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Extracellular pH modulates GABAergic neurotransmission in rat hypothalamus

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Abstract

Changes in extracellular pH have a modulatory effect on GABA_A receptor function. It has been reported that pH sensitivity of the GABA receptor is dependent on subunit composition and GABA concentration. Most of previous investigations focused on GABA-evoked currents, which only reflect the postsynaptic receptors. The physiological relevance of pH modulation of GABAergic neurotransmission is not fully elucidated. In the present studies, we examined the influence of extracellular pH on the GABA_A receptor-mediated inhibitory neurotransmission in rat hypothalamic neurons. The inhibitory postsynaptic currents (IPSCs), tonic currents, and the GABA-evoked currents were recorded with whole-cell patch techniques on the hypothalamic slices from Sprague-Dawley rats at 15–26 postnatal days. The amplitude and frequency of spontaneous GABA IPSCs were significantly increased while the external pH was changed from 7.3 to 8.4. In the acidic pH (6.4), the spontaneous GABA IPSCs were reduced in amplitude and frequency. The pH induced changes in miniature GABA IPSCs (mIPSCs) similar to that in spontaneous IPSCs. The pH effect on the postsynaptic GABA receptors was assessed with exogenously applied varying concentrations of GABA. The tonic currents and the currents evoked by sub-saturating concentration of GABA ([GABA]) (10 μ M) were inhibited by acidic pH and potentiated by alkaline pH. In contrast, the currents evoked by saturating [GABA] (1 mM) were not affected by pH changes. We also investigated the influence of pH buffers and buffering capacity on pH sensitivity of GABA_A receptors on human recombinant $\alpha 1\beta 2\gamma 2$ GABA_A receptors stably expressed in HEK 293 cells. The pH influence on GABA_A receptors was similar in HEPES- and MES-buffered medium, and not dependent on protonated buffers, suggesting that the observed pH effect on GABA response is a specific consequence of changes in extracellular protons. Our data suggest that the hydrogen ions suppress the GABAergic neurotransmission, which is mediated by both presynaptic and postsynaptic mechanisms.

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Keywords

protons; IPSC; acidification; hypothalamus; HEPES; buffer

INTRODUCTION

γ -aminobutyric acid (GABA), the predominant fast inhibitory neurotransmitter of the adult central nervous system (CNS), acts principally via the GABA_A receptors. Activation of the synaptic GABA_A receptors generates inhibitory postsynaptic currents (IPSCs), leading to hyperpolarization of the postsynaptic neurons and preventing them from firing action potentials. In addition to the synaptic transmission, the extrasynaptic receptors are activated by low extracellular GABA concentration (0.8–2.9 μ M) (Lerma et al., 1986), producing a widespread and sustained inhibition (tonic inhibition). Most brain functions employ combinations of both synaptic and tonic transmission. Tonic inhibition plays a crucial role in regulating neuronal excitability because it sets the threshold for action potential generation and integrates excitatory signals.

GABA_A receptor function is regulated by several endogenous ions, including physiological concentration of protons ($[H^+]$). Interstitial pH in mammals normally ranges between 7.36 and 7.44 (Bullock, 1990). It is known that pH in the brain changes with neural activity. Under various physiological conditions, brain pH may shift between 6.5 and 8.0 (Chesler, 1990, Chesler and Kaila, 1992). In certain pathological conditions such as stroke, seizure, hypoxia and ischemia, brain pH can shift up to 1 unit (Wang and Sonnenschein, 1955, Somjen, 1984, Siesjo et al., 1985, Carmeliet, 1999). It is well known that protons modulate neuronal excitability and this effect may be partially mediated through pH modulation of GABA_A receptors.

Our previous studies and other investigations have shown that GABA_A receptors are tightly regulated by extracellular pH. However, most studies focused on the pH effect on GABA-activated whole-cell currents recorded from recombinant or native preparations, which could not distinguish phasic and tonic inhibition components. A detailed elucidation of physiological relevance for pH modulation still remains insufficiently studied. While a few papers so far examined proton modulation of miniature inhibitory postsynaptic currents (mIPSCs) and GABA-evoked currents recorded from the neurons of hippocampus (Mozrzymas et al., 2003, Zhou et al., 2007), cerebellar granule (Dietrich and Morad, 2010) and spinal cord dorsal horn (Hugel et al., 2012), the proton effect on hypothalamic GABA_A receptors has never been studied. Furthermore, how proton modulates extrasynaptic GABA_A receptors is still unknown. The hypothalamus plays a vital role in endocrine, autonomic and behavioral functions (Meister, 1993). GABA suppresses the activity of hypothalamic neurons and has been suggested to be the dominant inhibitory neurotransmitter in the hypothalamus (Decavel and Van den Pol, 1990). Hypothalamic GABAergic neurotransmission through ionotropic GABA_A receptors has synaptic (phasic) and extrasynaptic (tonic) components (Park et al., 2007). In the present studies, we investigated pH modulation of GABAergic synaptic and extrasynaptic inhibition in the rat hypothalamic

areas. Our results demonstrated that protons modulate GABAergic synaptic and tonic currents in the rat hypothalamus.

EXPERIMENTAL PROCEDURES

Brain slice preparation

Hypothalamic slices were acutely prepared from postnatal day (P) 15–26 (day of birth=P1) Sprague-Dawley rats (either sex, Indianapolis, IN). The rat was anesthetized with isoflurane and rapidly decapitated. All procedures were conducted in accordance with the *National Institutes of Health Guide for Care and Use of Laboratory Animals*. All stages of brain dissection and tissue slicing were conducted in ice-cold (~4 °C) artificial cerebral-spinal fluid (ACSF) of the following composition (in mM): 124 NaCl, 5.0 KCl, 1.3 MgSO₄, 26 NaHCO₃, 1.24 KH₂PO₄, 2.4 CaCl₂ and 10 glucose; 300 mOsm and pH ~7.4 after equilibration with a 95% O₂/5% CO₂ gas mixture (carbogen). Thin hypothalamic slices (200 µm) were cut with a vibratome (VSL, World Precision Inst., Inc); slices were submerged in ACSF (22–25 °C) aerated with the carbogen gas mixture. Slices were stored at room temperature in carbogen bubbled ACSF until used.

Cloned receptors

Human embryonic kidney cell lines (HEK293) stably expressing recombinant human $\alpha 1\beta 2\gamma 2$ were used to investigate the effect of buffers on pH modulation of GABA_A receptors. Cells transiently expressing human glycine $\alpha 1$ receptors were also studied. HEK293 cell line was transiently transfected with recombinant receptor subunit using PolyJetTM DNA *In Vitro* transfection reagent (SignaGen Laboratories, Rockville, MD). Briefly, HEK293 cells were washed and placed in fresh Dulbecco's modified eagle medium containing 10% FBS and antibiotics (penicillin 100 U/mL). A 0.5 µg of human glycine $\alpha 1$ subunit cDNA was added to cells growing exponentially on poly-L-lysine coated coverslips placed in a 35-mm culture dish. Transfected cells were used for electrophysiological analysis 24–48 h after the transfection.

Electrophysiology

Whole-cell patch recordings were made at room temperature (22–25 °C) at a holding potential of –70 mV for brain slice or –60 mV for recombinant receptors. Patch pipettes of borosilicate glass (M1B150F, World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 7–8 MΩ. The pipette solution contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP; pH 7.2. For brain slice studies, a single slice was transferred to a recording chamber (~2 ml) and superfused continuously (7–10 ml/min, 22–25 °C) with external solution consisting of the following (in mM): 140 NaCl, 3.0 KCl, 2 MgCl₂, 10 HEPES, 2.4 CaCl₂, 10 glucose, 330 mOsm and pH 7.3. Individual hypothalamic neuron within the slice were visualized using an upright, fixed stage microscope (Nikon Optiphot-2UD) equipped with standard Hoffman modulation contrast (HMC) optics and a video camera system (Sony model XC-75 CCD video camera module, DOT-X monitor). Location of hypothalamic neurons studied in this investigation was identified to be in the posterior, dorsomedial, lateral, ventomedial and arcuate nuclei according to a stereotaxic atlas for adult rats (Paxinos and Watson, 1986) as

previously described (Huang and Dillon, 2002). Spontaneous GABAergic inhibitory postsynaptic currents (sIPSCs) were recorded in the whole-cell configuration in the presence of glutamate receptor antagonist kynurenic acid (1 mM, K3375, Sigma, St Louis, MO). Miniature IPSCs (mIPSCs) were isolated with additional tetrodotoxin (TTX, 0.5 μ M, T8024, Sigma, St Louis, MO). For studies on cloned receptors, a coverslip containing cultured cells was placed in a small chamber (~ 1.5 ml) on the stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5–8 ml/min, 22–25 °C) with the following external solution containing (in mM): 125 NaCl, 5.5 KCl, 0.8 MgCl₂, 3.0 CaCl₂, 10 HEPES, 10 glucose and pH 7.3. The currents (GABAergic IPSCs, tonic currents and GABA- or glycine-induced Cl⁻ currents) from the whole-cell configuration were obtained using a patch clamp amplifier (PC-501A, Warner Instruments, Hamden, CT) equipped with a 5101-01G headstage. Signals were filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), and sampled at 30 kHz using a Digidata 1200 and pClamp software (pClamp 6.0, Axon Instruments) and stored on a computer for offline analysis. The series resistance (R_s) was compensated online by 60–70% in voltage-clamp mode to reduce voltage errors. To monitor the possibility that access resistance changed over time or during different experimental conditions, at the initiation of each recording we measured and stored the current response to a 5 mV voltage pulse on our digital oscilloscope. This stored trace was continually referenced throughout the recording. The cells in which access resistance or baseline showed instability during recording period were not included in the analysis.

Experimental protocol

For the studies on hypothalamic slices, pH of external solutions was altered by addition of NaOH or HCl, and routinely checked before and during experiments. The osmolality of the control external medium (pH 7.3) was 330 ± 9.1 mOsm and was not changed in basic or acidic solutions (333 ± 7.3 and 348 ± 5.8 mOsm, respectively). Calculation with Nernst equation shows that reversal potential for Cl⁻ was apparently unaffected by pH adjustment (0.56 mV change by HCl). For the experiments with pH buffers on recombinant receptors, pH of external solution was set to desired pH value with NaOH. The osmolality of the solutions containing lower HEPES or MES was adjusted to ~330 mOsm by adding sucrose. To assess the pH effect, cells were first bathed in media that was set to the test pH, then GABA, bicuculline or glycine, dissolved in the same test pH solution, was applied from independent reservoirs by gravity flow for 10–20 sec to the cells via a Y-shaped tube positioned within 100 μ m of the cells or the membrane patch. With this system, the 10–90% rise time of the junction potential at the open tip was 12–51 ms (Huang and Dillon, 1999). Because in the whole-cell recordings, external solution of low pH elicited, as in other studies (Bevan and Yeats, 1991, Krishek et al., 1996, Zhai et al., 1998), a transient whole-cell inward current, recordings at various pH test values was made after the transient current had recovered and stabilized. In addition, IPSC activity recording or ligand application was at least 3 min after a complete washout of previous test pH or ligand application. The order of different pH testing was randomized to minimize time-dependent variables.

Data analysis

The GABA-activated currents were analyzed with Clampfit 6.0 (Axon Instruments). All spontaneous and miniature synaptic events were detected and analyzed using MiniAnalysis

6.03 (Synaptosoft, Decatur, GA). IPSC events were sampled for a 5-min continuous recording period for each neuron under control (pH 7.3), testing pH (pH 8.4 or 6.4) and washout condition (pH 7.3). IPSC events were detected automatically using the threshold criteria of root mean square (RMS) noise and then inspected visually for any aberrant currents. GABA-dependent IPSCs were analyzed by the rise time (10–90%), peak amplitude and charge transfer (area under IPSC waveform) parameters of the MiniAnalysis. The time constants of current decay were calculated with biexponential function fitting of the averaged trace of grouped IPSC events. The tonic currents were estimated with a shift in baseline current caused by bicuculline (10 μ M) application. The average baseline holding currents and the outward shift currents after applying bicuculline were determined by Origin 5.0 (Microcal Software, Inc, Northampton, MA). The tonic current was calculated by subtracting the average baseline current from the average outward shift current caused by bicuculline.

Differences between different pH situations were assessed by one way ANOVA test or Student's t-test (paired or unpaired). All data are represented as mean $\pm$ S.E.M. and $P < 0.05$ was considered statistically significant.

RESULTS

Effect of pH on spontaneous GABAergic IPSCs

Spontaneous inward synaptic currents were recorded with the whole-cell configuration at a holding potential of -70 mV. Block of ionotropic glutamate receptors with kynurenic acid (1 mM) allowed recording of essentially pure GABAergic IPSCs, which were completely blocked by bicuculline methiodide (10 μ M, Fig. 1A). The effect of extracellular pH was investigated using external media of which pH was set to 7.3 (control) or to testing pH (pH 8.4 or 6.4). Once the baseline was stable, GABAergic IPSCs were collected over a 5 min period before, during, and after the perfusion of testing pH media. A total of 15 successful recordings from 9 rats (P15–26, mean: P22) in which no run-down of IPSCs occurred and no change in series resistance was detected (see Method) were included in analysis of this study. Spontaneous IPSCs recorded in the control condition ($n=28$) had a highly variable amplitude of 30.1–174.7 pA (mean amplitude 65 ± 6.0 pA), a frequency of 0.14 – 1.43 Hz (mean = 0.33 ± 0.06 Hz).

In 13 out of 15 recorded cells (87%), spontaneous GABAergic IPSC activity exhibited an enhancement in alkaline pH and an inhibition in acidic pH. Change of extracellular pH produced a significant change in amplitude and frequency of sIPSCs. Specifically, as shown in Fig. 1B&C, the mean amplitude of sIPSCs was increased from 67 ± 8.4 pA in control pH to 87 ± 12 pA ($127 \pm 5.0\%$ of control) in alkaline pH (8.4), and reduced from 63 ± 8.8 pA at control to 44 ± 5.2 pA ($76 \pm 5.0\%$ of control) in acidic pH (6.4) ($n=14$, $p<0.05$, paired t-test). Meanwhile, as shown in Fig. 1D, alkaline pH decreased while acidic pH increased inter-event intervals of sIPSC. As a result, sIPSC frequency was increased to $192 \pm 45\%$ of control in pH 8.4 (from 0.26 ± 0.04 Hz to 0.48 ± 0.14 Hz), and reduced to $56 \pm 5.8\%$ of control in pH 6.4 (from 0.41 ± 0.10 to 0.21 ± 0.05 Hz) (Insert in Fig. 1D, $p<0.05$, paired t-test). The pH-induced changes in amplitude and frequency were returned to the control level

after reperfusion of the slices at pH 7.3, suggesting that the pH effect was fully reversible ($p > 0.05$, paired t-test, compared to control).

The alkaline pH also exerted a significant effect on kinetics of sIPSCs. Figure 2A showed that the 10–90% rise time significantly decreased to $88 \pm 3.8\%$ of control at pH 8.4 (from 2.54 ± 0.24 ms to 2.22 ± 0.23 ms), and increased to $115 \pm 6.9\%$ of control at pH 6.4 (from 2.69 ± 0.30 ms to 3.00 ± 0.31 ms) ($n=14$, $p < 0.05$, paired t-test). Consistent with the changes in amplitude and rise time, the IPSC area, which reflects the total charge transfer that occurs during each synaptic event, was markedly increased to $129 \pm 6.2\%$ of control at pH 8.4 (from 731 ± 94 mspA to 978 ± 158 mspA), and reduced to $74 \pm 4.5\%$ of control at pH 6.4 (from 605 ± 53 mspA to 436 ± 45 mspA). ($p < 0.01$, $n=14$, paired t-test) (Fig. 2B). After the test pH medium was switched to pH 7.3 medium, the kinetic parameters of IPSCs returned to the control level ($p > 0.05$, paired t-test, compared to control), suggesting that the effect of pH on sIPSC kinetics was reversible (Fig. 2). However, the decaying phase of sIPSCs including fast and slow time constants and their relative fractions was not significantly affected by changes in pH (Fig. 2C&D).

Effect of pH on miniature GABAergic IPSCs

It has been known that extracellular pH modulates the excitability of hypothalamic neurons. pH may modify the firing pattern of presynaptic interneurons and in turn alter the frequency of spontaneous IPSCs. To determine whether pH effect on IPSCs was caused by action potential-dependent mechanism, the experiments were repeated in the presence of TTX ($0.5 \mu\text{M}$) which blocks action potentials by binding to the voltage-gated Na^+ channels. In the control condition, the amplitude of TTX-resistant mIPSCs recorded from 15 hypothalamic neurons was smaller than that of sIPSCs (sIPSCs: 65 ± 6.0 pA, mIPSCs: 50.8 ± 5.7 pA, $p < 0.05$, unpaired t-test) from the rats with similar development stage. After removal of action potential-dependent IPSCs, protons exert the modulatory effect similar to spontaneous IPSCs. As shown in Fig. 3A–D, in all recorded cells ($n=8-9$), alkaline pH increased amplitude, frequency and charge transfer of mIPSCs significantly. Acidic pH had an opposite effect on mIPSC amplitude, frequency and charge transfer. Moreover, the pH modulatory effect was highly reversible upon returning to control pH (Fig. 3B). The 10–90% rise time and decaying phase of mIPSCs were not significantly altered by change in extracellular pH (Fig. 3D–F).

Effect of pH on tonic GABAergic current

We next wanted to examine whether a change in extracellular pH will regulate tonic GABA currents. Applying the GABA_A antagonist bicuculline (up to $50 \mu\text{M}$) into the chamber blocked sIPSCs, but hardly elicited tonic current. The lack of detection of tonic GABA currents is due to high perfusion rate of slices that may wash away the ambient transmitter, leading to a reduction or even elimination of the tonic current, particularly in cells on the surface of the submerged slices we used here (Glykys and Mody, 2007, Clarkson et al., 2010). To overcome this problem, 1 or $2 \mu\text{M}$ GABA was added to extracellular solution for continuous perfusion to mimic *in vivo* ambient extracellular GABA levels (Lerma et al., 1986). We blocked GABA_A receptors with bicuculline ($10 \mu\text{M}$) and measured the change in holding current. This revealed a “mimic” tonic current in the hypothalamic neurons under

different pH conditions. Over the pH range used in the study (pH 6.4–8.4), ionization state of bicuculline (pK_a 4.84) has little change (<3%) based on calculation with Henderson-Hasselbalch equation. Thus pH effect on bicuculline response could be attributable to proton influence on GABA_A receptors. In all recorded cells, tonic currents were increased in pH 8.4, and decreased in pH 6.4. As shown in Fig. 4, on average, alkaline pH (8.4) significantly increased tonic current by 183% (from 10.7 ± 1.7 pA to 19.7 ± 2.7 pA, $n=19$) while acidic pH (6.4) significantly suppressed the amplitude of tonic currents by 29% (from 12.4 ± 1.1 pA to 8.9 ± 1.4 pA, $n=13$, $p<0.01$, paired t-test). Moreover, the pH-induced changes were reversible upon returning to control pH.

Effect of pH on evoked GABA currents

In order to access the postsynaptic component of pH mediated modulation of GABAergic IPSCs, we also examined the GABA-activated currents from the same cells in which mIPSCs were recorded in different pH conditions. The whole-cell currents activated by saturating (1 mM) or low (10 μ M) [GABA] were recorded in the presence of TTX (0.5 μ M) and kynurenic acid (1 mM). As shown in Fig. 5A&B, the currents activated by 10 μ M GABA were markedly regulated by pH in all recorded cells. The average currents were increased to $211.7 \pm 32.5\%$ and decreased to $66.7 \pm 6.8\%$ of the control at pH 8.4 and pH 6.4, respectively ($n=10-11$, $p<0.05$, paired t-test). On contrast, change in pH had no effect on the currents activated by 1 mM GABA (Fig. 5C&D). The mean current amplitude was $100.3 \pm 1.01\%$ and $99.9 \pm 1.3\%$ of control at pH 8.4 and 6.4, respectively ($n=16-20$, $p>0.05$, paired t-test). Such concentration-dependence of pH modulation of GABA-activated currents is consistent with previous reports on recombinant and native receptor preparations (Zhai et al., 1998, Huang and Dillon, 1999, Li et al., 2003, Huang et al., 2004, Wang et al., 2005).

Effect of buffers on pH sensitivity of GABA_A receptor

As shown above, GABAergic neurotransmission which largely occurs at postsynaptic level was tightly regulated by extracellular pH under HEPES-buffered medium. Compared to CO₂/HCO₃⁻ buffered ACSF, HEPES-buffered medium has the advantages that its pH is not dependent on dissolved CO₂ or change in medium [HCO₃⁻], and it does not have extracellular alkalinization caused by HCO₃⁻ efflux through GABA_A receptor channels (Chen and Chesler, 1991, 1992). Thus HEPES buffered medium has been most commonly used as an alternate formulation of ACSF in electrophysiology studies including those on pH related physiological process. Recently, Hugel *et al* (2012) reported that the inhibition of GABA-activated currents in acidic pH condition is caused by protonated HEPES not by protons. Therefore, pH sensitivity of GABA_A receptors observed in HEPES-buffered medium may not be a specific consequence of change in extracellular pH. In order to address this important issue, we investigated the effect of different pH buffers on pH sensitivity of human $\alpha 1\beta 2\gamma 2$ GABA_A receptors stably expressed in HEK293 cells. First, we examined the direct effect of two common buffers, HEPES (pK_a 7.5) and 2-ethanesulfonic acid (MES, pK_a 6.1), on the currents activated by EC₃₀ GABA (6 μ M). Based on calculation with Henderson-Hasselbalch equation, it is estimated that the portion of the protonated HEPES is 61% at pH 7.3 and 93% at pH 6.4. If protonated HEPES is the actual inhibitor to GABA_A receptors, one would expect that the inhibition of GABA current by HEPES would

become greater with increasing portion of protonated HEPES at acidic pH. Fig. 6A&B show that 20 mM HEPES inhibited GABA currents to a similar extent under different pH conditions. On average, the GABA currents were reduced by 20 mM to $72 \pm 2.1\%$ at pH 7.3 and $74 \pm 1.7\%$ at pH 6.4 compared to the currents at 10 mM HEPES ($n=9$, $p>0.05$, paired t-test). GABA currents were further inhibited to $38 \pm 1.8\%$ of control by 40 mM HEPES ($n=13$) at pH 6.4, suggesting that HEPES effect was not saturated at 20 mM. In the case of MES, the protonated form of MES is 6% at pH 7.3 and 33% at pH 6.4. We tested whether the effect of MES on GABA receptors was influenced by a rise in protonated MES when pH was changed from pH 7.3 to 6.4. As shown in Fig. 6C&D, 10 mM MES reduced GABA currents to $90 \pm 1.1\%$ of control at pH 6.4, which is similar to that observed at pH 7.3 ($93 \pm 1.9\%$ of control, $n=8$; $p>0.05$, paired t-test). These data suggest that direct action of either HEPES or MES on GABA_A receptors is not dependent on their protonated status.

Next, we compared the pH sensitivity of GABA response in 10 mM HEPES-buffered medium vs. 20 mM HEPES buffering medium. It is estimated that change in pH from 7.3 to 6.4 causes a net increase of 3.2 mM ($32\% \times 10$) and 6.4 mM ($32\% \times 20$) protonated HEPES in 10 mM HEPES and 20 mM HEPES-buffered medium, respectively. If pH effect on GABA currents were mediated via protonated HEPES, one would expect that such pH effect would be greater in the medium buffered with a higher buffer concentration. As shown in Fig. 7, pH sensitivity of GABA_A receptors was not statistically different in the 10 mM HEPES vs 20 mM HEPES. At pH 6.4, GABA-activated currents were reduced to $71 \pm 1.9\%$ of control in 10 mM HEPES and $76 \pm 3.5\%$ in 20 mM HEPES ($n=9$, $p>0.05$, paired t-test). These data suggest that the inhibition of GABA current in acidic pH is a specific consequence of change in extracellular $[H^+]$ not protonated HEPES.

To further attribute the observed pH effect to extracellular protons not buffer used, we compared the sensitivity to pH 6.4 in 10 mM MES-buffered medium with that in 10 mM HEPES-buffered medium. As shown in Fig. 7, the inhibition of GABA currents by acidic condition in 10 mM MES-buffered medium was similar to that in 10 mM HEPES-buffered medium. The GABA-activated currents were reduced to $77 \pm 2.3\%$ of control for MES ($n=8$) and $71 \pm 1.9\%$ of control for HEPES ($n=9$; $p>0.05$, unpaired t-test). In view of the fact that MES has much less inhibition on GABA response than HEPES whereas pH sensitivity was elicited similarly in these two buffer media, these data further suggest that extracellular protons are responsible for the observed pH effect.

Effect of buffering capacity on pH sensitivity of GABA_A receptor

It has been reported that buffering capacity influences modulation of L-type Ca^{2+} channels by released vesicular protons after exocytosis in photoreceptor terminals (DeVries, 2001, Hirasawa and Kaneko, 2003, Palmer et al., 2003). Insufficient buffering could result in inability to “clamp” microenvironment of cells to the same pH as that of the extracellular solution. Buffer capacity is determined by buffer molar concentration at a given pH. To determine the concentration-response relationship of influence of buffering capacity on pH sensitivity, we examined pH sensitivity of GABA response during perfusion of the medium buffered with a series of HEPES concentrations varying from 1 to 20 mM. As shown in Fig. 8A&B, the sensitivity to acidic pH 6.4 was similar in the media buffered with 2.5–20 mM

HEPES ($p > 0.05$, one-way ANOVA analysis). However, pH sensitivity was significantly attenuated in 2 mM HEPES, and completely lost in 1 mM HEPES. The relative currents were $87 \pm 2.7\%$ of control in 2 mM HEPES ($n=6$) and $106 \pm 4.9\%$ in 1 mM HEPES ($n=20$, $p < 0.05$, unpaired t-test, compared to 2.5–20 mM HEPES). These data indicate that buffering capacity influences pH effect on GABA_A receptors. Furthermore, the minimum buffering that is adequate to reveal pH effect is 2.5 mM HEPES under our experimental conditions.

Next, to determine whether buffering capacity requirement is unique to HEPES buffer, we recorded pH sensitivity of GABA response during perfusion of MES-buffered medium using weak (1 mM) and strong buffering (10 mM). The inhibition of GABA currents by pH 6.4 was significantly diminished in 1 mM MES compared to 10 mM MES ($96 \pm 2.5\%$ of control at 1 mM vs $77 \pm 2.3\%$ at 10 mM, $p < 0.05$, unpaired t-test) (Fig. 8C&D). In addition, it has been reported that glycine receptors are inhibited by acidic pH (Laube et al., 2000, Chen et al., 2004, Chen and Huang, 2007). Consistent with a previous report (Hugel et al., 2012), we found that 10 mM HEPES had no effect on human glycine $\alpha 1$ receptors transiently expressed in HEK293 cells (data not shown). Since HEPES has no direct action on glycine receptors, pH sensitivity of glycine receptors is presumably mediated by protons not HEPES. As shown in Fig. 8C&D, the ability of acidic pH to inhibit glycine $\alpha 1$ receptors was dramatically attenuated when the cells were perfused with the medium containing 1 mM buffer. On average, the glycine-activated currents were reduced to $44 \pm 4.2\%$ of control in 10 mM HEPES and $67 \pm 3.5\%$ in 1 mM HEPES ($p < 0.05$, unpaired t-test). Thus we confirmed that influence of buffering capacity on proton-mediated effect could be replicated with an alternative buffer (MES) and another receptor (glycine $\alpha 1$ receptor).

Finally, since buffer has its greatest buffering capacity when pH is equal to its pK_a value, pH studies should ideally be performed using the buffer whose pK_a is close to testing pH, for example, HEPES (pK_a 7.5) for pH 7.3 and MES (pK_a 6.1) for pH 6.4. However, in the case of GABA_A receptors, pH effect could be interfered by difference in direct inhibition of buffers on the receptors. For example, as shown above (Fig. 6), MES has much less direct effect on GABA_A receptors than HEPES, which could ultimately undermine proton-induced effect when pH sensitivity of GABA_A receptors is assessed from same cells using different buffers. To reveal such interference, we recorded GABA currents in 10 mM HEPES- and 10 mM MES-buffered medium from same cells. As shown in Fig. 9, at pH 7.3, GABA currents recorded in MES-buffered medium were increased by 21% compared to those in HEPES-buffered medium ($n=6$, $p < 0.05$, paired t-test). This increase was due to a partial relief of relatively greater inhibition from HEPES, which would interfere with the observation on proton-induced inhibition (23–28%, tested with a MES or HEPES system. See Fig. 7). As a result, GABA currents were not significantly altered (relative current: $95 \pm 4.8\%$ of control, $n=10$, $p > 0.05$, paired t-test) after the external solution was changed from pH 7.3 buffered with 10 mM HEPES to pH 6.4 buffered with 10 mM MES (Fig. 9). These data suggest that pH-dependent modulation of GABA_A receptors has to be studied in the same buffer compositions to eliminate the interference from various buffers. Furthermore, in order to determine whether 10 mM HEPES-buffered medium delivers an appropriate pH buffering to reveal pH effect on GABA_A receptors, we recorded GABA currents in a mixture of HEPES (10 mM) and MES (10 mM) buffers to gain an optimal buffering capacity at pH 7.3 and pH

6.4 (adjusted with NaOH), respectively. Fig. 9 show that the effect of pH on GABA response with a mixture of HEPES and MES buffers was comparable to that with 10 mM HEPES buffer (relative current: $73 \pm 2.4\%$ with HEPES+MES; $71 \pm 1.9\%$ with HEPES. $p > 0.05$, unpaired t-test). Thus these data indicate that the buffer system used in our current studies is appropriate to disclose proton effect.

DISCUSSION

We have shown here that GABAergic synaptic neurotransmission in the hypothalamic area is modulated by extracellular pH. Alkaline condition enhances GABA_A receptor-mediated neurotransmission with an increase in frequency and amplitude of sIPSCs and mIPSCs. Acidic extracellular environment attenuates GABAergic inhibitory activity. pH regulates GABAergic synaptic transmission, both presynaptically and postsynaptically.

In the present studies, both spontaneous and miniature GABAergic IPSC frequency increase in alkaline pH and decrease in acidic pH. The pH-dependent change in IPSC frequency indicates alterations in GABA release probability from the presynaptic neurons. The observed pH effect on spontaneous IPSCs frequency could be partially attributed to neuronal excitability at presynaptic site which affects action potential-dependent release of neurotransmitter. Effects of protons on neuronal activity have been attributed to the modulation of a variety of neuronal ion channels and receptors (Tombaugh and Somjen, 1996, Kaila and Ransom, 1998). The similar pH effect on IPSC frequency still retained in mIPSCs after blockade of action potential-evoked IPSCs with TTX, indicating that constitutive GABA release at the presynaptic terminal is also regulated by extracellular pH. In fact, extracellular pH affects the frequency of miniature GABAergic or glycinergic IPSC (Li et al., 2003, Mozrzymas et al., 2003, Zhou et al., 2007) and NMDA EPSC (Chen et al., 1998) in a similar manner, further supporting the idea that proton-mediated basal neurotransmitter release may be mediated by a universal mechanism at presynaptic terminals. Voltage-gated Ca^{2+} channels could be ideal candidates for the target considering the fact that external pH regulates voltage-dependent Ca^{2+} channels (VDCCs), which involve in miniature neurotransmitter release via Ca^{2+} channels-coupling to synaptic vesicle exocytosis (Otsu and Murphy, 2003). It has been known that alkalinization facilitates whereas acidification inhibits calcium currents in a variety of cell types (Tombaugh and Somjen, 1996, Shah et al., 2001). Various neuronal VDCC subtypes are sensitive to extracellular pH, even modest pH shifts (Doering and McRory, 2007). Sitges and Rodriguez showed that increase in the baseline release of [^3H]GABA produced by external alkalinization is dependent on external Ca^{2+} (Sitges and Rodriguez, 1998). Acid-sensing ion channels (ASICs) are expressed throughout the nervous system and activated by extracellular proton, and regulates neuron excitability (Alvarez de la Rosa et al., 2003, Grunder and Chen, 2010). ASICs are also reported to be involved in neurotransmitter release. Presynaptic release probability of neurotransmitter was increased in hippocampal neurons from ASIC1 knockout mice (Cho and Askwith, 2008). Thus how pH modulates miniature neurotransmitter release is yet to be elucidated.

The effects of pH on IPSC amplitude and mimic tonic current are likely due to modulation of postsynaptic GABA_A receptors by protons. The studies on recombinant and native

GABA_A receptors indicate that pH sensitivity of GABA_A receptors varies from different preparations depending on the experimental conditions, animal species, brain regions and subunit compositions (Tang et al., 1990, Pasternack et al., 1992, Smart, 1992, Vyklicky et al., 1993, Krishek et al., 1996, Zhai et al., 1998, Huang and Dillon, 1999, Krishek and Smart, 2001, Wilkins et al., 2002, Mozrzymas et al., 2003, Feng and Macdonald, 2004, Huang et al., 2004, Wang et al., 2005, Mercik et al., 2006, Dietrich and Morad, 2010, Hugel et al., 2012). Most of these studies were conducted in CO₂/HCO₃⁻-free buffers such as HEPES. Notably, Hugel et al (2012) reported that pH sensitivity of GABA_A receptors observed in HEPES buffered medium is mediated by protonated HEPES not by extracellular protons. Their conclusion was largely drawn from inability of pH to regulate GABA response in the medium buffered with 1 mM HEPES or CO₂/HCO₃⁻ buffering system (Hugel et al., 2012). We presented several lines of evidence to argue this conclusion. 1) Direct inhibition of HEPES and MES on GABA response was not dependent on their protonated status; 2) pH sensitivity of GABA response was not affected by varying amount of protonated HEPES as measured with total [HEPES] from 2.5 to 20 mM; 3) pH effect on GABA currents was similar in the HEPES-buffered medium and in the medium buffered with MES which had much less direct effect than HEPES. In addition, it has been demonstrated that L-type Ca²⁺ channels inhibition by released vesicular protons after exocytosis was prominent in a weak buffering system with 3 mM HEPES or CO₂/HCO₃⁻ but greatly reduced by increasing concentrations of external HEPES. The most plausible explanation is that higher [HEPES] rapidly buffers endogenous proton shift, minimizing proton effect on Ca²⁺ channels (DeVries, 2001, Hirasawa and Kaneko, 2003, Palmer et al., 2003). In the present studies, we have shown that the minimum buffering that is able to deliver extracellular proton change to the receptors is 2.5 mM HEPES under our experimental conditions. The sensitivity to extracellular pH change was completely lost in GABA_A receptors and greatly diminished in glycine receptors in the medium buffered with 1 mM buffers, suggesting that 1 mM buffer reagents do not provide effective buffering capacity to “clamp” pH of microenvironment in extracellular space. As a result, pH-dependent process would not be adequately disclosed under the conditions with insufficient buffering capacity. Furthermore, as reported previously, HCO₃⁻ influx through GABA receptor channels causes extracellular alkalization (Chen and Chesler, 1991, 1992), which may compromise the extracellular proton effect. The pH effect on GABA response observed in CO₂/HCO₃⁻ buffered medium may be the mixed consequence upon two variables: protons and HCO₃⁻. Collectively, our data support the notion that pH effect of GABA response in HEPES-buffered medium is a specific consequence of changes in extracellular protons.

The results from our laboratory and others have shown that acidic pH inhibits GABA-activated currents recorded from different recombinant receptors and several brain regions (Smart, 1992, Zhai et al., 1998, Huang and Dillon, 1999, Li et al., 2003, Huang et al., 2004), suggesting that proton modulatory effect may be universal and not depend on GABA_A receptor subunit configurations. In the present studies, both spontaneous and miniature IPSC amplitude, mimic tonic and low [GABA]-evoked currents recorded from majority of the cells (>87%) were inhibited with increasing extracellular [H⁺] while the currents activated by saturating [GABA] were not affected. The studies from our lab (Huang and Dillon, 1999,

Huang et al., 2004) and others (Zhai et al., 1998, Li et al., 2003, Wang et al., 2005) have demonstrated that the H⁺ effect is competitive with respect to GABA and its competitive antagonist bicuculline. Furthermore, our previous studies suggest that pH modulation occurs in part by influencing GABA binding, either directly or allosterically (Huang et al., 2004). It is noted that [GABA] in the synaptic cleft has been estimated to reach as high as 0.3 to 3 mM (Maconochie et al., 1994, Jones and Westbrook, 1995). This may account for the fact that proton influence on IPSC amplitudes elicited by near saturating GABA is apparently less than that on those evoked by low GABA concentration. In the present studies, we demonstrated that similar to other CNS regions (Tombaugh, 1994, Velisek, 1998, Zhou et al., 2007), the acidic pH suppresses while alkaline promotes both phasic and tonic GABAergic transmission in the hypothalamus of rats. Given the fact that extracellular acidification commonly occurs during certain pathological conditions such as stroke, seizure, hypoxia, and ischemia (Wang and Sonnenschein, 1955, Somjen, 1984, Siesjo et al., 1985, Carmeliet, 1999), these results support the idea of a suppressed GABAergic inhibitory tone in the CNS, possibly as a contributing factor that leads to neuronal hyper-excitability to cause brain injuries.

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Highlight of the studies

- Extracellular pH modulates GABAergic neurotransmission in rat hypothalamus
- Acidic pH suppresses GABAergic IPSCs while alkaline pH has opposite effects
- Extracellular pH modulates tonic GABA currents
- pH effect on GABA response is a specific consequence of change in extracellular H⁺

Figure 1. Effect of pH on amplitude and frequency of spontaneous GABAergic IPSCs (sIPSCs) from rat hypothalamic neurons

A, GABAergic IPSC recordings. Representative traces were recorded before and after excitatory postsynaptic currents were blocked by kynurenic acid (KA, 1 mM). Abolishment of synaptic IPSCs with 10 μ M bicuculline confirms that IPSCs are mediated by GABA_A receptors. **B**, Representative traces were recorded from same cell through pH 7.3, 8.4 and 6.4. 1 mM KA was present in the external solution throughout. $V_H = -70$ mV. Note that the amplitude of sIPSCs was increased in pH 8.4 and decreased in pH 6.4 compared to pH 7.3. **C**, Combined cumulative probability plot of amplitude of sIPSCs from all cells recorded under control, pH 8.4 or pH 6.4. Insert shows summary data of pH effect on sIPSC amplitude. **D**, Cumulative probability plot of sIPSC intervals for all neurons recorded in pH 7.3, 8.4 and 6.4. Insert shows that the change in frequency of sIPSCs was statistically significant at pH 8.4 and pH 6.4. All values were normalized to the control at pH 7.3 (assigned as 100%). $n=14$, *: $p<0.05$; **, $p<0.01$, paired t-test, compared to control.

Figure 2. Effect of pH on kinetics of sIPSCs recorded from rat hypothalamic neurons
 sIPSCs were recorded from same cell at control, test pH (8.4 or 6.4) and washout. Summary graphs show a reversible pH dependent change in 10–90% rise time (**A**) and charge transfer (**B**). All values were normalized to control. $n=14$, *, $p<0.05$; **, $p<0.01$, paired t-test, compared to control. **C**, Mean values of the fast (τ_1) and slow (τ_2) time constant of decaying phase of sIPSCs recorded at different pH. **D**, Summary data of pH effect on relative fraction of fast (A1) and slow (A2) components of sIPSC decaying phase. The values of time constants and their components were obtained from the biexponential function fitting of an averaged trace of grouped IPSC events recorded at pH 7.3, 8.4 and 6.4. Note that pH did not significantly alter sIPSC decay kinetics.

Figure 3. pH Effect on miniature GABAergic IPSCs (mIPSCs) in rat hypothalamic neurons
A, Cumulative probability plot of amplitude of mIPSCs from all cells recorded under control, pH 8.4 or pH 6.4. **B**, Summary data on pH effect on mIPSC amplitude. Top traces show the average of all IPSC events recorded under control (thin solid line), test pH (bold line) and washout (dash line) from same cell. **C**, Cumulative probability plot of mIPSC intervals for all neurons recorded in pH 7.3, 8.4 and 6.4. **D**, Summary data of pH effect on mIPSC frequency, rise time and charge transfer. All values were normalized to control at pH 7.3. **E**, Mean values of the fast (τ_1) and slow (τ_2) time constant of decaying phase of

mIPSCs recorded at different pH. *F*, Summary data of pH effect on relative fraction of fast (A1) and slow (A2) components of mIPSC decaying phase. Each data point contains at least 8 cells. In B and D, *, $p < 0.05$; **, $p < 0.01$, paired t-test, compared to control.

Figure 4. pH effect on tonic GABA currents in rat hypothalamic neurons

A, Recording from a hypothalamic neuron at pH 7.3, 8.4 and 6.4. Low [GABA] (2 μ M) was added to external solution to mimic ambient extracellular [GABA]. Left panel shows focally applied GABA_A receptor antagonist bicuculline (10 μ M) caused an outward shift in the baseline, revealing a tonic current. **B**, Summary of pH effect on tonic GABA currents. Alkaline pH (8.4) increased while acidic pH (6.4) decreased the tonic GABA currents. n=13–19. **: p<0.01, paired t-test, compared to control.

Figure 5. pH effect on the evoked GABA currents from rat hypothalamic neurons

The currents activated by low GABA (10 μ M, **A&B**) and saturating GABA (1 mM, **C&D**) were recorded in pH 7.3, pH 8.4 and pH 6.4. All the currents are normalized to the control at pH 7.3 (assigned as 100%). Note that pH affects the response to low [GABA] but not to saturating [GABA]. n=10–11. **: p<0.01, paired t-test, compared to control.

Figure 6. Effect of buffers on GABA_A receptors does not depend on protonated buffers

The whole-cell Cl⁻ currents activated by EC₃₀ GABA (6 μ M) were recorded from HEK293 cells stably expressing human α 1 β 2 γ 2 GABA_A receptors. The percentage of protonated form of buffer (HEPES-H or MES-H) under pH 6.4 and 7.3 is calculated with Henderson-Hasselbalch equation, and indicated with black portion of the pie. **A**, Representative recordings show a similar inhibition of GABA currents by 20 mM HEPES at pH 7.3, and by 20 mM HEPES at pH 6.4. **B**, Summary of effect of HEPES on GABA-activated currents. All currents are normalized to control (10 mM HEPES). n = 9. **C**, Representative recordings show that the effect of 10 mM MES on GABA response at pH 6.4 is comparable to that at pH 7.3. **D**, Summary of effect of MES on GABA current at pH 6.4 and 7.3. All currents are normalized to absence of MES. n=8 for pH 6.4 and 7.3. p>0.05, paired t-test. These data suggest that direct action of buffers on GABA_A receptor is independent on their protonated status.

Figure 7. pH sensitivity of GABA_A receptors in MES-buffered and HEPES-buffered medium
Whole-cell Cl⁻ currents activated by EC₃₀ GABA were recorded from the cells expressing human $\alpha 1\beta 2\gamma 2$ GABA_A receptors. **A**, Representative recordings show the effect of pH on GABA-activated currents recorded in HEPES- and MES- buffered medium. **B**, Summary of effect of pH on GABA currents under HEPES- and MES- buffered medium. All currents are normalized to control pH (7.3). Note that pH sensitivity of GABA_A receptors is similar in 10 mM HEPES, 20 mM HEPES and 10 mM MES. n=6–9. p>0.05, unpaired t-test.

Figure 8. Effect of buffering capacity on pH sensitivity

A, The currents activated by 6 μ M GABA were recorded at pH 7.3 and 6.4 from cells expressing human $\alpha 1\beta 2\gamma 2$ GABA_A receptors during perfusion with the medium buffered with 1 or 2.5 mM HEPES. **B**, Summary data of pH sensitivity of GABA_A receptors during perfusion with varying HEPES concentrations from 1 to 20 mM. All currents are normalized to pH 7.3. The data of 10 mM and 20 mM HEPES are re-plotted from Fig. 7B for comparison. GABA currents were suppressed to a similar level at pH 6.4 in the media buffered with HEPES from 2.5 to 20 mM. However, the sensitivity to acidic pH was greatly reduced or completely lost in 2 and 1 mM HEPES-buffered medium. $n=6-20$. *, $p<0.05$, paired t-test, compared to pH. +, $p<0.05$, unpaired t-test, compared to any [HEPES] from 2.5 to 20 mM. **C**, Whole-cell Cl^- currents activated by EC₃₀ GABA or glycine were recorded from the cells expressing human $\alpha 1\beta 2\gamma 2$ GABA_A receptors or human $\alpha 1$ glycine receptors. Note that acidic pH 6.4 produced a remarkable reduction of GABA- and glycine-activated currents during perfusion with 10 mM MES- or HEPES-buffered medium. However, the inhibitory response to pH 6.4 was completely lost in GABA_A receptors in 1 mM MES-

buffered medium, and greatly attenuated in glycine receptors in 1 mM HEPES-buffered medium. **D**, Summary data of effect of buffers and buffering capacity on pH sensitivity of GABA_A and glycine receptors. All currents are normalized to pH 7.3. Each data point contains at least 5 cells. *, $p < 0.05$, unpaired t-test, compared between 1 mM and 10 mM buffers.

Figure 9. pH sensitivity of GABA_A receptors in the medium buffered with combination of HEPES and/or MES

A, GABA currents were recorded during perfusion of medium buffered with 10 mM HEPES alone, 10 mM HEPES (set pH at 7.3) and 10 mM MES (set pH at 7.3 or 6.4), or a combination of 10 mM HEPES and 10 mM MES (HEPES+MES, set pH at 7.3 or 6.4). **B**, Summary data of pH sensitivity of GABA_A receptors in the medium buffered with 10 mM HEPES alone, 10 mM HEPES or 10 mM MES (HEPES/MES) and combination of 10 mM HEPES and 10 mM MES. The data of 10 mM HEPES are re-plotted from Fig. 7B for

comparison. All currents are normalized to pH 7.3. Each data point contains at least 6 cells.
*, $p < 0.05$, paired t-test, compared to control.