The Role of ASIC1a in the Regulation of Synaptic Release Probability

THESIS

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Abstract

Extracellular pH plays an important role in neuronal signaling. As primary receptors of pH signals, acid-sensing ion channels (ASICs) are able to translate fluctuations in the extracellular pH into membrane potentials and calcium signals. ASICs and pH signaling are thought to play important roles in anxiety, affect, and pain, although the mechanism by which they are able to influence these processes remains poorly understood. During conditions of dysregulated pH aberrant ASIC activity is known to result in cellular dysfunction and death, making a mechanistic explanation of ASIC function of broad importance to our understanding of downstream consequences during pathophysiological circumstances. One significant role of ASIC is in its ability to modulate synaptic vesicle release, a property which may contribute to neuronal dysfunction secondary to disruptions in pH signaling. This study demonstrates that the mechanism of ASIC-dependent regulation of synaptic vesicle does not rely upon rapid local signaling, but rather requires several hours of ASIC block to be interrupted, suggesting that it may take place through the induction of gene regulation and cause global changes in cellular physiology. Similarly, our results suggest that ASIC1a may be responding to endogenous proton flux to accomplish this regulation, refining our understanding of the cause and context of ASIC1a activation in health and disease.

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Chapter 1: Introduction

1.1 Extracellular pH and Acid-Sensing Ion Channels

The extracellular pH of neural tissue is a dynamic variable that conveys complex information about the physiological state of the brain. The information encoded by these transient changes in pH is sampled by the downstream receptors and transformed into electrical potentials that subsequently modulate neuronal activity [1-5]. Among the myriad of endogenous pH receptors, acid-sensing ion channels (ASICs) are among the most specialized and direct in their ability to cause rapid physiological effects, and are capable of initiating long-term modulation of synaptic properties and modulate such diverse physiological processes as memory, anxiety, and pain [1, 2, 6-12]. Likewise, as a complex integrator of diverse physiological and metabolic processes, extracellular pH is sensitive to a number of pathological and pathophysiological changes, in many cases resulting in known secondary dysfunction through the unregulated activation of ASIC channels [13]. However, this crucial area of research into the physiological and potential pathophysiological role of ASICs in modulating the neural circuitry is limited by the lack of a molecular mechanism by which ASICs are able to accomplish this regulation, beginning with the induction of the relevant pH signal that causes ASIC activation and continuing

with the downstream molecular events following ASIC activation [1, 2]. Identification of these crucial components in the system of pH-mediated signaling will lay a crucial foundation for the development of tools to investigate a variety of disease states ranging from depression, to anxiety, to chronic pain and multiple sclerosis [7, 9, 14-16]

1.1.1 The origin and regulation of pH transients in the CNS

Although the extracellular pH is subject to endogenous buffering through the enzyme carbonic anhydrase (CA), transient perturbations of extracellular pH occur as protons or bicarbonate flow across cellular membranes [3, 4, 17-23]. The duration and amplitude of these transients are dependent upon spatial diffusion and the rate at which the perturbation can be buffered by the endogenous system [3-5, 17-23]. The sources of these transients include a diverse array of processes ranging from simple homeostatic regulation of intracellular ions, to an array of complex mechanisms of activity-dependent proton and bicarbonate flux [3].

Each of these sources contributes its distinct signature on the extracellular pH that evolves both spatially and temporally as ions diffuse and are acted upon by CA [22]. However, endogenous buffering through CA is not a monolithic process but varies with specific isoforms of the enzyme as well as the ionic basis of the pH transient being buffered [24]. Indeed, as a pH buffer CA may act as both a proton source as well as a proton sink, and inhibition of this enzyme has the result of potentiating pH transients derived from proton flux even as it diminishes pH transients derived from bicarbonate flux (**Figure 1.1**). This complex array of

processes operating simultaneously in a neuron produces a rich signal in the extracellular fluid that varies in time and space that can then be sampled by precise downstream receptors in order to adjust physiological responses.

1.1.2 Regulation of intracellular pH as a homeostatic process

The earliest work in understanding transmembrane proton flux was aimed at understanding simple homeostatic regulation of intracellular pH, irrespective of its role as a physiological signal in its own right. In the absence of active pH regulation within the cell, biophysical theory predicted that the proton gradient across a cell membrane should reflect the Donnan equilibrium, which assumes that as the chemical potential of each ionic species reaches the vanishing point at equilibrium. However, this prediction was shown to be false in the 1930's by a series of studies, which demonstrated that the intracellular environment is relatively more alkaline than the prediction at equilibrium, indicating that the intracellular pH is being regulated by some energy-requiring mechanism [25]. Despite this early result, it was not until the 1970's that the first acid extrusion mechanism was discovered. Eventually families of amiloride-sensitive Na^+/H^+ exchangers [26], Na^+-dependent HCO^{3-} transporters [27], and Cl^-/HCO^{3-} exchangers [28] were all found to take part in homeostatic maintenance of intracellular pH [3] by fluxing protons or bicarbonate across the cell membrane, respectively.

1.2 Proton and Bicarbonate flux in physiological activity

Subsequent work demonstrated that proton and bicarbonate fluxes across cell membranes, in addition to playing a homeostatic role in maintaining optimal pH for intracellular protein function, are also indicators of the physiological activity of neurons. Initial studies in rat hippocampus and turtle cerebellum showed that extracellular electrical stimulation of afferents produced alkaline shifts in the postsynaptic tissue [29, 30]. These studies led to the development of a rich literature examining the sources and significance of such activity-dependent mechanisms and uncovered a myriad of pH-modulating processes ranging from neurotransmitter currents , to glial depolarization [19, 31], to Ca^{2+} homeostasis [22].

1.2.1 Neurotransmitter Effects

Early reasoning linked activity-dependent alkaline transients to direct action of ionotropic receptors, as contemporaneous biophysical studies in excised patch demonstrated that $GABA_A$ channels have significant bicarbonate permeability [32]. Subsequent physiological studies demonstrated bicarbonate-dependent picrotoxin-sensitive extracellular alkaline transients after application of GABA, in a dose-dependent manner. These transients were also diminished in the presence of the carbonic anhydrase inhibitor acetazolamide. These findings are suggestive of an outward flux of bicarbonate along its electrochemical gradient and subsequent conversion to CO_2 via enzymatic action [17, 33-40]. Similar effects were discovered in the rat medulla on application of glycine, suggesting that glycine receptors are responsible for alkalinizations due to a similar bicarbonate permeability [41]. The results of these experiments led to the early hypothesis that activity-dependent

alkaline transients are caused by the activation of inhibitory receptors that leads to an increase in extracellular bicarbonate [17].

However, a series of experiments in the Chesler lab demonstrated that this effect by itself is not sufficient to explain the original observation of activity-dependent alkalinization, as these transients persisted in the cerebellum even in the absence of bicarbonate [30, 39, 40, 42], the presence of picrotoxin [39, 40], and they were known to be enhanced, rather than inhibited, by acetazolamide application [43]. Combined, these data suggested that activity-dependent alkalinization in this area is dependent upon an inward proton flux rather than the outward bicarbonate flux produced by the $GABA_A$ receptor. Based on these results, Chen and Chesler explored potential mechanisms involving the contribution of glutamate receptors and found that application of glutamate into the rat hippocampus produced dose-dependent alkaline transients that were enhanced, rather than inhibited by acetazolamide application, similar to the activity-dependent alkalinization from stimulation with extracellular electrodes [18]. These results are consistent with a glutamate-activated inward proton flux, though there is not yet consensus on the precise mechanism of this effect, with several candidate mechanisms currently under investigation [3-5].

Taken together these findings demonstrate that neurotransmission, in addition to creating a transient change in electrical potential across the cell membrane, also results in a transient change in pH on both sides of the membrane. Both neurotransmitter effects exist in a dose-dependent relationship with their respective ligands and produce physiologically relevant signals, though our

understanding of the significance of the pH transients remains in its infancy. Interestingly, however, both excitatory and inhibitory neurotransmission seem to result in similar extracellular alkaline transients in contrast to their opposing effects on membrane potential, suggesting that these transients may simply serve as an extracellular signature of neurotransmission in general. These neurotransmitter-dependent pH transients appear to contribute to the larger picture of activity-induced pH changes, and presumably are the predominant factor determining the local pH in the nano- and microdomain near to the postsynaptic receptor itself, potentially having an effect on nearby proton-sensitive receptors such as the NMDA or L-type calcium channels [44-47].

1.2.2 Effects of general neuronal activity on pH

Along with the neurotransmitter-specific components of activity-dependent pH transients, there are also mechanisms related to downstream effectors of neuronal stimulation. These pH-modulating processes include the acidic transients caused by the release of synaptic vesicles and the activation of K^+-dependent glial shifts, as well as alkaline transients from homeostatic Ca^{2+}/H^+ antiport [19, 22]

Due to the activity of V-ATPase in acidifying synaptic vesicles for neurotransmitter loading, synaptic vesicles release both protons as well as neurotransmitters into the synaptic cleft during vesicle fusion, and upon fusion the V-ATPase inserted into the plasma membrane continues to pump protons until recycled into a new synaptic vesicle [44]. This phenomenon has been observed in

motor neurons as well as retinal neurons where it is hypothesized to play an important physiological role in certain aspects of visual processing [48].

Likewise, extracellular application of K^+ has been shown to cause an acidic shift [49] that was later identified to be of glial origin [50, 51]. Further work in gliotic hippocampal slice with a minimal neuronal component demonstrated that K^+ concentrations comparable to the levels found during high frequency neuronal firing are sufficient to cause significant extracellular acidification of the surrounding tissue as a specific glial contribution to activity-dependent pH transients. The mechanism of this effect is thought to be due to glial depolarization resulting in the activation of electrogenic bicarbonate transport through the NBC-3 Na^+/HCO^{3-} antiporter [19].

Although the pH-modulating mechanisms previously mentioned are responsible for generating activity-dependent acidic transients, the most common finding in bulk extracellular solution (i.e. not in the micro- or nano-domains of the transporters) is activity-dependent alkalinization, suggesting that the acidic transients are outweighed in overall effect by an even greater alkaline transient. This alkaline transient has largely been attributed to the exchange of extracellular protons for intracellular Ca^{2+} via the plasma membrane Ca^{2+}-ATPase (PMCA), which works to maintain Ca^{2+} homeostasis during perturbations resulting from neuronal activity [52-54]. Recent work has demonstrated that the surface alkalinization elicited by voltage steps in neurons in hippocampal slice can be completely blocked by pipet application of the PMCA inhibitor carboxyeosin, demonstrating that surface alkalinization in this context is entirely dependent upon proton antiport through

PMCA [22]. Although, in the absence of neurotransmission and glial depolarization, the total contribution of PMCA to extracellular pH transients during neuronal activity was not determined, PMCA activity appears to be responsible for a large proportion, if not all, of voltage sensitive proton flux intrinsic to neurons.

Together these findings demonstrate that, in addition to neurotransmitter-specific effects on extracellular pH, complex mechanisms of proton and bicarbonate flux sensitive to membrane potential and physiological activity form an elaborate dance of extracellular pH transients. These pH transients reflect an integrated ionic signal in the extracellular space that provides information on the metabolic and physiological circumstances of the surrounding tissue.

1.3 Role of pH transients in modulating neuronal activity

The presence of such an ionic signal that integrates information about the physiological activity of neurons is, of course, hardly meaningful unless there is a receptor to receive that information and translate that signal into electrically coded information that can affect physiology. In fact, multiple sensors of this kind have been described, including canonical ionotropic receptors such as the NMDA, $GABA_A$, and AMPA receptors, but also proton-specific receptors such as the family of Acid-Sensing Ion Channels (ASICs) which are the focus of this work.

1.3.1 Regulation of glutamate receptors by extracellular pH

The effect of extracellular protons on glutamate receptors is one of overall inhibition. It was demonstrated in the early 1990's that extracellular protons inhibit

NMDA receptors with an pH_{50} of 7.0-7.3, indicating that at physiological pH close to half of NMDA receptors are inhibited in this way [45-47, 55]. Subsequent work indicated that activity-dependent alkaline transients were sufficient to boost NMDA current and lead to measurable changes in neuronal excitability [21], suggesting that along with membrane depolarization, activity-dependent pH transients may play a role in the function of the NMDA receptor as a "coincidence detector" of neuronal activity. Moreover, it was subsequently discovered that the characteristics of these particular transients are determined by a neuron specific form of carbonic anhydrase car14, which appears to otherwise have no role in macroscopic pH regulation [24]. Altogether, these findings suggest that the NMDA receptor is subject to a highly developed form of pH-dependent regulation that involves activity-dependent alkaline transients to relieve tonic inhibition, and relies on a unique CA isoform which may exist for the specific purpose of molding these particular pH transients. AMPA and kainite receptors are similarly inhibited with an IC_{50} of ~6.2 through a mechanism that involves a decrease in the open probability as well as enhancing receptor desensitization [56, 57], though at present they have not yet been shown to exhibit pH dependent effects during physiological conditions.

1.3.2 Regulation of GABA and glycine receptors by extracellular pH

Chloride channels such as the $GABA_A$ and glycine receptors show differing responses to extracellular acidification. $GABA_A$ channels exhibit complex proton dependent effects depending on the subunit composition [58]. The $\alpha_1\beta_1$ $GABA_A$ receptors show current potentiation in acidic pH and inhibition in alkaline pH,

whereas $\alpha_1\beta_1\gamma_2S$ show complete insensitivity to extracellular pH. Interestingly, the $\alpha_1\beta_1\gamma_2S\delta$ receptors demonstrate a bell-shaped response to extracellular protons in which *both* alkalinization *and* acidification cause current inhibition. Glycine receptors, on the other hand universally show current inhibition in the presence of acidic pH [59]. This diversity of effects of extracellular protons on ligand-gated chloride channels seems to indicate that different effects of extracellular pH transients may be desired at different cell types and cellular compartments depending upon the precise physiological context. However, despite this diversity of effects of extracellular protons on chloride currents, all $GABA_A$ currents are thought to be inhibited by intracellular alkalinization, as the increase of free bicarbonate inside of the cell shifts the reversal potential towards more depolarized values given the significant permeability of the channel to this species [60]. It might be speculated that a similar effect occurs with glycine receptors, which also have a significant permeability to bicarbonate, though the literature to date is silent on this matter.

1.3.3 Regulation of voltage-gated channels by extracellular pH

The sensitivity of L-type calcium channels to protons has been shown to play a significant role in the physiology of certain synapses, particularly in the retina where presynaptic L-type channels are the predominant subtype that cause vesicle fusion [61-65]. Early work in this area demonstrated that synaptic vesicle release at the cone-bipolar cell synapse in the retina caused a HEPES-sensitive inhibition presynaptic L-type calcium channel current [44], and that this inhibition, in turn,

may play an important role in the center-surround effect of retinal physiology by which certain inhibitory pathways allow for high resolution perception of visual details [48, 66]. Similarly, members of the family of voltage-gated sodium channels also demonstrate proton-dependent inhibition with an IC_{50} of ~6.0 [67] and members of the Kv1 family of potassium channels show closed-state inactivation by protons [68].

1.4 ASIC channels

Along with the previously discussed receptors, in which extracellular pH transients can be described as playing a modulatory role to affect neuronal physiology, extracellular acidification is the primary, and only known, endogenous signal for the gating of a family of ion channels known as Acid-Sensing Ion Channels (ASICS). ASICs are trimeric proton-gated cation channels that are able to translate changes in extracellular pH directly into excitatory potentials [2]. The presence of an excitatory acid-gated current was initially reported by Krishtal in the 1980's [29], but molecular identification of a specific channel didn't occur until work in the Lazdunski lab in the 1990's [69], and these channels have since been identified as integral components in a number of physiological and pathological processes [3, 5, 13]. Although functional ASIC channels in the central nervous system (CNS) may be composed of various trimeric combinations of the ASIC1a, ASIC2a, and ASIC2b subunits [2], the ASIC1a subunit is required for high-affinity proton sensing at a pH of 7.2, and is known to mediate acid-dependent increases in cytoplasmic calcium concentration [70]

The exquisite sensitivity of ASIC1a to protons coupled with the lack of any other known physiological ligand has suggested that ASIC1a functions primarily as an ionotropic receptor for endogenous protons utilizing the rich environment of fluctuating pH in nervous and other tissue [1]. Although the ASIC1a subunit is widely expressed throughout the nervous system, it is particularly enriched in areas of high synaptic density such as the amygdala, cerebellum, and hippocampus [11]. These findings resulted in a body of literature exploring the hypothesis that endogenous pH transients may play particularly important roles relative to fear and affect, motor behavior, and learning and memory through a role in synaptic physiology [11, 12], a view which has subsequently been supported by a sizeable body of literature detailing a number of physiological phenomena that require tissue acidification in the presence of the ASIC1a subunit [1, 7-10, 14]

1.4.1 ASIC1a in the amygdala

The literature on ASIC1a function in the amygdala is particularly well developed. Early observations demonstrated that the loss of the ASIC1a subunit results in defects in fear conditioning, as measured by freezing in response to a stimulus associated with foot-shock, which is an animal model of acquired anxiety [11]. A subsequent study demonstrated that overexpression of ASIC1a through the introduction of transgene is also sufficient to significantly increase responses to fear conditioning on this same assay [10], providing further evidence that ASIC1a may indeed play a role in cellular correlates of learning in the amygdala. Molecular characterization of the amygdala in animals before and after undergoing fear

conditioning demonstrated that the loss of ASIC1a is sufficient to prevent the expression of the immediate early gene *c-fos*, which is an important mediator of activity-induced gene regulation in neurons and a general marker of increased neuronal activity [9]. The early inference that these ASIC1a-dependent alterations in fear conditioning were specifically due to amygdalar expression of ASIC1a was initially made due to the historically accepted role of this structure in mediating fear responses as well as the observed high level of ASIC1a expression and effect on gene transcription in this tissue. This hypothesis was subsequently confirmed and narrowed to the region of the basolateral amygdala (BLA) through the ability of BLA specific viral transfection of ASIC1a to rescue diminished fear conditioning responses in ASIC1a knockout animals [9].

Along with its effect on learned fear responses, however, further work also demonstrated that knockout or pharmacological inhibition of ASIC1a is sufficient to prevent expression of unconditioned fear responses as well. An early study measured a decrease in acoustic startle amplitude, decreased center-avoidance in the open field test, and decreased freezing time when exposed to the predator odor TMT in ASIC1a knock-out mice, and confirmed a diminished TMT effect on animals treated with the ASIC1a inhibitor psalmotoxin-1 (PcTx-1) as well [14]. Another interesting type of unconditioned fear response induced by elevated arterial CO_2 appears to also be largely regulated amygdalar ASIC1a expression [8, 71]. In this study it was noted that inhalation of 10% CO_2 caused acidification of the mouse amygdala and resulted in a significant fear response in mouse models of behavior. However, this response was severely blunted in ASIC1a knockout mice or after

treatment with the ASIC1a inhibitors PcTx-1 and A-317567. Additionally, the mechanism of CO_2-dependent activation of ASIC1a was prevented by pretreating the amygdala with bicarbonate through an intracerebroventricular cannula, providing clear evidence of acid-dependent physiological mechanism of ASIC1a activation in the amygdala.

Along with fear and anxiety behavior, ASIC1a has been shown to play an important role in the contribution of the amygdala to depressive behavior. Genetic knockout or pharmacological inhibition of channels containing the ASIC1a subunit has been shown to decrease depressive behavior in mice as measured in the forced swim test and tail suspension tests and, moreover, focal reintroduction of ASIC1a into the basolateral amygdala through viral transfection is able to restore depressive behavior to wild-type levels [7].

1.4.2 ASIC1a in the hippocampus

The hippocampus was identified early as a location of high expression of ASIC1a [69, 72]. Subsequent work identified ASIC channels as the source of acid-induced action potentials and suggested that acid-induced excitation through ASIC channels may play an important role in hippocampal physiology [73]. More precise studies utilizing ASIC1 and ASIC2 knockout animals identified distinct populations of ASIC channels within the hippocampus whose properties were dependent upon the presence of ASIC2a [74]. Importantly, ASIC2a was identified as being able to prevent the calcium conductance seen in ASIC1a homomeric channels and later in ASIC1a/2b channels [75, 76].

Along with early data regarding the expression and calcium conductance of ASIC channels in the hippocampus, functional studies of the hippocampus demonstrated that ASIC1a plays an important role in cellular correlates of memory and had a demonstrable impact on learning behavior. ASIC1a knockout mice had defects in spatial learning and maintenance of LTP, which was attributed to a functional link between ASIC1a and the NMDA receptor [12]. However, these findings have not been reproduced and have recently become controversial [77], so it is presently unclear what the role of ASIC1a may be in the development of LTP. Despite this controversy, however, an interaction between ASIC1a and NMDA in cultured hippocampal cells has been shown to affect ASIC1a currents and result in increased neuronal death during ischemia [78], demonstrating that they are indeed functionally linked and may both take part in NMDA-dependent synaptic plasticity.

Separate from its potential effects on LTP, ASIC1a activity has also been shown to modulate the absolute number of synapses in hippocampal slice. Both knockdown of the ASIC1a transcript using siRNA [70] as well as activation with chronic acidosis [79] significantly decreased the number of dendritic spines, indicating that acid-dependent activation of ASIC1a can affect both the synaptic strength as well as synaptic density in the hippocampus, two important aspects of synaptic transmission. Additionally, the presence of ASIC1a is required for stress-dependent reduction of hippocampal *bdnf* transcript, an important indicator of neuronal activity and a crucial mediator in many models of depression [7].

In addition to regulating synaptic properties such as synaptic strength and dendritic spine density, hippocampal ASIC1a has also been shown to play a role in

acid-mediated seizure termination [71]. Application of the chemoconvulsant drug pentylenetetrazole causes longer seizure duration in wild-type mice than in ASIC1a knockout mice in whole animal studies, suggesting that the presence of ASIC1a plays a role is seizure termination. Likewise, application of pH 6.8 to hippocampal slices after induction of seizure-like activity is sufficient to disrupt seizures in wild-type animals, but not ASIC1a knockout animals, suggesting that this mechanism is indeed due to activity-dependent acidification. Although ASICs are excitatory channels in most instances, they may contribute to inhibitory effects both through second-messenger signaling related to their calcium conductance as well as through their preferential localization to inhibitory interneurons.

1.5 The significance of ASIC1a as a mediator of pathology

Just as tightly regulated pH transients are used as physiological signals with specific membrane-bound receptors, the molecular mechanisms that keep these processes in balance can fail during pathological conditions and lead to disrupted signaling and further injury. The development of pathological pH shifts in the CNS occurs secondary to a number of conditions, which result in inappropriate stimulation of ASICs and further exacerbation of injury. This process is directly analogous to the excitotoxicity produced during unregulated activation of glutamate receptors as a result of pathological processes such as stroke, traumatic injury, and neurodegenerative disease. Like dysregulation of glutamate signaling, dysregulation of pH signaling is found in an incredible number of pathological conditions, particularly those associated with ischemic hypoperfusion and chronic

inflammation [80]. Moreover, because pH transients arise from a diversity of activity-dependent phenomena as well from the metabolic sources in ischemia and inflammation, a number of pathophysiological conditions of abnormal neuronal activity may likewise result in dysregulation of pH signaling and result in abnormal ASIC1a signaling, perhaps providing a credible link between ASIC1a and psychiatric disorders such as depression, anxiety, and post-traumatic stress syndrome [1, 2, 7-10, 14, 15].

1.5.1 ASIC1a mediated injury in ischemic stroke

During ischemic conditions such as stroke, hypoxic conditions force the cells to enter into anaerobic respiration and produce lactic acid as a metabolic byproduct. Poor perfusion of these tissues then results in little washout of these and other acidic metabolic byproducts, resulting in a local acidification of the neural tissue [81-87]. This local acidification may reach a pH of 6.5–6.0 under normoglycemic conditions, and during hypoglycemic conditions it is possible for the pH to fall below 6.0 [81, 86, 88]. ASIC1a is sensitive to pH values as high as 7.2 and is maximally activated by pH 6.0 [1, 2]. Early experiments demonstrated that the presence of ASIC1a significantly increases the infarct size in intact mice after middle cerebral artery occlusion (MCAO) [89]. The mechanism of this effect was determined to be an acid-dependent ASIC1a-mediated pathway of neuronal death that is dependent upon the presence of extracellular Ca^{2+} and potentiated in the presence of oxygen-glucose deprivation (OGD). These results were subsequently replicated in cultured human cortical neurons [90]. These findings are consistent with the current model

of ASIC1a-dependent acidotoxicity in which prolonged tissue acidosis results in ASIC1a activation and subsequent conduction of extracellular Ca^{2+} into the cell which then initiates a process of Ca^{2+} dependent neuronal death [13]. In addition to this basic finding, neuroprotection after stroke can be accomplished by pharmacologically blocking ASIC1a with a significant effect up to 5 hours after injury, indicating that acid-mediated death during ischemic injury is an ongoing process that occurs due to the disruption of normal pH signaling in the brain following ischemic injury [91].

1.5.2 ASIC1a mediated injury in multiple sclerosis

Multiple sclerosis is a disease of autoimmune attack of the myelin sheath surrounding central neurons. It is often modeled in animals with experimental autoimmune encephalitis (EAE) by inoculating mice with brain extracts mixed with an adjuvant to form an immune response. It is known that the inflammatory lesions produced by EAE are accompanied by a local tissue acidosis of approximately 6.6 [15], a pH derangement which is sufficient to result in the unregulated activation of ASIC1a. This pH shift, which was speculated to be as a result of inflammation-induced hypoxia, was accompanied by a commensurate increase in the expression of hypoxia-inducible factor 1α (HIF-1α). Additionally, in inflamed EAE tissue the ASIC1a mRNA was found to be significantly upregulated, with expression being localized predominantly to the injured axons and injured oligodendrocytes [15, 16]. These results support hypothesis that ASIC1a may play a role in the pathological mechanism of axonal degeneration in EAE. Indeed, induction of EAE in ASIC1a

knockout mice demonstrated a significantly decreased level of disability using a blinded clinical scoring method, a finding that was replicated in wild-type animals pretreated with the ASIC inhibitor amiloride [15]. In vitro studies with optic nerve preparations demonstrated that incubation at pH 6.5 resulted in an ASIC1a-dependent decrease of axonal fibers, and ex vivo studies on EAE lesions in mice pretreated with amiloride demonstrated greater axon sparing than control [15]. Lastly, in human tissue taken from multiple sclerosis lesions ASIC1a was found to be preferentially expressed in injured axons and oligodendrocytes in regions of active demyelination, suggesting that it may play an equivalent acidotoxic role in the human disease as well [16].

1.6 ASIC1a as a regulator of vesicle release

Although ASIC1a is widely accepted as a receptor for endogenous pH transients that is able to affect synaptic properties and regulate learning, memory, and affect, there has been a remarkable dearth of mechanistic evidence about the nature of any such regulatory role. Early evidence linking ASIC1a activity to LTP through the influence of the NMDA receptor has recently been challenged [77], leaving little consensus as to how ASIC1a might play the regulatory role ascribed to it in behavioral studies. However, there is compelling evidence that an important role of ASIC1a in cellular physiology might be played through regulation of events surrounding vesicle release in the presynaptic terminal.

The first hint of such a relation was reported as an anti-nociceptive effect of the ASIC1a peptide inhibitor PcTx1 [92]. This study examined behavioral correlates of

pain behavior in mice after PcTx1 application and found that ASIC1a inhibition results in decreased pain behavior. Interestingly, however, the analgesia produced by PcTx1 administration did not show additivity with morphine. Moreover, chronic PcTx1 administration resulted in an identical course of physiological tolerance as morphine, suggesting that somehow pharmacological inhibition of ASIC1a may be resulting in downstream activation of opioid receptors. Indeed, pretreatment with the opioid receptor inhibitors naloxone, naloxonazine, and naltrindol demonstrated that the analgesic effects of PcTx1 are mediated through activation of the μ and δ opioid receptors, but not through a direct pharmacological effect of PcTx1, as these results were repeated using ASIC1a knockdown with antisense mRNA. Because ASIC1a is co-expressed in the dorsal horn with met-enkephalin, a potent agonist of both μ and δ opioid receptors, a mechanism of ASIC1a regulation of met-enkephalin release emerged as a potential pathway linking PcTx1 administration and pain behavior. Interestingly, measurement of met-enkephalin concentration in CSF extracts demonstrated a 7-fold increase after application of PcTx1, supporting the view that ASIC1a is involved in the release of this opioid peptide. Furthermore, genetic deletion of the preproenkephalin gene prevented the analgesic effect of PcTx1 completely.

The resulting picture from this study reveals a mechanism in which ASIC1a in the dorsal horn seems to exert tonic inhibition of met-enkephalin release, which can be relieved through the application of the ASIC1a inhibitor PcTx1. It is noteworthy that, as a cation channel, ASIC1a would be expected to *potentiate* release of co-expressed met-enkephalin through membrane excitation, so its role in *inhibiting*

peptide release suggests a more complicated mechanism. Though the authors do not speculate on the nature of such a mechanism, the net results are presumably limited to either regulating the absolute number of vesicles released, the concentration of met-enkephalin in individual vesicles, or extracellular met-enkephalin degradation, each of which would be consistent with the findings presented.

Parallel work on the role of ASIC1a in synaptic transmission in the Askwith lab revealed that genetic deletion of ASIC1a results in an increase *synaptic* vesicle release as well [93]. This work was based on evaluation of the paired-pulse ratio (PPR) in autaptic hippocampal culture in both wild-type and ASIC1a knockout neurons. The paired pulse ratio is a commonly used assay in which two action potentials are evoked in rapid succession and the decrement in post-synaptic current, normalized as a ratio, is used to estimate vesicle depletion. In neurons with a high probability of vesicle release the decrement in current will be greater than that in neurons with a low probability of vesicle release, a phenomenon that is simply due to a smaller remaining pool of vesicles available for release [94-99].

The major finding of this work was that ASIC1a knockout neurons demonstrate a *lower* PPR than wild-type, indicating a higher probability of vesicle release (**Figure 1.2,** reproduced from [93]). Although this finding, by itself, might be attributable to a simple change in the excitability of the neuron or the characteristics of the waveform that invades the presynaptic terminal, these possibilities were ruled out through an analysis of spontaneous synaptic vesicle release in the presence of the voltage-gated sodium channel blocker tetrodotoxin. In these studies it was further

found that the frequency of spontaneous vesicle release is greater in ASIC1a knockout neurons than in wild-type neurons (**Figure 1.3,** reproduced from [93]). Because in the absence of voltage-gated sodium channel activity the neuron is unable to become excited or transmit action potentials, this finding indicates that loss of ASIC1a expression is sufficient to alter properties of the presynaptic terminal itself.

Although the presynaptic terminal and the events leading up to the release of synaptic vesicles has been well studied, much about its workings remain unclear. Bernhard Katz, in his seminal 1954 paper "Quantal components of the end-plate potential" [100] proposed a model of postsynaptic current that relied on three separate variables: the number of vesicle release sites, the probability of vesicle release after an action potential, and the postsynaptic response to a single synaptic vesicle. This model, though subsequently clarified in some ways, has remained remarkably intact to this day. Importantly, the release probability at a synapse refers to the probability that at least one vesicle will be released from that presynaptic after an action potential, and as such it is sensitive to two different factors: the number of vesicles docked at that terminal, and the probability that any one vesicle will release, called the fusion probability. Beyond this outline, however, the precise factors influencing the fusion probability are still poorly understood. Fusion probability is a complex factor that relates to the properties of the voltage-gated calcium channels that produce that triggering calcium signal, the molecular identities of the calcium sensors which initiate fusion, and the phosphorylation

status of any number of coordinating proteins which act in concert during the complicated process of vesicle fusion [94-99].

With this great complexity in mind, the mechanism of ASIC1a inhibition in the regulation of release probability might be related to an increase in the number of docked vesicles, or ASIC1a may regulate any of the myriad factors involved in vesicle fusion itself. This question was addressed through the application of hypertonic sucrose to an autaptic neuron preparation, a treatment long known to result in the spontaneous fusion of all docked vesicles for the purpose of calculating the size of the ready releasable pool [101]. Interestingly, this treatment failed to reveal any significant difference between the ASIC1a knockout and the wild-type neurons (**Figure 1.4,** reproduced from [93]). This finding indicates that ASIC1a plays a role in the modulation of synaptic vesicle release through a direct effect on the fusion probability of the individual vesicles docked at the presynaptic terminal.

Importantly, although this study was carried out using ASIC1a knockout neurons, the effect has been demonstrated to be an authentic effect of ASIC1a activity rather than a result of a compensatory mechanism unique to the knockout animals as transfection of ASIC1a back into the knockout neurons was able to rescue the altered release probability back to wild-type levels (**Figure 1.5,** reproduced from [93]). The correlate of this observation is that pharmacological inhibition of ASIC1a using the tarantula venom peptide PcTx1 should also be able to result in a change in synaptic release probability just as it resulted in increased met-enkephalin release in dorsal horn neurons [92]. This experiment will be discussed further in chapter 2.

This result, although relative to a separate vesicle pool, is remarkably consistent with the view of ASIC1a as preventing the release of met-enkephalin in the dorsal horn of the spinal cord [92]. As an apparent modulator of intrinsic vesicle fusion machinery, ASIC1a might be expected to have similar effects on both synaptic and large dense-core neuropeptide-containing vesicles. According to this view, loss of ASIC1a activity, either through pharmacological or genetic manipulation, might lead to relatively widespread changes in neuropeptide and neurotransmitter release patterns and result in the myriad of dysfunctions seen in ASIC1a knockout animals, including alterations in pain sensitivity, but also relative to functions of the amygdala and hippocampus where ASIC1a is particularly strongly expressed. Moreover, as ASIC1a activity is sensitive to endogenous pH transients that are derived from a multitude of different metabolic and physiological processes, ASIC1a may be a final common pathway by which any number of abnormal conditions may influence properties of neuropeptide and synaptic vesicle release and potentially result in disorders such as anxiety or depression.

1.7 Hypothesis

Despite the concordance of these results and their potential implications for human disease, they also raise a number of perplexing questions. Although ASIC1a appears to be playing a role in vesicle release, no presynaptic expression of this channel has yet been described in normal tissue [16]. This suggests that ASIC1a activity is resulting in a global, rather than a local, effect on the cell. One potential explanation of this effect may be that ASIC1a is known to play a role in gene

expression in certain tissues, as evidenced by ASIC1a-dependent activation of the calmodulin-dependent kinase II [70] and subsequent expression of the immediate early gene *c-fos* in the amygdala as well as the growth factor *bdnf* in the hippocampus [7, 9] Another perplexing issue is that, although ASIC1a is known to be responsive to extracellular protons, there is no clear data regarding the origin and identity of the pH transients relevant to its activation. Indeed, activation of ASIC1a purely from endogenous pH transients has yet to be directly observed [2]. Relative to these issues, we hypothesize that ASIC1a regulates synaptic vesicle release through slow signaling pathways, to include pathways of gene regulation, and that endogenous ASIC1a is activated by pH transients whose characteristics will respond to inhibition of the enzyme carbonic anhydrase (**figure 1.6**).

Given the potential of ASIC1a to play a role in a number of mental and neurological disorders through its regulation of release probability, the present work is an attempt to describe the molecular underpinnings of this mechanism. Specifically, we hope to demonstrate that application of the ASIC1a inhibitor PcTx1 is sufficient to result in an increase in vesicle release probability, and that this effect results from ASIC1a-dependent activation of slow signaling pathways. Moreover, we hope to suggest that this process is dependent upon a mechanism of endogenous proton flux, perhaps providing a valuable insight to the molecular events leading up to and causing ASIC1a activation as well as the subsequent molecular events transducing that signal to changes in synaptic properties.

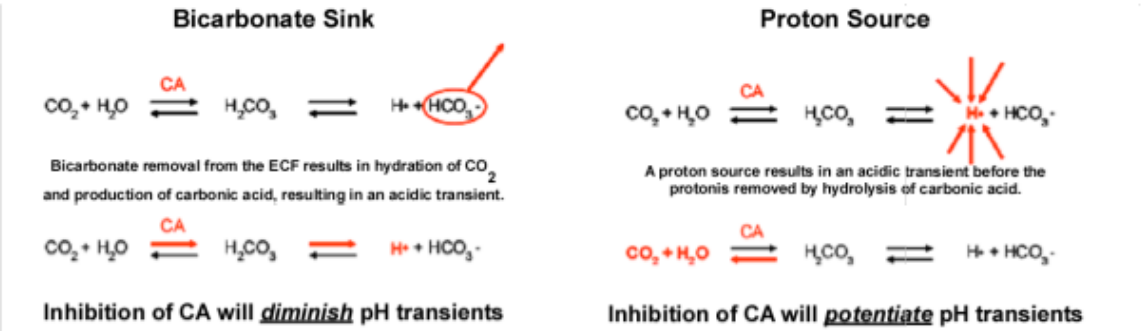

FIG 1.1 Mechanism of carbonic anhydrase in the production of pH transients

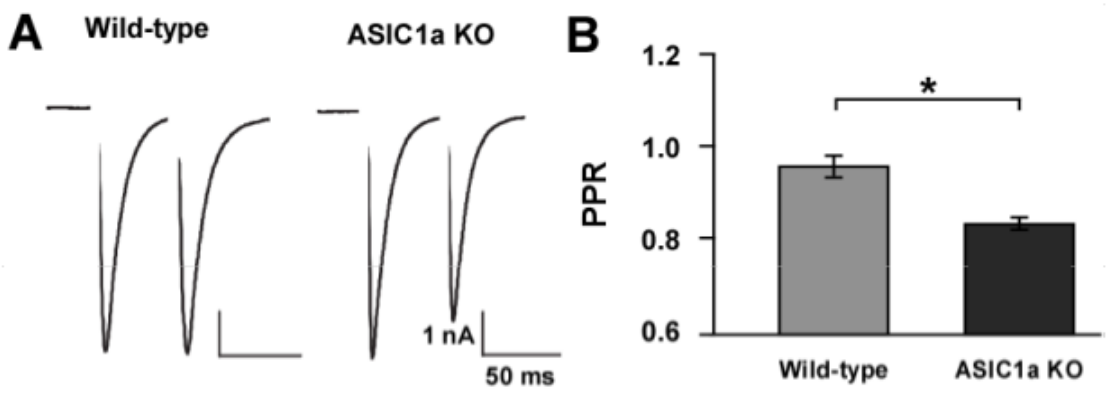

FIG 1.2 AMPAR paired pulse ratios (PPRs) A: Representative traces of AMPAR PPRs in wild-type and ASIC1a knockout neurons B: Quantification of PPR with an interpulse interval of 50 ms ($p < 0.05$)

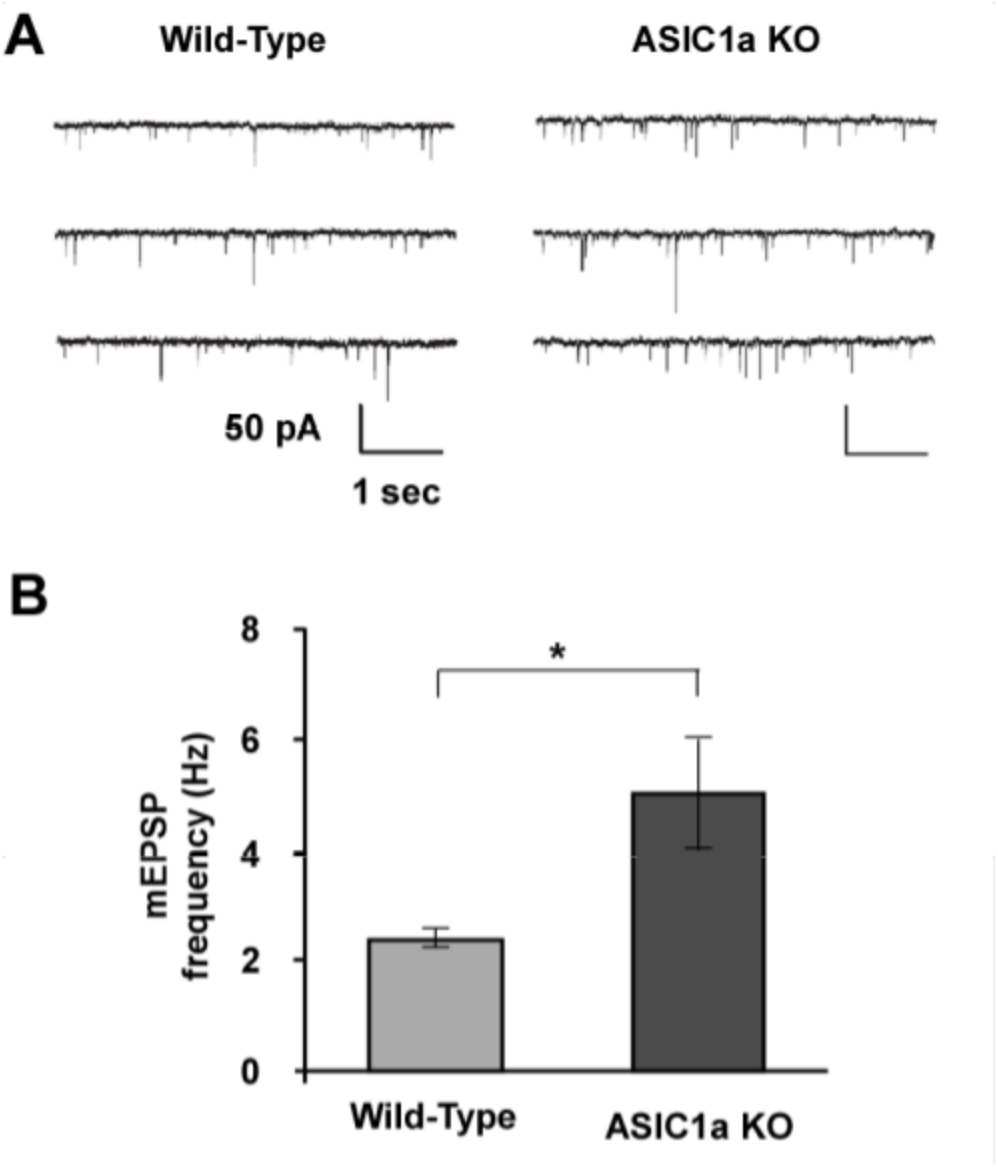

FIG 1.3 Spontaneous vesicle release A: Representative traces of wild-type and ASIC1a KO spontaneous release B: Quantification of mEPSP frequency ($p < 0.05$)

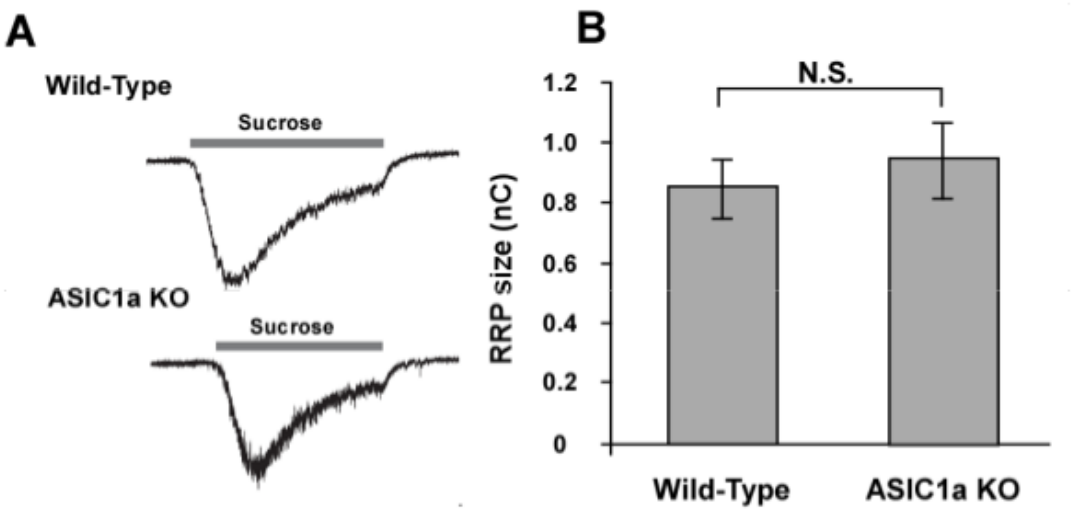

FIG 1.4 Calculation of RRP size using hypertonic sucrose (500 mM) A: Representative traces of sucrose dependent vesicle release in wild-type and ASIC1a neurons B: Charge transfer of sucrose-induced postsynaptic charge transfer calculated by integrating the transient portion of the postsynaptic curren (no significant difference)

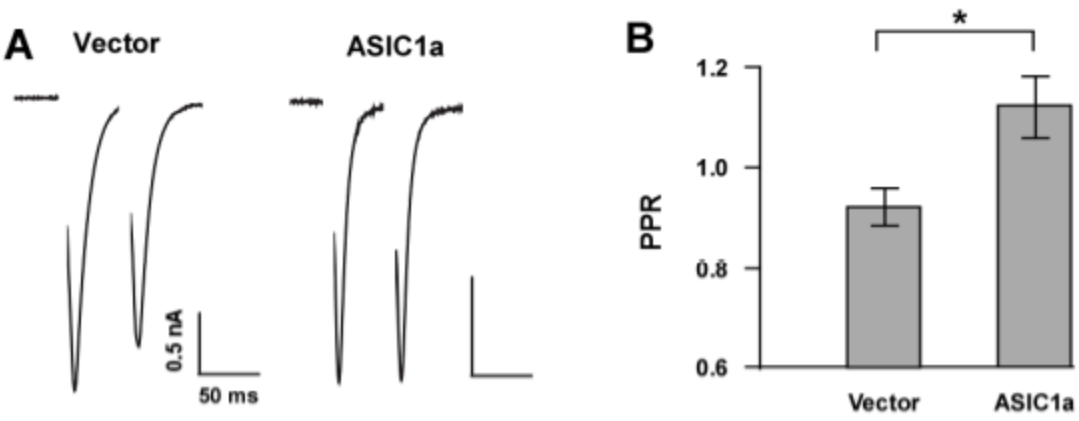

FIG 1.5 Rescue of PPR by reintroduction of ASIC1a into knockout A: Representative trace of postsynaptic currents in ASIC1a transfected neurons and vector control B: Quantification of PPR reveals a significant decrease in release probability in ASIC1a transfected neurons ($p < 0.05$)

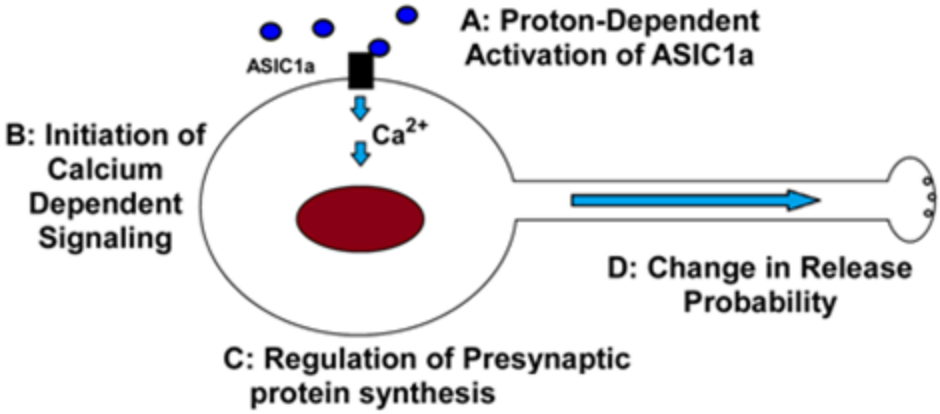

FIG 1.6 A model of ASIC1a dependent regulation of synaptic transmission in which proton-dependent activation of ASIC1a results in cellular signaling the causes alterations in protein synsthesis and subsequent changes in release probability

Chapter 2: Materials and Methods

2.1 Microisland culture of hippocampal neurons

Primary autaptic hippocampal neuron cultures were prepared using previously published methods [93]. Briefly, hippocampi were dissected from postnatal day 0-1 ASIC2 knockout pups and manually shredded. It should be noted that ASIC2 knockout mice develop and breed normally and there are no gross morphological brain abnormalities [12]. Hippocampal tissue was transferred into Leibovitz's L-15 medium containing 0.25 mg/mL bovine serum albumin and 0.38 mg/mL papain, and incubated for 12 minutes at 37°C with 95% O_2 and 5% CO_2 gently blown over the surface of the medium. Following incubation, the hippocampal tissue was washed three times with mouse M5-5 medium (Consisting of Earle's minimal essential medium with 5% fetal bovine serum, 5% horse serum, 0.4 mM L-glutamine, 16.7 mM glucose, 5,000 U/L penicillin, 50 mg/L streptomycin, 2.5 mg/L insulin, 16 nM selenite, and 1.4 mg/L transferrin) and triturated with glass pipettes. A fine mist of collagen (5 mg/10 mL) was spritzed onto the coverslips [102] and allowed to dry completely before being exposed to UV light for 2 hours. Hippocampal cells were plated onto these coverslips in 24 well dishes in M5-5

media at a density of approximately 250,000 cells per well. After 48-72 hours, 10 μM cytosine 1-β-D-arabinofuranoside (ARAC) was added to inhibit glial proliferation. Neurons were used from 14 to 21 days in culture.

2.2 Electrophysiology

To record H^+-gated and postsynaptic currents, we used the whole-cell voltage clamp technique. The extracellular solution contained 128 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.55 mM glucose, 10 mM MES, and 10 mM HEPES. Sodium hydroxide was used to adjust the pH of the extracellular solution to either pH 7.4 or pH 6.0. The intracellular pipette solution contained 121 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, and 300 μM Na_3GTP (pH 7.3). Patch electrodes were pulled with a P-97 micopipette puller (Sutter Instrument Co., Novato, CA) and fire-polished with a microforge (Narishige, East Meadow, NY). Micropipettes with 1-3 MΩ resistances were used for experiments. The membrane potential was held constant at -70 mV. Data were collected at 5 kHz using an Axopatch 200B amplifier, Digidata 1322A and Clampex 9 (Molecular Devices, Sunnyvale, CA). Neurons were continuously perfused with the extracellular solution from gravity-fed perfusion pipes at a flow rate of approximately 1 mL per minute. Perfusion pipes were placed 250 to 300 μm away from cells, and flow was directed toward the recorded cells to ensure fast solution exchange. H^+-gated currents were evoked by the exogenous application of pH 6.0 extracellular solutions.

To record paired action potential (AP)-evoked whole-cell postsynaptic currents, solitary autaptic neurons on microislands were selected, and stimulated every 10 seconds (0.1 Hz) with a transient depolarization of membrane potential from -70 mV to +10 mV for 2 ms. An extracellular solution containing 10 μM cyano-7-nitroquinoxaline-2,3-dione (CNQX) or 30 μM bicuculline was used to determine if the autaptic neuron was glutamatergic or GABAergic. GABAergic neurons were excluded from this study. Unless otherwise stated, all reagents were purchased from Invitrogen/Gibco (Carlsbad, CA), Sigma-Aldrich (St. Louis, MO), or Fisher Scientific (Waltham, MA). Data were analyzed using Clampfit 9 software (Molecular Devices). Data are presented as the mean ± the standard error of the mean (SEM). As appropriate, a two-tailed Student's t test and one-way ANOVA with Dunnet's post-hoc test were used for statistical analyses.

2.3 Application of PcTx1 peptide

Prolonged application of the ASIC1a inhibitory peptide PcTx-1 (100 nM) was accomplished by treatment of coverslips within the incubator for between 0 and 12 hours before recording.

During electrophysiological recordings, the ASIC1a inhibitory peptide Psalmotoxin (100 nM) was applied along with ECF in conventional gravity-fed perfusion pipes at a flow rate of approximately 1 mL per minute. Perfusion pipes were placed 250 to 300 μm away from cells and remained motionless throughout the PcTx1 application process, which lasted approximately 5.5 minutes during

experiments involving acute application and was continually perfused in experiments involving prolonged application.

Chapter 3: Results

3.1 Acute inhibition of ASIC1a does not alter postsynaptic current amplitude

Because genetic deletion of ASIC1a has been shown to result in an increase of release probability [93], we hypothesized that ASIC1a may potentially take part in local signaling in the presynaptic terminal. Because ASIC2 knockouts were previously shown to have normal release probability, to provide a homogenous population of ASIC1a homomeric receptors that are sensitive to the ASIC1a inhibitor PcTx1, we performed our experiments on ASIC2 knockout autaptic hippocampal culture. We found that application of the ASIC1a inhibitor PcTx-1 to isolated ASIC2 knockout autaptic neurons, though accomplishing a profound inhibition of ASIC1a by 2 minutes (**Figure 3.1**), did not alter normalized postsynaptic current amplitude (n=6) (**Figure 3.2**). This finding suggests that ASIC1a does not modulate release probability through rapid local signaling pathways. Current amplitudes were normalized to the first postsynaptic current amplitude before application of PcTx-1. Postsynaptic current amplitude would be expected to increase subsequent to an increase of release probability. Data are represented by mean +/- SEM.

3.2 Acute inhibition of ASIC1a does not alter paired pulse ratios

Along with PcTx-1 having no effect on post-synaptic current amplitude in isolated autaptic neurons, we further found that it failed to affect the paired pulse ratio (PPR) after ~ 5.5 minutes of application (n=6) (**Figure 3.3**). This finding suggests that ASIC1a does not modulate release probability through rapid local signaling pathways. The PPR was obtained by dividing the second post-synaptic current amplitude by the first. The PPR, as a measure of vesicle depletion, would be expected to decrease subsequent to an increase of release probability. Data are represented by mean +/- SEM.

3.3 Prolonged ASIC1a inhibition increases postsynaptic current amplitude

Incubation of ASIC2 knockout autaptic hippocampal culture for 0-12 hours demonstrated a significant increase in postsynaptic current density after 7 hours (**Figure 3.4**) ($p<0.05$, student's t-test; n=5 for control and n=9 for 7-12 hour treatment). This finding suggests that ASIC1a regulation of release probability occurs through a slow signaling pathway. Current density was measured by dividing the postsynaptic current amplitude by the cell membrane capacitance. Data is represented as mean +/- SEM.

3.4 Prolonged inhibition of ASIC1a decreases PPR

In agreement with the results relating to the effect of prolonged ASIC1a inhibition on postsynaptic current density, incubation in PcTx1 resulted in a significant decrease in PPR after 7 hours (**Figure 3.5**) ($p<0.029$ ANOVA with Dunnet's post-hoc test; n=5 for control, n=6 for 3-6 hours, and n=9 for 7-12 hour

treatment). This finding suggests that ASIC1a regulation of release probability occurs through a slow signaling pathway. Data is represented as mean +/- SEM.

3.5 Prolonged inhibition of CA results in decrease current density

In order to assay the potential sources of endogenous ASIC1a activation in culture, the CA inhibitor acetazolamide (10 μM) was applied for 8 hours to ASIC2 knockout autaptic hippocampal culture and the resulting postsynaptic current density was compared to vehicle control. Acetazolamide application resulted in a trend towards decreased postsynaptic current density (**Figure 3.6**) (n=4 for control and n=6 for acetazolamide treated; not yet significant). This finding suggests that inhibition of CA may increase ASIC1a activity and result in decreased release probability. These findings are suggestive of a mechanism of ASIC1a activation that is dependent upon outward flux of protons.

3.6 Prolonged inhibition of CA results in increased PPR

In agreement with findings indicating a trend towards decreased postsynaptic current density after 8 hours of CA inhibition, this treatment also resulted in a trend towards an increase in PPR (**Figure 3.7**) (n=4 for control and n=6 for acetazolamide treated; not yet significant). These findings together suggest that prolonged inhibition of CA results in a decrease in release probability, potentially through increased activation of ASIC1a

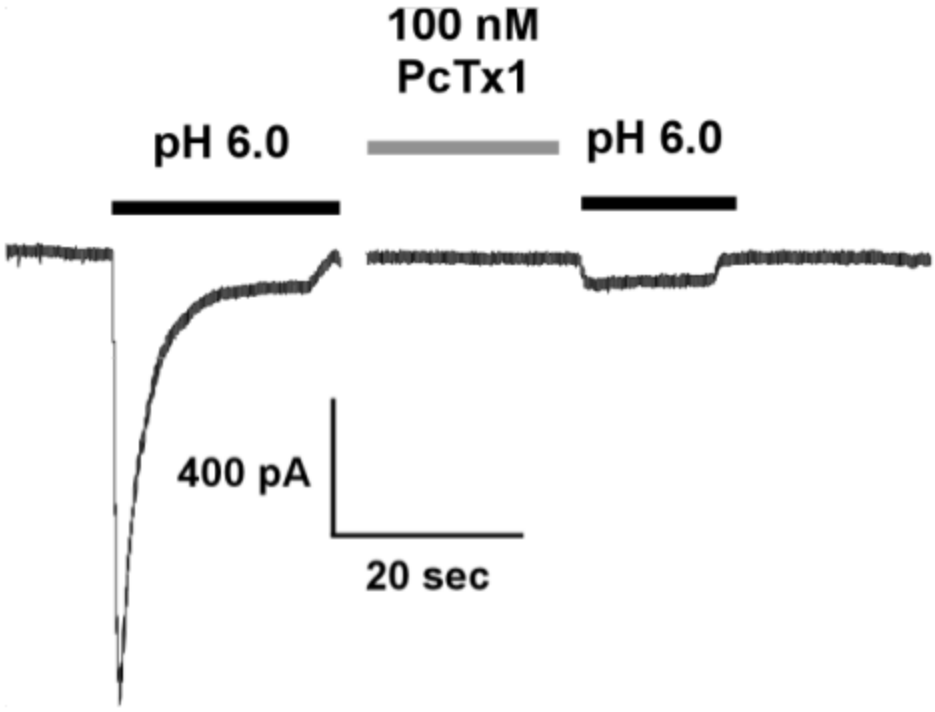

FIG 3.1 Representative trace showing the inhibition of ASIC1a after 2 minutes of incubation with PcTx1 in autaptic hippocampal culture

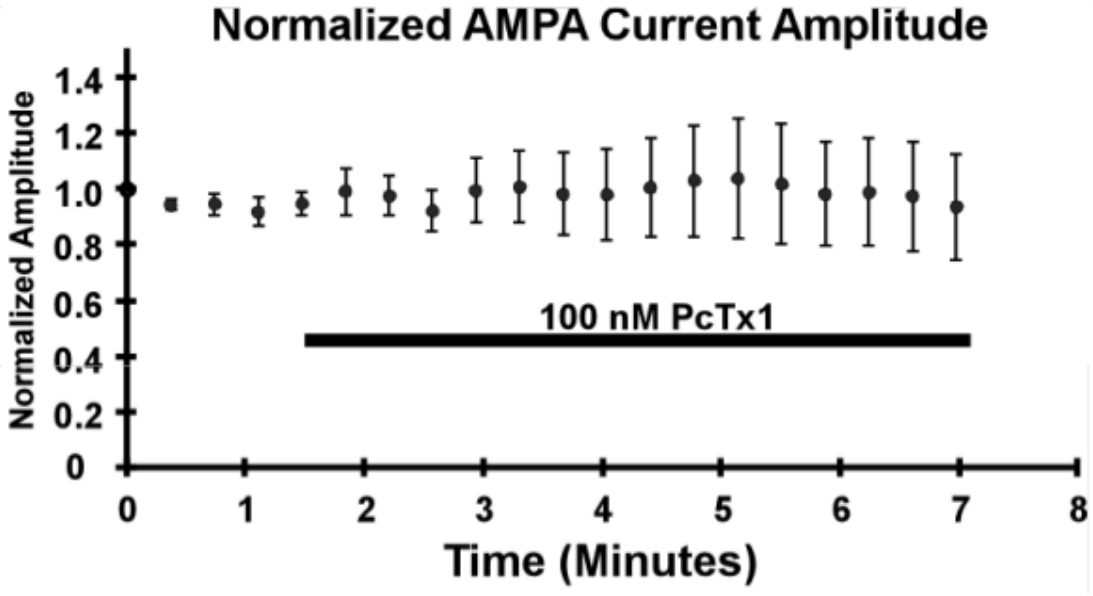

FIG 3.2 Application of 100 nM PcTx1 does not alter AMPA current amplitude in ASIC2 knockout autaptic hippocampal culture on DIV 14-21 at a stimulation frequency of 0.045 (n=6)

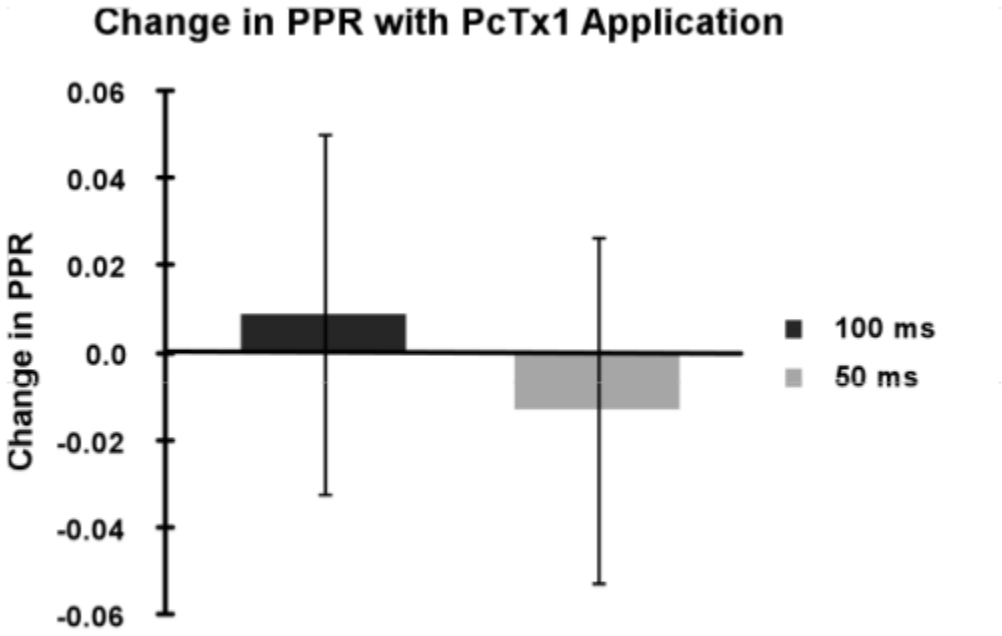

FIG 3.3 Application of 100 nM PcTx1 does not alter the PPR in autaptic cultured hippocampal neurons (n=6)

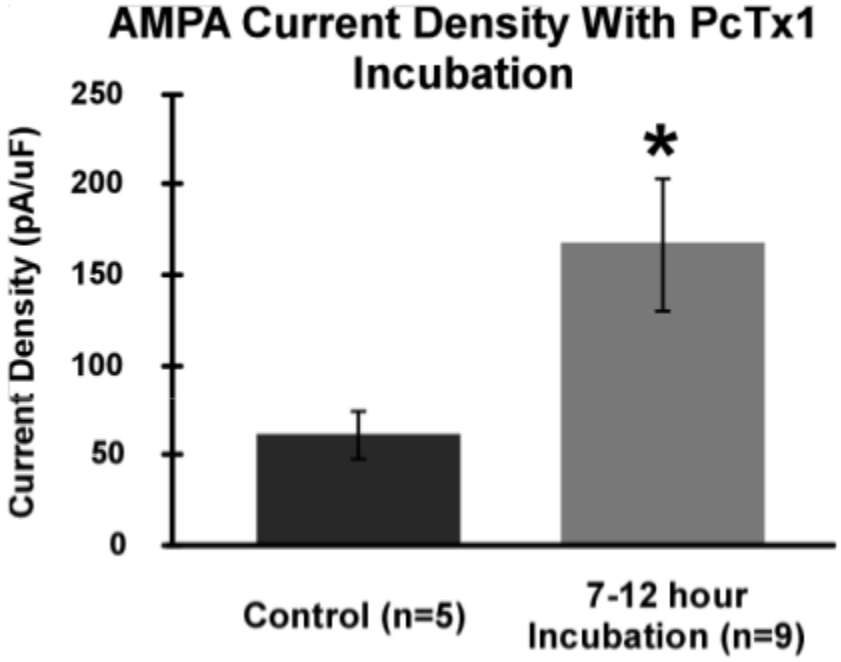

FIG 3.4 Incubation in 100 nM PcTx1 causes a significant increase in synaptic AMPAR current density after 7 hours of treatment ($p < 0.05$, student's t-test)

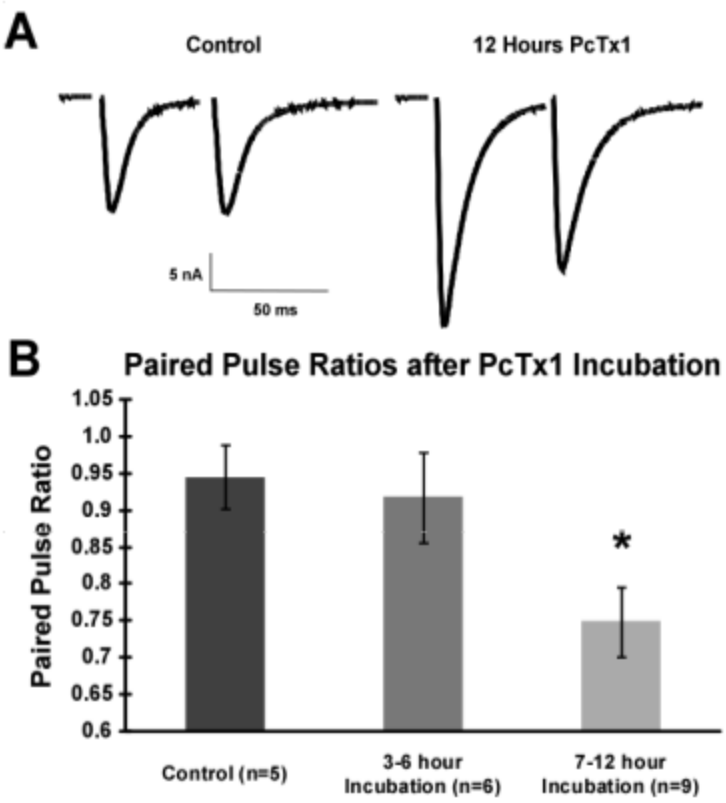

FIG 3.5 Incubation in 100 nM PcTx1 causes a significant decrease in PPR in autaptic hippocampal culture after 7 hours of treatment A: Representative trace demonstrating decreased PPR and increased current amplitude B: Quantification of PPR ($p < 0.029$, One-Way ANOVA, Dunnet's Post-Hoc)

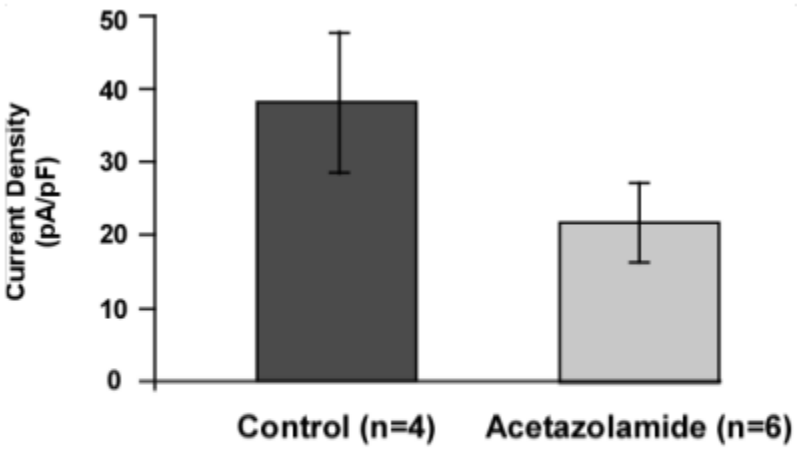

FIG 3.6 The effect of 8-hour acetazolamide incubation on postsynaptic current density. This figure demonstrates a trend toward a decrease in postsynaptic current density consistent with a decreased release probability. Not yet signficant ($p < 0.172$)

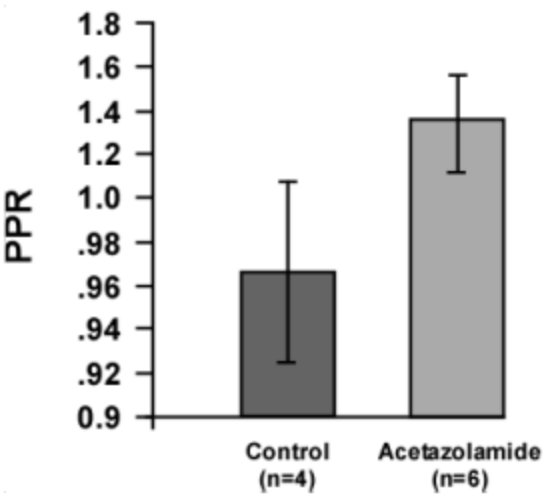

FIG 3.7 8 hours of incubation with 10 uM acetazolamide causes a trend towards increased PPR, consistent with a decrease in release probability. Not yet significant ($p < 0.171$)

Chapter 4: Discussion and Conclusions

This study was designed to explore the mechanism of ASIC1a modulation of synaptic release probability. Previous work has demonstrated that genetic knockout of ASIC1a is sufficient to result in an increase in release probability, but left unclear the possible mechanism of this effect, as well as the endogenous signal that might drive ASIC1a activity to achieve it [93]. Our hypothesis was that endogenous pH transients led to ASIC1a activity and subsequently resulted in slow systemic effects that modulated release probability. The reasoning behind this hypothesis was that ASIC1a has been shown to be expressed in the somatodendritic compartment, but not in undamaged axons, suggesting that ASIC1a could not participate in *local* signaling in the uninjured presynaptic terminal [16]. Likewise, ASIC1a activation is known to result in systemic effects, including global changes in dendritic spine density through the downstream activation of CaMKII [70], and is also known to be required for certain types of activity-dependent gene expression, such as *c-fos* in the amygdala after fear training and *bdnf* in the hippocampus after stress [7, 14]. Together these findings paint a picture of a pathway regulating global physiological properties in response to metabolic and physiologic events conveyed through the signal of extracellular pH. The significance of this study lies in the fact that ASIC1a is known to regulate the physiology of a number of diverse processes

such as anxiety, depression, and pain, and may play an important role in the development of certain types of psychiatric and somatic pathologies. Moreover, because ASIC1a is sensitive to extracellular pH, which itself responds to multiple distinct processes, ASIC1a-dependent regulation of cellular physiology may play the role of a final common pathway by which a number of metabolic or physiological derangement lead to the cellular dysfunction.

To address our hypothesis that ASIC1a regulates release probability through slow systemic signaling rather than rapid local signaling, we compared the effects of acute and prolonged ASIC1a inhibition on synaptic transmission. If ASIC1a were taking part in rapid local signaling, we would expect an increase in normalized postsynaptic current amplitude and a decrease in PPR immediately following channel inhibition, which occurs within 2 minutes of application of PcTx1 (**Figure 3.1**). However, we found that whereas acute block of ASIC1a with PcTx1 had no effect whatsoever on either normalized postsynaptic current amplitude (**Figure 3.2**) or PPR (**Figure 3.3**), prolonged block for over 7 hours significantly increased postsynaptic current density (**Figure 3.4**) and decreased PPR (**Figure 3.5**). These findings are consistent with a delayed increase in release probability after ASIC1a block, potentially due to a change in gene regulation followed by a delay from axonal transport to the presynaptic terminal.

This finding that ASIC1a modulation of release probability relies upon slow signaling through presumably global changes is well supported by the current literature. ASIC1a is known to result in global morphological changes in dendritic

spine density through the activity of CaMKII [70]. ASIC1a is known to alter gene transcription of the immediate early gene *c-fos* as well as the growth factor *bdnf* [7, 14]. Likewise, this result is consistent with the finding that ASIC1a is able to increase the release of the opioid peptide met-enkephalin, either through a similar mechanism of potentiated vesicle release, but even through the alternative mechanism of increased met-enkephalin production and vesicle loading.

To address our hypothesis that ASIC1a sensitivity to endogenous pH transients is crucial in its role as a modulator of synaptic properties, we compared an 8-hour treatment of the CA inhibitor acetazolamide to vehicle control. It should be noted that inhibition of CA is not expected to alter the basal pH of the extracellular solution, which is an equilibrium value strictly determined by the relative thermodynamic stabilities of the species involved, but rather affects only the amplitude and duration of the activity-dependent perturbations. However, because ASIC1a is sensitive only to transient perturbations in pH, requiring re-alkalinization in order to recover from desensitization, application of acetazolamide provides a unique opportunity to test the hypothesis that endogenous pH transients are activating ASIC1a in culture and will give insight into their ionic nature. Because inhibition of CA results in potentiation of pH transients created by proton flux, we would expect that if ASIC1a were being activated by this species of pH transient acetazolamide application would result in an overall decrease in release probability as channel activity increases. Likewise, because inhibition of CA results in inhibition of pH transients created by bicarbonate flux, we would expect that if ASIC1a were

being activated by this species of pH transient acetazolamide application would result in an overall increase in release probability as channel activity decreases, similar to the effect of PcTx1. Our data demonstrates a trend, not yet significant, towards a decrease in release probability after acetazolamide application, as indicated by an increase in PPR. These preliminary results suggest that ASIC1a may be responding to pH transients created by proton flux in order to result in modulation of vesicle release probability. This type of mechanism would be consistent with the activity of membrane-bound V-ATPase for synaptically localized ASICs or with the activity of Na^+/H^+ exchangers for somatically localized ASICs.

This study advances our understanding of the role that ASIC1a plays in synaptic physiology and provides us with a potential pathway that may contribute to a variety of psychiatric and somatic diseases. To the extent that the role of ASIC1a is to interpret changes in extracellular pH for the cell and translate them into membrane potentials and calcium signals, it will play a pivotal role in explaining the pathophysiological response to conditions of pathological disruption of extracellular pH. Classic conditions that prevent the maintenance of normal pH signaling, such as ischemia and inflammation, are already known to result in ASIC1a-dependent cellular injury, but the intricacies of physiological pH signaling are still barely understood. However, inasmuch as ASIC1a is important for normal function in a particular tissue, such as the amygdala, we can infer that endogenous pH transients play an important signaling role in that tissue as well. Therefore any dysregulation of pH signaling, from any number of potential metabolic or physiological

derangements, has the potential to cause secondary dysregulation of synaptic properties through the influence of ASIC1a. Much as ASIC1a may result in secondary injury during stroke or inflammation, it may also result in secondary synaptic disruption due to other alterations in neuronal activity or proton handling. Understanding the mechanism by which ASIC1a can accomplish this may play a crucial role in unraveling the pathogenesis of complex neurological and psychiatric disorders and presenting a new avenue for potential treatment.

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