Auto-phosphorylation of a voltage-gated K⁺ channel controls non-associative learning

Shi-Qing Cai¹, Yi Wang^{1,2}, Ki Ho Park¹,
Xin Tong, Zui Pan and Federico Sesti*

Department of Physiology and Biophysics, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ, USA

Here, we characterize a new K⁺ channel–kinase complex that operates in the metazoan *Caenorhabditis elegans* to control learning behaviour. This channel is composed of a pore-forming subunit, dubbed KHT-1 (73% homology to human Kv3.1), and the accessory subunit MPS-1, which shows kinase activity. Genetic, biochemical and electrophysiological evidence show that KHT-1 and MPS-1 form a complex *in vitro* and in native mechanosensory PLM neurons, and that KHT-1 is a substrate for the kinase activity of MPS-1. Behavioural analysis further shows that the kinase activity of MPS-1 is specifically required for habituation to repetitive mechanical stimulation. Thus, worms bearing an inactive MPS-1 variant (D178N) respond normally to touch on the body but do not habituate to repetitive mechanical stimulation such as tapping on the side of the Petri dish. Hence, the phosphorylation status of KHT-1–MPS-1 seems to be linked to distinct behavioural responses. In the non-phosphorylated state the channel is necessary for the normal function of the touch neurons. In the auto-phosphorylated state the channel acts to induce neuronal adaptation to mechanical stimulation. Taken together, these data establish a new mechanism of dynamic regulation of electrical signalling in the nervous system.

The EMBO Journal (2009) 28, 1601–1611. doi:10.1038/emboj.2009.112; Published online 23 April 2009

Subject Categories: signal transduction; neuroscience

Keywords: habituation; kinase; potassium channel; nervous system; neuronal adaptation

Introduction

Voltage-dependent potassium (K⁺) channels (Kv) regulate neuronal excitability by controlling the movement of K⁺ ions across the membrane in response to changes in cell voltage. Modulation of these channels by signalling molecules, such as protein kinases, represents a common mechanism by which

neurons adapt their electrical responses to a variety of external events. In this classic picture, K⁺ channels provide the substrate for enzymes and as such they are the passive endpoint of signalling cascades. This wisdom has recently been challenged by the discovery that K⁺ channels might also possess their own enzymatic activity, which is conferred by non-conducting accessory subunits, including mammalian Kvβ2, *Drosophila* Slob and *Caenorhabditis elegans* MPS-1 (Zeng *et al*, 2004; Cai *et al*, 2005; Weng *et al*, 2006).

MPS-1, which shows kinase activity, phylogenetically belongs to the KCNE family (Bianchi *et al*, 2003; Cai *et al*, 2005). The *kcne* genes encode integral membrane proteins with a single transmembrane span, which act to modulate the basic attributes of K⁺ channels, including permeation, gating, trafficking, abundance at the plasma membrane and pharmacology, and when genetically mutated, cause congenital and acquired disease (reviewed by McCrossan and Abbott, 2004). There are four KCNE homologues in *C. elegans*, named *mps* genes (Park *et al*, 2005). The founder of the sub-family, MPS-1, shares significant homology with human KCNE2 and KCNE4, suggesting that MPS-1 might be a mosaic of both. MPS-1 is expressed in the nervous system of the worm, including the amphid sensory neurons, touch-sensing neurons (ALM/PLM) and vulva. In the amphid neurons, MPS-1 assembles with the voltage-gated K⁺ channel KVS-1 to form a complex that contributes to both the maintenance and the sensitivity of those cells (Bianchi *et al*, 2003). By contrast, the partners for MPS-1 in touch neurons and in the vulva are not known. *In vitro* studies have shown that MPS-1 is able to phosphorylate KVS-1 and that its kinase activity acts to decrease the open probability of the channel (Cai *et al*, 2005).

The discovery that K⁺ channels are capable of endogenous enzymatic activity prompts the intriguing question as to which are the physiological roles of this new mode of K⁺ channel regulation. To address this fundamental question we investigated the physiological role of the kinase activity of MPS-1. Here we show that MPS-1 assembles with a voltage-gated K⁺ channel, dubbed KHT-1 (for K⁺ channel for Habituation to Tap), in the touch neurons of the worm. Our data provide a model that predicts that the phosphorylation status of KHT-1–MPS-1 is linked to distinct behavioural responses. In the basal, non-phosphorylated state the channel is important for the normal function of the touch neurons. When mechanical stimulation is repeated, such as continuous tapping on the side of the Petri dish, the channel auto-phosphorylates and as a consequence, the animal learns that taps are not important (habituation). Thus, the fact that the kinase activity of MPS-1 is specifically required for habituation behaviour identifies MPS-1 as a true learning-susceptibility gene.

Results

KHT-1 is a candidate partner of MPS-1

MPS-1 does not form functional channels on its own. Therefore, to elucidate the role that MPS-1 plays in

*Corresponding author. Department of Physiology and Biophysics, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, 683 Hoes lane, Piscataway, NJ 8854, USA. Tel.: +1 732 235 4032; Fax: +1 732 235 5038; E-mail: sestife@umdnj.edu

¹These authors contributed equally to this work

²Present address: Department of Genetics, Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, 1300 Morris Park Avenue, Bronx, NY 10461, USA

Received: 7 November 2008; accepted: 30 March 2009; published online: 23 April 2009

KHT-1	---MDSEYRIILNVGGVRHETYKHTLKKIPATRLSRLTTNLAN---	YDPVLNEYFFDRH	53
Kv3.1	MGQGDESERIVINVGGRHQTYRSTLRITLPGLRLAWLAEPDAHSHFDYDPRADEFFDRH		60
KHT-1	PGVFAQILNYYRTGKLHYPLDVCGLPFEELKYWGLDSNECEPCWMTLSGTRDTQDVLK		113
Kv3.1	PGVFAHILNYYRTGKLHCPADVCGPLYEELAFWGLIDETDVEPCWMTYRQHRDAEALD		120
KHT-1	TLD--ELDIEVDQTE--GP-----ELYKRFGEWEDDYHNDLSAWQKIKPRIWRLF		159
Kv3.1	SFGGAPLDNSADDADADGPGDSGDGEDELEMTKRLALSDSPDGRPGFWRRWQPRIWALF		180
KHT-1	DEPNSSRSAQFIAAISVFFLITAIIVFCLKTHPGLRIPELAPFGNFSRNHRSTSSRIHPA		219
Kv3.1	EDPYSSRYARYVAFASLFFILVSIITFCLETHE--RFNPITVNKTEIENVNGTQVRY--		238
KHT-1	QINIDKANSRPHPTFMYIETICNIWFTEILARFSSCPSRFEYLRAPVNIIDIVATLTFY		279
Kv3.1	-----REAETEAFITYIEGVCVWFTEFLMRVIFCPNKVEFIKNSLNIIDFVALPPFY		292
KHT-1	IDLLSSMFGATAD-----LEFFSIIRIMRLFKLTHHNSGLKILMHTFRASAKELMLLVF		333
Kv3.1	LEVGLSGLSSKAADKDLVGLFRVVRFRVIRLIRIFKLTRHFVGLRVLGHTLRASNEFLLLII		352
KHT-1	FLVLGVVVFASLVYVAERVES-----NEDNQFVSIPIGLWVAIVTMTTIGYGDITPHT		386
Kv3.1	FLALGVLI FATMIYYAERIGAQPNDPSASEHTHFKNIPIGFWWAVVTMTTLGYGDMYPQT		412
KHT-1	YLGRLIGSICALAGVLTIALPVPVIVSNFAMFYSHQTQARSKMPKKRR-----GVLSDMQ		440
Kv3.1	WSGMLVGAICALAGVLTIAMPPVPVIVNFGMYSLAMAKQKLPKKKKKHIPRPPQLGSPN		472
KHT-1	VAQHVKHAPVRNSNGPTQRKNMENTPLVSNHPTPHTLI--		479
Kv3.1	YCKSVVNSPHHSTQSDTCPLAQEILEINRAGRKPLRGMSI		513

Figure 1 KHT-1 is the *C. elegans* homologue of Kv3.1. KHT-1 and Kv3.1 alignment was calculated by ClustalW software (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html). There is 73 % homology. Red, green and blue colours indicate identity, strong similarity and low similarity, respectively.

mechanosensation, we sought to identify potential pore-forming subunits with which MPS-1 might interact in the touch neurons. We focused on predicted R186.5, here christened KHT-1, as this gene shows high homology to KVS-1 (55%). We cloned KHT-1 using a standard RACE technology, and its cDNA sequence was as predicted. The KHT-1 channel is the homologue of the human Kv3.1 (73 % homology, Figure 1), which is abundantly expressed in the brain, where it forms a complex with KCNE3 (Perney *et al*, 1992; McCrossan *et al*, 2003). Other likely candidates such as KQT-1, whose human homologue, KCNQ1, assembles with KCNE2 in the stomach and with KCNE1 in the heart and inner ear (Barhanin *et al*, 1996; Sanguinetti *et al*, 1996; Schulze-Bahr *et al*, 1997; Wei *et al*, 2005; Roepke *et al*, 2006) or EXP-2, which assembles with MPS-4 in the pharynx (Park and Sesti, 2007), failed to interact with MPS-1 in CHO cells (data not shown).

To determine whether KHT-1 and MPS-1 might form a complex, we initially expressed the two proteins in mammalian (CHO) cells. Thus, KHT-1 yielded robust surface expression (Figure 2A) and co-immunoprecipitated with MPS-1 (Figure 2B). The fact that MPS-1 and KHT-1 can interact prompted the question to as whether MPS-1 can phosphorylate KHT-1. To evaluate KHT-1 phosphorylation, we used anti-phosphoserine/phosphothreonine (anti-phosphoS/T) antibodies as done before (Cai *et al*, 2005). Figure 2C shows that wild-type MPS-1, but not the D178N MPS-1 mutant, were able to increase anti-S/T staining of KHT-1, suggesting that MPS-1 phosphorylates KHT-1. We conclude that in CHO cells, KHT-1 forms a stable complex with MPS-1 and provides a substrate for the kinase activity of the latter.

KHT-1 forms a complex with MPS-1 in the touch neurons

As our experiments indicated that KHT-1 and MPS-1 form a complex in CHO cells, we next investigated the possibility

that this complex could exist *in vivo*. Thus, we sought to identify neurons in which these two proteins are co-expressed, using GFP fusions. In transgenic worms expressing GFP driven by the *kht-1* promoter in a *kht-1* KO background (*P_{kht-1}::gfp*), intense GFP fluorescence was visible in the PLM touch neurons (Figure 3A), which also express MPS-1 (Bianchi *et al*, 2003), suggesting that KHT-1 and MPS-1 might form a complex in these cells. *P_{kht-1}::gfp* also yielded signals in intestine and in several neurons in the head and tail (data not shown), which are currently unidentified. To further explore the notion that MPS-1 and KHT-1 might form a complex in the PLM neurons, we assessed their surface co-localization in primary cells prepared from embryos, as morphological, electrophysiological and GFP reporter studies have shown that the differentiation, morphology and functional properties of cultured cells are similar to those observed *in vivo* (Christensen *et al*, 2002; Zhang *et al*, 2002; Suzuki *et al*, 2003). We constructed transgenic worms expressing the wild-type *mps-1* gene and a mutated gene encoding for the inactivated kinase fused to *gfp* in the C-terminus in a *mps-1* KO background (we name these genotypes, respectively, *P_{mps-1}::wild type-mps-1::gfp* and *P_{mps-1}::d178n-mps-1::gfp*, where *P_{mps-1}* is the *mps-1* promoter). Both constructs gave strong GFP signals in the cells where MPS-1 is normally expressed, including the PLM neurons (Supplementary Figure S-1A-B), indicating that wild-type and D178N fold and traffic to the plasma membrane normally and that inactivation of the kinase activity of MPS-1 has no effect on its expression. In Figure 3B a representative confocal image of a PLM neuron obtained from *P_{mps-1}::wild type-mps-1::gfp* embryos is shown. (Nomarski images of the broad field are shown in Supplementary Figure S-2.) In Figure 3C the same cell stained with a custom-made polyclonal antibody raised against KHT-1 is shown. Both subunits were detected in the dendrite and along the perimeter of the cell, indicating surface expression

Figure 2 KHT-1 and MPS-1 form a complex in mammalian cells. **(A)** Immunolocalization of KHT-1 at the surface of CHO cells transfected with KHT-1 cDNA and phase-contrast light transmission image of the field. Images were taken with an Olympus BX61 microscope equipped with Nomarski optics. **(B)** KHT-1 and c-Myc-MPS-1 epitope tagged to the c-Myc tag in the N-terminus co-immunoprecipitate in CHO cells. Lysates were immunoprecipitated using c-Myc-conjugated beads and western visualization was with anti-KHT-1 antibody. **(C)** CHO cells transfected with KHT-1-HA alone or with the indicated MPS-1 variants were immunoprecipitated using HA-conjugated beads. Western visualization with rabbit anti-phosphoserine/threonine (S/T) antibodies is shown in the top panel. Western visualization with anti-KHT-1 antibody is shown in the bottom panel.

Figure 3 KHT-1 and MPS-1 form a complex in native PLM neurons. **(A)** *kht-1* expression in PLM neurons was determined by a transcriptional gene reporter obtained by fusing ~3.5 kb of the promoter sequence of *kht-1* with GFP in a Fire vector pPD95.75. The figure shows side and top views using two distinct worms. The dendrites are indicated by arrows. **(B)** Confocal image of the plasma membrane of a PLM neuron cultured from embryos of *P_{mps-1}::wild type-mps-1::gfp* worms. **(C)** Confocal image of the same neuron in **(B)** stained with anti-KHT-1. **(D)** A merged image of the confocal images in **(B)** and **(C)**. The yellow colour indicates co-localization of KHT-1 and MPS-1 at the plasma membrane. **(E)** Co-immunoprecipitations of wild type MPS-1-GFP and D178N MPS-1-GFP with KHT-1. Worm lysates were immunoprecipitated with anti-KHT-1 antibody and western visualization was done using anti-GFP antibody. Controls were obtained from precipitates obtained using non-conjugated protein A beads. Images were taken with a Zeiss LSM 510 META confocal microscope and merged by ImageJ software.

as expected. Most importantly, their signals overlapped (Figure 3D), suggesting that KHT-1 and MPS-1 form a complex in the PLM neuron. (analogous results were obtained with *P_{mps-1}::d178n-mps-1::gfp*, Supplementary Figure S-3.) To confirm this notion we carried out native co-immunoprecipitations ($n = 3$ experiments). Thus, lysates of *P_{mps-1}::wild type-mps-1::gfp* and *P_{mps-1}::d178n-mps-1::gfp* worms were

immunoprecipitated with anti-KHT-1 antibody. Western blot analysis with anti-GFP antibody showed a specific band migrating with a molecular weight of ~52 kDa, which corresponds to the molecular weight of the MPS-1-GFP fusion protein, which was not detected in control samples (Figure 3E). Together these data indicated that KHT-1 and MPS-1 form a stable complex in the PLM neuron.

Figure 4 MPS-1 is an inhibitory subunit of KHT-1. (A) Representative whole-cell currents recorded in CHO cells transfected with KHT-1 cDNA. Voltage protocol (inset) was 1 s voltage steps from -80 mV to $+120$ mV (20 mV increments). (B) Current density–voltage (σ – V) relationships for KHT-1 channels alone expressed in CHO cells. $n = 27$. (C) Reversal potential of KHT-1 currents alone on isotonic replacement of sodium with various external potassium levels. Data fit to a Nernst function (equation (2)) gave $kT/e = 23.1 \pm 1.1$. $n = 11$ cells. (D) Normalized macroscopic conductances, G/G_{Max} (equation (3)) for KHT-1 channels alone. The theoretical line was constructed by fitting the data to the Boltzmann function (equation (4)) with 65.4 mV and $V_s = 16.1$ mV. $n = 27$ cells. (E) Representative whole-cell currents expressed in CHO cells transfected with KHT-1 and wild-type MPS-1 cDNA. (F) Representative whole-cell currents expressed in CHO cells transfected with KHT-1 and D178N MPS-1 cDNA. (G) σ – V relationships for KHT-1-MPS-1 channels (triangles) and KHT-1-D178N channels (circles). $n = 17$ and 18 cells, respectively. The dotted line is the (σ – V) relationship for KHT-1 channels alone. (H) G/G_{Max} relationships for KHT-1-MPS-1 channels (triangles) and KHT-1-D178N channels (circles). The theoretical line is the Boltzmann fit of the G/G_{Max} relationship for KHT-1 alone (panel D). Data are presented as mean $\pm$ standard error of the mean (s.e.m.). Statistically significant differences from control are indicated with $**P < 0.01$.

The kinase activity of MPS-1 acts to inhibit the KHT-1 current

Next, we characterized the functional properties of the KHT-1 current using the whole-cell configuration of the patch clamp. CHO cells transfected with KHT-1 alone expressed robust, voltage-dependent, rapidly activating K⁺ currents (Figure 4A and B). By progressive substitution of bath potassium with sodium, we found the KHT-1 channel to be selective for potassium, which shifted the reversal potential by $kT/e = 23.1 \pm 1.1$ mV, $n = 11$ (Figure 4C). The half-maximal voltage for activation, $V_{1/2}$, was 67.2 ± 5.4 mV and the slope, $V_s = 16.3 \pm 1.4$ mV, $n = 27$ (Figure 4D). Thus, *C. elegans* KHT-1 encodes a K⁺-selective channel with properties similar to mammalian channels of the Kv3 family, indicating functional conservation suggested by sequence homology. Next, we characterized the functional effects of MPS-1 on KHT-1. Cells co-transfected with wild-type MPS-1 and KHT-1 expressed currents, which were roughly 5-fold smaller than KHT-1 currents alone (Figure 4E and G). It should be noted that when we replaced wild-type MPS-1 with the constitutively inactive D178N mutant, the magnitude of the current returned to KHT-1-alone levels (Figure 4F and G). This was specifically due to the lack of enzymatic activity in the MPS-1 variant because the D to N mutation does not affect the expression of the protein nor its interactions with pore-forming subunits (Cai *et al*, 2005), which are mainly controlled by its transmembrane domain (Wang and Sesti, 2007). Neither wild-type MPS-1 nor D178N modified other gating attributes of the complex, such as activation. (Figure 4H, The theoretical line is the Boltzmann fit of KHT-1 alone.) We conclude that MPS-1 is an inhibitory subunit of

KHT-1 and that the inhibition of the KHT-1 current is expressly mediated by the kinase activity of MPS-1. It is interesting to observe that the kinase activity of MPS-1 acts similarly to inhibit the current of KVS-1 (Cai *et al*, 2005), underscoring not only a sequence but also functional homology in the two channels.

The KHT1-MPS-1 complex is required for mechanosensation

C. elegans senses gentle touch on the body mainly through two neuron types, the ALM and PLM neurons—located in the middle of the body and in the tail, respectively—both of which express MPS-1 (Driscoll and Kaplan, 1997; Bianchi *et al*, 2003). Therefore, to probe the physiological role of the kinase activity of MPS-1 in mechanosensation, we constructed transgenic worms expressing the wild-type gene and a mutated gene encoding for the inactivated kinase in an *mpps-1* null background (we name these genotypes, respectively, *P_{mpps-1}::wild type-mpps-1* and *P_{mpps-1}::d178n-mpps-1*) and assessed how these worms responded to mechanical challenges. By a gentle touch on the head or tail the worm responds by moving backward or forward, respectively. However, when the mechanical stimulus is repeated, for example by rhythmically tapping on the Petri dish, the worm habituates (Rankin *et al*, 1990; Rankin and Broster, 1992). Thus, we evaluated both the response of the transgenic animals to gentle touch (body-touch phenotype) and their ability to habituate to repetitive mechanical stimulation (tap phenotype). Figure 5A shows that parental worms (N2 genotype) responded nearly 100% of the times to gentle touches on the tail by moving forward. By contrast, the

Figure 5 The KHT1-MPS-1 complex is required for mechanosensation. (A) Responses to touches in the tail in parental (N2), *mps-1* null, *kht-1* null, *P_{mps-1}::wild type-mps-1* (wild type), *P_{mps-1}::d178n-mps-1* (d178n) genotypes. The *mec-4(d)* mutant, which induces necrotic touch-neuron death and therefore is touch insensitive, was used as control strain. $n \geq 50$ worms/genotype. (B) Response to touch on the head in the indicated phenotypes. $n \geq 50$ worms/genotype. Behavioural tests were carried out without knowledge of the worms' genotype. Data are presented as mean $\pm$ s.e.m. Statistically significant differences from control are indicated with **P* < 0.0 and ***P* < 0.01.

response of *mps-1* null worms was significantly diminished in agreement with earlier data, in which *mps-1* was inactivated by RNA interference (Bianchi *et al.*, 2003). Also, genetic ablation of *kht-1* caused defective forward response (Figure 5A), underscoring the active role played by this channel in mechanosensation. Furthermore, the defective phenotype of the null worms could not be ascribed to abnormal development of the touch neurons because they did not show developmental or morphological abnormalities (Supplementary Figure S-1B-D). The rescue of the wild-type *mps-1* gene in the null genotype fully restored mechanosensation, as expected (Figure 5A). It should be noted that inactivation of the kinase activity of MPS-1 (*P_{mps-1}::d178n-mps-1* genotype) did not affect the ability of the animal to respond to touch on the tail (Figure 5A). Nearly identical responses were recorded when the animals were touched on the head (reverse movement, Figure 5B). We conclude that the kinase activity of MPS-1 is not required for the basal function of the touch neurons. Interestingly, in both *kht-1*-null and *mps-1*-null worms forward and reverse movement were equally impaired. This is expected for MPS-1, which is expressed in the ALM and PLM neurons and might suggest that KHT-1 is also expressed and is functional in both cells. The lack of *P_{kht-1}*-driven GFP signals in the ALM neurons could be because of several factors—most importantly, the fact that GFP requires high levels of protein expression to yield detectable signals.

The kinase activity of MPS-1 is necessary for habituation to tap

On tapping the Petri dish every 5 s, N2 worms and *P_{mps-1}::wild type-mps-1* worms quickly habituated (Figure 6A and movie in Supplementary data). Similar results were obtained at ISI 2, 10 and 60 s (data not shown), in agreement with earlier reports (Rankin *et al.*, 1990; Rankin and Broster, 1992). Surprisingly, when *P_{mps-1}::d178n-mps-1* worms were subjected to the same training, they habituated considerably slower (Figure 6A and movie in Supplementary data. 2 s, 10 s and 60 ISI, data not shown) and retained a significant (~30%) residual response to tap. For quantification, we

fitted the habituation coefficient to a single exponential function and used the time constant of the exponential, τ_H , as a measure of the ability of the worms to habituate. Thus, parental and *P_{mps-1}::wild type-mps-1* genotypes habituated quickly (τ_H values ranging between 2–4 taps) in an ISI-dependent fashion (Figure 6B). In contrast, the number of taps necessary to habituate *P_{mps-1}::d178n-mps-1* worms was 10-fold higher and fairly independent of the ISI used during training. N2 and *P_{mps-1}::wild type-mps-1* worms could retain habituation for nearly 1 h (Figure 6C). Recovery was more rapid in worms trained at short (ISI = 5 s, Figure 6C) than at long ISI (ISI = 60 s, data not shown), ruling out possible effects of fatigue. In contrast, *P_{mps-1}::d178n-mps-1* worms recovered almost instantaneously (ISI = 5 s, Figure 6C; ISI = 60 s, data not shown). In reality, this behaviour did not reflect defective retention of habituation training, but rather was a consequence of the inability of these worms to habituate to tap. It is important to emphasize that the inability of the *P_{mps-1}::d178n-mps-1* genotype to habituate to tap was not caused by defective function of the touch neurons. In fact, we found that, as in other species, habituation to tap in *C. elegans* arises as a consequence of progressive desensitization to the stimulus (Figure 6D). Thus, the animals harbouring D178N MPS-1 could not habituate because they retained touch sensitivity during tapping. Consistent with this mechanism, when we tried to habituate *kht-1* null worms we found that the animals were less receptive to taps than normal worms, as though they were already desensitized (Figure 6E). Notably, *mps-1* KO worms showed a similar behaviour (Figure 6F), further suggesting that the two proteins form a complex in the touch neurons. It is likely that the weak response in the null genotypes was a consequence of the fact that both KHT-1 and MPS-1 are required for the normal function of the touch neurons, which therefore do not respond to tap when this current is suppressed.

Touch neurons express rapidly inactivating voltage-gated K⁺ currents

To gain insight into the molecular mechanisms underlying the effect of the KHT1-MPS-1 complex on mechanosensa-

Figure 6 The kinase activity of MPS-1 is necessary for habituation to tap. (A) Habituation to tap in N2 (hollow squares), *Pmps-1::wild type-mps-1* (filled squares) and *Pmps-1::d178n-mps-1* (hollow triangles) animals at ISI = 5 s. The theoretical lines were constructed by fitting the data to a single exponential function:

$$H.C. = A + Be^{-T/\tau_H} \quad (1)$$

where T is the number of taps, A and B are constants and τ_H is the time constant of the exponential. $n=7$ experiments/genotype. (B) τ_H coefficients as a function of the ISI for N2, *Pmps-1::wild type-mps-1* and *Pmps-1::d178n-mps-1* animals. Data represent averages of 5–7 experiments/genotype. (C) Recovery from habituation to tap in N2, *Pmps-1::wild type-mps-1* and *Pmps-1::d178n-mps-1* animals trained at 5 s ISI. Data represent averages of 3–4 experiments/genotype. (D) Body-touch phenotype in N2, *Pmps-1::wild type-mps-1* and *Pmps-1::d178n-mps-1* animals earlier trained with the indicated number of taps. $n=3$ –5 experiments/genotype. (E) Habituation to tap in the *kht-1*-null genotype. Dotted line represents N2 worms. ISI = 5 s. $n=5$ experiments. (F) Habituation to tap in the *mps-1*-null genotype. Dotted line represents N2 worms. ISI = 5 s. $n=7$ experiments. Behavioural tests were carried out without knowledge of the worms' genotype. Data are presented as mean $\pm$ s.e.m. Statistically significant differences from control are indicated with * $P<0.0$ and ** $P<0.01$.

tion, we characterized the electrophysiology of native cells cultured from embryos. In agreement with earlier reports (Suzuki *et al*, 2003), primary touch neurons expressed robust, inactivating currents, which were abolished on substitution of *N*-methyl-D-glucamine for K⁺ in the pipette solution (data not shown), suggesting that they were mainly carried by K⁺ ions. Representative currents, along with peak and steady-state current density–voltage relationships, are shown in Figure 7A and B. Thus, N2 currents showed half-maximal activation, $V_{1/2} = 65.5 \pm 5.0$ mV, with a slope factor, $V_s = 19.3 \pm 1.8$ mV (Figure 7C). Inactivation was moderately voltage-dependent (~ -0.064 ms/mV) and fast ($\tau \sim 7$ ms at +80 mV, Figure 7D). As expected, rescue of *wild-type* or *d178n mps-1* in the null background gave rise to currents that fully recapitulated the parental currents (Figure 7A–D). No substantial overexpression of MPS-1 occurred in the transgenic worms, consistent with the observation that the body-touch phenotype of *Pmps-1::wild type-mps-1* and *Pmps-1::d178n-mps-1* worms is normal. We also occasionally recorded non-inactivating, slowly activating currents (data not shown) in neurons of all genotypes. These currents had been earlier reported by others (Suzuki *et al*, 2003), and are probably the result of some degenerative process in the cell.

KHT-1 and MPS-1 contribute to the K⁺ current in the touch neurons

Currents expressed in the touch neurons of the *kht-1* null genotype are shown in Figure 7E. These currents inactivated,

with peak values roughly 20% smaller than those of the N2 current (Figure 7G). However, although a substantial steady-state component remained in native N2 currents after inactivation, in the *kht-1* null genotype, this steady-state component was markedly diminished (Figure 7G), suggesting that it was conducted by KHT-1. Therefore, we algebraically isolated the native KHT-1 current, I_{KHT-1} , by subtracting *kht-1* null currents from N2 currents (a representative example is shown in Figure 7F). Native I_{KHT-1} seemed qualitatively similar to cloned I_{KHT-1} , an impression that was corroborated by quantitative analysis. Thus, normalized I – V relationships of native and cloned I_{KHT-1} overlapped (Figure 7F). The $V_{1/2}$ was 64.4 ± 7.7 mV, $n=7$ in the native channel (data not shown), a value very close to the $V_{1/2} = 67.2 \pm 5.1$ mV shown by the cloned channel. In addition the slope factors of native and cloned channels, $V_s = 19.7 \pm 1.4$ mV, $n=7$ and $V_s = 16.5 \pm 0.7$ mV, respectively, were comparable, indicating that the native and the cloned channel show similar voltage dependence. Thus, we conclude that KHT-1 conducts a steady-state K⁺ current in the touch neurons. The electrophysiological analysis further strengthened the notion that KHT-1 is expressed in both ALM and PLM neurons because we did not record N2-like currents in cells of this genotype. If KHT-1 was expressed only in the PLM neurons, roughly 50% of the currents should have shown N2 characteristics. Next, we analysed the effect of ablating *mps-1* on the electrophysiological properties of the touch-neuron current. Representative *mps-1* null currents are shown in Figure 7E.

Figure 7 The KHT-1-MPS-1 complex contributes to the K⁺ current in the touch neurons. (A) Representative whole-cell currents elicited by single voltage jumps from -80 to $+120$ mV (inset) in N2, $P_{mps-1}::wild\ type-mps-1$ (wild-type) and $P_{mps-1}::d178n-mps-1$ (D178N) touch neurons. Currents were recorded 4 days after seeding. (B) Peak and steady-state σ - V relationships in touch neurons of N2 (hollow squares), $P_{mps-1}::wild\ type-mps-1$ (filled squares) and $P_{mps-1}::d178n-mps-1$ (triangles). $n = 22$, 15 and 17 cells, respectively. (C) G/G_{Max} relationships for N2, $P_{mps-1}::wild\ type-mps-1$ and $P_{mps-1}::d178n-mps-1$. Data are from $n = 22$, 15 and 17 cells, respectively. The theoretical line was calculated by fitting the N2 curve to the Boltzmann function (equation (4)) with 65.5 ± 5.0 mV and $V_s = 19.3 \pm 1.8$ mV. (D) Inactivation time constants for N2, $P_{mps-1}::wild\ type-mps-1$ and $P_{mps-1}::d178n-mps-1$ currents. Time constants were calculated by fitting macroscopic currents to a single exponential function (equation (1)). $n = 22$, 15 and 17 cells respectively. Linear fit of the data gave a slope of -0.064 ± 0.002 ms/mV. (E) Representative whole-cell currents elicited by single voltage jumps from -80 to $+120$ mV in $kht-1$ -null and $mps-1$ -null touch neurons. Currents were recorded 4 days after seeding. (F) Representative KHT-1 current obtained by algebraically subtracting $kht-1$ -null currents from N2 currents. Left, normalized current-voltage (I - V) relationships of native (hollow triangles) and heterologously expressed (filled triangles) KHT-1 currents. $n = 7$ and $n = 27$ cells, respectively. (G) Macroscopic peak and steady-state (hollow) current densities, σ , at $+120$ mV in N2, $kht-1$ -null and $mps-1$ -null touch neurons. $n = 22$, 18 and 25 cells respectively. (H) Window currents in N2, $kht-1$ -null and $mps-1$ -null touch neurons. Fractional currents, $I_{steady\ state}/I_{peak}$ were normalized and fitted to a modified Boltzmann function (equation (5)) with $V_{1/2} = 12.5 \pm 1.0$ mV and $V_s = 23.2 \pm 1.8$ mV for N2, $V_{1/2} = 6.1 \pm 0.5$ mV and $V_s = 19.6 \pm 2.0$ mV for $kht-1$ -null and -2.6 ± 0.4 mV and $V_s = 17.6 \pm 1.1$ mV for $mps-1$ -null. $n = 22$, 18 and 25 cells, respectively. Activation curves were calculated by fitting normalized conductances (G/G_{Max}) to the Boltzmann function (equation (4)) with respectively, $V_{1/2} = 65.5 \pm 5.0$ mV and $V_s = 19.3 \pm 1.8$ mV for N2, $V_{1/2} = 60.8 \pm 5.7$ mV and $V_s = 17.7 \pm 1.2$ mV for $kht-1$ -null and $V_{1/2} = 64.1 \pm 3.2$ mV and $V_s = 17.9 \pm 1.1$ mV for $mps-1$ -null. $n = 22$, 18 and 25 cells, respectively. The total areas of the window currents are, respectively, 2.3, 0.7 and 0.9 mV. Touch neurons (ALM and PLM) were marked by the $P_{mec-4}::gfp$ reported, which specifically expresses in these neurons. Data are presented as mean $\pm$ s.e.m. Statistically significant differences from control are indicated with $*P < 0.0$ and $**P < 0.01$.

Like in the *kht-1* null genotype, genetic ablation of *mps-1* suppressed the steady-state component of the current and moderately reduced the amplitude of the peak current (Figure 7G). Taken together, these data indicate that genetic ablation of *kht-1* or *mps-1* modifies the touch-neuron current in a similar manner. This suggests that MPS-1 is an essential component of the complex, which therefore cannot operate properly without MPS-1. This explains why these genotypes show similar mechanosensory defects.

The lack of KHT-1-MPS-1 decreases K⁺ flux

A decrease in the steady-state current is expected to reduce the net flux of K⁺ ions during depolarization and therefore to affect the neuronal output of the touch neuron. To quantify this effect we calculated the overlap in the relationships for channel activation and inactivation, referred to as the window current. The inactivation curve was given by the fractional current, ($I_{steady\ state}/I_{peak}$). The activation curve was given by the normalized peak conductance, G/G_{Max} . The window currents for N2, *kht-1*-null and *mps-1*-null genotypes are shown in Figure 7H; the area under the intersection of the activation/inactivation curves gives an estimate of the flux of potassium across the membrane during depolarization. The

area was significantly reduced in both the *kht-1*-null and *mps-1*-null genotypes compared with the N2 genotype (0.7 and 0.9 mV versus 2.3 mV). Together, these data suggest that genetic ablation of *kht-1* or *mps-1* results in decreased K⁺ flux and therefore, acts to hinder touch-neuron repolarization. This mechanism provides a likely explanation to as why *kht-1*-null and *mps-1*-null worms are touch defective.

Discussion

In this study we find that the bifunctional accessory subunit MPS-1 assembles with the pore-forming subunit KHT-1 in the touch neurons of *C. elegans* to form a complex that plays a crucial role in the functioning of these cells. KHT-1 and MPS-1 co-immunoprecipitated *in vivo* and *in vitro* and co-localized *in vivo*. Behavioural and electrophysiological analyses showed that genetic ablation of these genes caused similar mechanosensory defects and affected the current in the touch neurons in a similar manner. This suggests that the KHT-1-MPS-1 complex is functional *in vivo* and further, that it is assembled in the early stages of the secretory pathway, so that the lack of any subunit is sufficient to destabilize the entire complex. This seems to be a general property of the

MPS and the KCNE proteins (Bianchi *et al*, 2003; McCrossan *et al*, 2003; Chandrasekhar *et al*, 2006; Wang and Sesti, 2007).

The observation that transgenic worms bearing a inactivated kinase variant (D178N) retain normal touch sensitivity but habituate to taps only partially and slowly underscores the specific role of the kinase activity of MPS-1 in controlling this form of non-associative learning. Consistent with the fact that *P_{mmps-1::d178n-mmps-1}* worms, as well as *P_{mmps-1::wild type-mmps-1}* worms, show normal sensitivity to touch, the K⁺ currents expressed in the touch neurons of these genotypes recapitulated parental currents. Unfortunately, the small neurons and hydrostatic skeleton of *C. elegans* make physiological studies technically demanding, especially in live animals. For this reason we could not directly measure the KHT-1–MPS-1 current in habituated touch neurons. However, the evidence presented here provides a model that predicts that MPS-1 is inactive in the basal state and that repetitive stimulation initiates a cascade of events that eventually leads to auto-phosphorylation of KHT-1–MPS-1. This might imply that other, currently unidentified proteins might act to regulate the timing by which the kinase activity of MPS-1 is switched on, to phosphorylate KHT-1.

Inhibition of the KHT-1–MPS-1 current through auto-phosphorylation induced by repetitive mechanical stimulation or through genetic ablation of *kht-1* or *mmps-1* seems to be a central step to induce touch insensitivity and, therefore, habituation. Suzuki and collaborators showed that repetitive mechanical stimulation caused a progressive decrease in the electrical response of the touch neurons by a reduced Ca²⁺ influx conducted by the EGL-19 channel (Suzuki *et al*, 2003). In human heart, longer action-potential duration and slower [Ca²⁺]_i decline slow the recovery from inactivation of the L-type Cav1.2 calcium channel, thereby decreasing calcium current availability (Altamirano and Bers, 2007). It is plausible that the KHT-1–MPS-1 complex might similarly induce cumulative inactivation in the EGL-19 calcium channel—which, incidentally is the homologue of the human cardiac channel (90% homology to Cav1.2)—by prolonging the duration of the mechanoreceptor potential. Thus, auto-phosphorylation of the KHT-1–MPS-1 complex would diminish K⁺ flux, delay touch-neuron repolarization, induce cumulative inactivation in the EGL-19 calcium channel and as a consequence, dampen touch-neuron excitability.

Modification of K⁺ channels by protein kinases is a mechanism contributing to learning and memory formation in vertebrate and invertebrate organisms (Nestler and Greengard, 1983; Cohen, 1989; Krebs, 1994; Levitan, 1999; Biron *et al*, 2006; Tomioka *et al*, 2006). For example, in mammals, phosphorylation of Kv4.2 by protein kinase A, protein kinase C and of mitogen-activated protein kinase can modulate action-potential back propagation—a mechanism that acts to enhance the long-lasting synaptic plasticity (Magee and Johnston, 1997; Markram *et al*, 1997; Hoffman and Johnston, 1998; Johnston *et al*, 1999)—by decreasing channel's activity. In invertebrates, conditioning of the phototactic response in *Hermissenda crassicornis* has been linked to phosphorylation-mediated changes in K⁺ channel activity by protein kinase C and mitogen-activated protein kinase (Crow and Alkon, 1978; Farley and Alkon, 1980; Alkon *et al*, 1983; Farley and Auerbach, 1986; Neary and Alkon, 1986; Farley and Schuman, 1991; Etcheberrigaray *et al*, 1992; Crow and Forrester, 1993; Crow *et al*, 1998). There is evidence that

in *Drosophila* neural and behavioural plasticity might be mediated by modulation of EAG by calcium/calmodulin-dependent protein kinase II kinase (Griffith *et al*, 1994; Yao and Wu, 2001). These physiological processes share a common molecular mechanism: enhanced neuronal excitability through kinase-mediated decrease of a K⁺ current. Hence, significant similarity might exist between the role of K⁺ channels in determining non-associative learning in *C. elegans* and in other organisms, including mammals.

In conclusion, these data underscore a marked level of conservation between mammals and invertebrates in the mechanisms causing habituation. Considering that both KHT-1 and MPS-1 have human homologues that interact *in vivo* (Kv3.1–KCNE3) and *in vitro* (Kv3.1–KCNE2/3 McCrossan *et al*, 2003; Lewis *et al*, 2004), we speculate that the KHT-1–MPS-1 complex might represent a precursor of mammalian systems in which the K⁺ channel and the protein kinase have evolved to become separate entities.

Materials and methods

Strains

Strain used were Bristol (N2), *mmps-1(ok1376)* (*mmps-1* null; outcrossed four times), *shw-3(ok1884)* (*kht-1* null, outcrossed 4 times) and *mec-4(u231)* (*mec-4(d)*). We constructed *mmps-1(ok1376)(wild type)(myo-2::gfp)*, termed *P_{mmps-1::wild type-mmps-1}*, *P_{mmps-1::mmps-1(ok1376)(d178n)(myo-2::gfp)}*, termed *P_{mmps-1::d178n-mmps-1}*, *mmps-1(ok1376)(wild type::gfp)*, termed *P_{mmps-1::wild type-mmps1::gfp}*, *mmps-1(ok1376)(d178n::gfp)*, termed *P_{mmps-1::d178n-mmps-1::gfp}*, *mmps-1(ok1376)(mec-4::gfp)* and *shw-3(ok1884)(mec-4::gfp)*.

Molecular biology

The wild-type *mmps-1* gene plus its promoter (*P_{mmps-1::wild type-mmps-1}*, 3836bp) were amplified from genomic DNA by PCR using the following primers: 5'-CCCAAGCTTGAATGTGGCTGCTCAATCGAAGG TACCC-3' and 5'-CGCGGATCCGTCACGCTAAAGCTAAATGATTATCG TCA-3' and were inserted in the Fire vector pPD95.75 using *Hind* III and *Bam*HI restriction sites. The *P_{mmps-1::d178n-mmps-1}* construct was constructed by PCR using the wild-type construct as template. To obtain the *P_{mmps-1::wild type-mmps-1::gfp}* and *P_{mmps-1::d178n-mmps-1::gfp}* constructs, the STOP codon in the wild-type and d178n constructs was mutated to glutamine by PCR, in-frame with the start codon of *gfp*.

Cloning of KHT-1 was carried out with a Smart Race kit (Clontech) using poly(A)⁺ mRNA extracted from total *C. elegans* RNA with the Oligotex kit (Qiagen). The primer for KHT-1 5'-RACE was TCTGTACTGCGTGCCCACTTCTAAACATTCT and that for 3'-RACE was AGTCATCCTCCCAACCAAATCGTTTGTACAGT. cDNA was amplified by PCR and inserted in pCl-neo vector (Promega) for functional expression in CHO cells. The full sequence of KHT-1 was communicated to GenBank, which issued the accession number DQ185514. KHT-1-HA was epitope tagged by replacing the terminal stop codon with nucleotides encoding hemagglutinin (HA) residues (YPYDVP-DYA-STOP). MPS-1 was tagged by inserting the c-Myc sequence (ISMEQKLISEEDLN) (Wang and Sesti, 2007). The constructs were subcloned into pCl-neo vector (Promega) for expression in CHO cells. All sequences were confirmed by automated DNA sequencing. Transcripts were quantified with spectroscopy and compared with control samples separated by agarose gel electrophoresis stained with ethidium bromide.

Construction of transgenic animals

For transgenic expression of *P_{mmps-1::wild type-mmps-1}* and *P_{mmps-1::d178n-mmps-1}*, the constructs were injected into the syncytial gonads of adult *mmps-1(ok1376)* hermaphrodites. Transformant lines each for *P_{mmps-1::wild type-mmps-1}* and *P_{mmps-1::d178n-mmps-1}* were stabilized by a mutagenesis-induced integration into a chromosome, by irradiating 40 animals with 4000 rads of γ-ray for 40 min. The progeny were checked for 100% transmission of the marker (*myo-2::gfp*) and also for the presence of the transgene by PCR amplification. Two lines for *mmps-1(ok1376)(wild type)(myo-2::gfp)*

(#8 and #9) and two lines for *mps-1(ok1376)(d178n)(myo-2::gfp)* (#4 and #8) were outcrossed four times. The data presented in this study were obtained from lines #8 and #4, respectively (all four lines gave similar results in gentle body touch assays). For electrophysiology lines #8 and #4, *mps-1(ok1376)* and *shw-3(ok1884)* were outcrossed with *Pmec-4::gfp* transgenic worms that express GFP in the touch neurons under the *mec-4* promoter, and the progenies were selected for homozygous transmission of the transgenes. For construction of *P_{mps-1}::wild-type-mps-1::gfp* and *P_{mps-1}::d178n-mps-1::gfp*, we proceeded as described above, except that lines were not integrated.

Immunoprecipitations and co-immunoprecipitations

CHO cells. For immunoprecipitations, CHO cells were plated/transfected with cDNA as described before (Bianchi *et al*, 2003) and used 24–36 h post transfection. CHO cells were washed with 10 ml ice-cold PBS and lysed with ~2 ml ice-cold RIPA buffer (50 mM TRIS pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% IGEAL CA-630, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, freshly-added 10 mM iodoacetamide, phosphatase and protease inhibitors) for 30 min at 4 °C. Cell lysates were centrifuged for 60 min at 4 °C and the supernatant was mixed with HA-conjugated beads (Roche) and rocked at 4 °C for 3 h. Beads were washed thrice with ice-cold TBST and incubated in SDS sample buffer at ~90–95 °C for 15 min.

For co-immunoprecipitation, cells were lysed with 1% NP-40 buffer, 50 mM TRIS pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% IGEAL CA-630, phosphatase and protease inhibitors (Calbiochem). Cell lysates were centrifuged for 60 min at 4 °C and the supernatant mixed with c-Myc-conjugated beads and rocked at 4 °C for 3 h. Beads were washed thrice with ice-cold 1% NP-40 buffer and then incubated in SDS sample buffer at ~90–95 °C for 15 min.

Native. *P_{mps-1}::wild type-mps-1::gfp* and *P_{mps-1}::d178n-mps-1::gfp* worms were harvested and collected in 30% sucrose at 1000 rpm for 5 min. The pelleted worms were washed 2 times in cold water in a 15 ml conical Falcon tube. Worms were lysed in 3 ml lysis buffer (50 mM Tris, 150 mM NaCl, protease inhibitor, 0.1 mM PMSF, pH 7.6) by sonication (2-s pulses with an interval of 30 s chill between two pulses). The lysate was centrifuged at 18 000 rpm for 30 min. The supernatant was removed and the pellet was resuspended in 1% NP-40 buffer (50 mM Tris, 150 mM NaCl, protease inhibitor, 0.1 mM PMSF pH 7.6), rocked at 4 °C for 1 h and then centrifuged at 18 000 rpm for 30 min at 4 °C. The supernatant was incubated with 50 µl protein A beads for 2 h. The beads were then centrifuged at 18 000 rpm at 4 °C and the supernatant was transferred to a fresh tube. In all, 4 µl of anti-KHT-1 antibody (from rabbit) was added to the supernatant and incubated for 3 h. Then 50 µl protein A beads were added, and rocked at 4 °C for 2 h. The beads were collected by centrifugation at 18 000 rpm at 4 °C, washed thrice with NP-40 buffer and then incubated in SDS sample buffer at 90–95 °C for 15 min. Western visualization was carried out with anti-GFP antibody from mouse (Invitrogen).

Behavioural assays

Age synchronization. Nematodes were grown in standard 10 cm NGM plates + OP50 *Escherichia coli* until a large population of gravid adults was reached (3–5 days). The animals were collected in 50 ml Falcon tubes washed in M9 buffer (22 mM KH₂PO₄, 22 mM NaH₂PO₄, 85 mM NaCl, 1 mM MgSO₄) and treated with 10 volumes of basic hypochlorite solution (0.25 M NaOH, 1% hypochlorite freshly mixed). Worms were incubated at room temperature for 10 min, the eggs (and carcasses) collected by centrifugation at 400 g for 5 min at 4 °C, incubated overnight in M9 buffer and seeded on standard NMG plates.

Behavioural tests were carried out 'blind' that is the experimenter did not know the worms' genotype:

Gentle body touch. Experiments were carried out as described earlier (Bianchi *et al*, 2003). Briefly, single worms were picked to individual plates and tested six times for response to light touch to the head and tail with an eyelash. Responses to head/tail touch were recorded as backward/forward movement. The overall response of each group of worms to touch was expressed as the average percentage of times the worms responded.

Habituation to tap. The experiments were carried out by two investigators: one analysed the worm's behaviour under a stereo-microscope (provided with a grid that allowed to distinguish 1/8 worm's length) and delivered the taps, and the other set the ISI and recorded the scores. Individual worms were transferred to the test plate (bacteria free) and allowed to rest for 15–20 min to recover from habituation caused by the transfer. Taps (impulse ~10 mN s) were delivered manually by raising the Petri dish (2.5 cm) and dropping it. The elevation of the dish was controlled by an L-shaped glass rod, placed vertically at the base of the microscope. The habituation coefficient, *HC*, was defined as the backward distance travelled by the worm in response to a tap. Large responses (distance ≥ 1/2 of the animal's length) scored *HC* = 1. Medium responses (1/4 < distance < 1/2 and/or stops) scored *HC* = 0.5. Distances < 1/4 of the animal's length scored *HC* = 0. N2 and *P_{mps-1}::d178n-mps-1* worms were also filmed (representative movies are shown in Supplementary data) and analysed using ImageJ software. Taps were delivered by hitting the Petri dish on the side at ISI = 5 s. τ_H values were $\tau_H = 3.1 \pm 0.7$ ($n = 11$) and $\tau_H = 23.1 \pm 6.1$ ($n = 13$, $P < 0.021$) for N2 and D178N, respectively (data not shown). These values are in good agreement with τ_H values obtained manually.

Recovery from habituation. To evaluate recovery from habituation, the worms were habituated by a train of 30 taps (block 1) and then 2, 5, 10, and 30 min after habituation training the same treatment was repeated (block 2). Training was carried at 5 s and 60 s ISI. Recovery was expressed as the percent increase of the average initial *HC* (calculated by averaging the *HC* in response to the first and second tap after training).

A standard experiment consisted of three independent tests with groups of 10 or more animals in each group. (This number was generally sufficient to achieve statistical significance because the homogeneous genetic background of the nematodes limits individual behavioural differences.) Individual responses were averaged and s.e.m. calculated.

Electrophysiology

Heterologous expression system. CHO cells were plated/transfected as described before (Bianchi *et al*, 2003) and used for 24–36 h post transfection. The bath solution consisted of 4 mM KCl, 100 mM NaCl, 10 mM Hepes (pH = 7.5 with NaOH), 1.8 mM CaCl₂ and 1.0 mM MgCl₂. Pipette solution consisted of 100 mM KCl, 10 mM Hepes (pH = 7.5 with KOH), 1.0 mM MgCl₂, 1.0 mM CaCl₂, and 10 mM EGTA (pH = 7.5 with KOH).

Primary cultures. Cultured cells were prepared as described before (Park *et al*, 2005). Briefly, gravid adult worms were lysed using 0.5 M NaOH and 1% NaOCl. Released eggs were washed thrice with sterile egg buffer containing 118 mM NaCl, 48 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂ and 25 mM Hepes (pH 7.3, 340 mosM), and adult carcasses were separated from washed eggs by centrifugation in sterile 30% sucrose. Eggshells were removed by resuspending pelleted eggs in a sterile egg buffer containing 1 unit/ml chitinase at room temperature for 1.5 h. Embryos were resuspended in L-15 cell culture medium containing 10% foetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin (Sigma) and dissociated by gentle pipetting. Intact embryos, clumps of cells and larvae were removed from the cell suspension by filtration. Dissociated cells were plated on glass cover slips pre-coated with peanut lectin (0.1 mg/ml) dissolved in water. For electrophysiological recordings, bath solution consisted of 145 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 5 mM MgCl₂, 10 mM Hepes/NaOH (pH 7.50) and 20 mM D-glucose. The pipette solution contained 125 mM potassium gluconate, 18 mM KCl, 0.7 mM CaCl₂, 2 mM MgCl₂, 2 mM MgATP, 10 mM EGTA/KOH and 10 mM Hepes/KOH (pH 7.5). Currents were repeatedly elicited 3–5 times (with the same voltage stimulus) and digitally averaged online. Leak currents were recorded in the cell-attached configuration before establishing the whole-cell configuration and were digitally subtracted during analysis. Data were recorded with an amplifier Axopatch 200B (Axon) a PC (Dell) and Clampex software (Axon). Data were filtered at $f_c = 1$ kHz and sampled at 2.5 kHz.

Immunolabelling of cultured touch neurons

Cells were washed thrice with PBS and fixed with paraformaldehyde (4% in PBS) for 15 min at room temperature. After fixing, cells

were washed thrice for 5 min with PBS. Cells were incubated with 0.1% Triton X-100 in PBS for 5 min, then washed thrice with PBS and blocked for 1 h at room temperature with 5% non-fat dry milk in PBS plus 0.1% Tween 20. Cells were incubated in fresh M9 media with anti-KHT-1 antibody (1:100 dilution) at room temperature for 1.5 h. Cells were then incubated with a Cy3-conjugated anti-rabbit secondary antibody (1:2000, in 5% non-fat dry milk in PBS plus 0.1% Tween 20), for 1.5 h at room temperature, and subsequently washed thrice for 5 min with PBS + 0.1% Tween 20. The samples were analysed and photographed by our departmental Zeiss LSM 510 META confocal microscope.

Data analysis

Mathematical functions. The Nernst equation was given by

$$V_{\text{rev}} = \frac{kT}{ze} \ln \left(\frac{[K^+]_o}{[K^+]_i} \right) \quad (2)$$

where k is the Boltzmann constant, T the temperature in Kelvin, e is electronic charge and $[K^+]_o$ and $[K^+]_i$ are, respectively, the concentration of K⁺ in the bath solution and in the pipette solution.

Macroscopic conductances were calculated as

$$G_{\text{rev}} = \frac{I}{V - V_{\text{rev}}} \quad (3)$$

where V and I are, respectively, the test voltage and the corresponding current. V_{rev} is the theoretical reversal potential calculated by the Nernst function (equation (2)).

The Boltzmann function was given by

$$\frac{1}{1 + \exp((V_{1/2} - V)/V_s)} \quad (4)$$

References

- Alkon DL, Acosta-Urquidí J, Olds J, Kuzma G, Neary JT (1983) Protein kinase injection reduces voltage-dependent potassium currents. *Science* **219**: 303–306
- Altamirano J, Bers DM (2007) Effect of intracellular Ca²⁺ and action potential duration on L-type Ca²⁺ channel inactivation and recovery from inactivation in rabbit cardiac myocytes. *Am J Physiol Heart Circ Physiol* **293**: H563–H573
- Barhanin J, Lesage F, Guillemare E, Fink M, Lazdunski M, Romey G (1996) K(V)LQT1 and Isk (minK) proteins associate to form the I(Ks) cardiac potassium current. *Nature* **384**: 78–80
- Bianchi L, Kwok SM, Driscoll M, Sesti F (2003) A potassium channel-MiRP complex controls neurosensory function in *Caenorhabditis elegans*. *J Biol Chem* **278**: 12415–12424
- Biron D, Shibuya M, Gabel C, Wasserman SM, Clark DA, Brown A, Sengupta P, Samuel AD (2006) A diacylglycerol kinase modulates long-term thermotactic behavioral plasticity in *C. elegans*. *Nat Neurosci* **9**: 1499–1505
- Cai SQ, Hernandez L, Wang Y, Park KH, Sesti F (2005) MPS-1 is a K(+) channel beta-subunit and a serine/threonine kinase. *Nat Neurosci* **8**: 1503–1509
- Chandrasekhar KD, Bas T, Kobertz WR (2006) KCNE1 subunits require co-assembly with K(+) channels for efficient trafficking and cell surface expression. *J Biol Chem* **281**: 40015–40023
- Christensen M, Estevez A, Yin X, Fox R, Morrison R, McDonnell M, Gleason C, Miller III DM, Strange K (2002) A primary culture system for functional analysis of *C. elegans* neurons and muscle cells. *Neuron* **33**: 503–514
- Cohen P (1989) The structure and regulation of protein phosphatases. *Annu Rev Biochem* **58**: 453–508
- Crow T, Forrester J (1993) Down-regulation of protein kinase C and kinase inhibitors dissociate short- and long-term enhancement produced by one-trial conditioning of *Hermisenda*. *J Neurophysiol* **69**: 636–641
- Crow T, Xue-Bian JJ, Siddiqi V, Kang Y, Neary JT (1998) Phosphorylation of mitogen-activated protein kinase by one-trial and multi-trial classical conditioning. *J Neurosci* **18**: 3480–3487
- Crow TJ, Alkon DL (1978) Retention of an associative behavioral change in *Hermisenda*. *Science* **201**: 1239–1241
- Driscoll M, Kaplan J (1997) Mechanotransduction. In *C. elegans II*, Riddle DL, Blumenthal T, Meyer BJ, Priess JR (eds) Cold Spring Harbor: Cold Spring Harbor Laboratory Press
- Etcheberrigaray R, Matzel LD, Lederhendler II, Alkon DL (1992) Classical conditioning and protein kinase C activation regulate the same single potassium channel in *Hermisenda crassicornis* photoreceptors. *Proc Natl Acad Sci USA* **89**: 7184–7188
- Farley J, Alkon DL (1980) Neural organization predicts stimulus specificity for a retained associative behavioral change. *Science* **210**: 1373–1375
- Farley J, Auerbach S (1986) Protein kinase C activation induces conductance changes in *Hermisenda* photoreceptors like those seen in associative learning. *Nature* **319**: 220–223
- Farley J, Schuman E (1991) Protein kinase C inhibitors prevent induction and continued expression of cell memory in *Hermisenda* type B photoreceptors. *Proc Natl Acad Sci USA* **88**: 2016–2020
- Griffith LC, Wang J, Zhong Y, Wu CF, Greenspan RJ (1994) Calcium/calmodulin-dependent protein kinase II and potassium channel subunit eag similarly affect plasticity in *Drosophila*. *Proc Natl Acad Sci USA* **91**: 10044–10048
- Hoffman DA, Johnston D (1998) Downregulation of transient K⁺ channels in dendrites of hippocampal CA1 pyramidal neurons by activation of PKA and PKC. *J Neurosci* **18**: 3521–3528
- Johnston D, Hoffman DA, Colbert CM, Magee JC (1999) Regulation of back-propagating action potentials in hippocampal neurons. *Curr Opin Neurobiol* **9**: 288–292
- Krebs EG (1994) The growth of research on protein phosphorylation. *Trends Biochem Sci* **19**: 439
- Levin IB (1999) Modulation of ion channels by protein phosphorylation. How the brain works. *Adv Second Messenger Phosphoprotein Res* **33**: 3–22
- Lewis A, McCrossan ZA, Abbott GW (2004) MinK, MiRP1, and MiRP2 diversify Kv3.1 and Kv3.2 potassium channel gating. *J Biol Chem* **279**: 7884–7892
- Magee JC, Johnston D (1997) A synaptically controlled, associative signal for Hebbian plasticity in hippocampal neurons. *Science* **275**: 209–213

where V is the test voltage and $V_{1/2}$ and V_s are constants. Fractional currents $I_{\text{steady}}/I_{\text{peak}}$ were fitted to a modified Boltzmann function:

$$A + \frac{B}{1 + \exp((V_{1/2} - V)/V_s)} \quad (5)$$

where A and B are two constants.

Statistical analysis

Quantitative data are presented as mean ± s.e.m. The level of significance was calculated using Student's *t*-test for single comparisons and ANOVA for multiple comparisons, and corrections carried out using a Tukey means comparison test with 95% confidence interval level using OriginPro 7.5 software. Statistical significance was accepted when the level of significance, P , was lesser than 5% ($P < 0.05$).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Drs Andrew Jauregui and Maureen Barr for their help with filming the worms. The *mps-1(ok1376)* and *shw-3(ok1884)* strains were a gift from the *C. elegans* Gene Knockout Consortium. The *Pmec-4::gfp* strain was a kind gift from Dr Monica Driscoll. We thank Dr John Lenard for critical reading of the manuscript. This work was supported by a NIH grant (R01GM68581) to FS. S-QC and FS designed the research. S-QC and YW carried out research and analysed data. KHP carried out habituation tests and electrophysiology in CHO cells. ZP helped with co-localization. XT constructed the wild-type and d178n constructs. FS wrote the paper.

- Markram H, Lubke J, Frotscher M, Sakmann B (1997) Regulation of synaptic efficacy by coincidence of postsynaptic APs and EPSPs. *Science* **275**: 213–215
- McCrossan ZA, Abbott GW (2004) The MinK-related peptides. *Neuropharmacology* **47**: 787–821
- McCrossan ZA, Lewis A, Panaghie G, Jordan PN, Christini DJ, Lerner DJ, Abbott GW (2003) MinK-related peptide 2 modulates Kv2.1 and Kv3.1 potassium channels in mammalian brain. *J Neurosci* **23**: 8077–8091
- Neary JT, Alkon DL (1986) Protein phosphorylation and associative learning in *Hermissenda*. *Acta Biochim Biophys Hung* **21**: 159–176
- Nestler EJ, Greengard P (1983) Protein phosphorylation in the brain. *Nature* **305**: 583–588
- Park KH, Hernandez L, Cai SQ, Wang Y, Sesti F (2005) A Family of K⁺ Channel Ancillary Subunits Regulate Taste Sensitivity in *Caenorhabditis elegans*. *J Biol Chem* **280**: 21893–21899
- Park KH, Sesti F (2007) An arrhythmia susceptibility gene in *Caenorhabditis elegans*. *J Biol Chem* **282**: 19799–19807
- Perney TM, Marshall J, Martin KA, Hockfield S, Kaczmarek LK (1992) Expression of the mRNAs for the Kv3.1 potassium channel gene in the adult and developing rat brain. *J Neurophysiol* **68**: 756–766
- Rankin CH, Beck CD, Chiba CM (1990) *Caenorhabditis elegans*: a new model system for the study of learning and memory. *Behav Brain Res* **37**: 89–92
- Rankin CH, Broster BS (1992) Factors affecting habituation and recovery from habituation in the nematode *Caenorhabditis elegans*. *Behav Neurosci* **106**: 239–249
- Roepke TK, Anantharam A, Kirchhoff P, Busque SM, Young JB, Geibel JP, Lerner DJ, Abbott GW (2006) The KCNE2 potassium channel ancillary subunit is essential for gastric acid secretion. *J Biol Chem* **281**: 23740–23747
- Sanguinetti M, Curran M, Zou A, Shen J, Spector P, Atkinson D, Keating M (1996) Coassembly of KvLQT1 and MinK (IsK) proteins to form cardiac IKs potassium channel. *Nature* **384**: 80–83
- Schulze-Bahr E, Wang Q, Wedekind H, Haverkamp W, Chen Q, Sun Y, Rubie C, Hordt M, Towbin JA, Borggreffe M, Assmann G, Qu X, Somberg JC, Breithardt G, Oberti C, Funke H (1997) KCNE1 mutations cause jervell and Lange-Nielsen syndrome. *Nat Genet* **17**: 267–268
- Suzuki H, Kerr R, Bianchi L, Frokjaer-Jensen C, Slone D, Xue J, Gerstbrein B, Driscoll M, Schafer WR (2003) *In vivo* imaging of *C. elegans* mechanosensory neurons demonstrates a specific role for the MEC-4 channel in the process of gentle touch sensation. *Neuron* **39**: 1005–1017
- Tomioka M, Adachi T, Suzuki H, Kunitomo H, Schafer WR, Iino Y (2006) The insulin/PI 3-kinase pathway regulates salt chemotaxis learning in *Caenorhabditis elegans*. *Neuron* **51**: 613–625
- Wang Y, Sesti F (2007) Molecular Mechanisms Underlying KVS-1-MPS-1 Complex Assembly. *Biophys J* **93**: 3083–3091
- Wei AD, Butler A, Salkoff L (2005) KCNQ-like potassium channels in *Caenorhabditis elegans*. Conserved properties and modulation. *J Biol Chem* **280**: 21337–21345
- Weng J, Cao Y, Moss N, Zhou M (2006) Modulation of voltage-dependent Shaker family potassium channels by an aldo-keto reductase. *J Biol Chem* **281**: 15194–15200
- Yao W, Wu C (2001) Distinct roles of CaMKII and PKA in regulation of firing patterns and K(+) currents in *Drosophila* neurons. *J Neurophysiol* **85**: 1384–1394
- Zeng H, Fei H, Levitan IB (2004) The slowpoke channel binding protein Slob from *Drosophila melanogaster* exhibits regulatable protein kinase activity. *Neurosci Lett* **365**: 33–38
- Zhang Y, Ma C, Delohery T, Nasipak B, Foat BC, Bounoutas A, Bussemaker HJ, Kim SK, Chalfie M (2002) Identification of genes expressed in *C. elegans* touch receptor neurons. *Nature* **418**: 331–335