^- current for each oocyte. Data points were fitted to a linear regression (slope = 2.3).

it showed a shift in reversal potential to more negative potentials in 20 mM $CaCl_2$ ($\Delta V = -28$ mV, $n = 6$), indicating that Ca^{2+} is the permeating ion; and fourth, it inwardly rectified, as does the MEC-4(d) channel Na^+ current (**Fig. 3e**).

We also found that adding the MEC-10(d) subunit to the channel complex altered the amiloride inhibition constant (K_i) for the Ca^{2+} current in the same way that it alters the amiloride K_i for the Na^+ current[10] (**Fig. 3f**). Similarly, returning to measurements on the endogenous Ca^{2+}-activated Cl^- current (which is insensitive to amiloride up to a concentration of 100 μM; ref. 30) as a readout of Ca^{2+} currents permeating MEC-4(d) channels, we recorded K_i values similar to those reported[10] for the MEC-4(d)-dependent Na^+ current ($K_i = 0.37$ and 6.7 μM with and without MEC-10(d), respectively; **Fig. 3g**). These data support the idea that the MEC-4(d) channel complex itself directly conducts the amiloride-sensitive Ca^{2+} current that we isolated.

Notably, the voltage dependence of the amiloride block in $CaCl_2$ did not parallel that of the MEC-4(d) channel in NaCl at very negative voltages (**Fig. 3h**). This observation suggests that amiloride begins to interfere with the permeating Ca^{2+} ion at these voltages[32]; thus, amiloride and Ca^{2+} may share a common binding site. On the basis of the shift in reversal potential, we calculated a permeability ratio of Ca^{2+} over Na^+ (PCa/PNa) of 0.22 ± 0.04 ($n = 9$), and 0.22 ± 0.02 for the complex lacking MEC-10(d) ($n = 9$; **Fig. 3e**) and we obtained the following divalent permeability series for the MEC-4(d) channel complex: PCa = PMg > PSr > PBa >> PZn.

Ca^{2+} conductance is dependent on the MEC-4(d) subunit

We next determined systematically which subunits of the MEC-4(d) channel complex are required for Ca^{2+} current. Previous work has shown

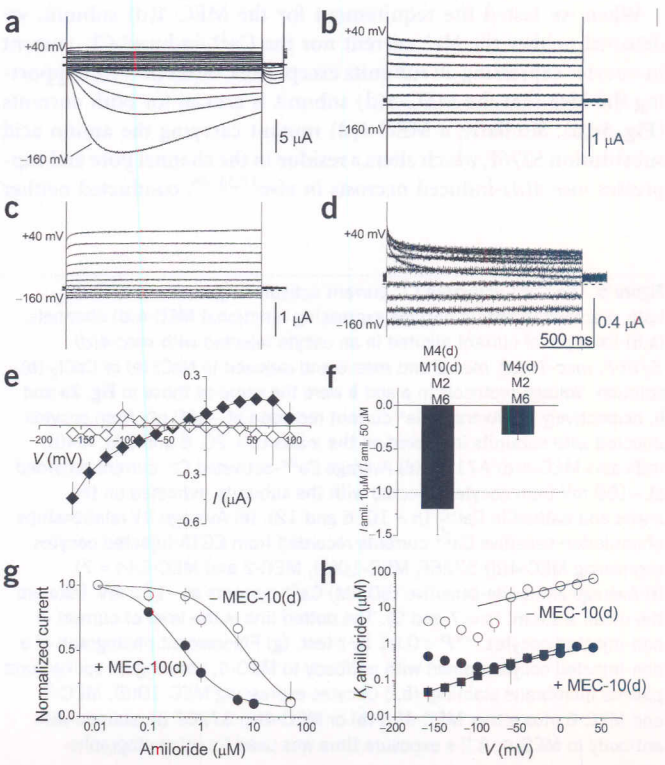

Figure 3 MEC-4(d) channels conduct Ca^{2+}. (**a**) Ca^{2+}-activated Cl^- currents were elicited in an oocyte injected with *mec-4(d)*, *mec-10(d)*, *mec-2* and *mec-6*. Tail currents are due to repolarization at −30 mV. (**b**) The same oocyte was then injected with few nanoliters of 250 mM EGTA through the recording pipette (to a final concentration of ~5 mM) and stimulated with the same voltage protocol (from −160 to + 40 mV from a holding potential of 0 mV). (**c**) Currents remaining after exposing the same oocyte to 10 μM amiloride. (**d**) Amiloride-sensitive current obtained by subtracting the current in **c** from that in **b**. (**e**) Average *I/V* relationships of amiloride-sensitive currents recorded from EGTA-injected oocytes expressing MEC-4(d), MEC-2 and MEC-6 (◆; $n = 9$), and from oocytes not injected with *mec* RNA (◇; $n = 4$). (**f**) Ratio of Ca^{2+} current elicited by 10 μM amiloride to that elicited by 50 μM amiloride in oocytes expressing the indicated subunit compositions ($n = 4$). Dotted line indicates a ratio of 1. (**g**) Amiloride dose-response curves (at −160 mV) for Ca^{2+}-activated Cl^- currents in oocytes injected with *mec-4(d)*, *mec-2* and *mec-6* with (+M10(d); $n = 6$) or without (−M10(d); $n = 6$) *mec-10(d)*. K_i values were 0.37 μM for +M10(d) and 6.7 μM for −M10(d). (**h**) Voltage dependence of the amiloride blockade in **g**. Data were also recorded in NaCl solution for oocytes injected with *mec-4(d)*, *mec-10(d)*, *mec-2* and *mec-6* (+M10(d) in NaCl; $n = 6$). The smooth line is a fit using a Woodhull model[32] ($\delta = 0.40$ for +M10(d), 0.34 for −M10(d) and 0.50 for +M10(d) in NaCl).

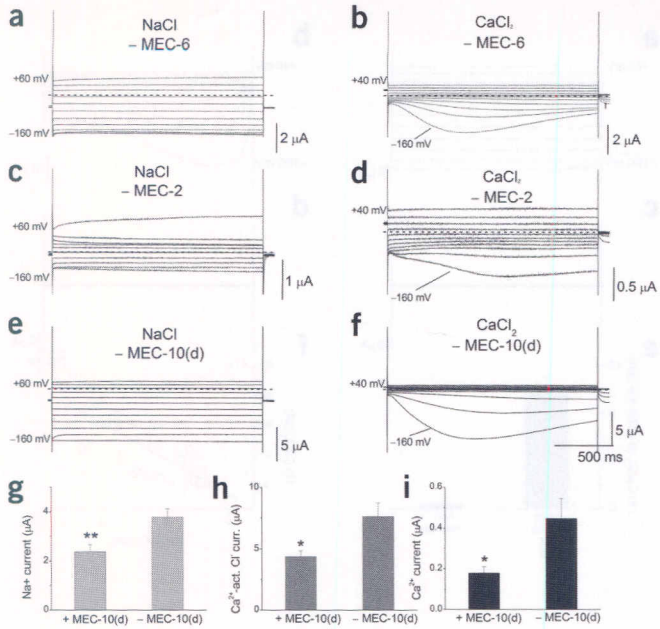

Figure 4 The MEC-4(d) subunit is required for Ca^{2+} permeability. (**a**) Example of Na^+ currents elicited by voltage steps from –160 to + 60 mV from a holding potential of –30 mV in an oocyte injected with *mec-4(d)*, *mec-10(d)* and *mec-2* and exposed to NaCl solution. (**b**) The same oocyte was exposed to a solution in which NaCl was replaced with $CaCl_2$. The current was activated by voltage steps from –160 to + 40 mV from a holding potential of 0 mV. (**c**) Na^+ currents elicited in an oocyte injected with *mec-4(d)*, *mec-10(d)* and *mec-6* using the voltage protocol in **a**. (**d**) The same oocyte was exposed to $CaCl_2$ solution using the voltage protocol in **b**. (**e**) Na^+ currents elicited in an oocyte injected with *mec-4(d)*, *mec-2* and *mec-6* using the voltage protocol in **a**. (**f**) The same oocyte was exposed to $CaCl_2$ solution using the voltage protocol in **b**. (**g**) Effect of coexpressing MEC-10(d) on the MEC-4(d) Na^+ current amplitude. Shown is the average Na^+ current amplitude at –100 mV in oocytes injected with *mec-4(d)*, *mec-2* and *mec-6* with or without *mec-10(d)* ($n = 20$ for both). (**h**) Effect of coexpressing MEC-10(d) on the Ca^{2+}-activated Cl^- current ($n = 10$ and $n = 12$, respectively). Currents were recorded at –160 mV. (**i**) Effect of coexpressing MEC-10(d) on the MEC-dependent Ca^{2+} current ($n = 9$ for both). Currents were recorded at –160 mV. $^*P < 0.05$ and $^{**}P < 0.01$ by *t*-test.

that the addition of either stomatin-like MEC-2 or paraoxonase-like MEC-6 to the MEC-4(d) subunit expressed in *Xenopus* oocytes decreases the PLi/PNa ratio, suggesting that amino acid residues in MEC-2 and MEC-6 may line the channel pore[10,11]. We found that omission of either MEC-2 or MEC-6 still permitted the Ca^{2+}-dependent Cl^- current to be activated, indicating that neither subunit is essential for Ca^{2+} permeability (**Fig. 4a–d**). As is true for the Na^+ current, however, omission of either MEC-2 or MEC-6 lowered the Ca^{2+}-activated Cl^- current amplitude. Tests for the contribution of the MEC-10(d) subunit showed that MEC-10(d) is also not essential for the influx of Ca^{2+} (**Fig. 4e,f**); however, the addition of MEC-10(d) diminished the Ca^{2+}-activated Cl^- current and the isolated amiloride-sensitive Ca^{2+} current amplitude in a manner that paralleled its effects on the Na^+ current (**Fig. 4g–i**).

When we tested the requirement for the MEC-4(d) subunit, we detected neither the Na^+ current nor the Ca^{2+}-induced Cl^- current in oocytes expressing all subunits except MEC-4(d) ($n = 6$), supporting the idea that the MEC-4(d) subunit is crucial for both currents (**Fig. 5c,d**). Similarly, a MEC-4(d) mutant carrying the amino acid substitution S276F, which alters a residue in the channel pore and suppresses *mec-4(d)*-induced necrosis *in vivo*[17,33–36], conducted neither the Na^+ nor the Ca^{2+} current ($n = 16$; **Fig. 5a–f**), despite its expression at the plasma membrane (**Fig. 5g–i**). We conclude that the Ca^{2+} current induced by MEC-4(d) channels does not result from nonspecific activation of endogenous *Xenopus* oocyte channels; instead, functional MEC-4(d) subunits are essential for generating this Ca^{2+} current.

The MEC-4(d) channel conducts Ca^{2+} in touch neurons

Because direct electrophysiological recording from *C. elegans* touch receptor neurons is not technically feasible and the PCa/PNa ratio is relatively small such that *in vivo* measurement of Ca^{2+} influx through MEC-4(d) is an extreme practical challenge (at best), we used imaging protocols that report Ca^{2+} influx in cultured touch neurons to address whether the MEC-4(d) channel conducts Ca^{2+} *in vivo*[13].

We used *crt-1*-null mutations to block necrosis[5] in cultures of touch neurons expressing MEC-4(d) (**Supplementary Fig. 1** online). We cultured cells from *crt-1(null);mec-4(+)* and *crt-1(null);mec-4(d)* mutants,

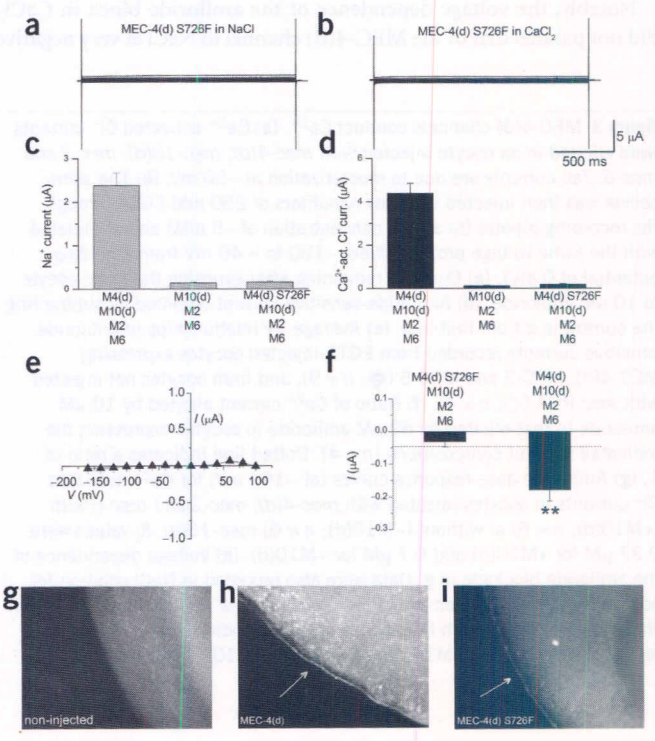

Figure 5 The Ca^{2+}-activated Cl^- current activated by exposure to $CaCl_2$ bath is activated only in oocytes expressing functional MEC-4(d) channels. (**a**,**b**) Example of current elicited in an oocyte injected with *mec-4(d) S726F*, *mec-10(d)*, *mec-2* and *mec-6* and exposed to NaCl (**a**) or $CaCl_2$ (**b**) solution. Voltage protocols in **a** and **b** were the same as those in **Fig. 2a** and **b**, respectively. (**c**) Average Na^+ current recorded at –100 mV from oocytes injected with subunits indicated on the *x*-axis ($n = 20$, 6 and 16). M4(d) indicates MEC-4(d) A713T. (**d**) Average Ca^{2+}-activated Cl^- current recorded at –160 mV from oocytes injected with the subunits indicated on the *x*-axis and bathed in $CaCl_2$ ($n = 10$, 6 and 12). (**e**) Average *I*/*V* relationships of amiloride-sensitive Ca^{2+} currents recorded from EGTA-injected oocytes expressing MEC-4(d) S726F, MEC-10(d), MEC-2 and MEC-6 ($n = 7$). (**f**) Average amiloride-sensitive (50 μM) Ca^{2+} currents at –160 mV. Data are the mean ± s.e.m. ($n = 7$ and 9). The dotted line is the level of current in non-injected oocytes. $^{**}P < 0.01$ by *t*-test. (**g**) Fluorescent photograph of a non-injected oocyte stained with antibody to MEC-4, showing no background plasma membrane staining. (**h**,**i**) Oocytes expressing MEC-10(d), MEC-2 and MEC-6 plus either MEC-4(d) (**h**) or MEC-4(d) S726F (**i**), stained with antibody to MEC-4. A 2-s exposure time was used for all photographs.

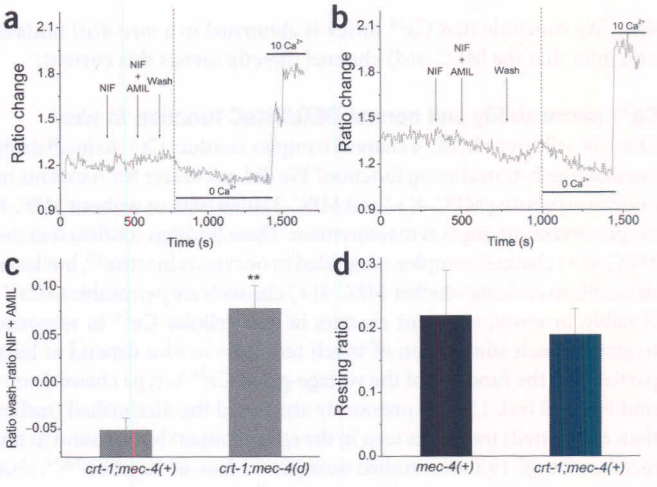

Figure 6 Cultured *mec-4(d)*-expressing touch receptor neurons show an amiloride-sensitive Ca^{2+}-current not present in *mec-4(+)* neurons. (**a**,**b**) Left, *crt-1(null);mec-4(+)* (**a**) and *crt-1(null);mec-4(d)* (**b**) cultured touch neurons were imaged for about 5 min in standard extracellular saline solution and perfused first with 20 μM nifedipine (NIF) for 3 min and then with 20 μM nifedipine plus 10 μM amiloride (AMIL) for 2.5 min. The amiloride was then washed out. The ratio change is the YFP/CFP value reported by the cameleon sensor, which reflects intracellular Ca^{2+} (refs. 13,50). The slow change in intracellular Ca^{2+} reflects the perfusion rate used in these experiments. Right, both neurons were also subjected to calibration (see **Supplementary Fig. 2** online). The resting ratio in the *crt-1(null);mec-4(+)* neuron (**a**) was 21% of the maximal ratio change, corresponding to about 200 nM Ca^{2+}. The resting ratio of the *crt-1(null);mec-4(d)* neuron (**b**) was 14% of the maximal ratio change. No statistically significant difference between the resting ratios of the two neurons was found (not shown). (**c**) Comparison of average ratio change after application of amiloride in *crt-1(null);mec-4(+)* and *crt-1(null);mec-4(d)* cultured touch neurons. The ratio change was measured as the ratio at the end of the wash minus the ratio at the end of amiloride application. Experimental points were fitted from the beginning of nifedipine application to the end of the wash by polymodial regression to determine the ratio levels. $^{**}P < 0.01$ by *t*-test. (**d**) Average percentage (fraction of 1) of the maximal ratio change, representing the resting Ca^{2+} level for *mec-4(+)* ($n = 18$) and *crt-1(null);mec-4(+)* ($n = 8$) touch neurons. Data are the mean ± s.e.m.

identified the touch neurons (which also expressed cameleon from the *mec-4* promoter and were therefore fluorescent) and compared the cameleon-reported Ca^{2+} changes when we manipulated amiloride. We included nifedipine in these experiments to control for the nonspecific effects of amiloride on voltage-gated Ca^{2+} channels and to eliminate possible secondary currents (**Fig. 6a,b**). Whereas nifedipine did not affect intracellular Ca^{2+} levels, the application of amiloride produced a marked transient decrease in intracellular Ca^{2+} in *crt-1(null);mec-4(d)* neurons that was not observed in *crt-1(null);mec-4(+)* touch neurons (**Fig. 6b,c**). These results indicate that death-blocked *mec-4(d)* neurons express an amiloride-sensitive Ca^{2+} current that is not present in *mec-4(+)* neurons.

Taken together, our data on Ca^{2+} currents carried by heterologously expressed MEC-4(d) channels and our *in vivo* cameleon studies suggest the MEC-4(d) channel may initiate necrosis by directly conducting Ca^{2+} into the touch neurons.

crt-1 mutants have normal $[Ca^{2+}]_i$

Null alleles of calreticulin and mutant ER Ca^{2+} release channels are potent suppressors of *mec-4(d)*-induced necrosis[5]. Two alternative models could explain how a deficiency in Ca^{2+}-storing calreticulin might block neuronal death: first, without calreticulin cytoplasmic Ca^{2+} levels might remain low, such that even constitutively active MEC-4(d) channels cannot raise cytoplasmic Ca^{2+} to a toxicity threshold; and second, resting cytoplasmic Ca^{2+} levels might be maintained without calreticulin but, in the absence of significant ER Ca^{2+} stores, additional Ca^{2+} release from ER would be impaired, preventing a neurotoxic increase in Ca^{2+}. The cameleon Ca^{2+} reporter enabled us to distinguish between these two models.

We measured resting Ca^{2+} by comparing the yellow fluorescence protein (YFP) to cyan fluorescence protein (CFP) fluorescence ratio in the cells during perfusion with 2 mM $CaCl_2$ (a physiological saline) to the maximal ratio change (**Fig. 6a,b**). We calculated the resting Ca^{2+} level in *crt-1* mutant neurons to be about 200 nM, which was the same as in wild-type touch neurons (**Fig. 6d** and **Supplementary Fig. 2** online). We conclude that calreticulin deficiency does not markedly alter resting concentrations of intracellular Ca^{2+}. Because resting levels are normal in the calreticulin-null mutant, the suppression of death is more likely to be conferred by preventing exceptional release of Ca^{2+} from the ER.

DISCUSSION

A new view of MEC-4(d) neurotoxicity

The mechanisms by which aberrant ion channel activity elicits neuronal death are a chief focus of research on neuronal injury and disease. Here we have investigated the molecular mechanism by which a hyperactive DEG/ENaC Na^+ channel becomes neurotoxic. Our studies have ruled out a role for VGCCs as a secondary Ca^{2+} source in death initiation and have shown that the MEC-4(d) channel itself directly conducts Ca^{2+}. We have also generated data supporting the idea that a cataclysmic release of ER-derived Ca^{2+} is likely to be a central component in necrosis initiation. Our data hold implications for both the normal function of DEG/ENaCs and for the neurotoxicity associated with channel hyperactivation.

Necrosis-inducing MEC-4(d) channels are permeable to Ca^{2+}

We obtained the following evidence that the heterologously expressed MEC-4(d) channel complex can conduct Ca^{2+}. First, the MEC-4(d) complex induced a large current in the presence of $CaCl_2$ that showed signature features of the previously characterized endogenous Ca^{2+}-activated Cl^- current in *Xenopus* oocytes[31]. Second, the amplitudes of the MEC-4(d)-dependent Na^+ current and the induced endogenous Ca^{2+}-activated Cl^- current were correlated in several, distinct experiments. Third, the amiloride sensitivity signatures for both the Na^+ and Ca^{2+}-activated Cl^- currents matched. Fourth, the recording of subtracted currents identified a MEC-4(d)-dependent Ca^{2+} current ($PCa/PNa \approx 0.2$) that showed amiloride sensitivity and MEC-10 dampening effects identical to those of the MEC-4(d)-dependent Na^+ current. Last, the nonlinear voltage dependency of amiloride block in $CaCl_2$ indicated interference between amiloride and the permeating Ca^{2+} ion at negative voltages, which is possible only if Ca^{2+} enters the channel[32]. Our data on interference between amiloride and Ca^{2+} also suggest that this ion uses part of the amiloride-binding site (perhaps G717; see **Supplementary Fig. 3** online) to enter and to pass through the pore. Because a change in the extracellular $CaCl_2$ concentration shifts the reversal potential of this current, and because Na^+ is absent from the extracellular bath in these experiments, these data clearly establish Ca^{2+} as the ion carried by the isolated MEC-4(d)-dependent current.

Using the cameleon Ca^{2+} sensor, we found evidence of amiloride modulation of intracellular Ca^{2+} only in touch neurons expressing *mec-*

4(d). We conclude that Ca^{2+} influx is abnormal in a *mec-4(d)* mutant and infer that the MEC-4(d) channel directly carries this current.

Ca^{2+} permeability and normal DEG/ENaC function *in vivo*

Does the wild-type MEC-4 channel complex conduct Ca^{2+} to mediate its normal touch-transducing function? We did not detect Na^+ currents in oocytes expressing MEC-4(+) and MEC-2 either with or without MEC-6 despite several attempts at measurement. These findings confirm that the MEC-4(+) channel complex assembled in oocytes is inactive[10], but leave us unable to evaluate whether MEC-4(+) channels are permeable to Ca^{2+}. Notably, however, transient changes in intracellular Ca^{2+} in response to gentle touch stimulation of touch receptors *in vivo* depend at least partially on the function of the voltage-gated Ca^{2+} L-type channel subunit EGL-19 (ref. 13). We previously attributed the diminished (rather than eliminated) transients seen in the *egl-19* mutant background to the fact that the *egl-19* alleles studied were partial-loss-of-function[13,37]. Our identification of an amiloride-sensitive Ca^{2+} current in *mec-4(d)* touch neurons, however, raises the possibility that some of the brief Ca^{2+} influx induced by gentle touch may be contributed directly by the MEC-4(+) channel. Ca^{2+} permeability of MEC-4(+) channels and its role in gentle touch sensation could be determined in future experiments by cameleon measurements of *in situ* exposed touch neurons, in which extracellular Ca^{2+}, nifedipine and EGL-19 function could be manipulated to determine their impact on Ca^{2+} transients. Alternatively, a mutant MEC-4(d) that uncouples Na^+ and Ca^{2+} permeability might be identified and tested for its impact on normal touch sensation.

Is Ca^{2+} permeability a common feature of DEG/ENaC family members? The only other *C. elegans* degenerin channel (out of 28 encoded by the *C. elegans* genome) that has been electrophysiologically characterized is the UNC-105 muscle channel, which seems to be Ca^{2+} impermeable[38]. Mammalian αENaC subunits have been reported to be Ca^{2+} impermeable, although Ca^{2+} permeability can be conferred by substituting an aspartic acid or cysteine residue for a highly conserved serine residue (S589) situated in the pore-lining domain[35]. Data on the Ca^{2+} permeability of neuronally expressed ASIC channels are limited and sometimes contradictory[39–44]. Notably, however, studies on ASIC1a channels, which are predominately expressed in the central nervous system, are localized to synapses and have been implicated in synaptic plasticity, have been shown to conduct Ca^{2+} (refs. 21,22). Given the profound importance of intracellular Ca^{2+} in short- and long-term regulation of neuronal activity, we speculate that DEG/ENaC Ca^{2+} conductance might have more crucial physiological roles in neuronal function than was previously suspected.

A potential role of Ca^{2+} influx in necrosis initiation

Our data suggest that a previously unexpected influx of Ca^{2+} via the MEC-4(d) channel contributes to necrosis activation. Even though the Ca^{2+} currents are relatively small and the PCa/PNa ratio is only about 0.2, this Ca^{2+} permeability is likely to be significant for an ion channel that remains constitutively open. In support of this possibility, the electrophysiological characterization of mutant MEC-4(d) subunits encoding a substitution at a second site corresponding to those isolated as *in vivo* intragenic revertants of the *mec-4(d)* necrosis-inducing phenotype indicated that these channels were either inactive (MEC-4(d) S726F) or showed a markedly reduced inward current (MEC-4(d) G717E; see **Supplementary Fig. 3** online).

Notably, none of the six distinct mutant MEC-4(d) channels that we have electrophysiologically characterized so far (D714N, G717E, S726F, S726C, S726D and A745T, all within or near the channel pore) uncouples Na^+ from Ca^{2+} conductance. Thus, we cannot specifically distinguish whether Na^+ or Ca^{2+} influx or both are required for initiating death. Nonetheless, given the well-known role of Ca^{2+} in regulating neuronal viability, the simplest model is that an excessive Ca^{2+} influx has a central role in signaling Ca^{2+} release from the ER.

A dynamic role for Ca^{2+} release in neuronal necrosis

In vivo analysis of MEC-4(d)-induced necrosis has shown that neurotoxicity depends on ER Ca^{2+} stores primarily held by Ca^{2+}-binding protein calreticulin[5]. Our *in vivo* measurements of resting Ca^{2+} levels in neurons lacking calreticulin showed unaffected baseline levels of Ca^{2+} and ruled out the possibility that *crt-1* neurons, in which death is strongly suppressed, experience a general homeostatic lowering of intracellular Ca^{2+}. The important implication of this finding is that the requirement for calreticulin ER Ca^{2+} storage and release proteins in necrosis most probably reflects their participation in a dynamic release event that is elicited in response to an inappropriate influx of ions across the plasma membrane. Notably, in mouse calreticulin knockout fibroblasts, resting cytoplasmic Ca^{2+} is maintained but the ability to release ER Ca^{2+} from stores is markedly diminished[45], supporting the idea that similar cellular effects of calreticulin deficiency occur in nematode and mammalian neurons.

We thus propose the following model for MEC-4(d)-induced neurotoxicity. The MEC-4(d) mutant channel conducts excess Na^+ and Ca^{2+} into the touch neuron, thereby provoking Ca^{2+} release from the ER. The MEC-4(d)-imported influx of Ca^{2+} could directly activate the ER Ca^{2+} release channels and/or could activate G-protein-coupled receptors and/or Ca^{2+}-dependent phospholipases, thereby increasing IP3, which in turn would activate the IP3 receptor. The outcome would be a neurotoxic increase in cytoplasmic Ca^{2+} derived from ER stores, which would activate calpain death proteases and other downstream destructive processes. Although this detailed molecular mechanism has been worked out in nematodes, we note that it shares several features with mammalian necrosis models and is likely to apply to the potential neurotoxicity of mammalian ASICs[22] or other plasma membrane Ca^{2+} channels that are activated inappropriately or excessively.

Ca^{2+} permeability of DEG/ENaCs in pathological conditions

In pathological conditions in which extracellular Ca^{2+} or acidosis are increased, such as seizures, cerebral ischemia and physical injury, the Ca^{2+} permeability of DEG/ENaC channels can become cytotoxic. Notably, studies have shown that native Ca^{2+}-conducting ASIC1a channels contribute to the rise in intracellular Ca^{2+} that occurs during acidosis and that they contribute significantly to ischemic injury[21,22]. Thus, the toxicity of excessively activated neuronally expressed DEG/ENaCs is conserved from nematodes to humans. Amiloride, a common blocker that is used in the clinic for other purposes, might have value as a pharmaceutical approach to reduce neuronal damage from ischemic insults.

It should be also noted that, although the DEG/ENaC family of ion channels has not been specifically implicated in human neurodegenerative conditions, mutant subunits that are hyperactivated or inappropriately regulated are clear candidates for potential roles in neurodegenerative conditions. Similarly, allelic variation in this channel family may underlie susceptibility to stroke damage and neuronal survival. Given the conserved aspects of degenerin and ASIC function, continued analysis of genetic and physiological components in *C. elegans* may provide insight into neuronal injury mechanisms relevant to the design of intervention strategies in humans.

METHODS

***C. elegans* strains and growth.** Nematodes were grown according to standard protocols[46], except that enriched peptone plates seeded with *Escherichia coli*

strain NA22 were used for growth for egg preparations. Cameleon experiments were done with touch neurons cultured from *crt-1(bz29)* and *crt-1(bz29);mec-4(u231)* eggs[5] expressing yellow cameleon version 2.12 under the control of the *mec-4* promoter[13].

Oocyte expression and electrophysiology. The *mec-2*, *mec-4(d)* and *mec-10(d)* expression clones have been described[10] and were prepared in bacterial strain SMC4 (ref. 10). We cloned *mec-6* into pGEM. Mutations at second sites introduced by PCR were confirmed by sequencing. Capped RNAs were synthesized using a T7 mMESSAGE mMACHINE kit (Ambion), purified on RNAeasy columns (Qiagen), and checked for size, integrity and concentration. Stage V–VI oocytes were manually defolliculated after 2 h of collagenase treatment (2 mg/ml in Ca^{2+}-free OR2 solution[47]) of *Xenopus laevis* ovaries (NASCO). Oocytes were injected with 52 nl of complementary RNA (cRNA) mix to a final amount of 5 ng per oocyte of each cRNA except for *mec-6*, which was injected at 1 ng per oocyte. We incubated oocytes in OR2 at 20 °C for at least 4 d before making recordings.

Currents were measured using a GeneClamp 500B two-electrode voltage clamp amplifier (Axon Instruments) at 20 °C. Electrodes (0.3–1 MΩ) were filled with 3 M KCl or 3 M KCl plus 250 mM EGTA, and oocytes were perfused with a NaCl solution containing 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM HEPES (pH 7.2) or a $CaCl_2$ solution containing 73 mM $CaCl_2$, 2 mM KCl and 10 mM HEPES (pH 7.2). For experiments in which the extracellular Ca^{2+} was lowered to 20 mM, the solution contained 20 mM $CaCl_2$, 2 mM KCl, 159 mM NMDG chloride and 10 mM HEPES (pH 7.2). Divalent cation permeability experiments used salines containing 73 mM XCl_2, 2 mM KCl and 10 mM HEPES (pH 7.2), where X was Mg^{2+}, Sr^{2+}, Ba^{2+} or Zn^{2+}. We used the pCLAMP suite of programs (Axon Instruments) for data acquisition and analysis. Currents were filtered at 200 Hz and sampled at 1 kHz.

Immunocytochemistry. Paraformaldehyde-fixed oocyte slices were stained with antibody to MEC-4 (ref. 9; diluted 1:50 in 1% bovine serum albumin in PBS plus 0.1% Tween 20) as described[47]. The secondary antibody was Cy2-conjugated goat anti-rabbit (diluted 1:2,000; Jackson ImmunoResearch). After staining, slices were mounted with Vectorex medium (Vector) and photographed with an Axiplan 2 microscope (Zeiss) equipped with a digital camera.

***C. elegans* primary cultures.** We prepared *C. elegans* primary cultures from eggs as described[48]. VGCC blockers were added to the culture media immediately after dissociation.

Cameleon-based determination of intracellular Ca^{2+}. Two-day-old cells were placed in a recording chamber (Warner Instruments) and perfused with an extracellular saline solution (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM D-Glucose; pH 7.2 and 340 mOsm) for at least 5 min before being superfused with the same solution containing 20 μM nifedipine with or without 10 μM amiloride. We determined the minimal and maximal ratios as described[49,50]. The baseline Ca^{2+} concentration was determined by averaging the ratio level during the 5-min perfusion with standard saline and then calculating the percentage of the maximal ratio change between 0 and 10 mM Ca^{2+} that the ratio represented.

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

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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