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Acid-sensing ion channel 1a is required for mGlu receptor dependent long-term depression in the hippocampus

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Running title: Role of ASIC1a in mGlu receptor-dependent LTD

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Abstract

Acid-sensing ion channels (ASICs), members of the degenerin/epithelial Na⁺ channel superfamily, are widely distributed in the mammalian nervous system. ASIC1a are highly permeable to Ca²⁺ and are thought to be important in a variety of physiological processes, including synaptic plasticity, learning and memory. To further understand the role of ASIC1a in synaptic transmission and plasticity, we investigated metabotropic glutamate (mGlu) receptor-dependent long-term depression (LTD) in the hippocampus. We found that ASIC1a channels mediate a component of LTD in P30-40 animals, since the ASIC1a selective blocker psalmotoxin-1 (PcTx1) reduced the magnitude of LTD induced by application of the group I mGlu receptor agonist (S)-3,5-Dihydroxyphenylglycine (DHPG) or induced by paired-pulse low frequency stimulation (PP-LFS). Conversely, PcTx1 did not affect LTD in P13-18 animals. We also provide evidence that ASIC1a is involved in group I mGlu receptor-induced increase in action potential firing. However, blockade of ASIC1a did not affect DHPG-induced polyphosphoinositide hydrolysis, suggesting the involvement of some other molecular partners in the functional crosstalk between ASIC1a and group I mGlu receptors. Notably, PcTx1 was able to prevent the increase in GluA1 S845 phosphorylation at the post-synaptic membrane induced by group I mGlu receptor activation. These findings suggest a novel function of ASIC1a channels in the regulation of group I mGlu receptor synaptic plasticity and intrinsic excitability.

Key words: ASIC, hippocampus, electrophysiology, LTD, mGlu receptors.

Abbreviations

aCSF, artificial cerebrospinal fluid; AP, action potential; ASIC, acid sensing ion channel; EPSC_{AMPA}, excitatory post-synaptic current AMPA-mediated; DRG, dorsal root ganglion; EPSC_{NMDA}, excitatory post-synaptic current NMDA-mediated; LTD, long-term depression; mGlu, metabotropic glutamate; PP-LFS, paired-pulse low frequency stimulation; PcTx1, psalmotoxin-1.

1. Introduction

Acid-sensing ion channels (ASICs) are cationic receptors, members of the degenerin/epithelial channel superfamily. In the central nervous system (CNS), ASIC channels are widely distributed in neurons, where they form homotrimeric and heterotrimeric complexes of ASIC1a, ASIC2a and ASIC2b subunits [1-4]. ASIC1a is the critical determinant of ASIC channel composition in the brain, which allows the entry of Na⁺ and Ca²⁺ into cells [3,5-7]. Several observations indicate that ASICs are located post-synaptically, especially on dendritic spines, although they can be found also on dendrites and cell bodies [1,8-10]. It has been shown that ASIC subunits interact with scaffolding proteins, such as post-synaptic density protein 95 and protein interacting with C-kinase-1 [10-12].

Several studies highlight a role of ASIC1a in different physiological conditions including synaptic plasticity, learning and memory, transmission of nerve impulses as well as in pathological settings such as ischemia, neuronal injury and epilepsy [8,13-15]. It has been demonstrated that ASICs are required for long-term potentiation (LTP) at cortico-basal lateral amygdala synapses, and are critical for associative fear learning and memory [16]. On the other hand, the role of ASICs in the hippocampus is still not clear [8,13,17,18] and may vary depending on the experimental conditions used. Recently, it was demonstrated that ASICs activity is potentiated by group I mGlu receptors in the peripheral nervous system [19]. In the light of these findings, we here investigated the role of ASIC1a in group I mGlu receptor-mediated synaptic plasticity in hippocampus.

In order to examine the role of ASIC1a in mGlu receptor-dependent LTD and intrinsic excitability at the hippocampal pyramidal neurons Schaffer collateral-CA1 synapse we used psalmotoxin-1 (PcTx1), a selective inhibitor of ASIC1a channels [12,20-22]. We have also investigated the underlying biochemical mechanisms involved in the interplay between group I mGlu receptors and ASIC1a.

2. Materials and methods

2.1. Slice preparation

All experiments followed international guidelines on the ethical use of animals from the EU Directive 2010/64/EU and the UK Animals (Scientific Procedures) Act 1986. C57BL6/J mice (13-18

or 30-40 days old) were killed by decapitation and brains were rapidly dissected out and parasagittal hippocampal slices (250-400 μm) were cut with a vibratome (VT 1200S, Leica) in cold (0°C) artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 124; KCl 3; MgSO_4 1; CaCl_2 2 H_2PO_4 ; Na 1.25; NaHCO_3 26; glucose 10; saturated with 95% O_2 , 5% CO_2 (pH 7.4), and left to recover for 1 h in ACSF at $30 \pm 2^\circ\text{C}$.

2.2. Electrophysiology

Individual slices were placed in a recording chamber, on the stage of an upright microscope (Zeiss, Germany) and submerged in a continuously flowing (3 ml/min) solution at 28°C ($\pm 0.2^\circ\text{C}$). Individual neurons were visualized through a 40 $\times$ water-immersion objective (Olympus, Japan) connected to infrared video microscopy (Hamamatsu, Japan). Borosilicate glass electrodes (3–7 $\text{M}\Omega$), pulled with a PP 83 Narishige puller, were filled with a solution containing the following (in mM): CsCH_3SO_3 115; CsCl 10; KCl 10; CaCl_2 0.45; EGTA 1; Hepes 10; QX-314 5; $\text{Na}_3\text{-GTP}$ 0.3; Mg-ATP 4.0; pH adjusted to 7.3 with CsOH or K-Gluconate 135; KCl 10; MgCl_2 2; CaCl_2 0.05; EGTA 0.1; HEPES 10; Mg-ATP 4; Na-GTP 0.3, pH 7.3 with KOH.

Whole-cell voltage clamp (at -70 mV holding potential) or current clamp experiments were carried out with a MultiClamp 700B or Axopatch 200B amplifier (Axon Instruments, Foster City, CA), filtered at 1kHz and digitized (10kHz). The excitatory post-synaptic currents (EPSCs) were elicited at 0.033 Hz with a glass pipette filled with aCSF, placed in CA1 *stratum radiatum* to stimulate Schaffer collateral fibers. EPSC_{AMPA} and EPSC_{NMDAR} were obtained in presence of GABA_A (Picrotoxin 100 μM) and NMDA (MK-801 10 μM) or AMPA (CNQX 10 μM) receptor antagonists, respectively. The mGlu receptor-LTD was induced by bath-application of DHPG 50 μM for 10 min or by a paired-pulse low frequency stimulation protocol (PP-LFS: 900 pulses, 15 min) of Schaffer collateral fibers.

Action potential firing was studied in the presence of GABA_A, AMPA and NMDA receptor antagonist, using injection of a constant current pulse (300 ms; typically 350 pA) that induced steady-state depolarization and maintained regular firing of action potentials (APs) throughout the pulse.

2.3. Measurement of polyphosphoinositide hydrolysis in hippocampal slices

Group I mGLU receptor-stimulated polyphosphoinositide (PI) hydrolysis was measured in hippocampal slices obtained from C57BL6/J male mice at 40 days of age. Hippocampus was

dissected out and incubated at 37 °C under constant oxygenation for 30-45 min in Krebs-Henseleit (Sigma-Aldrich, Italy) buffer equilibrated with 95% O₂, 5% CO₂ to pH 7.4. Thirty μ l of gravity packed slices were then incubated for 60 min in 250 μ l buffer containing 1 μ Ci of myo-[3H]-inositol. Slices were incubated with LiCl (10 mM, for 10 min) followed by DHPG (200 μ M) in the presence or absence of PcTx1 (100 ng/ml). After one hour, slices were washed with ice-cold buffer and the incubation was stopped by the addition of 900 μ l of methanol:chloroform (2:1). After further addition of 300 μ l chloroform and 600 μ l water, the samples were centrifuged at low speed to facilitate phase separation. The upper aqueous phase was loaded into Dowex 1-X-8 columns for the separation of [3H]-InsP. Columns were washed twice with water, once with a solution of sodium tetraborate (5 mM) and sodium formate (40 mM) to elute cyclic InsP and glycerophosphoinositols, and then with ammonium formate (200 mM) and formic acid (100 mM) for the elution of [3H]-InsP [23]. Values are expressed as the ratio between the radioactivity measured in the [3H]InsP fraction and the protein content. Proteins were quantified by the Lowry method [24].

2.4. Western blotting

Western blot analysis was performed in hippocampal tissue from parasagittal slices obtained using standard methods. For each experiment (four independent experiments), four early adult mice (P30-40) were used and slices were equally distributed for each experimental condition. After recovery from cutting procedure, parasagittal hippocampal slices were kept in a holding chamber containing aCSF, saturated with 95% O₂ -5% CO₂ at 28 °C ($\pm$ 0.2 °C) and known concentration drugs: DHPG (50 μ M, 10 min), DHPG+PcTx1 (PcTx1 100 ng/ml, 10 min followed by PcTx1 plus DHPG 50 μ M, 10 min), PcTx1 (100ng/ml, 20 min), DHPG+PcTx1+MPEP (MPEP 10 μ M+PcTx1 100ng/ml, 10 min followed by MPEP+PcTx1+DHPG, 10 min) DHPG+PcTx1+JNJ (JNJ 10 μ M+PcTx1 100ng/ml, 10 min followed by JNJ+PcTx1+DHPG, 10 min) all experimental conditions including picrotoxin (100 μ M) and MK-801 (10 μ M).

Biochemical subcellular fractionation was performed accordingly to the protocol reported in [25]. Hippocampal slices were homogenized in 0.32 M ice-cold sucrose at pH 7.4 containing (in mM) 1 HEPES, 1 MgCl₂, 1 EDTA, 1NaHCO₃, and 0.1 PMSF, complete set of phosphatases inhibitors (Serva, Germany) and proteases inhibitors (Serva, Germany).

The homogenized tissue was centrifuged at 1000 x g for 10 min, in order to discard nuclei-associated membranes in the pellet. The supernatant was centrifuged at 3000 x g for 15 min to

obtain a crude membrane fraction, subsequently re-suspended in 1 mM HEPES containing phosphatases and proteases inhibitors and centrifuged at 50.000 rpm for 1 h.

The pellet obtained was re-suspended in a buffer containing 75mM KCl and 1% Triton X-100, and again centrifuged at 50.000 rpm for 1 h. The resulting supernatant is referred to as Triton soluble fraction (TSF), whereas the final pellet re-suspended in 20 mM HEPES plus phosphatases and proteases inhibitors is the Triton Insoluble Fraction (TIF).

As previously reported [25], TIF has been used instead of the classical post-synaptic density (PSD) because the amount of the starting material was very limited.

Equal amount of TIF proteins (~15 µg for each condition) were resolved by 10% SDS-polyacrylamide gels and blotted onto PVDF membrane (Serva, Germany). The resulting blot was blocked for 1 h at room temperature using Tris-buffered saline-Tween (t-TBS) (M) Tris, 0,02; NaCl, 0,15; Tween 20, 0,1%) containing 5% skimmed milk. The membranes were then incubated overnight at 4°C with specific antibodies: rabbit anti-GluA1 1:1000 (Signalway Antibody, USA), rabbit anti-p-GluA1 (Ser845) 1:1000 (Cell Signaling, USA), according to the manufacturer's protocol. After 50 min washing in t-TBS, the blots were incubated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit IgG secondary antibodies 1:3000 (UCS Diagnostic). After 50 min rinsing in t-TBS, immunoreactive bands were detected by enhanced chemiluminescence (ECL; WESTAR, Cyanagen, Italy).

2.5. Statistical analysis

Electrophysiological and biochemical data are represented as mean values $\pm$ S.E.M. Statistical significance was evaluated by Student's t-test paired or unpaired between last 10 min following delivery of the conditioning protocol. *n* represents the number of neurons and an average of 2 neurons per animal was used. Polyphosphoinositide hydrolysis was evaluated by One-Way ANOVA + Fisher's PLSD test. Statistical analysis for western blot experiments was performed with GraphPad PRISM 4 (GraphPad Software, USA). *n* represents the number of experiments. One-way ANOVA followed by Newman-Keuls Multiple Comparison Test was carried out when intergroup comparisons were required. Statistical significance was set at $p < 0.05$.

2.5. Drugs

6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (S)-3,5-Dihydroxyphenylglycine (DHPG), dizocilpine (MK-801), picrotoxin, JNJ16259685 and MPEP were purchased from Sigma-Aldrich (Milan, Italy);

psalmotoxin-1 (PcTx1) was purchased from Hello Bio (Bristol, UK) or Alomone Labs (Israel). When drugs were dissolved in DMSO, the final concentration of DMSO did not exceed 0.5%. [³H]-Myo-inositol (18 Ci/mmol) was purchased from Perkin Elmer (Milan, Italy).

3. Results

3.1. ASIC1a are involved in mGlu receptor-dependent LTD of EPSC_{AMPA} in early adult animals

Activity-dependent LTD of EPSC_{AMPA} and EPSC_{NMDA} that relies on mGlu receptors has been described at Schaffer collateral-CA1 synapse [26-28]. Here, using whole-cell patch clamp recordings, we confirm previous data showing that mGlu-LTD of both EPSC_{AMPA} and EPSC_{NMDA} transmission can be induced pharmacologically at CA1 hippocampal synapses. Accordingly, in juvenile (P13-P18) C57BL6/J mice, bath-applied DHPG (50 μ M, 10 min) produced LTD of both EPSC_{AMPA} ($64 \pm 10\%$; $n=9$; Fig. 1A₁) and EPSC_{NMDA} ($57 \pm 5\%$; $n=4$; Fig. 1A₂). Both forms of synaptic plasticity were insensitive to the effects of the ASIC1a blocker PcTx1 (100 ng/ml). In fact, LTD of EPSC_{AMPA} was $59 \pm 1\%$ ($n=7$, $p>0.05$; Fig. 1A₁) and LTD of EPSC_{NMDA} was $65 \pm 4\%$ ($n=4$, $p>0.05$; Fig. 1A₂).

Since a developmental switch occurs in the synaptic mechanisms underlying hippocampal mGlu-LTD [29], we performed experiments in slices obtained from early adult mice. Differently to what observed in juvenile mice, in slices obtained from early adult mice (P30-P40), PcTx1 was able to reduce, in a concentration dependent manner, the mGlu-LTD of EPSC_{AMPA} induced by bath-application of DHPG (control $60 \pm 5\%$, $n=9$ vs. PcTx1 $26 \pm 5\%$, $n=10$, $p<0.001$; Fig. 2A₁), while no change was observed for the mGlu-LTD of EPSC_{NMDA} (control $57 \pm 14\%$, $n=4$ vs. PcTx1 $52 \pm 7\%$, $n=4$, $p>0.05$; Fig. 2A₂). Notably PcTx1 did not impact pre-established mGlu-LTD of EPSC_{AMPA} (control $60 \pm 5\%$, $n=9$ vs. PcTx1 $57 \pm 7\%$, $n=6$, $p>0.05$; Fig. 2B), suggesting that ASIC1a impairs mGlu receptor function *per se*, thereby affecting mGluR-mediated LTD maintenance signaling. Furthermore, we tested the ability of PcTx1 to affect activity-dependent mGlu-LTD of EPSC_{AMPA} induced by PP-LFS [24]. Notably, PcTx1 was able to decrease also this form of synaptic plasticity (control $43 \pm 5\%$, $n=7$ vs. PcTx1, $14 \pm 5\%$, $n=9$, $p<0.001$; Fig. 2C). To confirm the role of ASIC1a in regulating the mGlu-LTD of EPSC_{AMPA} in early adult mice, we performed recordings using the non-selective ASIC blocker amiloride (100 μ M). Similarly to PcTx1, bath-application of amiloride was able to significantly reduce the mGlu-LTD of EPSC_{AMPA} (control $60 \pm 5\%$, $n=9$ vs. amiloride $25 \pm$

8%, $n=9$, $p<0.001$; Fig. 2D₁), while no change was observed for the mGlu-LTD of EPSC_{NMDA} (control $57 \pm 14\%$, $n=4$ vs. PcTx1 $54 \pm 10\%$, $n=5$, $p>0.05$; Fig. 2D₂).

Overall these data suggest that ASIC1a are required for mGlu-LTD of EPSC_{AMPA} in the hippocampus of early adult mice.

3.2. ASIC1a are implicated in the DHPG-mediated increase of intrinsic excitability of CA1 pyramidal neurons

Besides the involvement of group I mGlu receptors in hippocampal LTD, activation of these receptors increases excitability of CA1 pyramidal neurons [30]. Thus, we extended our analysis investigating the potential contribution of ASIC1a in modulating DHPG-mediated increase of firing discharge induced by depolarizing current steps [28]. Figure 3A shows representative trace recordings of the firing frequency induced by different concentrations of PcTx1, bath-applied for 20 min. In agreement with recent observations [31], PcTx1 was able to reduce in a dose-dependent manner the number of APs evoked by a 300 ms current pulse of 350 pA (control $n=6$ vs. PcTx1 30 ng/ml $n=6$, $p>0.05$; control $n=6$ vs. PcTx1 100 ng/ml $n=6$, $p<0.001$; control $n=6$ vs. PcTx1 300 ng/ml $n=6$, $p<0.001$) (Fig. 3A). Next, we studied the effect of PcTx1 on the enhancement of firing discharge induced by group I mGlu receptors activation [28,31,32]. Accordingly, bath-applied DHPG (50 μ M, 10 min) increases the number of APs evoked by depolarizing current pulses ($n=7$, 350 pA $p<0.05$; 50 pA $p<0.05$; -250 pA $p>0.05$) and this leads also to a significant enhancement of total postsynaptic depolarization as measured by the area under the curve ($n=7$, 350 pA $p<0.05$; 50 pA $p<0.01$; -250 pA $p>0.05$). Of note, to unmask a possible link between ASIC1a and mGlu receptors, we applied PcTx1 at 30 ng/ml, a concentration that was still able to reduce DHPG-LTD on the one hand, and was ineffective in modulating firing discharge induced by depolarization alone on the other. Under this condition, PcTx1 was able to reverse the DHPG-mediated increase of firing ($n=6$, 350 pA $p<0.05$; 50 pA, $p<0.05$; -250 pA $p>0.05$) and of area under the curve ($n=6$, 350 pA $p<0.05$; 50 pA $p<0.05$; -250 pA $p>0.05$) (Fig. 3B).

Collectively, these data suggest that at least some actions of group I mGlu may occur through ASIC1a in the hippocampus of early adult mice.

3.3. PcTx1 does not affect polyphosphoinositide hydrolysis but prevents GluA1 phosphorylation induced by DHPG

In light of the functional crosstalk between group I mGlu receptors and ASIC1a, we next examined the effects of DHPG alone or in the presence of PcTx1 100 ng/ml, on the enhancement of PI hydrolysis in hippocampal slices from early adult mice. PI hydrolysis represents the canonical signal transduction pathway activated by mGlu1/5 receptors [33]. As expected, addition of DHPG to hippocampal slices increased [3H]InsP formation by about 2-fold compared to control (control n=3 vs. DHPG n=3; $p<0.05$). The increase in [3H]-InsP accumulation was not affected by incubation with PcTx1 (control PcTx1 n=4 vs. DHPG+ PcTx1 n=4; $p<0.05$ (Fig. 4A) and no change was observed in basal [3H]InsP level ($p>0.05$), suggesting that some other mechanism underlies the functional crosstalk between ASIC1a and group I mGlu receptors.

To investigate whether the reduction of mGlu-LTD of EPSC_{AMPA} caused by ASIC1a blockade in pyramidal neurons is dependent on reduction of AMPA receptor internalization, we performed biochemical experiments to measure the phosphorylation of AMPA-GluA1 receptor subunit at the post-synaptic site in hippocampal slices treated with DHPG, DHPG + PcTx1 or PcTx1 alone. Using a well-established post-synaptic-enriched biochemical preparation [25], we isolated the triton insoluble fraction from pharmacological treated brain slices. In line with previous results [34], we found that the levels of p-GluA1/GluA1 ratio were significantly increased upon DHPG treatment compared to control (control n=4 vs. DHPG n=4; $p<0.01$) (Fig. 4B). PcTx1 was able to reverse the DHPG-mediated increase in GluA1/GluA1 ratio (DHPG n=4 vs. DHPG+PcTx1 n=4; $p<0.05$ (Fig. 4B). Moreover, to discriminate between group I mGlu receptor subtypes, mGlu1 or mGlu5 receptor antagonists were added to DHPG+PcTx1. The p-GluA1/GluA1 ratio was significantly reduced in slices treated either with the mGlu5 antagonist MPEP (DHPG n=4 vs. DHPG+PcTx1+MPEP n=4; $p<0.05$), or with the mGlu1 antagonist JNJ (DHPG n=4 vs DHPG+PcTx1+JNJ n=4; $p<0.05$) compared to DHPG treatment alone (Fig. 4C).

Altogether, these results demonstrate ASIC1a is able to regulate post-synaptic AMPA-GluA1 subunit phosphorylation, suggesting that a functional interaction between ASIC1a and AMPA receptors might occur during mGlu-LTD.

4. Discussion

The idea that protons can function as a neurotransmitter has existed for some time [35-37], however the precise role of ASICs in synaptic transmission and neuroplasticity remains still elusive [16,37-39]. In fact, it has been suggested that ASIC1a may contribute to certain forms of

synaptic plasticity in the hippocampus, although results are controversial [8,17,18]. Here we show for the first time that ASIC1a plays a critical role in mGlu receptor-dependent LTD of AMPA receptor-mediated transmission in CA1 pyramidal neurons of early adult mice. A recent study demonstrated that PcTx1 did not affect hippocampal LTD induced by low-frequency electrical stimulation or (RS)-3,5-DHPG. The difference between this and our study might rely on experimental variables including electrophysiological approach used (multi-electrode array vs. patch-clamp recordings); animal age (>40 days old vs. ≤P40 days old); temperature (30°C vs. 28°C) concentration of PcTx1 (100 nM vs. 100 ng/ml); and use of different enantiomer, concentration (100 μM vs. 50 μM) and time of perfusion (20 min vs. 10 min) of DHPG. Finally, differently from Liu and colleagues [18], we have investigated different components of excitatory transmission following the pharmacological isolation of NMDA or AMPA receptor component. In agreement with this, experiments showing the contribution of ASIC1a to mGlu-LTD have been observed only in early adult mice, a time in which a post-synaptic mechanism of expression of mGlu-LTD in mature spines has been suggested [40]. On the other hand, immature spines which lack ASICs [41], typically express a pre-synaptic form of LTD [40].

In addition, we have examined the effect of PcTx1 on the DHPG-mediated increase in membrane excitability. We show that ASIC1a are implicated in the mGlu receptor-mediated excitability of CA1 pyramidal neurons, further demonstrating that a functional crosstalk between ASIC1a and group I mGlu receptors occurs, though the mechanisms remain to be elucidated. We then extended the analysis to agonist-stimulated PI hydrolysis, which represents the classical signal transduction pathway activated by group I mGlu receptors [33]. Although PcTx1-treated hippocampal slices expressed similar [3H]InsP formation following DHPG application, it is possible that the effect here observed might be anatomically restricted to area CA1 thus too small to be detected by immunoblot analysis. Moreover, there is growing evidence that a variety of biased signaling modalities might affect GPCR signaling including group I mGlu receptors [42,43]. In particular, canonical mGlu5 receptor signaling might be influenced by ligand bias, location bias and cell type bias, as well as by age-related alterations and differences in receptor isoforms and formation of multimeric complexes with other GPCRs [44]. Also, there is evidence that mGlu1/5 receptors may trigger both PLC-dependent and independent changes in intracellular calcium which in turn may regulate mGlu-LTD through calmodulin and calmodulin-dependent kinases [45].

Whether the ASIC-mediated depolarization enhances the effect of mGlu receptors or rather

mGlu receptors regulate an H⁺-related process still needs to be explored. In this line, several conductance mechanisms contributing to group I mGlu receptor-mediated depolarization in CA1 pyramidal neurons have been previously described [46-51].

Noteworthy, here we show that ASIC1a regulates post-synaptic AMPA-GluA1 subunit phosphorylation following mGlu-LTD, suggesting that a functional interaction between ASIC1a and AMPA receptors might occur in specific forms of synaptic plasticity.

Overall, data here presented further confirm that ASICs can mediate proton signaling at synaptic sites [52] and reveal for the first time a link between ASIC1a channels and mGlu receptors. Of note, recent evidence published by Gan and colleagues [19] demonstrated a crosstalk between group I mGlu receptor and ASIC3 in dorsal root ganglion (DRG) neurons. In particular, group I mGluRs activation of nociceptive DRG neurons is able to sensitize ASICs hence contributing to acidosis-evoked pain. Therefore, in light of this novel peripheral mechanism, it is reasonable to assume that this interplay might occur also at central synapses possibly regulating synaptic plasticity events.

Further clarification of the molecular mechanisms underlying the interaction between these two pathways would add a new dimension to the role of ASICs in synaptic function under different conditions. On the one hand, this study highlights a novel role of ASICs in hippocampal synaptic plasticity and in cellular responses to mGlu receptors activation, opening a stream of investigation on their possible role in regulating cognitive processes; on the other it might shed new light on the possible contribution of these channels to pathological events at the synapse. Accordingly, several reports have previously shown the involvement of ASICs in ischemic neuronal injury, traumatic neuronal multiple sclerosis and Parkinson's disease [52]. Therefore, ASICs are emerging as potential therapeutic targets for the treatment of several brain disorders [53]. Future studies are required to understand whether similar coupling between ASICs and mGlu receptors is likely to contribute to synaptopathology as well.

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References

- [1] García-Añoveros J, Derfler B, Neville-Golden J, Hyman BT, Corey DP. BNaC1 and BNaC2 constitute a new family of human neuronal sodium channels related to degenerins and epithelial sodium channels. *Proc. Natl. Acad. Sci. USA* 1997; 94:1459-1464.
- [2] Price MP, Snyder PM, Welsh MJ. Cloning and expression of a novel human brain Nap channel. *J. Biol. Chem.* 1996; 271, 7879e7882.
- [3] Waldmann R, Champigny G, Bassilana F, Heurteaux C, Lazdunski M.. A proton-gated cation channel involved in acid-sensing. *Nature* 1997; 386:173-177.
- [4] Jasti J, Furukawa H, Gonzales EB, Gouaux E. Structure of acid-sensing ion channel 1 at 1.9 Å resolution and low pH. *Nature* 2007; 449, 316e323.
- [5] Wu LJ, Duan B, Mei YD, Gao J, Chen JG, Zhuo M, Xu L, Wu M, Xu TL. Characterization of acid-sensing ion channels in dorsal horn neurons of rat spinal cord. *J. Biol. Chem.* 2004; 279:43716-43724.
- [6] Yermolaieva O, Leonard AS, Schnizler MK, Abboud FM, Welsh MJ. Extracellular acidosis increases neuronal cell calcium by activating acid-sensing ion channel 1a. *Proc. Natl. Acad. Sci. USA* 2004; 101:6752-6757.
- [7] Sherwood TW, Lee KG, Gormley MG, Askwith CC. Heteromeric acid-sensing ion channels (ASICs) composed of ASIC2b and ASIC1a display novel channel properties and contribute to acidosis-induced neuronal death. *J Neurosci.* 2011; 31:9723-9734.
- [8] Wemmie JA, Chen J, Askwith CC, Hruska-Hageman AM, Price MP, Nolan BC, Yoder PG, Lamani E, Hoshi T, Freeman JH Jr, Welsh MJ. The acid-activated ion channel ASIC contributes to synaptic plasticity, learning, and memory. *Neuron* 2002; 34:463-77.
- [9] Zha XM, Wemmie JA, Green SH, Welsh MJ. Acid-sensing ion channel 1a is a postsynaptic proton receptor that affects the density of dendritic spines. *Proc Natl Acad Sci U S A.* 2006; 103:16556-61.
- [10] Zha XM, Costa V, Harding AM, Reznikov L, Benson CJ, Welsh MJ. ASIC2 subunits target acid-sensing ion channels to the synapse via an association with PSD-95. *J Neurosci.* 2009; 29:8438-46.
- [11] Hruska-Hageman AM, Wemmie JA, Price MP, Welsh MJ. Interaction of the synaptic protein PICK1 (protein interacting with C kinase 1) with the non-voltage gated sodium channels BNC1 (brain Na⁺ channel 1) and ASIC (acid-sensing ion channel). *Biochem J.* 2002; 361:443-50.

- [12] Baron A, Deval E, Salinas M, Lingueglia E, Voilley N, Lazdunski M. Protein kinase C stimulates the acid-sensing ion channel ASIC2a via the PDZ domain-containing protein PICK1. *J Biol Chem.* 2002; 277:50463-8.
- [13] Wemmie JA, Askwith CC, Lamani E, Cassell MD, Freeman J Jr, Welsh MJ. Acid-sensing ion channel 1 is localized in brain regions with high synaptic density and contributes to fear conditioning. *J Neurosci.* 2003; 23: 5496-502.
- [14] Gao J, Duan B, Wang DG, Deng XH, Zhang GY, Xu L, Xu TL.. Coupling between NMDA receptor and acid-sensing ion channel contributes to ischemic neuronal death. *Neuron* 2005; 48:635-46.
- [15] Ziemann AE, Schnizler MK, Albert GW, Severson MA, Howard MA, Welsh MJ, Wemmie J A. Seizure termination by acidosis depends on ASIC1a. *Nat. Neurosci.* 2008; 11: 816-822.
- [16] Du J, Reznikov LR, Price MP, Zha XM, Lu Y, Moninger TO, Wemmie . JA, Welsh MJ. Protons are a neurotransmitter that regulates synaptic plasticity in the lateral amygdala. *Proc Natl Acad Sci U S A.* 2014; 111: 8961-6.
- [17] Wu PY, Huang YY, Chen CC, Hsu TT, Lin YC, Weng JY, Chien TC, Cheng IH, Lien CC. Acid-sensing ion channel-1a is not required for normal hippocampal LTP and spatial memory. *J Neurosci.* 2013; 33:1828-32.
- [18] Liu MG, Li HS, Li WG, Wu YJ, Deng SN, Huang C, Maximyuk O, Sukach V, Krishtal O, Zhu MX, Xu TL. Acid-sensing ion channel 1a contributes to hippocampal LTP inducibility through multiple mechanisms. *Sci Rep.* 2016; 6, 23350.
- [19] Gan X, Wu J, Ren C, Qiu CY, Li YK, Hu WP. Potentiation of acid-sensing ion channel activity by peripheral group I metabotropic glutamate receptor signaling. *Pharmacol Res.* 2016; 107:19-26.
- [20] Escoubas P, Bernard C, Lambeau G, Lazdunski M, Darbon H.. Recombinant production and solution structure of PcTx1, the specific peptide inhibitor of ASIC1a proton-gated cation channels. *Protein Sci.* 2003; 12:1332-43.
- [21] Chen X, Kalbacher H, Gründer S. The tarantula toxin psalmotoxin 1 inhibits acid-sensing ion channel (ASIC) 1a by increasing its apparent H⁺ affinity. *J Gen Physiol.* 2005; 126:71-9.
- [22] Zambelli VO, Pasqualoto KFM, Picolo G,. Chudzinski-Tavassi AM, Cury Y. Harnessing the knowledge of animal toxins to generate drugs. Review Article *Pharmacol Res.* 2016; In Press.
- [23] Nicoletti F, Iadarola ML, Wroblewski JT, Costa E. Excitatory amino acid recognition sites coupled with inositol phospholipid metabolism: Developmental changes and interaction with α -adrenoceptors, *Proc Natl Acad Sci USA* 1986; 83:1931-1935.
- [24] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol

reagent. *J Biol Chem.* 1951; 193:265-75.

[25] Gardoni F, Picconi B, Ghiglieri V, Polli F, Bagetta V, Bernardi G, Cattabeni F, Di Luca M, Calabresi P. A critical interaction between NR2B and MAGUK in L-DOPA induced dyskinesia. *J Neurosci.* 2006; 11:2914-22.

[26] Kemp N, Bashir ZI. NMDA receptor-dependent and -independent long-term depression in the CA1 region of the adult rat hippocampus in vitro. *Neuropharmacology* 1997; 36:397-9.

[27] Bhouri M, Farrow PA, Motee A, Yan X, Battaglia G, Di Menna L, Riozzi B, Nicoletti F, Fitzjohn SM, Bashir ZI. mGlu1 receptor-induced LTD of NMDA receptor transmission selectively at Schaffer collateral-CA1 synapses mediates metaplasticity. *J Neurosci.* 2014.; 34:12223-9.

[28] Davies CH, Clarke VR, Jane DE, Collingridge GL. Pharmacology of postsynaptic metabotropic glutamate receptors in rat hippocampal CA1 pyramidal neurones. *Br J Pharmacol.* 1995; 116: 1859-69.

[29] Nosyreva ED, Huber KM. Developmental Switch in Synaptic Mechanisms of Hippocampal Metabotropic Glutamate Receptor-Dependent Long-Term Depression. *J Neurosci.* 2005; 25:2992–3001.

[30] Gereau RW, Conn PJ. Roles of specific metabotropic glutamate receptor subtypes in regulation of hippocampal CA1 pyramidal cell excitability. *J Neurophysiol.* 1995; 74:122-9.

[31] Mango D, Barbato G, Piccirilli S, Panico MB, Feligioni M, Schepisi C, Graziani M, Porrini V, Benarese M, Lanzillotta A, Pizzi M, Pieraccini S, Sironi M, Blandini F, Nicoletti F, Mercuri NB, Imbimbo BP, Nisticò R. Electrophysiological and metabolic effects of CHF5074 in the hippocampus: protection against in vitro ischemia. *Pharmacol Res.* 2014; 81:83-90.

[32] Chuang SC, Zhao W, Young SR, Conquet F, Bianchi R, Wong RK. Activation of group I mGluRs elicits different responses in murine CA1 and CA3 pyramidal cells. *J Physiol.* 2002; 541:113-21.

[33] Ireland, DR, Abraham WC. Group I mGluRs increase excitability of hippocampal CA1 pyramidal neurons by a PLC-independent mechanism. *J Neurophysiol.* 2002; 88:107-16.

[34] Delgado JY, O'dell TJ. Long-term potentiation persists in an occult state following mGluR-dependent depotentiation. *Neuropharmacology* 2005; 48:936-48.

[35] Nicoletti F, Bockaert J, Collingridge GL, Conn PJ, Ferraguti F, Schoepp DD, Wroblewski JT, Pin JP. Metabotropic glutamate receptors: from the workbench to the bedside. *Neuropharmacology* 2011; 60:1017-41.

[36] Caldwell L, Harries P, Sydlik S, Schwiening CJ. Presynaptic pH and vesicle fusion in *Drosophila* larvae neurones. *Synapse* 2013; 67: 729-40.

- [37] Wang TM, Holzhausen LC, Kramer RH. Imaging an optogenetic pH sensor reveals that protons mediate lateral inhibition in the retina. *Nat Neurosci.* 2014; 17:262-8.
- [38] Highstein SM, Holstein GR, Mann MA, Rabbitt RD. Evidence that protons act as neurotransmitters at vestibular hair cell-calyx afferent synapses. *Proc. Natl. Acad. Sci. U. S. A.* 2014; 111, 5421e5426.
- [39] Beg AA, Ernstrom GG, Nix P, Davis MW, Jorgensen EM. Protons act as a transmitter for muscle contraction in *C. elegans*. *Cell* 2008; 132, 149e160.
- [40] Kreple CJ, Lu Y, Taugher RJ, Schwager-Gutman AL, Du J, Stump M, Wang Y, Ghobbeh A, Fan R, Cosme CV, Sowers LP, Welsh MJ, Radley JJ, La Lumiere RT, Wemmie JA. Acid-sensing ion channels contribute to synaptic transmission and inhibit cocaine-evoked plasticity. *Nat Neurosci.* 2014; 8: 1083-91.
- [41] Alvarez de la Rosa D, Krueger SR, Kolar A, Shao D, Fitzsimonds RM, Canessa CM. Distribution, subcellular localization and ontogeny of ASIC1 in the mammalian central nervous system. *J Physiol.* 2003; 546:77-87.
- [42] Dhami GK, Ferguson SS. Regulation of metabotropic glutamate receptor signaling, desensitization and endocytosis. *Pharmacol Ther.* 2006; 111:260-71.
- [43] Pupo AS, Duarte DA, Lima V, Teixeira LB, Parreiras-e-Silva LT, Costa-Neto CM. Recent updates on GPCR biased agonism Review Article *Pharmacol Res.* 2016; In Press
- [44] Jong YJ, Sergin I, Purgert CA, O'Malley KL. Location-dependent signaling of the group 1 metabotropic glutamate receptor mGlu5. *Mol Pharmacol.* 2014; 86:774-85.
- [45] Sethna F, Zhang M, Kaphzan H, Klann E, Autio D, Cox CL, Wang H. Calmodulin activity regulates group I metabotropic glutamate receptor-mediated signal transduction and synaptic depression. *J Neurosci Res.* 2016; 94: 401-8.
- [46] Charpak S, Gähwiler BH, Do KQ, Knöpfel T. Potassium conductances in hippocampal neurons blocked by excitatory amino acid transmitters. *Nature* 1990; 347:765–767.
- [47] Guérineau NC, Gähwiler BH, Gerber U. Reduction of resting K⁺ current by metabotropic glutamate and muscarinic receptors in rat CA3 cells: mediation by G-proteins. *Journal of Physiology* 1994; 474: 27-33.
- [48] Pozzo-Miller ID, Petrozzino JJ, Connor JA. G protein-coupled receptors mediate a fast excitatory postsynaptic current in CA3 pyramidal neurons in hippocampal slices. *J Neurosci* 1995; 15:8320–8330.
- [49] Congar P, Leinekugel X, Ben-ari Y, Crépel V. A long- lasting calcium-activated nonselective

cationic current is generated by synaptic stimulation or exogenous activation of group I metabotropic glutamate receptors in CA1 pyramidal neurons. *J Neurosci.* 1997; 17:5366–5379.

[50] Chuang SC, Bianchi R, Wong RKS. Group I mGluR activation turns on a voltage-dependent inward current in hippocampal pyramidal cells. *J Neurophysiology* 2000; 83:2844–2853.

[51] Chuang SC, Bianchi R, Kim D, Shin HS, Wong RKS.. Group I metabotropic glutamate receptors elicit epileptiform discharges in the hippocampus through PLC β 1 signaling. *J Neurosci.* 2001; 21: 6387–6394.

[52] Huang Y, Jiang N, Li J, Ji YH, Xiong ZG, Zha XM. Two aspects of ASIC function: Synaptic plasticity and neuronal injury. *Neuropharmacology* 2015; 94:42-8.

[53] Radu BM, Banciu A, Banciu DD, Radu M. Acid-Sensing Ion Channels as Potential Pharmacological Targets in Peripheral and Central Nervous System Diseases. *Adv Protein Chem Struct Biol.* 2016; 103:137-67.

Figure legends

Figure 1. ASIC1a are not involved in mGlu receptor-dependent LTD in juvenile mice.

(A) Superimposed pooled data showing EPSC_{AMPA} (A1) and EPSC_{NMDA} (A2) illustrating the effect of PcTx1 (100 ng/ml) on mGlu receptor-dependent LTD in juvenile (P13-18) mice. Representative EPSC traces were taken at the time indicated by number.

Figure 2. ASIC1a contribute to mGlu receptor-dependent LTD in early adult mice.

(A) Superimposed pooled data showing the effect of PcTx1 (100 ng/ml) on EPSC_{AMPA} (A1) and EPSC_{NMDA} (A2) of DHPG-LTD in early adult (P30-40) mice. Bar chart in the inset (A1) illustrates the effect of increasing concentrations of PcTx1 (10-100 ng/ml) on the magnitude of EPSC_{AMPA} of DHPG-LTD. On top, representative EPSC traces were taken at the time indicated by the number.

(B) Superimposed pooled data illustrating the effect of PcTx1 (100 ng/ml) after induction of EPSC_{AMPA} of DHPG-LTD. (C) Superimposed pooled data showing the effect of PcTx1 (100 ng/ml) on EPSC_{AMPA} of mGlu-LTD induced by a PP-LFS protocol. (D) Superimposed pooled data showing the effect of the non-selective ASIC blocker amiloride on EPSC_{AMPA} and (D1) EPSC_{NMDA} (D2) of DHPG-LTD.

Figure 3. PcTx1 reduces DHPG enhanced excitability in CA1 pyramidal neurons.

(A) Left panel illustrates representative trace recordings in response to depolarizing and hyperpolarizing current steps before and after bath-application of PcTx1 (100 ng/ml). Right panel shows bar charts (mean $\pm$ S.E.M.) showing inhibition of firing discharge (as % of control) at different concentrations of PcTx1 (30-300 ng/ml). (B) Representative trace recordings in response to depolarizing and hyperpolarizing current steps are shown before and after DHPG application with or without PcTx1 (30 ng/ml). Bar charts (mean $\pm$ S.E.M.) illustrate area (% of control) and number of action potentials (nAP) in the presence of DHPG alone or in combination with PcTx1 (30 ng/ml).

Figure 4. PcTx1 does not affect polyphosphoinositide hydrolysis but prevents GluA1 (Ser845) phosphorylation induced by DHPG.

(A) The scheme shows the intracellular pathway mediated by mGlu1/5 receptors. Exogenous DHPG binds to cell surface mGlu1/5 receptors and activates phospholipase C (PLC), through Gq proteins. PLC in turn hydrolyzes the phosphatidylinositol 4,5-bisphosphate (PIP₂), pre-labeled with 1 μ Ci of myo-[³H]-inositol, to yield the second messenger IP₃. Lithium ions inhibit inositol monophosphatase (IMPase) to allow accumulation of [³H]-InsP. I, inositol, IP, inositol monophosphate; IP₃, inositol trisphosphate. DHPG-stimulated PI hydrolysis in hippocampal slices in the absence or presence of PcTx1. Values represent mean $\pm$ S.E.M. (n=3-4). *p<0.05 (One-Way ANOVA + Fisher's PLSD test) vs. the respective basal values, #p>0.05 control vs. PcTx1 under basal conditions. (B) Representative western blots with relative quantification. Values represent mean $\pm$ S.E.M. (n=4). **p<0.01 (One-Way ANOVA + Newman-Keuls) vs. control, §p<0.05 DHPG vs. DHPG+PcTx1. On top, representative western blots are shown. (C) Representative western blots with relative quantification. Values represent mean $\pm$ S.E.M. (n=4). **p<0.01 (One-Way ANOVA + Newman-Keuls) vs. control, §p<0.05 DHPG vs. DHPG+PcTx1+MPEP or DHPG+PcTx1+JNJ. On top, representative western blots are shown.

A1 **A2**

A1 **A2** **B** **C** **D1** **D2**

A **B**

A **B** **C**