Hyperpolarization-activated currents control the excitability of principal neurons in the basolateral amygdala

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Abstract

Anxiety is thought to be influenced by neuronal excitability in basolateral nucleus of the amygdala (BLA). However, molecules that are critical for regulating excitability of BLA neurons are yet to be determined. In the present study, we have examined whether hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels, which mediate the depolarizing cation current, can control the neuronal excitability. HCN channel-like activity appeared to be detected in BLA principal neurons. ZD7288, a specific blocker for HCN channels, increased the input resistance of membrane, hyperpolarized resting membrane potential, and enhanced action potential firing in BLA principal neurons. The blockade of HCN channels facilitated temporal summation of repetitively evoked excitatory postsynaptic potentials, suggesting that suppression of HCN channel activity in principal neurons can accelerate the propagation of synaptic responses onto the axon hillock. Thus, our findings have laid foundation for studies to reveal how HCN channel activity in BLA principal neurons regulates anxiety *in vivo*.
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The importance of the amygdala in autonomic, endocrine, and motor responses to affective stimuli and affective conditioning has long been recognized [1–3]. The amygdala is composed of more than 10 nuclei that are linked to one another [4]. In particular, the basolateral amygdala (BLA) has been known to drive and modulate affective behavior such as anxiety. Electrical and chemical stimulation of the BLA enhance anxiety [5,6], whereas lesions or inactivation of the BLA region reduces affective responses to conditioned stimuli [7,8]. For example, intra-BLA administration of glutamate antagonists of the *N*-methyl-D-aspartate (NMDA) [9,10] or non-NMDA [11,12] type receptors reduces anxiety. Blockade of GABA_A receptor-mediated inhibition in the BLA elicits increases in heart rate, blood pressure, and experimental anxiety [13–15]. Thus, emotional states appear to be tightly related with electrical excitability of the BLA.

The hyperpolarization-activated cation current (I_h) was first identified in cardiac sinoatrial node cells as a pacemaker current [16,17]. I_h has been also found in many cell types in the CNS [18]. I_h is produced by a family of ion channels called hyperpolarization-activated and cyclic-nucleotide-gated channel (HCN channel). Four HCN channel genes (HCN1–HCN4) have been cloned in mammals and they show very different biophysical properties [19,20].

I_h mediates repetitive firing in neurons and cardiac myocytes [21,22]. In addition, roles of I_h in the regulation of resting membrane potential, membrane input resistance, synaptic plasticity, and dendritic integration have been reported [23–27]. Increasing evidence implicates I_h in activity-dependent changes of neuronal excitability [28,29], in

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motor learning and memory [30], and in certain pathological conditions such as epilepsy or neuropathic pain [31,32]. Although it has been reported that I_h was also found in the BLA in rats [33], the involvement of I_h in patho-physiological states of the amygdala is yet to be determined. In the present study, we have addressed the questions of what types of neurons in the BLA express HCN channels and how I_h contributes to the modulation of intrinsic excitability and the processing of synaptic inputs in BLA neurons.

Materials and methods

Slice preparation. All procedures were approved by the Kyunghee University Animal Care and Use Committee. Male Sprague–Dawley rats (3–5 weeks of age) were decapitated, and brains were rapidly removed and placed into cold oxygenated (95% O_2, 5% CO_2) sucrose solution with the following composition (in millimolar): 175 sucrose, 20 NaCl, 3.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, and 11 glucose. Coronal slices of 300 μm thickness were cut with vibroslicer (Campden Instrument, London, UK) and left to adapt to room temperature (21–23 °C) for 1 h in oxygenated artificial cerebrospinal fluid (ACSF, in millimolar): 120 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, 11 glucose, and 2 $CaCl_2$. The slices were then transferred to the recording chamber, where they were fully submerged, continuously perfused with ACSF at a flow rate of 1.2–1.5 ml/min, and maintained at 33 ± 1 °C.

Electrophysiology. All recordings were performed with the patch-clamp technique in whole-cell configuration, using an EPC10 amplifier (HEKA Elektronik). Patch-clamp pipettes were pulled (model PP-83, Narishige Scientific Instrument Lab.) from borosilicate glass of outer diameter 1.2 mm (Warner Instruments) and had a tip resistance of 3–4 MΩ when filled with internal solution. The internal solution contained (in millimolar): 120 K-gluconate, 10 Hepes, 1 $MgCl_2$, 5 NaCl, 0.2 EGTA, 2 Mg-ATP, and 0.3 Na-GTP; pH was adjusted to 7.2 with KOH. Neurons used for recording located in dorso-central part of the BLA were visualized using infrared differential interference contrast (IR-DIC) video microscopy with a 40× magnification water-immersion objective (BX51WI, Olympus). After whole-cell configuration the series resistance was regularly monitored and a maximum series resistance of 15 MΩ was tolerated. Only neurons that had a resting membrane potential of at least −60 mV were used. Excitatory postsynaptic potentials (EPSP) were evoked with an electric stimulus (150 μs duration) using a bipolar stimulating electrode placed on basal part of the LA, just on the border of the BLA. Temporal summation of EPSP was evoked with stimuli at 25 Hz. ZD7288 (10 μM, Tocris Cookson) was applied via superfusion in the ACSF for 10–15 min.

Data analysis. All data were expressed as means ± SEM. Data were compared using unpaired *t*-test with GraphPad PRISM 4 (GraphPad software Inc.). Statistical significance was defined at the level of $p < 0.05$. Data were acquired and analyzed with Pulse/pulsefit v.8.67 (HEKA Elektronik), Igor Pro v.5.04B (Wavematrics) and Clampfit 8.2.0.235 (Axon Instrument).

Immunohistochemistry. Mice were anesthetized with isoflurane and transcardially perfused with pre-chilled PBS (pH 7.4). The brains were dissected out and rinsed in cold PBS. After the washing, the brains were embedded and then kept frozen at −80 °C. Coronal sections (6 μm thick) were prepared using cryostat microtome (Leica). The samples were air-dried overnight, fixed in cold acetone at −20 °C for 10 min and then air-dried for 1 h. Following washing in PBS, the tissue sections were incubated with blocking solution (PBS containing 10% normal goat serum, 0.1% Tween 20) for 1 h at room temperature. Then the samples were incubated with anti-HCN1 antibody (1:200, Alomone Labs) or anti-NeuN antibody (1:500, Chemicon) in the blocking solution at 4 °C overnight. Following washing with PBS containing 0.1% Tween 20, the samples were incubated with appropriate secondary antibodies. For ABC staining, samples were then processed using ABC Elite kit (Vector Laboratories) according to the manufacturer's manual. For immunofluorescence detection, the samples were then incubated with streptavidin-Texas Red and FITC-conjugated anti-rabbit antibody (Jackson Immunochemicals). Following mounting, the samples were analyzed under a fluorescence microscope (Axioplan 2, Zeiss).

Results

Basic electrophysiological properties of BLA neurons

Two main neuronal types are present in the BLA, i.e., pyramidal-like principal neurons that are glutamatergic and local circuit interneurons that are GABAergic [34,35]. Principal neurons constitute the majority (85–90%) of the BLA neuronal population and the cells were classified as principal neurons based on the pyramidal shape of their somata and their ability to show spike frequency adaptation in response to current injection [34,36]. To examine basic electrophysiological properties of the two types of BLA neurons, whole-cell patch-clamp recordings were made from 85 neurons located in the BLA. Most of the neurons (78 out of 85 cells; 92.8%) were found to be principal neurons showing fired trains of action potentials with varying degrees of spike frequency adaptation in response to prolonged depolarizing current injection (Fig. 1A, principal neuron) as previously described [36]. Hyperpolarizing currents injections in the principal neurons produced a depolarizing 'sag' in steady state membrane potential, indicating I_h activation (Fig. 1A, principal neuron). The rest (7 out of 85; 8.2%) showed no frequency adaptation in current-clamp recordings and often contained spontaneous EPSPs (Fig. 1A, Interneuron), which are typical characteristics of interneurons [36].

In voltage-clamp recordings, while interneurons were devoid of inward currents with I_h characteristics (Fig. 1B, interneuron), principal neurons had prominent I_h elicited by hyperpolarizing-voltage steps with a predominant fast exponential component and a smaller slow exponential component ($\tau_{fast} = 24.28 \pm 0.39$ ms and $\tau_{slow} = 227.60 \pm 13.01$ ms at −140 mV, $n = 78$, Fig. 1B, principal neurons). The kinetic description implies the predominance of HCN1 channels, because the HCN1 subtype shows faster kinetics than the others in exogenous expression systems [37]. An immunocytochemical study also revealed prominent expression of HCN1 channels in the BLA compared to the other amygdaloid nuclei (Fig. 2A). The result is consistent with higher level of I_h recorded from principal neurons in the BLA relative to the other nuclei (BLA, 417.3 ± 15.82 pA ($n = 78$); central nucleus, 97.53 ± 23.55 pA ($n = 15$); lateral nucleus, 189.1 ± 13.02 pA ($n = 13$) at −140 mV. The expression of HCN1 channels in the BLA was prominent in the surface of soma and neuronal processes (Fig. 2B).

Contribution of I_h to intrinsic properties of BLA principal neurons

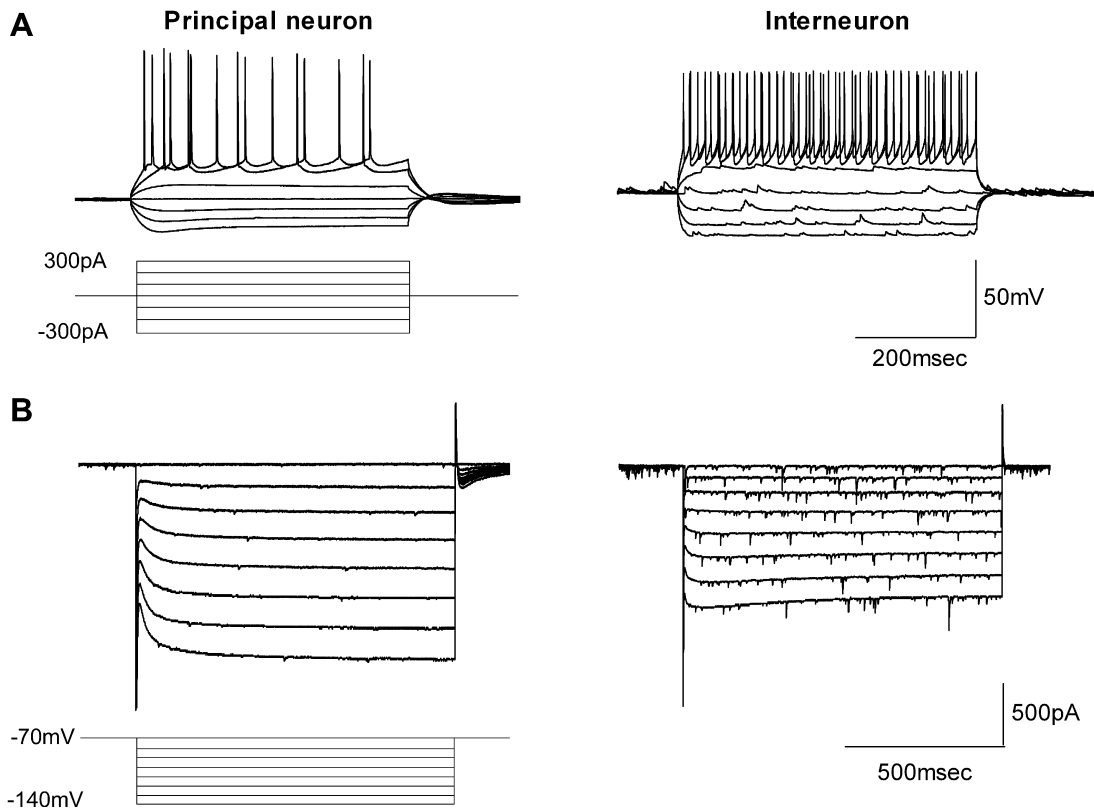To test whether I_h contributes to electrical membrane properties of BLA principal neurons,

Fig. 1. Electrophysiological characterization of BLA neurons. (A) Superimposed voltage responses to series of negative and positive current pulses (−300 to +300 pA in 50 pA increments) in a principal neuron and an interneuron. (B) Superimposed current responses recorded in whole-cell mode elicited by hyperpolarizing-voltage steps from −140 to −70 mV in a principal neuron and an interneuron.
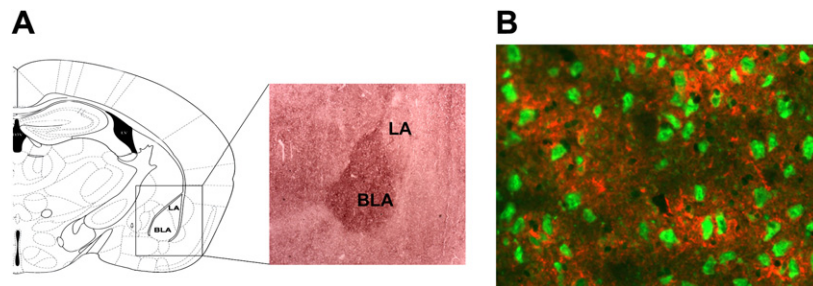

Fig. 2. Localized expression of HCN1 channels in BLA neurons. (A) To examine which area in the amygdala expresses HCN1 channels, mouse brain sections were processed for immunostaining using ABC kit. Note the BLA area is intensely stained. (B) The brain sections were double stained with anti-NeuN (green) and anti-HCN1 (red) antibodies. BLA area is shown. Note the HCN1 immunoreactivity is detected in the surface of soma and neuronal processes. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

hyperpolarization-activated currents, resting membrane potentials, input resistance, and firing rate of action potentials were examined in the presence or absence of 10 μM ZD7288, the specific I_h blocker [38]. ZD7288 (10 μM) inhibited the inward I_h currents evoked by hyperpolarizing-voltage steps, and it also blocked the depolarizing-voltage sag elicited by hyperpolarizing current injection (Fig. 3A). Current injection resulted in a greater voltage response in the presence of ZD7288 than in controls (Fig. 3B). I_h blockade by ZD7288 produced a significant increase in the frequency of action potentials (Fig. 3B and C). As predicted, application of ZD7288 produced an increase in input resistance (before = 66.41 ± 1.52 MΩ, $n = 78$; after = 108.2 ± 4.10

MΩ, $n = 25$; $p < 0.0001$, unpaired t-test), and it also hyperpolarized resting membrane potential (before = −65.31 ± 0.42 mV, $n = 78$; after = −71.98 ± 0.79 mV, $n = 25$, $p < 0.0001$, unpaired t-test). These results confirm that the electrical membrane properties of BLA principal neurons depend considerably on ZD7288-sensitive I_h (i.e., a decrease in I_h results in an increase in overall excitability).

Contribution of I_h to spread and summation of EPSPs in BLA principal neurons

Previous studies have shown that I_h is present at high densities of pyramidal neurons in CA1 region of the hippo-
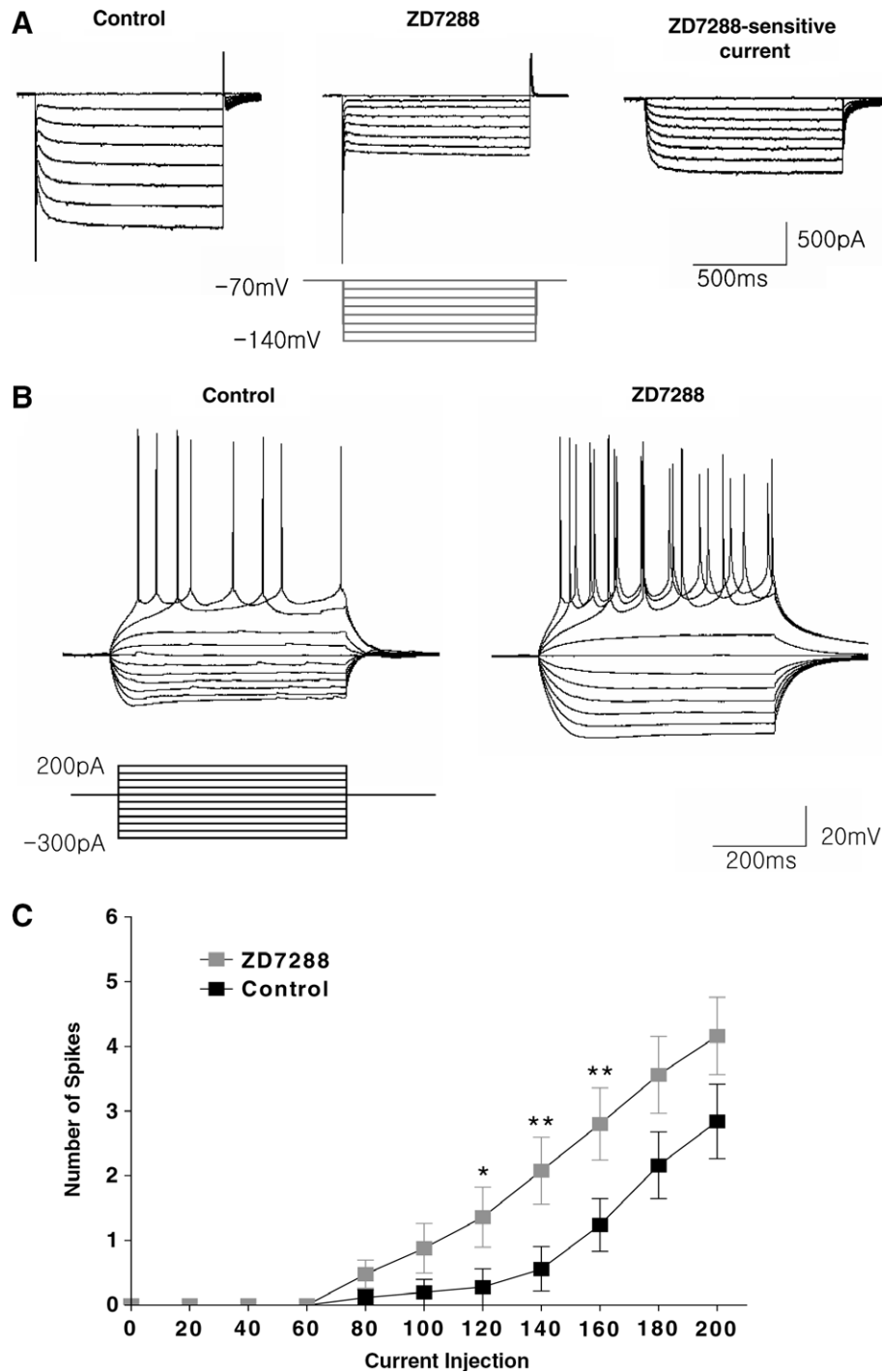
Fig. 3. Effect of ZD7288 on electrical membrane properties of BLA principal neurons. (A) Currents were evoked by hyperpolarizing-voltage steps from −140 to −70 mV. Bath application of 10 μM ZD7288 blocked slow hyperpolarization-activated currents, and I_h was isolated by subtracting current traces before and after addition of ZD7288. (B) Voltage responses were recorded by current steps from −300 to +200 pA in the presence or absence of 10 μM ZD7288. Blockade of I_h resulted in a greater voltage response; increased input resistance and increased number of action potentials. (C) Number of action potential spikes was counted as a function of depolarizing current injection with duration of 500 ms. Block of I_h significantly increased action potential firing rate ($n = 25$; $^*p < 0.05$ or $^{**}p < 0.005$).

campus and the neocortex, and that I_h plays a role in temporal summation of EPSPs in the pyramidal neurons [23,25]. In particular, dendritic I_h has been proposed to reduce postsynaptic responsiveness to excitatory synaptic inputs [27,39,40]. To test whether I_h in BLA principal neurons also contribute to modulation of synaptic response to

excitatory inputs, we evoked excitatory postsynaptic potentials (EPSPs) in BLA principal neurons in the presence or absence of ZD7288 (10 μM) by stimulating afferents along the border between the lateral amygdala (LA) and the BLA (Fig. 4A). When EPSPs were elicited by single stimuli, application of ZD7288 had no significant effects on EPSP
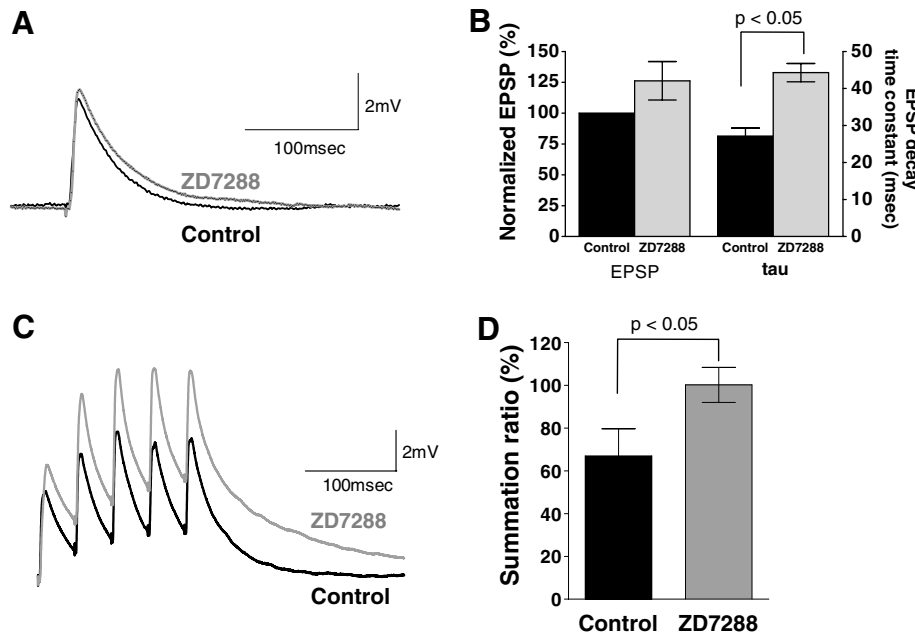
Fig. 4. Effect of ZD7288 on EPSPs evoked in BLA principal neurons. (A) Single EPSP was synaptically evoked by bipolar electrode located at the border of LA-BLA and recorded at resting membrane potential in the presence or absence of 10 μM ZD7288. Average traces of 10 consecutive sweeps were presented. (B) Pooled data for effect of ZD7288 on EPSP amplitude and EPSP decay time constant. EPSP amplitudes were normalized by those without ZD7288 and decay time constants were measured by fitting the traces with mono-exponential function ($n = 11$; $p < 0.05$). (C) Trains of EPSPs elicited by 25 Hz synaptic stimulations were recorded at resting potentials in the presence or absence of 10 μM ZD7288. Average traces of 10 consecutive sweeps were presented. (D) Pooled data for effect of ZD7288 on temporal summation of EPSPs. EPSP amplitude was measured as the peak voltage and summation ratio was expressed as a percentage increase in synaptic depolarization during a train of 5 EPSPs; i.e., summation ratio $= [(EPSP5 - EPSP1)/EPSP1]$, where EPSP5 is the peak voltage during the fifth and EPSP1 during the first EPSP in the train. ZD7288 significantly increased temporal summation ($n = 11$; $p < 0.05$).

amplitudes (1.263 ± 0.156-fold increase with ZD7288, $n = 11$; $p = 0.1077$; Fig. 4B), but it significantly prolonged the decay time constants of EPSPs (27.15 ± 2.16 ms for control, $n = 11$; 44.29 ± 2.48 ms for ZD7288, $n = 11$; $p < 0.05$; Fig. 4B).

We next tested whether the prolonged decay of EPSPs could contribute to modulation of somato-dendritic temporal summation. Temporal summation was determined by applying a brief train of synaptic stimulation at 25 Hz (Fig. 4C). Application of ZD7288 resulted in an increase in the temporal summation ratio of repetitively evoked synaptic responses (66.97 ± 12.69% for control, $n = 11$; 100.20 ± 8.18% for ZD7288, $n = 11$; $p < 0.05$; Fig. 4D). These data suggest that I_h plays a important role in fastening EPSP decay, and that reduced availability of I_h prolongs EPSP decay, thereby enhancing propagation of voltage responses, especially temporally integrated responses, to the axon hillock.

Discussion

Our results demonstrate that: (i) HCN channels are locally expressed in BLA principal neurons; (ii) suppression of HCN channel activity increases the intrinsic excitability of the principal neurons; and (iii) blockade of HCN channels enhanced the summation of EPSPs in the principal neurons. These results suggest that under condition where

HCN channels are blocked (or closed), electrical excitability of BLA principal neurons is dramatically increased. Conversely, opening of HCN channels attenuates the excitability. Thus, electrical excitability of BLA principal neurons might be dramatically regulated through the modulation of HCN channel activity, consistent with the previous findings shown in principal neurons in the cortex and the hippocampus [23,25,27].

Recent studies have proposed possible physiological roles of HCN channels in the context of their attenuation effect on neuronal excitability. In the hippocampus, HCN1 channels constrained spatial memory and plasticity by regulating dendritic integration of distal synaptic inputs onto CA1 pyramidal neurons [40]. In the prefrontal cortex, working memory networks were strengthened by inhibiting HCN channels [41]. Pathologically, altered HCN channels have been reported to produce aberrant activity of neurons and thus facilitate generation of seizure activity [42]. To our knowledge, a functional role of HCN channels in the amygdala has not been examined. The present findings clearly show that modulation of HCN channel activity can selectively tune the excitability of principal neurons in the BLA. Considering that emotional states are tightly related with electrical excitability of the BLA [5–8], modulation of HCN channel activity might be involved in regulating anxiety and affective responses. In fact, we found that intra-BLA infusion of ZD7288, which enhanced the

excitability of BLA neurons, increased anxiety under the elevated plus maze test (our unpublished observation).

The best-known modulator of HCN channels is cAMP, which directly binds to C-terminal cyclic-nucleotide-binding domain, shifting voltage dependence of HCN channel opening in a depolarization direction [22]. In addition, there are several pieces of experimental evidence that other modulators are present, independent of cyclic nucleotide. For example, protein tyrosine kinases have been implicated in the modulation of HCN channels [43–45]. p38 MAPK has been also established as a modulator of HCN channels [46]. Recently, phosphatidylinositol-4,5-biphosphate has been recently reported to allosterically open HCN channels by shifting voltage-dependent channel activation toward depolarized voltages [47]. Inhibition of HCN channels, which renders BLA principal neurons hyper-excitable, might be relevant to pathological states of the amygdala such as anxiety and mood disorders. In light of this, it will be interesting to explore signaling pathways regulating HCN channel activity of BLA principal neurons under pathological and physiological conditions.

Acknowledgments

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