Acid-sensing ion channel 1a (ASIC1a) has been shown to play important roles in synaptic plasticity, learning and memory. Here we identify a crucial role for ASIC1a in long-term depression (LTD) at mouse insular synapses. Genetic ablation and pharmacological inhibition of ASIC1a reduced the induction probability of LTD without affecting that of long-term potentiation in the insular cortex. The disruption of ASIC1a also attenuated the extinction of established taste aversion memory without altering the initial associative taste learning or its long-term retention. Extinction of taste aversive memory led to the reduced insular synaptic efficacy, which precluded further LTD induction. The impaired LTD and extinction learning in ASIC1a null mice were restored by virus-mediated expression of wild-type ASIC1a, but not its ion-impermeable mutant, in the insular cortices. Our data demonstrate the involvement of an ASIC1a-mediated insular synaptic depression mechanism in extinction learning, which raises the possibility of targeting ASIC1a to manage adaptive behaviours.
Acid-sensing ion channel (ASIC) family comprises six isoforms (ASIC1a, 1b, 2a, 2b, 3 and 4) encoded by four genes. These proton-gated channels are formed by three identical or different subunits, with varying pH sensitivity, ion selectivity and pharmacological characteristics depending on subunit compositions. ASIC1a-containing channels are the major ASICs in the central nervous system, where ASIC1a null neurons exhibit no acid-evoked current. The importance of ASIC1a has been well documented in rodent models of ischaemic neuronal death, chronic pain, seizure termination and neurodegenerative diseases. ASIC1a is also implicated in affective disorders, including anxiety and depression. However, despite the rich information about its neuronal expression and contributions to pathological processes, the physiological role(s) of ASIC1a in brain neurons remains largely unknown.

Increasing evidence supports the critical role of ASIC1a in synaptic transmission and plasticity. Given its postsynaptic localization, ASIC1a has been postulated to be activated by synthetically released protons during normal neurotransmission, serving as the main synaptic proton receptor to regulate high-frequency stimulation or theta burst stimulation (TBS)-induced long-term potentiation (LTP) in hippocampus and amygdala, and contribute to fear learning. ASIC1a promotes spine density in hippocampus, but inhibits spine density, alters the excitatory synaptic receptor function and constrains cocaine-evoked plasticity in nucleus accumbens, suggesting complex roles of ASIC1a in modulating synaptic plasticity and behavioural adaptation.

The complexity of ASIC1a function may reflect region-specific participation in different forms of synaptic plasticity and behavioural regulation. ASIC1a is highly expressed in neurons in the insular cortex, but its function there is unclear. Insular cortex is critical for cognition and emotion control, including sensory integration, chronic pain, emotional processing and gustatory recognition memory. It is best known for its role in taste learning and processing aversively motivated learning tasks, that is, conditioned taste aversion (CTA), a form of associative learning where the subject associates a novel taste (conditioned stimulus) with a subsequent transient visceral illness (unconditioned stimulus, US). At the synaptic level, LTP in insular cortex is known to contribute to the acquisition of CTA, but its molecular and synaptic mechanisms underlying CTA memory extinction remain undetermined.

Here we examine the role of ASIC1a in synaptic plasticity in the adult mouse insular cortex using multiple-electrode array slice recording, with the MED64 probe placed within the insular cortical slice as shown in Fig. 1a,b. We recorded the responses of the rostral insular region at the level of the corpus callosum connection to stimulation in the deep layers (V and VI) of the insular slice (the red dot in Fig. 1b). Current injection at the stimulation site typically elicited field excitatory postsynaptic potentials (fEPSPs) in a fraction of channels (>10 channels) of the 64-channel array. These are referred to as activated channels.

Conistent with the previous study, low-frequency stimulation (LFS) in insular cortical slices from WT mice induced a significant depression of fEPSPs in the majority of activated channels (Fig. 1c; Supplementary Fig. 3a–c), hence designated as LTD–LTD. This was abolished by NMDAR antagonist D-AP5 and therefore also referred to as NMDAR-dependent LTD (NMDAR-LTD). The induction ratio, defined as the percentage of LTD-positive channels among all activated channels, was 70.5 ± 4.9% (Fig. 1d; Supplementary Fig. 3d,e) in WT, but significantly decreased to 20.9 ± 5.7% in ASIC1a KO mice (P = 1.156E−05 versus WT; Fig. 1d; Supplementary Fig. 3f–j).

For channels that displayed LTD, the loss of ASIC1a did not appear to affect its magnitude, as the mean LTD magnitude of all LTD-positive channels were not significantly different between WT and ASIC1a KO groups (Supplementary Fig. 4a). The fEPSP amplitudes of activated channels did not influence the probability of LTD induction. When only channels with the strongest responses from individual slices were used for quantification, the mean fEPSP slopes at 50–60 min following LFS were significantly decreased in WT, but not ASIC1a KO groups (Supplementary Fig. 4b). Furthermore, neurons in both superficial (layer II–III) and deep (layer V–VI) layers of insular cortex exhibited reduced LTD induction (LTD induction ratios: superficial, WT: 73.7 ± 7.4%, ASIC1a KO: 24.9 ± 11.0%, P = 0.0019; deep: WT: 69.9 ± 6.1%, ASIC1a KO: 18.9 ± 8.5%, P = 0.0002; Supplementary Figs 4c,d and 8a,b) in ASIC1a KO mice. Together, these data suggest a global role of ASIC1a in the probability of insular LTD induction, but not LTD magnitudes in individual neurons, and this effect is independent of the fEPSP amplitude and cortical layer specificity of insular neurons.

To further establish the role of ASIC1a in insular LTD, we used a conditional ASIC1a KO line (ASIC1a<sup>flx/flx</sup>) to which an adenov-associated virus (AAV) that contained the coding sequence for either green fluorescence protein (GFP, AAV-Ctrl) or Cre recombinase (AAV-Cre) was injected bilaterally into insular cortices ~1 month before slice recording by the MED64 system.
Western blotting showed that the injection of AAV-Cre reduced ASIC1a protein expression in insular cortex as compared with AAV-Ctrl (Supplementary Figs 5a, b and 13f). The LTD induction ratio reduced from $80.0 \pm 4.3\%$ for AAV-Ctrl- to $15.9 \pm 5.5\%$ for AAV-Cre-injected ASIC1a$^{flox/flox}$ mice ($P = 2.177 \times 10^{-6}$ versus AAV-Ctrl; Fig. 1e, f).

**Figure 1 | ASIC1a is important for LFS-evoked LTD in insular cortex.** (a) Schematic diagram showing the location of a MED64 probe placed on the coronal insular slice (left) and the arrangement of the $8 \times 8$ recording array. (b) Light microscopy photograph showing the location of the MED64 probe relative to the insular cortex and layer (I to V and VI) designation. The red dot indicates the stimulation site. (c-e, g, i) Time courses of fEPSP slope changes in response to LFS (1 Hz, 900 pulses) at insular slices under conditions indicated. Inset: representative fEPSP traces at the time points indicated by numbers in the graph. Scale bar, 100 μm, 10 ms. (d, f, h, m) Summary of number of activated channels per slice and those that showed LTD in insular synapses. NS, not significant, *$P < 0.05$, ***$P < 0.001$, (d) $P = 0.1570$ and 0.0005, $n = 7-9$ slices per 7-8 mice; (f) $P = 0.7632$ and 3.854E$-05$, $n = 6-7$ slices per 4 mice each; (h) $P = 0.2660$ and 0.0194, $n = 5-6$ slices per 3-5 mice, compared between two groups; (m) activated: $P = 0.0700$, 0.0787 and 0.7211, LTD: $P = 0.0002$, 0.6495 and 0.0004, $n = 6-8$ slices per 4-8 mice, compared with Ctrl versus ASIC1a, Ctrl versus ASIC1a-HIF and ASIC1a versus ASIC1a-HIF, respectively, unpaired Student's t-test. *(i) Schematics of AAV vectors engineered to express a control construct (EGFP), ASIC1a or a pore-dead mutant (32HIF34AAA) of ASIC1a. Hsynapsin I, the human synapsin I promoter that drives expression in neurons. (j) An example of AAV-mediated EYFP expression in the insular cortex (left). The slice was counterstained with DAPI (4',6-diamidino-2-phenylindole). A brain atlas at the similar brain section level that contains the insular cortex (Bregma +0.98 mm) is shown at right. (k) A representative image at high magnification showing EYFP expression in individual insular neurons.
We next examined the effect of pharmacological blockade of ASIC1a-containing channels on LFS–LTD in insular cortex. Psalmotoxin 1 (PcTX1, 100 nM), an inhibitor of homomeric ASIC1a and heteromeric ASIC1a/2b channels, was applied to the slice at ~15 min before and continued during the LFS delivery. Heat-inactivated (boiled) PcTX1 was used as a control. Neither PcTX1 nor boiled PcTX1 altered basal excitatory synaptic transmission (Fig. 1g); however, the LTD induction ratio decreased from 72.4 ± 8.4% for boiled PcTX1 to 17.9 ± 9.3% for PcTX1-tretated slices (P = 0.0018; Fig. 1h). These results confirm the critical role of ASIC1a in insular LTD and rule out the possibility that LTD deficit in the ASIC1a KO mice might be caused by developmental compensation.

Re-expression of ASIC1a in ASIC1a null mice rescues LFS–LTD. To test whether restoration of ASIC1a expression in the insular cortices of ASIC1a KO mice could rescue LTD induction, we prepared an AAV that contained the coding sequence for enhanced yellow fluorescence protein (EYFP) protein linked to the N terminus of mouse ASIC1a through a 'self-cleaving' 2A-peptide driven by the human synapsin 1 promoter (AAV-ASIC1a; Fig. 1i) for neuron-specific expression. AAV-ASIC1a and the control virus (AAV-Ctrl; Fig. 1i) were bilaterally injected into the insular cortices of ASIC1a KO mice (Fig. 1j,k) ~1 month before slice recording. The injection of AAV-ASIC1a, but not AAV-Ctrl, successfully rescued LFS–LTD (Fig. 1l), with the induction ratio increased from 14.9 ± 6.3% for AAV-Ctrl to 69.9 ± 5.4% for AAV-ASIC1a (P = 2.513E-05 versus AAV-Ctrl; Fig. 1m). Moreover, expression of a non-conducting mutant, ASIC1a-HIF (32HIF34 mutated to 33AAA34, Fig. 1i), which has been shown to be devoid of channel conductance due to pore dysfunction35, failed to rescue the impaired insular LTD induction in ASIC1a KO mice (Fig. 1l), with the induction ratio (24.0 ± 8.2%, P = 0.3976; Fig. 1m) not significantly different from that of AAV-Ctrl. These data, thus, support the critical contribution of ASIC1a and its ion conductance to insular LTD and again rule out developmental compensation, as a contributing factor of impaired LTD in the mutant mice.

Importance of pH dynamics for LFS–LTD in insular cortex. As proton-gated channels, ASIC1a most likely contributes to LFS–LTD in response to pH fluctuations during synaptic activities. To test the influence of pH dynamics on LTD induction, we increased the buffering capacity of extracellular activities. To test the influence of pH dynamics on LTD induction, we increased the buffering capacity of extracellular activities. To test the influence of pH dynamics on LTD induction, we increased the buffering capacity of extracellular activities. To test the influence of pH dynamics on LTD induction, we increased the buffering capacity of extracellular activities.

ASIC1a is critical for DHPG-induced LTD in insular cortex. We further looked into the role of ASIC1a in another form of synaptic depression induced by bath application of group I metabotropic glutamate receptor agonist, 3,5-dihydroxyphenylglycine (DHPG, 100 μM), which is resistant to inhibition by NMDAR antagonist and thus referred to as NMDAR-independent LTD36. Notably, DHPG-induced LTD (DHPG-LTD) was also disrupted in the mutant animals (Fig. 2a). The induction ratio of insulin DHPG-LTD was decreased from 82.2 ± 5.6% for WT littermates to 20.4 ± 9.2% for ASIC1a KO mice (P = 0.0004; Fig. 2b). Importantly, the expression of AAV-ASIC1a, but not AAV-Ctrl, in insular cortices of ASIC1a KO mice restored DHPG-LTD induction ratio to the WT level (AAV-ASIC1a, 82.2 ± 5.8%; AAV-Ctrl, 15.3 ± 9.4%, P = 0.0001; Fig. 2c,d). Thus, ASIC1a is important for the development of both NMDAR-dependent and NMDAR-independent LTD in mouse insular cortex.

ASIC1a is not required for LTP induction in insular cortex. We also examined the effect of ASIC1a deletion/inhibition on LTP, another form of synaptic plasticity in insular cortex. Consistent with the previous study38, TBS in WT insular cortical slices induced a rapid and long-lasting potentiation of synaptic response in multiple sites (Fig. 2e; Supplementary Fig. 7a–c). However, the TBS-induced LTP reached similar levels between slices prepared from WT and ASIC1a KO mice (24.0 ± 7.4% for WT and 21.2 ± 6.5% for ASIC1a KO samples (P = 0.6711; Fig. 2f), and no cortical layer specificity (Supplementary Fig. 8c,d). Moreover, the ASIC1a inhibitor, PcTX1, did not affect TBS induction of LTP in insular cortical slices from WT mice (Fig. 2g), with an LTD induction ratio of 60.9 ± 6.6% (Fig. 2h), comparable to that of untreated WT samples (P = 0.1822). These results indicate that ASIC1a is unlikely involved in insular LTD induction by TBS, which contrasts sharply to its pronounced contribution to insular LTD induction as described above.

Insular LTD is selectively abolished by CTA extinction. In light of the selective role of ASIC1a in insular LTD, we sought to determine the specific insular cortex-regulated behavioural responses to which LTD might have a critical contribution. As stated in the Introduction, insular cortex is important for CTA, a form of aversetly motivated taste learning task30–33. While insular LTD has been linked to CTA acquisition34,35, little is known about the role of insular LTD in this process. Given the usually opposing roles of LTD and LTP in synaptic plasticity, we suspected that insular LTD might play a role in CTA extinction. As such, insular cortical slices from mice subjected to CTA training, including acquisition, retention and extinction, might exhibit altered LTD induction, depending on the specific process, in which insular LTD executes its function.

To induce CTA, mice were presented in the drinking water with saccharin as conditioned stimulus, which was then followed by the intraperitoneal injection of LiCl to cause malaise (US). The establishment of CTA was confirmed using aversion index, which compares the consumption of water and saccharin solution in two drinking bottles presented to the conditioned animal during the 30-min drinking period in subsequent days (Supplementary Fig. 9a). With this protocol, most animals acquired aversion index values between 80–90% after conditioning (see below). To extinguish CTA, the animals were allowed to drink only the saccharin solution (extinguished) during the 30-min drinking period for 7 days, whereas the control animals were given water only (CTA) to maintain the taste aversion (Fig. 3a). As shown in Fig. 3b, the CTA control mice maintained the high aversion index, while the extinguished animals exhibited a markedly reduced aversion index, indicating the extinction of CTA by the consumption of saccharin without the association with LiCl injection.

Analysing insular cortical slices from the above mice, we found that the CTA controls without extinction exhibited normal LTD–LTD (Fig. 3c), with an induction ratio of 68.5 ± 7.5% (Fig. 3d). For DHPG-induced LTD, the induction ratio was 80.3 ± 2.6% (Fig. 3f). Both of these values were comparable with those from naive mice (Figs 3d and 3b), indicating that insular LTD is not involved in the acquisition and retention of CTA. On the other hand, slices from the CTA-extinguished mice displayed diminished LTD to LFS (Fig. 3c) or DHPG (Fig. 3e). The LTD
Loss of ASIC1a selectively impairs CTA extinction. Given the importance of ASIC1a in insular LTD induction, the ASIC1a null mice would be expected to exhibit normal CTA acquisition and retention, but a dysfunction in CTA extinction. Indeed, CTA acquisition was comparable between WT and ASIC1a KO mice. On the day of conditioning, the two groups of mice consumed similar amounts of saccharin solution (saccharin solution intake: WT, 1.93 ± 0.11 g, n = 15; ASIC1a KO: 1.74 ± 0.15 g; n = 14; P = 0.3020). On the first day after conditioning, both groups developed aversion to saccharin (Fig. 4a), implying that ASIC1a does not play a significant role in CTA acquisition and retrieval. Over the next 28 days, the retention of CTA memory was determined on days 3, 7, 14 and 28, and shown to be similar between WT and ASIC1a KO mice (Fig. 4b), indicating that just like their WT littermates, the ASIC1a null mice were able to retain the normal CTA memory for at least 1 month.

By contrast, the ASIC1a KO mice failed to show a reduction in recent CTA memory following repeated choice test in the absence of LiCl, whereas WT mice exhibited CTA extinction within the second to third days of the choice test (Fig. 4c). Similarly, extinction of remote CTA memory, examined on days 15–21 after the conditioning, was also disrupted in the ASIC1a KO mice (Fig. 4d). These results indicate that ASIC1a selectively contributes to extinction, but not acquisition or retention of CTA.

As a control, we confirmed that the ASIC1a null mice had intact taste discrimination. Using the two-bottle unconditioned taste preference test44, we examined their preference for four taste solutions (sweet, salty, bitter and acidic) over water and found no difference between WT and ASIC1a KO littermates (Supplementary Fig. 9b–e).

ASIC1a in insular cortex is critical for CTA extinction. To establish insular cortex as the critical site of ASIC1a action on CTA extinction, we again employed the conditional ASIC1aflx/flx line as described above. Strikingly, AAV-Cre injection (Fig. 5a) resulted in greatly diminished CTA extinction in ASIC1aflx/flx mice as compared with AAV-Ctrl injection (Fig. 5b). By contrast, the injection of AAV-Cre in hippocampal CA1 region did not alter CTA extinction (Supplementary Figs 10a–d and 13g). These results support a specific role for insula-expressed ASIC1a in CTA extinction.

induction ratios were 19.3 ± 11.4% (Fig. 3d; P = 0.0049) and 11.4 ± 5.5% (Fig. 3f; P = 1.982E-07) in response to LFS and DHPG, respectively, which were significantly different from that of CTA controls. These results demonstrate that CTA extinction but not its acquisition or retention abolishes insular LTD.

The loss of LTD could result from blockade of mechanism(s) involved in LTD induction or a saturation effect that occludes subsequent LTD. To distinguish between these possibilities, we compared synaptic efficacy in insular cortical slices from CTA control and extinguished mice. We reasoned that if the LTD induction mechanism was disrupted, the basal excitatory synaptic efficacy should not differ between the two groups, whereas if LTD induction mechanism was disrupted, the basal excitatory synaptic efficacy in CTA extinguished samples (WT, 1.93 ± 0.11 g, n = 15; ASIC1a KO: 1.74 ± 0.15 g; n = 14; P = 0.3020). On the first day after conditioning, both groups found no difference between WT and ASIC1a KO littermates.
We then tested whether acute inhibition of ASIC1a in the insular cortex could affect CTA extinction. PcTx1 (10 pmol) or vehicle was injected bilaterally into insular cortices immediately after the two-bottle choice test on each day for three consecutive days beginning 3 days after the conditioning (Fig. 5c). The administration of PcTx1 (Fig. 5d; Supplementary Fig. 5c) significantly attenuated CTA extinction as compared with the vehicle (Fig. 5e). In contrast, injecting PcTx1 (5 pmol) into the hippocampal CA1 region had no effect on CTA extinction. PcTx1 (10 pmol) or vehicle was injected bilaterally into insular cortices immediately after the conditioning (Fig. 5c), and led to attenuation of CTA extinction in WT mice, and in CaMKII-Cre mice, the virus resulted in CTA memory impairment (Fig. 5n) or ASIC1a (Fig. 5o) was detected in WT mice injected with the vehicle. In contrast, in CaMKII-Cre mice, the virus resulted in CTA memory impairment (Fig. 5n) or ASIC1a (Fig. 5o; Supplementary Fig. 13b) was observed in a subpopulation of insular neurons of forebrain45, robust expression of mCherry (Fig. 5n) was still effective at inhibiting CTA extinction in WT mice, and in CaMKII-Cre mice, the virus resulted in CTA memory impairment (Fig. 5n). When injected into insular cortices of CaMKII-Cre mice, in which Cre recombinase is expressed in pyramidal neurons of forebrain45, robust expression of mCherry (Fig. 5n) and ASIC1a (Fig. 5o; Supplementary Fig. 13b) was observed in a subpopulation of insular neurons. No expression of mCherry (Fig. 5n) or ASIC1a (Fig. 5o) was detected in WT mice injected with the same virus. The shRNA contained in this AAV (Fig. 5m) was still effective at inhibiting CTA extinction in WT mice, as evidenced by the retention of high aversion index (Fig. 5p). By contrast, in CaMKII-Cre mice, the virus resulted in CTA memory extinction to a similar extent as in control animals (Fig. 5p). Collectively, the above results establish a pivotal role of insular ASIC1a in extinction of taste aversion.

As an alternative approach to eliminate ASIC1a expression, we generated an AAV construct that expresses a short hairpin RNA (shRNA) targeting ASIC1a, driven by the U6 promoter (Fig. 5i). After verification of its effectiveness in Chinese hamster ovary (CHO) cells (Supplementary Figs 11 and 13h), AAV-ASIC1a-shRNA or a negative control virus (AAV-NC-Ctrl) was injected into the insular cortices of WT mice (Fig. 5h). AAV-ASIC1a-shRNA markedly decreased ASIC1a protein levels in the insular cortex (P = 5.651E − 05, n = 4–6; Fig. 5j; Supplementary Fig. 13a) and led to attenuation of CTA extinction as compared with AAC-NC-Ctrl injected animals (Fig. 5k). We also performed a cell-type-specific rescue experiment by expressing exogenous ASIC1a in excitatory neurons together with the ASIC1a shRNA in excitatory neurons with the AAV vector with ASIC1a shRNA also contained a double-flxed inverted orientation coding sequence for shRNA-resistant mCherry-2A-ASIC1a (reading as ASIC1a*), which is only expressed in the presence of Cre recombinase (Fig. 5m). When injected into insular cortices of CaMKII-Cre mice, in which Cre recombinase is expressed in pyramidal neurons of forebrain45, robust expression of mCherry (Fig. 5n) and ASIC1a (Fig. 5o; Supplementary Fig. 13b) was observed in a subpopulation of insular neurons. No expression of mCherry (Fig. 5n) or ASIC1a (Fig. 5o) was detected in WT mice injected with the same virus. The shRNA contained in this AAV (Fig. 5m) was still effective at inhibiting CTA extinction in WT mice, as evidenced by the retention of high aversion index (Fig. 5p). By contrast, in CaMKII-Cre mice, the virus resulted in CTA memory extinction to a similar extent as in control animals (Fig. 5p). Collectively, the above results establish a pivotal role of insular ASIC1a in extinction of taste aversion.

**ASIC1a acts through GSK3β in insular cortex.** We further examined the key signalling pathway involved in insular LTD and CTA extinction. NMDAR-LTD in insular cortex requires protein phosphatase 1/2A (ref. 39), which was shown to regulate hippocampal NMDAR-LTD induction via glycogen synthase kinase-3β (GSK3β)46,47. Thus, we measured whether GSK3β was
activated by stimulating NMDAR in insular cortex and whether such an effect required ASIC1a. Insular cortical slices were treated with NMDA (50 μM, 3 min). Western blotting revealed GSK3β dephosphorylation at Ser-9, that is, activation on NMDA stimulation of insular cortices from WT, but not ASIC1a KO mice (Fig. 6a,b; Supplementary Fig. 13c). In addition, an enzymatic assay showed that NMDA increased GSK3β activities only in samples from WT, but not those from ASIC1a KO mice (Fig. 6c). Therefore, NMDAR stimulation of insular cortex activates GSK3β in an ASIC1a-dependent manner.

We then applied a selective GSK3β inhibitor (CT99021, 1 μM)48,49 to insular cortical slices before and during LFS delivery. While CT99021 treatment for up to 20 min had no effect on basal excitatory synaptic transmission (Fig. 6d), it disrupted the development of LFS-LTD (Fig. 6d), with the induction ratio reduced to 26.6 ± 9.2% from 70.5 ± 4.9% for untreated control slices (P = 0.005; Fig. 6e). Moreover, bilateral injection of CT99021 (200 pmol) into insular cortices of WT mice immediately after each two-bottle choice test on days 3–6 after conditioning (Fig. 6f) strongly attenuated CTA extinction (Fig. 6g). Thus, insular GSK3β signalling is also essential for CTA extinction, in addition to LTD induction.

Next, we examined whether ectopic expression of a constitutively active form of GSK3β (Ser-9 mutated to Ala, S9A) in the insular cortex could rescue the impaired LTD and CTA extinction in the ASIC1a KO mice. Injection of AAV-GSK3β-S9A (Fig. 6h) resulted in the increased GSK3β activity as compared with AAV-GSK3βb (Fig. 6i), which also successfully rescued LFS-LTD (Fig. 6j), with the induction ratio reached 71.8 ± 4.2%, significantly higher than that achieved by AAV-GSK3βb (22.6 ± 4.8%, P = 3.165E–06; Fig. 6k). Furthermore, AAV-GSK3β-S9A but not AAV-GSK3βb (Fig. 6l) restored CTA extinction of recent memory (Fig. 6m). Thus, our results indicate that GSK3β signalling in insular cortex after CTA acquisition is crucial for LTD induction and CTA extinction.

**Figure 4** | ASIC1a is important for extinction but not acquisition or retention of CTA memory. WT and ASIC1a KO mice were subjected to CTA on day 0. The acquisition of CTA memory was tested on the second day (day 1, a) and the retention examined on days 3, 7, 14 and 28 (b) (protocols shown in the upper panels) by the two-bottle choice test and quantified as aversion index (bottom panels). For retention test, the animals were given only water after the conditioning day until the test day. For extinction test (c,d), animals were given water and the saccharin solution for 30 min on each test day and aversion indices were obtained for seven consecutive days beginning either day 1 after CTA acquisition (for extinction of recent memory, c) or day 15 after CTA acquisition and two bottles of water provided on days 1–14 (for extinction of remote memory, d). Data for day 1 in c were the same as in a, regraphed again for comparison. n = 8–16, NS, not significant, *P < 0.05, **P < 0.1, ***P < 0.001, P = 0.2916 (a); 0.7197, 0.3763, 0.1644 and 0.6585 (b); 0.0256, 0.0031, 0.0080, 0.0441, 0.0200 and 0.0082 (c); 0.0308, 0.0005, 0.0096, 0.0417 and 0.0186 (d). WT versus ASIC1a-KO, unpaired Student’s t-test. (c) Group, F(1,203) = 42.041, P < 0.001; test day, F(6,203) = 2.207, P = 0.044; interaction, F(6,203) = 0.847, P = 0.535; (d) group, F(1,161) = 34.259, P < 0.001; test day, F(6,161) = 2.795, P = 0.013; interaction, F(6,161) = 2.049, P = 0.063, two-way analysis of variance.
extinction in the ASIC1a null mice (Fig. 6m). Therefore, a failure to activate GSK3β in insular cortex represents the major deficit in the ASIC1a KO mice that led to the diminished LTD and CTA extinction.

**Insular LTD is required for CTA extinction.** To establish that ASIC1a-dependent LTD in insular cortex is involved in CTA extinction, we investigated the behavioural consequence of blocking insular LTD in vivo. As a general mechanism of LTD\(^5\),
AMPAR endocytosis can be blocked by either intracellular application of a GluA2 mimetic peptide, GluA2-3Y (refs 50,51), or extracellular administration of a fluorescent infusion peptide FITC-Tat-GluA2-3Y (Tat-3Y; Fig. 7a), which contains a TAT peptide sequence derived from HIV to facilitate membrane penetration. Its negative control, FITC-Tat-GluA2-3A (Tat-3A; Fig. 7a) does not exert these effects. Application of Tat-3Y (1 μM), but not Tat-3A (1 μM), before and during LFS delivery, blocked LTD induction in insular cortical slices (Fig. 7b), with the induction ratios of 16.0 ± 7.1% and 78.0 ± 6.1% for Tat-3Y- and Tat-3A-treated slices (P = 5.900E−05; Fig. 7c), respectively, indicating that GluA2-dependent AMPAR endocytosis represents

**Figure 6 | GSK3β signalling is involved in ASIC1α-mediated insular LTD induction and CTA memory extinction.** (a,b) Representative images (a) and quantification (b) of western blots. n = 6. **P < 0.01, NS, not significant, P = 0.0021 and 0.5035, Ctrl versus NMDA, unpaired Student’s t-test. (c) GSK3β activities. n = 5, NS, not significant. **P < 0.01, ***P < 0.001 and 0.8486, Ctrl versus NMDA, unpaired Student’s t-test. (d) Time courses of fEPSP slope changes in response to LFS for insular cortical slices prepared as indicated. Scale bar, 100 μV, 10 ms. (e,k) Summary of number of activated channels per slice and those that showed LTD. NS, not significant. **P < 0.01, ***P < 0.001, (e) P = 0.6266 and 0.0014, n = 6–9 slices per 6–8 mice; (k) P = 0.4077 and 0.0001, n = 7–8 slices per 4–5 mice, compared between two groups, unpaired Student’s t-test. The Ctrl data in e were taken from Fig. 1d and regraphed for comparison. (f,l) The behavioural protocols used for g,m, respectively. (g,m) Time courses of aversion indices over a 4-day period for animals subjected to the CTA extinction protocol. n = 6–10. **P < 0.05, ***P < 0.001, P = 0.0010, 0.0003 and 0.0001 (g), 0.0055, 0.0009 and 0.0025 (m), compared between the two treatment groups, unpaired Student’s t-test. (g) Group, F(3,80) = 40.479, P < 0.001; test day, F(3,80) = 2.452, P = 0.07; interaction, F(3,80) = 4.109, P = 0.009. (m) Group, F(3,82) = 33.279, P < 0.001; test day, F(3,82) = 9.134, P < 0.001; interaction, F(3,82) = 6.084, P = 0.001, two-way analysis of variance. (h) Schematics of AAV vector constructs. (i) Injection of AAV-GSK3β-S9A increased GSK3β enzymatic activity in lysates from insular cortices as compared with the injection of AAV-GSK3β. n = 4–5. **P < 0.01, P = 0.0057, unpaired Student’s t-test.
a key step in insular LTD induction, similar to that in other brain regions\(^5\)

Finally, we assessed the effect of Tat-3Y (100 pmol) on CTA extinction (Fig. 7d). The successful delivery of the peptide was validated by the fluorescein isothiocyanate (FITC) fluorescence in insular cortex (Fig. 7e). Mice that received Tat-3Y displayed a striking deficiency in CTA extinction, as compared with those that received vehicle alone or Tat-3A (Fig. 7f). This result supports the notion that insular LTD is necessary for CTA extinction. Together, our data demonstrate that ASIC1a in the insular cortex confers a particular form of synaptic plasticity, which underlies its selective engagement in CTA extinction (Supplementary Fig. 12).

**Discussion**

The foregoing data demonstrate that ASIC1a plays a critical role in insular LTD (Figs 1 and 2) and CTA extinction (Figs 4 and 5) through a mechanism that requires GSK3b signalling (Fig. 6). Multiple lines of evidence support a functional link between insular LTD and CTA extinction. First, in insular cortices of CTA-extinguished WT animals, AMPAR-mediated excitatory synaptic transmission was reduced and further LTD induction was severely hampered (Fig. 3). The reduced probability of LTD induction in insular cortical slices from CTA-extinguished mice could reflect an occlusion effect of already reduced synaptic strength or a decreased ASIC1a function due to channel desensitization, both of which might have occurred during CTA extinction.
extinction learning. Second, inhibiting AMPAR endocytosis (Fig. 7), a general LTD mechanism or GSK3β signalling, is critical for insular LTD (Fig. 6a–g) prevented CTA extinction. Third, disrupting ASIC1a not only inhibited CTA extinction, but also strongly decreased the probability of insular LTD induction, and both of these were rescued by expressing constitutively active GSK3β in insular neurons (Fig. 6i–m). Therefore, insular LTD, which is strongly dependent on ASIC1a and mediated via GSK3β, is involved in CTA memory extinction. The participation of a synaptic depression mechanism in insular cortex for CTA extinction is reminiscent of previous studies that implicate LTD synaptic depression mechanism in insular cortex for CTA GSK3β and both of these were rescued by expressing constitutively active GSK3β. Third, through not fully characterized molecular events, extinction is reminiscent of previous studies that implicate LTD synaptic depression mechanism in insular cortex for CTA GSK3β and both of these were rescued by expressing constitutively active GSK3β. Thus, the new synaptic role of ASIC1a revealed here will inspire additional studies to further elucidate mechanisms of ASIC1a regulation on synaptic plasticity in various brain areas.

Behaviourally, the ASIC1a-dependent insular LTD is involved in extinction learning of aversive taste memory. This novel mechanism fills a major gap in our knowledge about taste learning. Previously, NMDAR-mediated, experience-dependent modifications of synapse strength as well as transcriptional and translational machineries, at insular cortex, have been shown to be indispensable underpinnings for taste memory formation. However, the mechanism for CTA extinction was poorly understood. It was thought that insular cortex is important for subserving CTA extinction via a dissociable molecular machinery from CTA learning, as pharmacological inhibition of either protein synthesis or β-adrenergic receptors, but not NMDAR in insular cortex blocked CTA extinction. Our finding that ASIC1a selectively contributes to CTA extinction, but not acquisition or retention (Fig. 4) supports this notion and adds important new knowledge to the understanding of taste learning and memory.

Methods

Animals. Animal care and the experimental protocols were approved by the Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine, Shanghai, China (permit number: DLAS-MP-ANIMI01-05). To reduce the experimental variability, age-matched littermate pairs resulting from heterozygous crosses were used for all experiments. All behavioural measurements were performed in awake, unrestrained, mice (male, 2–3 months old, C57BL/6) in a sound- and light-attenuated, temperature-controlled environment. The conventional global ASIC1a KO mice were the generous gifts of Professor Michael J. Welsh (Howard Hughes Medical Institute, University of Iowa, Iowa City, IA, USA). The conditional ASIC1a-cre mice were graciously provided by Professor Cheng-Chang Lien (National Yang-Ming University, Taiwan). The CaMKII-Cre mice were kindly provided by Professor Joe Z. Tsien (Georgia Regents University, Augusta, GA, USA). All behavioural measurements were performed by raters blinded to the genotype and treatment. Three to four animals were housed per cage and maintained on a 12 h light/dark cycle with food and water ad libitum except where indicated otherwise. All experimental manipulations were performed during the light-on phase of the cycle in accordance with the institutional guidelines.

Unconditioned taste preferences. The unconditioned taste preferences were tested according to a previous report with modifications. Full details of unconditioned taste preferences are in Supplementary Methods Online.

CTA test. The CTA tests were performed as described previously and modifications, where the conditioned taste stimulus (sodium saccharin solution) was paired with an aversive US (LiCl injection; Supplementary Fig. 9a). Mice were adapted for ~1 week in individual cages, in which they had access to water and no sodium saccharin. On day 1, 5, 7, 14 and 28 after conditioning, the two-bottle choice test was administered to determine the degree of CTA acquisition and retention (Fig. 4a,b). Animals were given water (but no sodium saccharin) during the 30-min drinking
After the slice had been allowed to recover for 20 min, one of the 64 available planar microelectrodes was selected for stimulation by visual observation through a charge-coupled device camera (DP70; Olympus) connected to the inverted microscope (Fig. 1b). For test stimulation, monopolar, biphasic constant-current pulses (0.2 ms in duration) generated by the data acquisition software (MOBIUS, Precision Alpha-Med Sciences) were applied to the deep layer of the insular slice at 0.008 Hz. The iEPSPs evoked at the remaining 63 channels of the 64-channel amplifier, displayed on the monitor screen and stored on the hard disk of a microcomputer for offline analysis. After selection of the best stimulation site and stabilization of the baseline synaptic responses, an input–output curve was first determined using the measurements of the iEPSP slope or the number of activated channels (output) in a series of ascending stimulation intensities from 8 to 24 μA in 2 μA steps (input).

For LTP induction, after the baseline synaptic responses were stabilized for at least 1 h, a TBS protocol (10 bursts at 5 Hz, 4 pulses at 100 Hz for each burst) was given at a stimulation intensity that was adjusted to elicit 40–60% of the maximal responses. After TBS, the response was repeatedly delivered once every min for at least 3 h to allow long-term monitoring of insular LTP induction and maintenance. For LTD induction, the intensity of the test stimulus was adjusted to elicit 40–60% of the maximum response. Stable baseline responses were then recorded for at least 20 min before delivery of the LFS protocol (1 Hz, 900 pulses, with the same intensity) to the deep layer of the insular slice. After LFS, the test stimulus was repeatedly delivered every min for at least 1 h to monitor any change in LTD expression. For chemically evoked LTD, after stabilization of the baseline responses, DHPG (100 μM, Tocris Bioscience) was applied for 20 min through perfusion, which was followed by washout for at least 50 min. To test the effects of GSK3β inhibition on LTD induction, a specific GSK3β inhibitor (1 μM, SB-366791A) was applied from 20 min before the LFS protocol and sustained for a total duration of 35 min as indicated (Fig. 6d). To test the effects of blockade of GluA2 endocytosis on LTD induction, Tat-3Y or Tat-3A (both 1 μM, GL Biochem Ltd) was applied from 20 min before the LFS protocol and sustained for a total duration of 35 min as indicated (Fig. 7b). To test the effects of increasing pH buffering capacity on LTD induction, a well oxygenated (95% O2, 5% CO2, v/v) and highly-pH-buffering extracellular solution containing (in mM): 79 NaCl, 2.5 KCl, 10 g-glucose, 1 mgMgSO4, 2 CaCl2, 1 NaH2PO4, pH 7.35–7.45, was applied from 20 min before the LFS protocol and sustained for a total duration of 35 min as indicated (Supplementary Fig. 6).

All multi-channel electrophysiological data were analysed offline by the MED64 Modius software. For quantification of LTP and LTD data, the slope of iEPSPs was measured and normalized to that of the initial one and expressed, as percentage change from the baseline level. The numbers of activated channels (that is, with the iEPSP amplitude going over ~20 μV) versus LTD-like (iEPSP slope increased by at least 20% of baseline) channels were counted and the induction ratios of LTD/LTP calculated as follows:80,81 (number of LTD-like channels/number of all activated channels) x 100%.

**Generation of AAV vectors.** AAV vectors (pAAV-MCS) carrying the full length of WT or mutant (S2HIIE+AAA) mouse ASIC1a cDNA or the full length of WT or mutant (S9A) human GSK3β were constructed with the coding sequence of EYFP fused to the N terminus of ASIC1a or GSK3β using the ‘self-cleaving’ 2A-peptide, which can separate two proteins apart during the protein translation42. The transcription of the fusion protein EYFP-2A-ASIC1a, EYFP-2A-GSK3β or EYFP-2A-GSK3β-S9A was driven by human synapsin 1 (synapsin I) promoter to specifically limit the expression to neurons. The construct was then packaged into AAV2/8 chimeric virus with AAV8 capsids and AAV2 ITR (inverted terminal repeat) element. Once expressed in vivo, EYFP-2A and ASIC1a (or GSK3β) should be expressed separately and leave only a single protein residue of ASIC1a (or GSK3β). The control AAV vector (AAV-Ctrl) was constructed by sub-cloning the EGF-coding sequence to the pAAV-MCS vector following the synapsin I promoter. The similar strategy was used to construct the AAV vector for expression of Cre recombinase driven by the synapsin I promoter.

To make the shRNA constructs, oligonucleotides that contained 21-base sense and antisense sequences targeting mouse ASIC1a (GenBank accession: NM_000597.1, sense sequence, 5’-GGACATTCCAGGACTTGCATA-3’; antisense sequence, 5’-GTTCCTCCGACAGTGTCCTG-3’) were connected with a hairpin loop followed by a poly(T) termination signal. For initial testing of the efficacy of the shRNA, the full-length ASIC1a cDNA was transfected into cultured neurons using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. The transfected neurons were then observed at four different time points: 1 day and 2 days after transfection and lysed in the lysis buffer. The relative expression of the shRNA was determined by qRT-PCR.

**Preparation of multi-electrode probe.** A commercial 64-channel multistate recording system (MED64; Alpha-Med Sciences) was used for extracellular field potential recordings6. Full details are in Supplementary Methods Online.

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supernatants were collected for western blotting. Finally, the AAV vectors were injected bilaterally into the insular cortex. The stereotaxic coordinates according to the mouse brain atlas were: anteroposterior, +0.98 mm; lateral, ±3.55 mm; dorsalventral, and –3.60 mm. One injection (1 μl) was made on each side of the insular cortex using microelectrodes connected with a microinjector pump (KDS 310, KD Scientific) at a rate of 0.1 μl/min -1. Microelectrodes were left in situ for an additional 10 min to allow the injectant to diffuse. Mice were allowed to recover for 4 weeks before behavioural analysis and the injection sites were examined at the end of the experiments. Brain slices from animals injected with the viruses were examined directly using fluorescent microscopy.

Surgical procedures and drug microinjection. Animals anaesthetized with 1% pentobarbitosal sodium and placed in a stereotaxic apparatus (RWD Life Science) were implanted bilaterally with a 26-Gauge guide cannula aimed at 0.50 mm above the dorsalventral, and –3.60 mm. One injection (1 μl) was made on each side of the insular cortex using microelectrodes connected with a microinjector pump (KDS 310, KD Scientific) at a rate of 0.1 μl/min -1. Microelectrodes were left in situ for an additional 10 min to allow the injectant to diffuse. Mice were allowed to recover for 4 weeks before behavioural analysis and the injection sites were examined at the end of the experiments. Brain slices from animals injected with the viruses were examined directly using fluorescent microscopy.

Real-time reverse transcription PCR. Insular cortices of WT and ASIC1a KO mice were dissected and total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific). Four micrograms of total RNA were used as a template for cDNA synthesis and amplification with the SuperScript III First-stand Synthesis System (Thermo Fisher Scientific) according to the manufacturer’s instructions. Full details are in Supplementary Methods Online.

Preparation of PSD fractions. The purification of the PSD fraction was performed based on the previous studies with some modifications. Full details are in Supplementary Methods Online.

NMDA stimulation of insular cortical slices. NMDA stimulation of brain slices was performed as previously described with minor modifications. Insular cortical slices (300 μm) were incubated in ACSF for 1 h at 31 °C before NMDA application. One half of each slice was incubated with NMDA (50 μM) for 3 min at 31 °C, while the other half was incubated in ACSF without NMDA as the control. After incubation, the insular region from each slice was dissected and then homogenized in the lysis buffer on ice. The lysates were used for western blotting with a phospho-GSK3β antibody that recognizes phosphorylation at Ser-9 or the GSK3β activity assay (see below).

Western blotting. Protein samples from different regions of mouse brain, including insular cortex and other areas, were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride filters. The filters were incubated overnight at 4 °C with appropriate antibodies. Secondary antibodies conjugated to horseradish peroxidase were added to the filters and then visualized in ECL solution. The visualization was performed via the ImageQuant LAS 4000 mini Molecular Imaging System (GE Healthcare Life Sciences), and the Image J software (NIH) was used for the analysis of band intensity. Antibodies used were as follows: ASIC1α (1:500; Santa Cruz, sc-13905), β-actin (1:1,000; Chemicon, Cat # MAB1501), GAPDH (1:1,000; KangChen, Cat # KC-5G4), GluA1 (1:1,000; Epitomics, Cat # 3861-1), GluA2 (1:1,000; Epitomics, Cat # 3520-1), GluN1 (1:1,000; BD Systems, Cat # PS3011B), GluN2A (1:1,000; Millipore, Cat # 07-632), GluN2B (1:1,000; Millipore, Cat # MAB5220), PSD-95 (1:1,000; Epitomics, Cat # 2366-1), pGSK3β-Ser9/pGSK3α-Ser21 (1:1,000; Cell Signaling Technology, Cat # 8566) and GSK3β (1:1,000; Cell Signaling Technology, Cat # 12456).

GSK3β activity assay. The GSK3β Activity Assay Kit (GENMED) was used according to the manufacturer’s instructions. The principle of the colorimetric method is based on the activity of GSK3β to phosphorylate the target sequence GPHRSTPESRAA in the presence of ATP and then through reactions that involve pyruvate kinase and lactate dehydrogenase to oxidize NADH (reduced nicotinamide adenine dinucleotide) into NAD (nicotinamide adenine dinucleotide). The absorbance was read at 340 nm.

Reagents. Most of the drugs and chemicals used in these experiments were purchased from Sigma-Aldrich unless where indicated otherwise. The lysis buffer used for protein extraction contained 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 3 mM NaF, 1 mM β-glycerophosphate, 1 mM Na3VO4, and 10% glycerol, with protease inhibitors and phosphatase inhibitors.

Statistical analyses. No statistical methods were used to predetermined sample sizes, but the sample sizes used are similar to those generally employed in the field. The experiments were collected and processed randomly. All behavioural tests and analysis were blindly conducted. Variance was similar between the groups being statistically compared and data were normally distributed supporting the use of parametric statistics. Data were analysed with Student’s t-test or two-way repeated-measures analysis of variance, followed by Fisher’s least significant difference post hoc comparison, where appropriate. P < 0.05, represent significant differences. All summary values in the text and figures represent mean ± s.e.m. Data analyses were performed using SPSS statistical program version 10.0.

Data availability. The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information files or from the authors on request.

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Author contributions
T.-L.X., F.L., W.-G.L., M.-G.L. and M.X.Z. designed the project. W.-G.L., S.D. and J.D. performed behavioural testing and analysis. Y.-M.L., S.S. and J.D. performed Western blotting. T.T.H. generated the floxed ASIC1a mice. Y.L., S.S. and J.D. and T.-M.L. performed protein purification. C.L. and Y.M.L. performed enzymatic activity assay. M.-G.L. and W.-G.L. did electrophysiological recordings and analysis. W.-G.L. and M.-G.L. and T.-L.X. wrote the manuscript. All authors read and approved the final manuscript.

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