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ASIC3, a sensor of acidic and primary inflammatory pain

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Acid-sensing ion channels (ASICs) are cationic channels activated by extracellular acidosis that are expressed in both central and peripheral nervous systems. Although peripheral ASICs seem to be natural sensors of acidic pain (e.g., in inflammation, ischaemia, lesions or tumours), a direct demonstration is still lacking. We show that ~60% of rat cutaneous sensory neurons express ASIC3-like currents. Native as well as recombinant ASIC3 respond synergistically to three different inflammatory signals that are slight acidifications (~pH 7.0), hypertonicity and arachidonic acid (AA). Moderate pH, alone or in combination with hypertonicity and AA, increases nociceptors excitability and produces pain suppressed by the toxin APETx2, a specific blocker of ASIC3. Both APETx2 and the *in vivo* knockdown of ASIC3 with a specific siRNA also have potent analgesic effects against primary inflammation-induced hyperalgesia in rat. Peripheral ASIC3 channels are thus essential sensors of acidic pain and integrators of molecular signals produced during inflammation where they contribute to primary hyperalgesia.

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Introduction

Acid-sensing ion channels (ASICs) are cationic channels activated by extracellular protons (Waldmann *et al*, 1997a, b; Wemmie *et al*, 2006; Lingueglia, 2007). Four genes encoding seven subunits (ASIC1a, ASIC1b, ASIC1b2, ASIC2a, ASIC2b, ASIC3 and ASIC4) have been identified so far in mammals. Functional channels result from the association of the different ASIC subunits into trimers (Jasti *et al*, 2007) leading to homomeric or heteromeric channels (Lingueglia *et al*, 1997; Alvarez de la Rosa *et al*, 2002; Benson *et al*, 2002; Hesselager *et al*, 2004). ASICs are predominantly neuronal channels, expressed in central (CNS) and peripheral (PNS) nervous systems. Whereas ASIC1a and ASIC2 are widely present in both CNS and PNS, the expression of ASIC1b and ASIC3 is restricted to peripheral sensory neurons (Waldmann *et al*, 1997a; Chen *et al*, 1998; Bassler *et al*, 2001).

As extracellular acidosis correlates with pain sensations (Steen *et al*, 1995a; Issberner *et al*, 1996; Reeh and Steen, 1996), ASICs have been proposed to sense extracellular acidifications occurring in pathological conditions such as inflammation, ischaemia, haematomas, fractures and lesions as well as in postoperative states (Krishtal and Pidoplichko, 1981; Waldmann *et al*, 1997a, b; Waldmann and Lazdunski, 1998; Woo *et al*, 2004). Indeed, experiments performed in healthy human volunteers (Ugawa *et al*, 2002; Jones *et al*, 2004) using non-specific blockers such as amiloride or non-steroidal anti-inflammatory drugs (NSAIDs) (Waldmann *et al*, 1997b; Voilley *et al*, 2001) and behavioural experiments in rats using a non-discriminative ASIC blocker (A-317567) (Dube *et al*, 2005) both support a function of ASICs in acid-induced cutaneous pain.

However, data obtained from ASIC knockout mice have failed to demonstrate a clear function of these channels in acidic or primary inflammatory pain (Price *et al*, 2001; Chen *et al*, 2002; Ikeuchi *et al*, 2008; and our own unpublished results) but have shown an effect on secondary mechanical hyperalgesia (related to central sensitization in the spinal cord) in injured or inflamed muscle and joint (Price *et al*, 2001; Sluka *et al*, 2003; Sluka *et al*, 2007; Ikeuchi *et al*, 2008). Therefore, the participation of peripheral ASICs to acid-induced cutaneous pain and primary inflammatory hyperalgesia remains an open question.

In this work, we show that rat cutaneous sensory neurons display a large amount of ASIC1a and ASIC3-like currents when stimulated by moderate acidosis (i.e., around pH 7.0). We then demonstrate the involvement of peripheral ASIC3 in sensing cutaneous acidic pain in normal and inflammatory conditions.

Results

DRG neurons innervating the skin exhibit a high level of ASIC3-like current

We have investigated native ASIC currents activated by moderate acidifications in rat skin dorsal root ganglion (DRG) neurons stained by retrograde labelling with the fluorescent dye DiI (Figure 1A). The very moderate pH value used in these experiments (pH 6.6) was chosen to mainly activate ASIC1-like and ASIC3-like currents, as ASIC2-like and TRPV1 currents have been described to be activated by more drastic acidifications (Tominaga *et al*, 1998; Lingueglia, 2007). These neurons have a resting membrane potential of -55.0 ± 1.8 mV and a membrane capacitance of 39.6 ± 1.8 pF ($n = 42$ and 43, respectively, data from four

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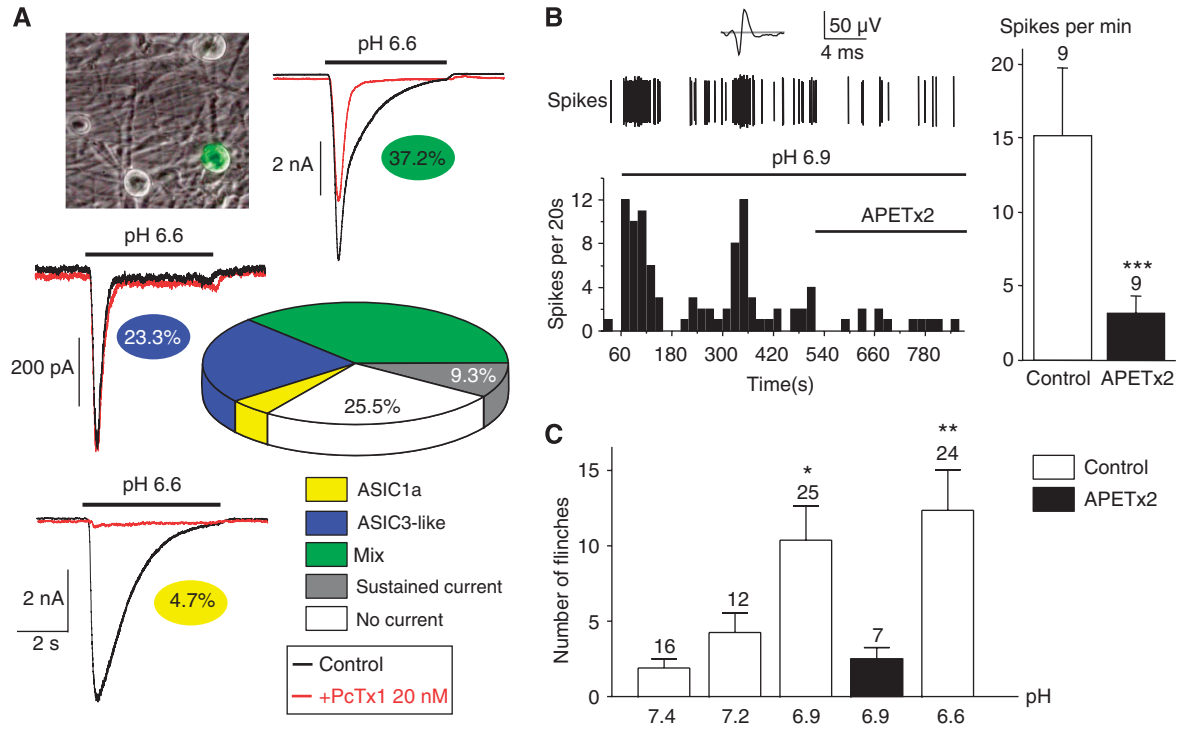

Figure 1 ASIC3 senses cutaneous acidic pain in rat. (**A**) Quantification and analysis of pH 6.6-evoked ASIC currents recorded at −80 mV from rat skin DRG neurons using the PcTx1 toxin. Skin DRG neurons in primary culture were identified using fluorescence microscopy after retrograde labelling with DiI (see the image on the top left). The respective percentages of the different current types are highlighted under each current trace and on the graph (data obtained from a total of 43 neurons). (**B**) Exemplar response of a CM-fibre to pH 6.9 (spikes) with the corresponding time plot of the spike-frequency shown below. The firing of action potential is maintained at pH 6.9, and application of APETx2 10 µM (bar) inhibits the response. The top trace shows the average action potential. Average spike frequency at pH 6.9 and pH 6.9 with APETx2 10 µM ($n=9$) is represented on the left. (**C**) Effect of moderate subcutaneous acidification on pain behaviour in rat (determined as the number of flinches of the injected hind paw; see Materials and methods). The injected solution (NaCl 0.9% + 20 mM HEPES) was buffered at pH values ranging from 7.4 to 6.6. Condition in which APETx2 10 µM was added to the injected solution is represented in black (*$P<0.05$ and **$P<0.01$, significantly different from pH 7.4, Kruskal–Wallis test followed by a Dunn's *post hoc* test).

different cultures), corresponding to estimated neuron diameters ranging from 20 to 45 µm. We found that 65.8 ± 6.3% of these skin DRG neurons exhibit a transient pH 6.6-induced ASIC-like current (4 of 7, 8 of 11, 8 of 10 and 8 of 15 neurons; Figure 1A), with a mean amplitude of −60.3 ± 16.0 pA/pF ($n=28$). Within the remaining skin DRG neurons, pH 6.6 induces either no current ($n=11$) or only a small sustained current (−1.2 ± 0.5 pA/pF, $n=4$). The ratio of ASIC-like current is not dramatically changed by the culture conditions; 70% (7 of 10 neurons) and 65.2% (15 of 23 neurons) of DRG neurons have a pH 6.6-evoked ASIC-like current after 24–48 h or 24–72 h of culture, respectively. Molliver *et al* (2005) have previously found that 11% of the skin afferent neurons have a functional pH 6.8-evoked ASIC-like current after 24–48 h of culture, whereas 28% are positively stained for ASIC3 by immunohistochemistry. The discrepancy with our data is most probably explained by differences in experimental procedure (rat upper back skin versus dorsal face of the hind paw, neurons of diameter <25 µm versus neurons of diameter ranging from 20 to 45 µm, pH 6.8- versus pH 6.6-evoked ASIC-like currents and threshold >300 pA compared with 30 pA in our study).

To discriminate between the different pH 6.6-evoked ASIC-like currents within the skin DRG neurons, we used Psalmotoxin 1 (PcTx1), a potent and selective inhibitor of homomeric ASIC1a channels (Escoubas *et al*, 2000). We show in Figure 1A that 4.7% (2 of 43) of these neurons express a current that is largely inhibited by PcTx1 (inhibition >90%). This current is identified as flowing through native ASIC1a homomers, and it has the same inactivation kinetics as the recombinant ASIC1a current expressed in the F-11 cell line ($\tau_{inactivation}=1.6\pm0.4$ s, $n=2$ versus $\tau_{inactivation}=1.7\pm0.09$ s, $n=10$; see Supplementary Figure 1). Then, 23.3% of the neurons (10 of 43) exhibit a PcTx1-insensitive current (inhibition <10%). This current is identified as an ASIC3-like current, and its inactivation kinetics are similar to that of recombinant ASIC3 expressed in F-11 cells ($\tau_{inactivation}=0.2\pm0.01$ s, $n=14$ versus $\tau_{inactivation}=0.4\pm0.09$ s, $n=9$; see Supplementary Figure 1). Finally, 37.2% of the neurons (16 of 43) have partially PcTx1-sensitive currents (10% ⩽ inhibition ⩽ 90%). This native mix current has an inactivation time constant ($\tau_{inactivation}=1.2\pm0.2$ s, $n=16$) that is reduced by the treatment with PcTx1 to a value ($\tau_{inactivation}=0.5\pm0.1$ s, $n=16$) not significantly different from that of recombinant ASIC3 and native ASIC3-like currents (see Supplementary Figure 1). This current is therefore considered as resulting from a mix of ASIC1a homomeric and ASIC3-like currents. Taken together, these data demonstrate that ASIC3-like currents are the most highly expressed ASIC currents activated by moderate acidifications in skin DRG neurons. They are present in 60.5% (26 of 43) of the skin sensory neurons.

Inhibition of ASIC3 removes cutaneous pain produced by moderate acidosis

To measure the contribution of the ASIC3 channel to nociceptor activation in response to moderate acidification, we have recorded unmyelinated single C-fibre activity with the nerve–skin preparation (Reeh, 1986; Alloui *et al*, 2006). When challenged with a moderate acidification to pH 6.9, a total of 51% of skin rat C-fibres show significant activation ($n=17$ of 33 fibres, $P<0.001$, Wilcoxon test) and 41% are activated by an exposure to pH 6.6 ($n=9$ of 22 fibres, $P<0.01$, Wilcoxon test). The spike discharge is irregular with bursts of activity, and some fibres show a delayed onset, but the activity is maintained as long as the pH is kept acidic (Figure 1B). In all the fibres tested ($n=9$), the sea anemone toxin APETx2 (a specific ASIC3 blocker; Diochot *et al*, 2004) at 10 μM suppresses the pH 6.9-induced spike activity ($P<0.01$, Wilcoxon test), confirming ASIC3 as the major pH-sensor of nociceptive fibres for moderate acidosis (Figure 1B).

Investigation of the pain behaviour of rats following subcutaneous injections of moderately acid solutions (pH 7.4, 7.2, 6.9 and 6.6) in one of the hind paws (Figure 1C) shows a significant pain behaviour at pH 6.9 (flinching score increasing from 1.9 ± 0.6 at pH 7.4 to 10.4 ± 2.3 at pH 6.9, $n=16$ and 25 respectively, $P<0.05$, Kruskal–Wallis test followed by a Dunn's *post hoc* test). The pain behaviour in rats fails to develop when APETx2 is co-injected together with the pH 6.9 solution (Figure 1C). Together, these results demonstrate that ASIC3 is a key sensor of cutaneous pain produced by moderate acidification.

Hypertonicity increases neuronal excitability in skin DRG neurons through an effect on ASIC3

In inflamed or injured tissue, several potent mediators meet in the interstitial fluid and form an inflammatory exudate (Steen *et al*, 1995b), the content of which is acidic and hyperosmotic (Vakili *et al*, 1970). We have thus investigated the effect of hyperosmolarity on the ASIC currents in skin DRG neurons evoked by moderate acidification (i.e., pH 7.0). We show that hyperosmolarity (600 mosmol kg^{-1} with mannitol) strongly enhances the pH 7.0-evoked ASIC current in skin DRG neurons (increased by $95\pm35\%$, $n=8$, $P<0.01$, paired Student's *t*-test; Figure 2A). This leads to an increase of neuronal excitability by triggering more action potentials in current-clamped neurons (Figure 2B). The percentage of neurons in which APs are triggered following a pH 7.0 application is 37.5% (3 of 8 neurons), and all of them display an increase of the firing rate when hyperosmolarity or arachidonic acid (AA) was combined with pH 7.0. ASIC3 and ASIC1a are responsible for most of the currents activated by moderate acidification in rat cutaneous DRG neurons (Figure 1). To precisely determine which of these ASIC isoform(s) is involved in this effect, the ASIC3 and ASIC1a channels were heterologously expressed in the F-11 DRG cell line (Francel *et al*, 1987; Deval *et al*, 2006). Figure 2C shows that hyperosmolarity (600 mosmol kg^{-1} with mannitol) significantly potentiates the ASIC3 current evoked by a shift from pH 7.4 to 7.2 (increase of $148\pm20\%$, $n=20$, $P<0.001$, paired Student's *t*-test). Conversely, the same external acidification to pH 7.2 failed to produce any significant ASIC1a current from ASIC1a-transfected cells and hyperosmolarity was without effect (Figure 2C, lower panel). The control (measured in isotonic conditions) and the enhanced (measured in 600 mosmol kg^{-1} hypertonic conditions) pH 7.2-induced currents recorded from ASIC3-transfected cells have the same reversal potential (49.4 ± 0.7 and 45.9 ± 3.4 mV respectively, $n=3$, $P=0.48$, paired *t*-test; see Figure 2D), confirming the specificity of the effect on ASIC3. Interestingly, hyperosmolarity (600 mosmol kg^{-1}) had no effect on pH 6.6-evoked ASIC3 current and was also without effect on the pH 6.6-evoked ASIC1a current (Figure 2E). Hyperosmolarity therefore seems to affect preferentially the persistent, non-inactivating ASIC3 window current (Yagi *et al*, 2006). The osmotic activation of pH 7.2-induced ASIC3 current was almost maximal when external osmolarity reached 600 mosmol kg^{-1} (Figure 2F). These results indicate that hyperosmolarity potentiates ASIC3 current, but not ASIC1a, at pH 7.2 probably through an effect on the window current.

Moderate acidosis, hypertonicity and AA synergistically affect ASIC3 to produce cutaneous pain

Arachidonic acid is known to positively affect ASIC currents (Allen and Attwell, 2002; Smith *et al*, 2007). We show here that AA increases the native ASIC current induced by a shift from pH 7.4 to 7.0 ($+172\pm65\%$, $n=6$, $P=0.06$, paired Student's *t*-test). This effect leads to an increased excitability of the skin DRG neurons by increasing the triggering of AP (Figure 3A). We have further explored the pH sensitivity of the AA effect on ASIC1a and ASIC3 channels transfected in F-11 cells. As observed for the effect of hypertonicity, AA potentiates the pH 7.2-evoked ASIC3 current ($+547\pm103\%$, $P<0.0001$, $n=23$, Wilcoxon test), whereas it has no effect on ASIC1a at the same pH (Supplementary Figure 2A). However, the kinetics of the two effects (i.e., hypertonicity and AA) are different. The effect of hypertonicity (co-application with the pH drop) is immediate, whereas the effect of AA needs a few minutes to be fully established (Supplementary Figure 2A). We have observed that AA also increases ASIC1a. The activation is larger at pH 7.0 ($+183\pm54\%$, $n=5$, $P=0.06$, Wilcoxon test) than at pH 6.6 ($+21\pm15\%$, $n=2$), but remains much lower than that observed for ASIC3 (increase of $493\pm84\%$, $n=9$, $P<0.001$, Wilcoxon test, and of $40\pm22\%$, $n=7$, $P=0.45$, paired Student's *t*-test at pH 7.0 and 6.6, respectively; see Supplementary Figure 2B). The potent effect of AA on the ASIC3 current essentially results from a shift of the pH dependence of activation towards less acidic values ($pH_{1/2}$ for activation shifted from 6.68 ± 0.01 to 6.84 ± 0.01, $n=5$ and 3 cells, respectively, Figure 3B). No significant effect of AA is observed on the pH dependence of the inactivation curve. As a consequence of these differential effects of AA on the pH dependence of activation and inactivation, the non-inactivating ASIC3 window current is strongly enhanced in the presence of AA (Figure 3C, upper panel). This leads to an activation of the ASIC3 channel at resting physiological pH (i.e., pH 7.4; see Figure 3C, lower panel). Figure 3D shows that the effects of AA and hypertonicity on ASIC3 currents are synergistic, demonstrating that this channel is built to integrate different signals such as moderate acidification, hypertonicity and AA that are found in inflammatory conditions.

In good agreement with the latter results, combining hypertonicity (NaCl 2%, ~600 mOsm kg^{-1}) and AA (10 μM) significantly increases the flinching score of rats (Figure 3E)

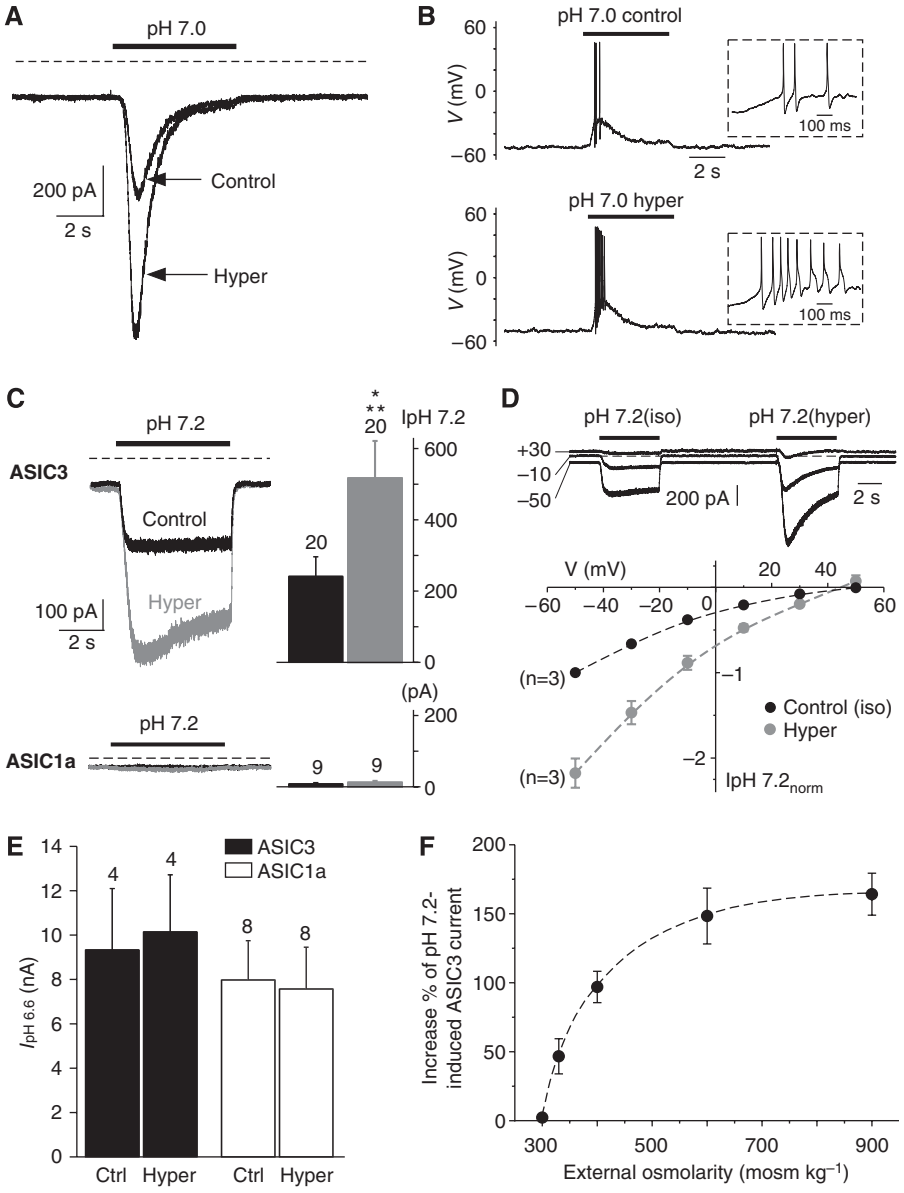

Figure 2 The potentiating effect of hypertonicity on native pH 7.0-evoked ASIC current is mainly mediated by ASIC3. (**A**) Typical ASIC current, recorded from a skin DRG neuron under voltage clamp at −80 mV, induced by a shift from pH 7.4 to 7.0. This current was strongly potentiated when a hypertonic solution (600 mosmol kg^{-1} with mannitol; see Materials and methods) was co-applied with pH 7.0. (**B**) Current clamp experiment performed on the same neuron as in A. The depolarization induced by the pH 7.0-evoked ASIC current was sufficient to trigger three action potentials (APs). Co-application of the hypertonic solution together with the pH drop led to an increase of the number of APs triggered. Time-scale magnifications of these APs are shown within the dotted rectangles. (**C**) Effect of hyperosmolarity (600 mosmol kg^{-1} with mannitol; see Materials and methods) on pH 7.2-induced ASIC1a and ASIC3 currents recorded at −50 mV from F-11-transfected cells. The number of experiments (n) is indicated above each bar (***$P<0.001$, paired Student's t-test). (**D**) Current–voltage relationship of the pH 7.2-induced ASIC3 current obtained from F-11-transfected cells before (control, isotonic) and during hyperosmotic shocks. Typical current traces are shown above the I/V curve. (**E**) Hyperosmolarity (600 mosmol kg^{-1} with mannitol) was without effect on both pH 6.6-induced ASIC3 and ASIC1a currents. (**F**) The increase in the percentage of the pH 7.2-induced ASIC3 current is represented as a function of external osmolarity. The hyperosmotic solutions were obtained by the addition of mannitol ($n=5$–20).

induced by moderate acidosis (i.e., pH 6.9; from 10.4 ± 2.3, $n=25$ to 20.4 ± 2.5, $n=25$, $P<0.05$, Kruskal–Wallis test followed by a Dunn's *post hoc* test). This increase in pain behaviour is largely prevented by co-injection of the ASIC3 blocker APETx2, whereas the homomeric ASIC1a blocker PcTx1 has no significant effect (Figure 3E). Taken together, all these results strongly suggest that the activation of peripheral ASIC3, but not homomeric ASIC1a, by inflammatory mediators contributes to inflammatory pain.

ASIC3 contributes to the development of CFA-induced primary inflammatory pain

To demonstrate more directly the function of ASIC3 in cutaneous inflammatory pain, the effect of APETx2 and PcTx1 were tested on a rat model of inflammatory pain. Four hours after the induction of inflammation by CFA injection in the hind paw, a significant heat hyperalgesia appears (Figure 4A). The heat hyperalgesia does not develop when the ASIC3 blocker APETx2 is co-injected with CFA,

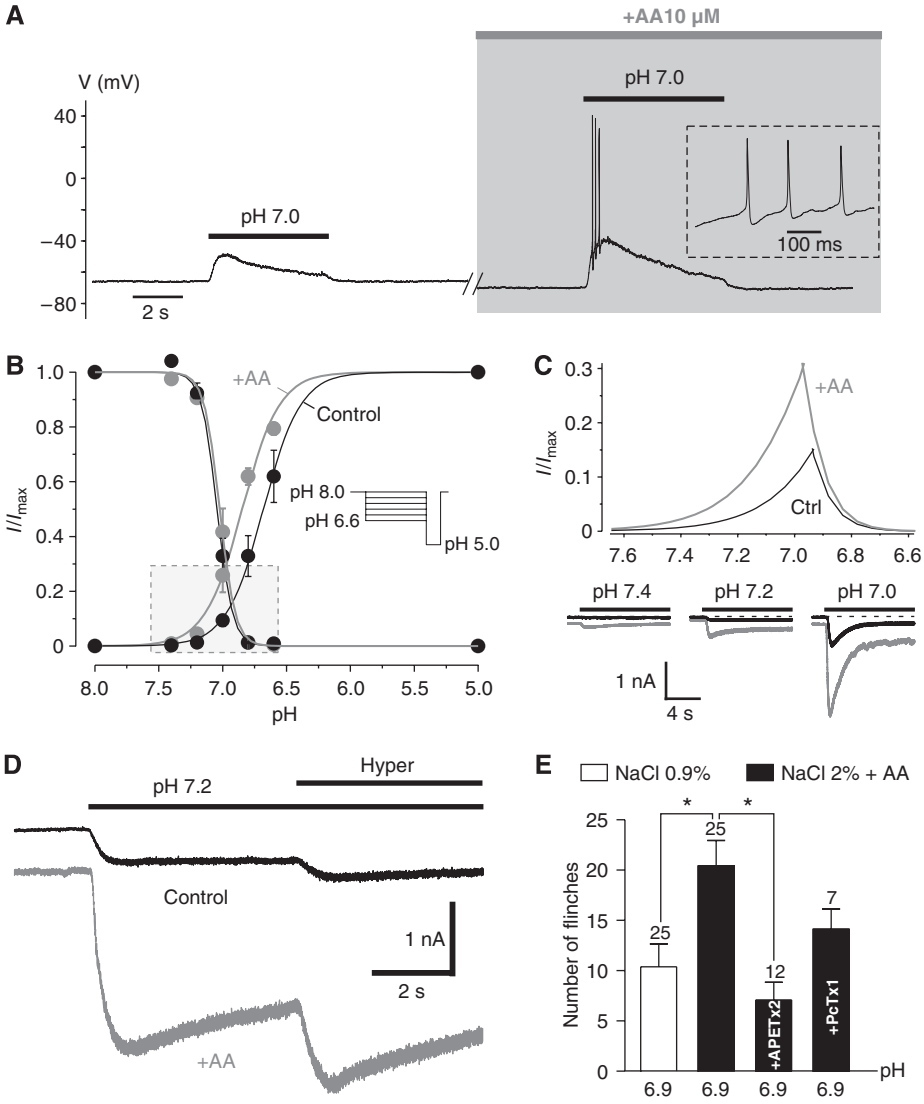

Figure 3 ASIC3 senses different inflammatory signals to produce cutaneous pain. (**A**) Current clamp experiment showing that arachidonic acid (AA) increased the excitability of a skin DRG neuron in response to a depolarization induced by a pH 7.0-evoked ASIC current. Time-scale magnifications of the APs triggered are shown within the dotted rectangle. Note that pH 7.0-induced ASIC current leads to a membrane depolarization near the AP threshold, which is not always sufficient to produce firing (left panel). (**B**) pH-dependent activation and inactivation curves were obtained from F-11 cells transfected with ASIC3, at −80 mV, according to the protocol shown in inset ($n = 3$–5). The framed rectangle indicates a part of the graph where the activation and inactivation curves overlap (window current). (**C**) Magnification of the framed zone shown in B indicating that the non-inactivating ASIC3 window current is strongly enhanced by AA (upper panel). The effect of AA on ASIC3 current induced by moderate acidifications is also represented (lower panel). (**D**) Representative whole-cell recording from transfected F-11 cells of a pH 7.2-induced ASIC3 current at −80 mV showing the synergistic effect of AA (10 µM) and hypertonicity (600 mosmol kg^{-1} with sucrose). (**E**) The effect on pain behaviour in rat of subcutaneous injections of acid (pH 6.9), hyperosmolarity and AA 10 µM together are compared with the effect of pH 6.9 alone. Conditions in which APETx2 10 µM or PcTx1 60 nM were added to the injected inflammatory cocktail are indicated on the bargraph. The number of experiments (n) is indicated above each bar (*$P < 0.05$, Kruskal–Wallis test followed by a Dunn's *post hoc* test).

whereas PcTx1 has no significant effect. The implication of ASIC3 in inflammatory thermal hyperalgesia is further confirmed by intrathecal injections of an siRNA targeting the ASIC3 channel before the induction of inflammation. A marked and specific knockdown of ASIC3 expression at the mRNA level has been demonstrated in lumbar DRGs after intrathecal injections of this siRNA (Supplementary Figure 3). In pain behaviour experiments, these injections prevent CFA-induced heat hyperalgesia, whereas the corresponding scramble siRNA used as a control is without effect (Figure 4B). These results directly demonstrate that ASIC3, but not homomeric ASIC1a, has an important function in primary inflammatory heat hyperalgesia at the peripheral level in rats.

Discussion

Protons are direct activators of nociceptors (Steen *et al*, 1992). Studies conducted both in humans and animals have shown a positive correlation between pain and tissue acidity. Perfusion of acidic solutions or iontophoresis of protons into the skin produces pain in humans (Steen *et al*, 1995a; Ugawa *et al*, 2002; Jones *et al*, 2004), and ASIC channels seem to be the best candidates to sense this cutaneous acidic pain (Ugawa *et al*, 2002; Jones *et al*, 2004). Recent results with transcutaneous iontophoresis, a non-invasive method, are particularly illustrative (Jones *et al*, 2004). They have shown that amiloride, a blocker of ASIC channels

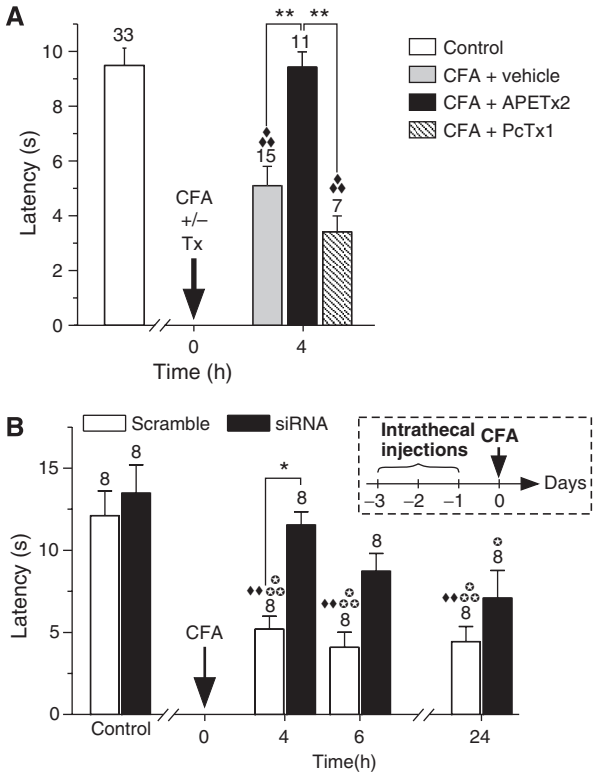

Figure 4 ASIC3 is a detector of cutaneous inflammatory pain in rat. (**A**) Effect of APETx2 20 μM and PcTx1 120 nM on CFA-induced thermal hyperalgesia in rat. Hind paw withdrawal latencies were measured at 50°C (see Materials and methods), and the time at which inflammation was induced (t_0) is indicated by the arrow (**$P<0.01$; ♦♦♦$P<0.001$, significantly different from control, Kruskal–Wallis test followed by a Dunn's *post hoc* test). (**B**) Effect of intrathecal injections (see the inset for the protocol) of ASIC3 siRNA on the CFA-induced heat hyperalgesia described in *A* (* and ✪$P<0.05$; ♦♦$P<0.01$; ✪✪✪$P<0.001$, significantly different from scramble and siRNA control, respectively; one-way ANOVA followed by a Tukey's *post hoc* test).

(Waldmann *et al*, 1997b), and NSAIDs, which also inhibit this class of channels (Voilley *et al*, 2001), significantly decrease acidic pain. They have also demonstrated that skin desensitized by repeated capsaicin application shows no significant reduction in acid-induced pain. This latter result strongly suggests that the acid detection is not through the capsaicin receptor TRPV1 (Jones *et al*, 2004). This conclusion is fully consistent with previous observations using direct perfusion of acidic solutions into human skin (Ugawa *et al*, 2002), which strongly suggested that acidic pain elicited by pH values between 7.4 and 6 was not significantly associated with the TRPV1 channel but was blockable by amiloride. The only limit of this interesting series of papers is that neither amiloride nor NSAIDs are specific inhibitors of ASIC channels, and that besides ASIC and TRPV1 channels, other types of ionic channels could be involved.

Results obtained in humans have yet had no parallel in mice. Deletion of ASIC3 channels in this animal species has failed to indicate a clear function of this channel in pain behaviour associated with cutaneous acidosis or inflammation (Price *et al*, 2001; Chen *et al*, 2002). Silencing of ASIC3 using a dominant-negative subunit has even led to an increased sensitivity to inflammatory stimuli (Mogil *et al*, 2005). Interpretation of these results is complicated by the fact that (i) mice express relatively low levels of ASIC channels in their DRGs (Leffler *et al*, 2006; Lin *et al*, 2008; and unpublished data from this laboratory) as compared with other animal species such as rats (this article), (ii) deleting or silencing ASIC genes might be associated with the appearance of compensatory mechanisms.

We report here that subcutaneous injections of moderately acidic solutions elicit pain in rats within the same pH range (pH ~6.9) as in humans (Steen *et al*, 1995a), and that this effect depends on ASIC3, but not homomeric ASIC1a. Consistent with this *in vivo* observation, we have shown that DRG neurons innervating rat skin display a high level of ASIC-like currents, which, when they are activated by slight acidification (pH 7.0), depolarize the neurons and trigger APs. We also show that very moderate acidifications also induce a significant increase in skin C-fibres firing, which is totally inhibited by the ASIC3-specific toxin APETx2. This demonstrates that ASIC3 is the leading receptor for moderate acidosis in skin nociceptors and participates in the signalling of acidic pain in rat.

Inflammation is one of the pain conditions that produce local tissue acidosis which, in principle, can be detected by ASIC channels. Inflammation also induces a large increase of ASIC channel expression in rat sensory neurons, particularly ASIC3. The nociceptor level of ASIC3 mRNA is increased by more than 15 times in CFA-evoked inflammation (Voilley *et al*, 2001). In addition, in primary cultures of DRG neurons, a mixture of the proinflammatory mediators NGF, serotonin, interleukin-1 and bradykinin increases the number of ASIC-expressing neurons as well as ASIC-like current amplitude in these neurons (Mamet *et al*, 2002). NGF has a particularly important function in regulating ASIC3 gene expression (Mamet *et al*, 2002, 2003). Besides this transcriptional regulation, post-translational modulation of ASIC3 by proinflammatory mediators also takes place. ASIC3 channel activity is increased, for instance, through the PKC pathway by compounds such as serotonin and bradykinin (Deval *et al*, 2004; Lingueglia, 2007). ASIC activity is increased by nitric oxide (NO), a mediator that reaches high levels in inflammation (Cadiou *et al*, 2007). Inflammation is also associated with hypertonicity (Vakili *et al*, 1970; Hamamoto *et al*, 2000), which modulates the activity of several ionic channels involved in pain perception, such as TRPV4 and TREK-1 (Alessandri-Haber *et al*, 2005; Alloui *et al*, 2006). We show here that hypertonicity increases the ASIC3 channel activity. Another important property of inflammation is that it dramatically increases levels of expression of phospholipases A2 (Vadas *et al*, 1993) that catalyses phospholipid hydrolysis with an increased production of AA. AA was shown previously to directly activate ASIC channels (Smith *et al*, 2007), and this work shows that ASIC3 is more sensitive than ASIC1a to AA. The very important observation about the effects of hypertonicity and AA is that they develop for very slight acidifications not far from the physiological pH (pH 7.4). In the presence of a hypertonic medium or AA, acidifications of only 0.2–0.4 pH units, which are easily attained in many pain-related situations such as inflammation, ischaemia, haematomas, fractures and lesions as well as in post-operative states (Issberner *et al*, 1996; Reeh and Steen, 1996; Woo *et al*, 2004), suffice to generate relatively large ASIC3-like currents and trigger action potential generation in

cultured DRG neurons. The mechanism of this effect has been analysed here in detail for AA. AA produces an alkaline shift of the pH dependence of the activation process and, by doing so, enhances a non-inactivating window current. In fact, in the presence of AA, the ASIC3 channel gains a small activity at physiological pH (pH 7.4).

The other interesting observation is that the effects of AA and hypertonicity on ASIC3 current are synergistic. In agreement with this *in vitro* effect, we have observed that injection of a hypertonic solution together with AA significantly increases pain produced by moderate acidosis. This pain enhancement is dramatically decreased by APETx2, the selective blocker of ASIC3, and not by PcTx1, the selective blocker of homomeric ASIC1a, indicating the important function of the ASIC3 channel in such a process. At this stage, it became important to investigate how these data could be extrapolated to real conditions of inflammation. Inflammation was produced by CFA injection, which led to primary heat hyperalgesia, and this hyperalgesia was drastically reduced by the ASIC3 blocker APETx2 injected subcutaneously, which only access cutaneous nociceptors. It was also drastically reduced when, before triggering the inflammation state, intrathecal injections of an siRNA against ASIC3 had induced a knockdown of ASIC3 expression in lumbar DRGs.

All these results taken together demonstrate the important function of ASIC3 in primary pain generation associated with moderate acidosis and inflammation. Therefore, blocking ASIC channels at different levels of the sensory system would clearly be beneficial in relieving pain. Central inhibition of ASIC1a acting upstream of the opiate system (Mazzuca *et al*, 2007) is well adapted for treating all types of pain and, in particular, chronic pain such as neuropathic pain. Local inhibition of the ASIC3 channel that has an important function downstream of the multiple stimuli associated with inflammation is clearly another option to treat inflammatory pain.

Materials and methods

F-11 cell line culture and transfection

The F-11 cell line (Francel *et al*, 1987; Deval *et al*, 2006) was cultured at 37°C under 5% CO_2 at a density of 50 000 cells per 35-mm-diameter Petri dish. The culture medium contained Ham's F-12 medium (Invitrogen) supplemented with 15% fetal bovine serum (ICN Biomedicals), 1 × HAT (sodium hypoxanthine, aminopterin and thymidine), 200 μg ml^{-1} allo-4-hydroxy-L-proline (Sigma-Aldrich) and 1% antibiotics (penicillin and spreptomycin, Gibco). One day after plating, cells were transfected with ASIC1a or ASIC3 cDNAs using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions, with a mix of the pCI-ASIC1a and pIRES$_2$-EGFP vectors (1:2 ratio) or the pCI-ASIC3 and pIRES$_2$-EGFP vectors (1:10 ratio). Cells were used for patch-clamp experiment 2–4 days after transfection. F-11 cells natively express low levels of ASIC1a homomeric channels. However, this level of endogenous current recorded at −50 mV is very low ($I_{pH\ 6.6} = 1.20 \pm 0.15$ pA/pF, $n = 7$) compared with the level of the ASIC3 or ASIC1a currents recorded here after transfection ($I_{pH\ 6.6} = 322.01 \pm 78.44$ pA/pF, $n = 7$ and 310.63 ± 49.80 pA/pF, $n = 4$, respectively).

Retrograde labelling of skin afferents

Dorsal root ganglion neurons innervating skin from Wistar rats (8–11 weeks) were labelled by subcutaneous injections of 5 × 1 μl of the fluorescent dye DiI (5% in DMSO, Molecular Probes) into the dorsal faces of hind paws. Dye was injected 2 weeks before killing the rat to prepare DRG primary culture.

Primary culture of labelled dorsal root ganglia neurons

Lumbar DRG L3–L6 were dissected bilaterally from the previously DiI-injected rats and enzymatically dissociated with 0.1% collagenase. Cells were then plated on collagen-coated 35-mm Petri dishes and maintained in culture at 37°C (95% air/5% CO2) in DMEM containing 5% fetal calf serum. Electrophysiological experiments were carried out 1–8 days after plating.

Electrophysiology

We used the whole-cell configuration of the patch clamp technique to measure membrane currents (voltage clamp) or membrane potentials (current clamp). Recordings were made at room temperature using an RK-400 amplifier (Bio-Logic Science Instruments) with a 3-kHz low-pass filter (Krohn-Hite). Data were sampled at 10 kHz, digitized by a Digidata 1322A A-D/D-A converter (Axon Instruments) and recorded on a hard disk using pClamp software (version 9; Axon Instruments). Patch pipettes (1–4 MΩ) contained (in mM) the following: 135 KCl, 2.5 Na_2-ATP, 2 $MgCl_2$, 2.1 $CaCl_2$, 5 EGTA, 10 HEPES (pH 7.25 with KOH). Various pH-buffered solutions and drugs of interest were applied to individual patch-clamped cells using a homemade microperfusion system driven by microsolenoid valves (Sirai, Italy) allowing rapid solution changes. The control bath solution contained (in mM) the following: 145 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES (pH 7.4 with NaOH). MES was used instead of HEPES to buffer solution pH ranging from 6 to 5 and ASIC currents were induced by shifting one out of eight outlets of the microperfusion system from the pH 7.4 control solution to an acidic test solution. For the experiments performed on cultured DRG neurons, glucose (10 mM) was added to the control bath solution. Hypertonic conditions were obtained by adding mannitol or sucrose to the external bathing solutions as indicating in the text.

Single C fibre recording

The isolated skin–saphenous nerve preparation and single C-fibre recording technique was used as described previously (Alloui *et al*, 2006). The skin of the hind paw of 8- to 14-weeks-old male rat was dissected with the saphenous nerve. The skin was superfused with warm (~30°C) synthetic interstitial fluid, in mM: NaCl 107, KCl 3.48, $NaHCO_3$ 26.2, NaH_2PO_4 1.67, $CaCl_2$ 1.53, $MgSO_4$ 0.69, Na-gluconate 9.64, glucose 5.5, sucrose 7.6, HEPES 10, pH adjusted to 7.4, 6.9 and 6.6 with NaOH, saturated with O_2/CO_2—95%/5%. The receptive field of an identified C-fibre was searched by mechanical probing of the skin and further characterized for mechanosensitivity with calibrated von Frey filaments. This protocol implies that all C-fibres were mechanosensitive. Conduction velocity was <1.3 ms. C-fibres receptive fields were isolated with a thick-walled elrin ring (diameter 400 μm) inside which solutions and toxin were applied through local perfusion pipes of a CL-100 bipolar temperature controller (Warner Instrument). Recordings were band-pass-filtered between 60 Hz and 2 kHz and sampled at 10 kHz on computer with pClamp 9 software (Axon Instrument). Action potential were analysed with Clampfit software (Axon Instrument).

Nociceptive behaviour in rats

Adult (7–8 weeks) male Wistar rats (Charles River) were kept with a 12-h light/dark cycle and with *ad libitum* access to food and water. Rats were acclimated for at least 1 week before experiments. For nociceptive behaviour experiments, rats were placed in a transparent observation chamber where they were acclimated for 20–30 min. They were then gently restrained, while 20 μl of a saline solution (0.9 or 2% NaCl + 20 mM HEPES, $7.4 \leqslant pH \leqslant 6.6$ supplemented or not with 10 μM AA and/or ASIC-specific toxins; see figure legends) was administered subcutaneously into the dorsal face of the hind paw using a 30-gauge needle connected to a 100-μl Hamilton syringe. Nociceptive behaviour (i.e., number of flinches) was counted over a 5-min period starting immediately after the injection (Alessandri-Haber *et al*, 2005).

Inflammation-induced heat-hyperalgesia in rats

Heat sensitivity of adult male Wistar rats (Charles River) was assessed by measuring hind paw withdrawal latency from a hot plate at 50°C (Bioseb, France). Rats were acclimated to the experience room for at least 30 min, and each measurement was made in duplicate. A first measure was performed before the induction of inflammation. Rats were then anesthetized (isoflurane 2.5%) and 150 μl of complete Freund's adjuvant (CFA), diluted 1:1

with saline, and containing either toxins (PcTx1 120 nM or APETx2 20 μM final concentration) or water was injected (26-gauge needle) subcutaneously into the plantar face of one hind paw. The withdrawal latency of the injected hind paw was then measured on the hot plate at 50°C, 4, 6 and 24 h after CFA injection.

In vitro evaluation and in vivo injection and validation of the siRNA

The siRNA sequence targeting rat ASIC3 (no. 1121, CTACACGC-TATGCCAAGGA) has been inserted into the siRNA expression vector pCi-U6-eCMV-neo as short hairpin RNA. This sequence showed no significant homology with other known rat sequences according to the Genbank database. This vector has been locally developed from the pCI neo vector (Promega) by replacing the *Sgf*I/*Not*I fragment (which includes the CMV promoter) with a cassette containing the U6 promoter that was amplified by PCR from the vector developed by Sui *et al* (2002). The pCi-U6-eCMV-neo vector contains a U6 promoter, a CMV enhancer and a neomycin resistance gene for stable transfection. shRNAs were inserted into the *Apa*I and *Eco*RI sites downstream of the U6 promoter. COS cells were transfected by the DEAE-dextran method (Deval *et al*, 2006), with the shRNA constructs together with a vector coding for N-terminal Myc-tagged ASIC3 at a 20:1 ratio. Cells were lysed between 48 and 72 h after transfection and processed for western blot analysis to assess the level of ASIC3 protein. The blots were probed with the anti-Myc A14 antibody (1:500; Santa Cruz Biotechnology) and a monoclonal antibody against actin (clone AC-40; 1:1000; Sigma) as a loading control.

Cy3-labelled siRNA no. 1121 and its corresponding scramble (no. 1121S; GCTCACACTACGCAGAGAT) synthesized by MWG Biotech (Germany) were injected in rats by intrathecal bolus to the lumbar region of the spinal cord once a day for 3 days before the induction of inflammation with CFA. Each 10-μl injection corresponded to 2 μg of siRNA complexed with i-Fect siRNA transfection reagent (Neuromics) at a ratio of 1:4 (w:v) (Luo *et al*, 2005), following the supplier's suggested protocol. siRNA uptake in lumbar DRGs was monitored by fluorescence microscopy on cryostat sections 24 h after a single intrathecal injection. The specificity of the effect was evaluated in lumbar dorsal root ganglia by quantitative reverse-transcription PCR 24 h after the last injection. L5 and L6 ganglia were removed, RNA was extracted with the RNeasy micro kit (Qiagen) and cDNA was prepared with cloned AMV First-Strand cDNA synthesis kit (Invitrogen) and used for qPCR in a Light-Cycler480 (Roche Products). The primers used are as follows: 18S aagtccctgccctttgtacaca/gatccgagggcctcactaaac; ASIC1a ctatcaccacgtcac-caagc/agtgtgacagcagggaaggt; ASIC1b ggagttggatgagggtgatg/tggagggta-cagctgttgg; ASIC2a gaccctctgcaacctcaatg/cagcgtggtacaagtcgttg; ASIC2b agtggttccgcaaactgg/ctgatgccctcgaagtgg; ASIC3 cacccaatgacttgcactgg/taggcagcatgttcagcagg; TRPV1 agcagcagtgagacccctaa/gaagtagaagatgcg cttgaca. Results were normalized against 18S and converted to fold induction relative to vehicle controls.

Statistical analysis

Data analysis was performed using Microcal Origin 6.0 and GraphPad Prism 4.03 softwares. Data are presented as mean ± s.e.m. and statistical differences between sets of data were assessed using either parametric or non-parametric tests followed by *post hoc* tests when appropriate.

Supplementary data

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

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