The Role of Protons in Synaptic Transmission in Rat Cortical Neurons

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Abstract

Acid Sensing Ion Channels (ASICs) have recently been identified as inhibitors of voltage and calcium gated ion channels (BK) (Petroff et al. 2008), and their overexpression has been shown to reduce seizure elongation and severity in mice (Ziemenn et al. 2008). ASICs regulate BK channel activity in accordance with pH variation (Petroff et al. 2008), and seizures are concurrent with increased acidity in the brain (Ziemenn et al. 2008). Influx of Na⁺ through the ASIC pore domain has been speculated to cause membrane potential repolarization that reduces seizure proliferation (Vukicevic and Kellenburger, 2004), but this is counterintuitive to what is known about Na⁺ causing membrane potential depolarization (Grunder and Chen 2010). Modifying BK subunits has been shown to produce temporal lobe seizures, paroxysmal dyskinesia, and generalized epilepsy (Alioua et al. 2008), indicating repolarization of neuronal membranes plays a significant role in these disorders.

In order to examine how ASIC regulation of BK affects repolarization in neuronal synapses, perforated patch clamp was used to examine membrane fluctuation in rat cortical neurons. Postsynaptic repolarization time constraint increased by 154% when buffering was increased from 2.5 mM HEPES to 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic), indicating ASIC inhibition of BK can have a dramatic effect on synaptic transmission. Irregularity in normal ASIC function may be an underlying factor in brain disease, and needs to be explored as a target for pharmaceutical control. To date, pharmacological studies have explored blocking Na⁺ influx through the pore domain of ASICs (Grunder and Chen et al. 2010), but no attention has been given to its ligand regulation of BK. ASIC/BK interactions offer opportunities for new approaches in controlling synaptic transmission in those suffering from seizures.
A THESIS

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by

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Introduction

The frontal cortex (Fig 1) is responsible for planning behavioral responses to external and internal stimuli, and is regarded as a major brain region involved in working memory (Buchsbaum, 2004). Schizophrenia, depression, obsessive-compulsive disorder, and frontal-lobe epilepsy are associated with functional abnormalities in this brain region (Buchsbaum, 2004; Farrant et al 2005). These disorders are far from being deciphered due to technological limitations and challenges involved when performing in vivo experiments.

Fig 1: Frontal cortex of the human brain.

Regional functionality of the brain is difficult to examine because of spacial and temporal limitations of current technology. For example, while functional MRI presents excellent spacial resolution, its temporal limitation is an obvious limiting factor when examining regional brain function. Lack of definitive physiological evidence for causes of psychological disorders has resulted in reliance upon psychological diagnosis, which has yielded comorbidity (Brown et al 2001; Newmann et al 1996) and controversial treatment (Altshuler et al 1995). It is clear science is a long way from bridging the gap between physiological and psychological levels.
Epilepsy studies bring us a step closer to discovering how physiological changes at the cellular level affect the entire organism because onset, duration, and progression of seizures can be clearly measured (Ziemenn et al. 2008) after medication is given. However, treatment for seizures has left one third of patients with little or no improvement (Reid et al. 2010), and debilitating long-term side effects are evident in consumption of some medications (Reid et al. 2010). These medications have mostly involved altering communication in chemical synapses.

Chemical synapses serve to convey signals from one neuron to the next, and are targets for pharmacological control. Presynaptic signals in the form of action potentials cause a release of neurotransmitters at the presynaptic terminal of a neuron, which act upon the postsynaptic membrane of subsequent neuron(s). Depending on the neurotransmitter released, these signals may result in an excitatory or inhibitory effect upon the postsynaptic membrane. There is a smaller likelihood of the postsynaptic membrane initiating an action potential if inhibitory effects are produced, while the opposite is true of excitatory postsynaptic potentials. Controlling transduction of these signals can have profound effects on an organism.

Inhibitory postsynaptic potentials are a result of lowering the postsynaptic membrane potential, thereby lowering the likelihood of producing an action potential. Gabba-aminobutyric acid (GABA) (Fig 2) reuptake inhibitors are commonly used for this purpose in the treatment of anxiety disorders, seizures, convulsions, and as part of the anesthetic cocktail during surgery. GABA is an inhibitory neurotransmitter which acts upon the postsynaptic membrane through ligand gated ion channels or G-protein coupled receptors. This interaction causes Cl⁻ ions to flow into the cell or K⁺ ions to flow out of the cell, both of which will lower, or hyperpolarize, the resting membrane potential. GABA reuptake inhibitors act upon the channels that reload GABA into the presynaptic neuron, thereby allowing GABA to remain in the synaptic cleft. GABA will continue to hyperpolarize the postsynaptic membrane as long as GABA reuptake inhibitors are present, thereby inhibiting the production of action potentials.
Excitatory postsynaptic potentials are a result of depolarizing the postsynaptic membrane potential, thereby increasing the likelihood of producing an action potential. Glutamate (Fig 3) is the most abundant neurotransmitter in the central nervous system that generates excitatory postsynaptic potentials by allowing the flow of Na\(^+\) into the postsynaptic neuron in a ligand-gated manner. This Na\(^+\) influx serves to depolarize the membrane, which also allows the activation of voltage-gated Ca\(^{2+}\) channels. The influx of Na\(^+\) and Ca\(^{2+}\) contributes to membrane depolarization, which increases the chance of generating an action potential. Ca\(^{2+}\) also has second messenger functions once inside the postsynaptic neuron. It particular, Ca\(^{2+}\) will bind to voltage gated and Ca\(^{2+}\) activated potassium channels (BK), which will allow the efflux of K\(^+\) to repolarize the membrane in a negative feedback mechanism.

Fig 3: Glutamate – the most common neurotransmitter known to cause excitatory postsynaptic potentials in the central nervous system.

It has recently been discovered that BK channel activation is not only regulated only by voltage changes and Ca\(^{2+}\) binding. Acid sensing Ion Channels (ASICs) (Fig 4) in close proximity to BK channels, serve as a third regulator of BK function (Petroff et al 2008). ASICs have an
extracellular domain that is shown to inhibit BK at pH 7.4, while releasing inhibition at pH 6.0 (Fig 5) (Petroff et al 2008). Acidification of the synaptic cleft occurs when H⁺ is released by synaptic vesicles in conjunction with neurotransmitters (Ahdut-Hakohen et al 2004). Neurosecretory vesicle contents are low in pH because they use the H⁺ concentration gradient generated by V-ATPase to load neurotransmitters (Grasnier 2000). The potential energy generated by the H⁺ ions inside the vesicle allows neurotransmitters to be loaded in an exchanger system (Grasnier 2000; Melkikh and Seleznev, 2007). Hydrogen ions affecting ASICs, which in turn release BK inhibition, may act as another feedback system regulating synaptic transmission by increasing the rate of postsynaptic potential repolarization (Petroff et al 2008).

Fig 4: Homology model of ASIC1a (Bargeton and Kellenberger, 2010).
ASICs are found throughout the membrane of cortical neurons (Wemmie et al. 2006), but acidification is localized to the synaptic cleft (Ahdut-Hakohen et al 2004; Ziemann et al 2008). Therefore, ASICs should constantly inhibit BK throughout the entire neuron, except the synaptic cleft in normal physiological conditions. If ASICs release BK inhibition at the synaptic cleft from vesicle discharge of H⁺, perforated patch clamp can be used to measure changes in excitatory postsynaptic potentials carried to the soma of the neuron. Varying the extracellular solution buffer from 2.5 mM HEPES to 20 mM HEPES should produce slower repolarization rates in postsynaptic potentials, since BK should remain inhibited in a high buffer concentration. HEPES is a very fast buffer that will serve to quickly chelate H⁺ ions in the synaptic cleft.

It has been shown that ASICs are involved in seizure modification by decreasing duration and progression in wild type compared with ASIC1a knockout mice (Ziemann et al 2008). However, current research has given little recognition to ASIC affects upon BK in action potential or postsynaptic potential regulation, which could have profound effects upon seizure activity. Unfortunately, Na⁺ influx modification, which is a characteristic of activated ASICs, was incorrectly speculated to be the main contributor to seizure reduction (Vukicevic and
Kellenburger, 2004). Speculating that the influx of Na$^+$ causes repolarization seems counterintuitive to what is known about basic membrane polarity (Grunder and Chen 2010). Na$^+$ is known to be the greatest contributor to membrane depolarization, which would cause more frequent action potential firing during influx. ASIC inhibition of BK at normal pH, compared with release of inhibition at lowered pH, should have been considered as the likely explanation for alterations in repolarization.

Since ASICs and BK are present along the entirety of the neuronal membrane, release of BK in a low pH seizure environment will allow a faster repolarization compared with normal conditions. The increased repolarization reduces the time of the refractory period, which will allow the neuron to fire more frequently. Increased frequency of firing can be speculated to increase the duration and progression of seizures.

In a recent study examining the action potential generation of hippocampal neurons in mice, researchers speculated that lowered pH generated less frequent action potential firing possibly due to the ASIC modification of Na$^+$ influx (Vukicevic and Kellenburger, 2004). In other words, the researcher claimed that lowered pH may cause an increase of Na$^+$ influx through ASICs, which could possibly lead to a reduction in action potential firing. There was no mention of ASIC inhibition of BK in speculating the cause of the collected data possibly because ASIC/BK interactions were not characterized until 2008 (Petroff et al 2008). The data below shows the research collected, and is a clear example of ASIC releasing inhibition of BK in lowered pH (Fig 6). Furthermore, the data contradicts the researchers’ conclusions. It is clearly evident that action potentials are more frequent, to the point of becoming indistinguishable from one another. Na$^+$ should not be attributed as the cause of this observation because Na$^+$ influx causes depolarization, not repolarization. ASIC/BK interactions need to be considered altering repolarization rates.
Surprisingly, these researchers did not measure the half-width (Fig 7) of the action potential data, which would give a clear indication that repolarization of action potentials in firing clusters was increased in lowered pH. Increased repolarization can be explained by ASIC release of BK inhibition in acidic environments. Inhibition of BK should slow repolarization, allow a longer duration of the refractory period, thereby decreasing the possibility of action potential firing, as seen in pH 6.9.
Fig 7: Half-width of an action potential is the difference in time between depolarization and repolarization at 50% of spike height (Bean, 2007).

Previous patch clamp studies have altered the pH of the extracellular bath solution, but this affects the ASICs along the entirety of the neuron. There has yet to be a study showing the effects of the interactions in the synaptic cleft only. Changing the extracellular buffer concentrations of cultured cortical neurons should provide insight into the role of ASIC/BK interactions in the synaptic cleft, while maintaining inhibition throughout the rest of the cell. By maintaining the pH at 7.4 in the extracellular solution, only H+ ions released by vesicles should be present in the synaptic cleft. A high HEPES concentration should remove these ions quickly, before they have the ability to cause ASIC release of BK inhibition. A low HEPES concentration should allow H+ ions released to relieve BK inhibition, while limiting the potential of affecting other ASICs throughout the rest of the cell membrane. Therefore, this experimental design should not affect action potential repolarization, and will only produce changes in postsynaptic potentials (Ho: $\mu_{\text{tau} 2.5 \text{mM}} = \mu_{\text{tau} 20 \text{mM}}$, Ha: $\mu_{\text{tau} 2.5 \text{mM}} \neq \mu_{\text{tau} 20 \text{mM}}$, $\mu=$mean Tau, IV: [HEPES], DV: Tau).
Materials and Methods

Cell Culture

E18 Sprague Dawley Rat cortex purchased from Brain Bits® was shipped in Hibernate EB, and cells were plated on Poly-L-Lysine coated dishes within 3 days. The cells were plated at a recommended density of 16,000 cells/cm², grown for a minimum of ten days in NbActiv1 medium (Neurobasal, B27, GlutaMAX), and incubated at 37°C, 5% CO₂, 95% humidity, and ambient O₂. Half of the cell media was changed every 3-4 days with fresh NbActiv1 medium. Cells were grown for ten days to allow sufficient growth of axons, dendrites, and synapses in the neuronal network (Fig 8 and 9), which allowed the measurement of spontaneously generated postsynaptic potentials and action potentials.

Fig 8: Auto fluorescence of Rat cortical neurons after 12 days of growth.
**Electrophysiology**

Membrane potential was measured using the perforated patch technique with front-loaded extracellular solution containing amphotericin. All borosilicate glass, fire polished pipettes were from Sutter Instrument Company, with an inner diameter of 0.50 mm and an outer diameter of 1.0 mm. Pipettes were pulled using Sutter Instrument Company’s Flaming/Brown micropipette puller model P-87 (Fig 10), with resistance of 3-9M ohms once filled with intracellular solution and immersed in the bath solution. Pipette resistance was automatically corrected prior to seal acquisition, as was whole cell and fast capacitance. The intracellular solution was composed of 2 mM K-ATP, 10 mM HEPES, 150 mM $K_2$SO$_4$, with a concentration of 280 mmol/kg. The bath solution was composed of 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 5 mM glucose, 1.5 mM CaCl$_2$, and 2.5 mM HEPES, with a concentration of 340 mmol/kg. The experimental solution was identical, with the exception of 20 mM HEPES instead of 2.5 mM HEPES.
An open diamond bath chamber, AgCl₂ reference electrode, Sutter instrument Company’s MPC-385 micromanipulator system, and Axon Instruments’ CV-7B headstage were used to acquire cell patches (Fig 11). Data was collected through Axon CNS Digidata 1440A for analog to digital conversion, and amplified through Axon CNS Multiclamp 700B (Fig 12). The user interface was Clampex software for electrophysiology protocol application, while a Cool Snap EZ camera attached to a Nikon-TiS confocal microscope (Fig 13) using Nikon NIS Elements software was used to visually acquire cells. Data was sampled at 20,000Hz.
Fig 12: Axon CNS Digidata 1440A for analog to digital conversion of signals, and Axon CNS Multiclamp 700B amplifier.

Fig 13: Cool Snap EZ camera attached to a Nikon-TiS fluorescent microscope.
Clampfit software was used to extract data into excel format. The Levenberg-Marquardt format to extract standard exponential fit data was applied to extract postsynaptic potential decay times (Fig 14). This fit function was used to determine the Tau decay time of postsynaptic potentials. The Levenberg-Marquardt application allowed consistent extraction when exponential tails varied in length. Tau is the time it takes for 66% of the exponential curve to rise or fall, and was used to indicate the rate of postsynaptic decay.

\[
f(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i} + C
\]

Fig 14: Exponential fit function used to obtain Tau decay time.

Results

Tau decay times of neurons increased significantly in extracellular solution with 20mM HEPES when compared with the 2.5 mM HEPES control (Fig 15). The Shapiro-Wilk test confirmed normality for Tau decay data in 11 cells (n=11). The null hypothesis was confirmed with w=0.872823, and P=0.0011, and would have been rejected if W<0.850, with a=0.05. Box plots visually confirm the normality test, and an outlier was indicated in the 20mM treatment (Fig 16).
Fig 15: Mean tau decay times of postsynaptic potentials recorded in varying buffer concentrations (error bars indicate standard deviation).

Fig 16: Tau decay time distribution between conditions. Error bars indicate data within 1.5 times the interquartile range.

To further explore the difference between treatments, a paired t-test was completed between Tau measurements of the 2.5 mM extracellular solution and 20 mM extracellular solution (Fig 17). A significant difference was found ($t_{(10)} = -5.95, p = .0001$) between treatments. Figure 16 visually represents the confidence in rejecting the null hypothesis, where the kernel (red) represents collected data, while the normal line (blue) represents where the data line should be if accepting the null hypothesis. To further illustrate the increase in Tau decay rate between treatments, each cell was plotted to show individual increases (Fig 18). Note that
the decay time of postsynaptic potentials in each cell showed an increase in each degree of freedom. The null hypothesis of equality in difference between 2.5 mM HEPES and wash treatments was confirmed ($t_{(10)}=0.54, p=0.6029$) (Fig 19).

Fig 17: T-test distribution between [low HEPES] (2.5mM) and [high HEPES] (20mM).

Fig 18: Paired profiles for Tau decay time increase in each individual cell.
There was no significant change in postsynaptic potential amplitude measurements between treatments (p=0.311, student’s t-test) (Fig 20). This is contrary to previously observed amplitude measurements when using current clamp (Fig 21). This points to the possibility that current clamp is necessary to record rise times, but counterintuitive to record the slower decay times. This difference should be further explored using softpanel, a device that allows manual injection of current. This will allow manual control over the resting membrane potential, while giving the user the option of ceasing current injection once action potential trains are observed.
Fig 20: Amplitude measurements of postsynaptic potentials used in Tau decay time exponential fit function. No current injection was used to acquire this data.

Fig 21: Amplitude of postsynaptic rise times measured in current clamp (unpublished data, Petroff).

To ensure that postsynaptic potential Tau decay times were generated from the synaptic cleft only, half-amplitude of action potentials was extracted from collected data (Fig 22).
paired t-test confirmed the lack of difference between treatments, with $t = 1.08$ and $p = 0.3049$ (Fig 23). The null hypothesis would have been rejected if $t > 1.812$. There was no significant difference between treatments, which indicates only ASIC inhibition of BK at the postsynaptic cleft affected membrane potential. This also confirms that the treatments in this experiment were affecting the synaptic cleft only. The hydrogen ions that acted upon ASICs in the synaptic cleft likely originated from vesicle release.

**Action Potential 1/2 Width**

![Graph showing action potential half-width between treatments of varied HEPES concentration.](image)

**Buffer Concentration of Extracellular Solution**

**Fig 22:** Action potential half-width between treatments of varied HEPES concentration.

**Distribution of Difference: lowHEPES - highHEPES**

![Graph showing distribution of difference between low and high HEPES treatments.](image)

**Fig 23:** Paired t-test results from half-width data collection.
Discussion

ASIC will inhibit BK in normal pH, and release inhibition in low pH (Petroff et al. 2007). Neurotransmitter release is accompanied by H⁺ ion release from synaptic vesicles (Ahdut-Hakohen et al. 2004; Ziemann et al. 2008), since a high H⁺ ion concentration is needed for loading (Grasnier 2000). This release of hydrogen ions will lower the pH in the synaptic cleft, releasing BK inhibition, causing faster repolarization of postsynaptic potentials. If the H⁺ ions are chelated by HEPES before they can interact with ASICs, BK will remain inhibited. This inhibition will decrease the rate of postsynaptic potential repolarization.

Average postsynaptic potential decay times more than doubled, from 68.4 ms to 173.8 ms, when a highly buffered extracellular solution was applied (p=.0001, Student’s t-test), confirming the hypothesis. This is likely due to BK/ASIC interactions in the synaptic cleft only, so half-width of action potentials was measured. There was no significant difference on action potential half-width between treatments (p=0.3049, student’s t-test), indicating action potential repolarization was not affected by altering buffer concentration. Therefore, only ASICs in the synaptic cleft were affected by varied buffer treatments.

The GABA system and voltage gated Na⁺ channels have been common targets for anti-seizure medications (Grunder and Chen 2010). The goal of targeting the GABA system and Na⁺ channels is simply to lower the resting membrane potential to reduce the likelihood of seizure occurrence. Unfortunately, the key components of seizure occurrence, action potential trains, have been documented to occur even when the resting membrane potential is normal or low (Fig 24). Using these medications will serve to reduce seizure occurrence, but not eliminate them altogether. Long-term side effects have also become a significant point of debate between quality of life and future mental health (Reid et al. 2010).
The negative feedback mechanism in the synaptic cleft involving ASIC regulation of BK has not been utilized as a target for pharmacological control, yet this investigation has shown the interaction to be dramatic. Postsynaptic decay time constraints were increased by 154% with fast buffering of the synaptic cleft, clearly indicating ASICs has a major role in repolarization. Furthermore, lack of change in postsynaptic potential amplitude and action potential half-width shows only ASICs in the synaptic cleft were affected. This new evidence shows that regulation of BK has a much greater effect on postsynaptic membrane potential than ASICs’ role of Na$^+$ influx. Pharmaceutical control of this system must be utilized in anti-seizure medication to further evolve treatment.

There are three classes of ASIC inhibitors that have been characterized to date (Grunder and Chen et al. 2010): metal ions, polypeptide toxins, and small-molecule inhibitors. Each serve to reduce depolarization by blocking Na$^+$ influx. Coupling this mechanism with an additional pharmaceutical that has the ability to keep BK uninhibited from ASICs should serve to reduce seizure onset. This combination could help frontal-lobe epileptics that have been unable to eradicate seizure onset, as well as reduce duration and progression in case they do occur.
References


**Image Sources**

Frontal cortex picture:  
[http://brainandgender.files.wordpress.com/2011/03/prefrontal_cortex.jpg](http://brainandgender.files.wordpress.com/2011/03/prefrontal_cortex.jpg)

Glutamate molecule picture:  
[http://upload.wikimedia.org/wikipedia/commons/thumb/f/ff/Glutamins%C3%A4ure_-_Glutamic_acid.svg/256px-Glutamins%C3%A4ure_-_Glutamic_acid.svg.png](http://upload.wikimedia.org/wikipedia/commons/thumb/f/ff/Glutamins%C3%A4ure_-_Glutamic_acid.svg/256px-Glutamins%C3%A4ure_-_Glutamic_acid.svg.png)
GABA molecule picture: ![Gamma-Aminobutters%C3%A4ure - gamma-aminobutyric acid.svg](http://upload.wikimedia.org/wikipedia/commons/thumb/4/4e/Gamma-Aminobutters%C3%A4ure_-_gamma-aminobutyric_acid.svg/232px-Gamma-Aminobutters%C3%A4ure_-_gamma-aminobutyric_acid.svg.png)

Patch clamp image: ![Patch clamp](http://www.icb.ufmg.br/~lbcd/grupod/tecnica.html)