Title: Increasing SK2 Channel Activity Impairs Associative Learning

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Abstract

Enhanced intrinsic neuronal excitability of hippocampal pyramidal neurons via reductions in the postburst afterhyperpolarization (AHP) has been hypothesized to be a biomarker of successful learning. This is supported by considerable evidence that pharmacological enhancement of neuronal excitability facilitates learning. However, it has yet to be demonstrated that pharmacological reduction of neuronal excitability restricted to the hippocampus can retard acquisition of a hippocampus-dependent task. Hence, the present study was designed to address this latter point using a small conductance potassium (SK) channel activator NS309 focally applied to the dorsal hippocampus. SK channels are important contributors to intrinsic excitability, as measured by the medium postburst AHP. NS309 increased the medium AHP and reduced EPSP width of CA1 neurons in vitro. In vivo, NS309 reduced the spontaneous firing rate of CA1 pyramidal neurons and impaired trace eyeblink conditioning in rats. Conversely, trace eyeblink conditioning reduced levels of SK2 channel mRNA and protein in the hippocampus. Therefore, the present findings indicate that modulation of SK channels is an important cellular mechanism for associative learning and further support postburst AHP reductions in hippocampal pyramidal neurons as a biomarker of successful learning.

Key Words

Afterhyperpolarization, intrinsic excitability, NS309, trace eyeblink conditioning
Introduction

The learning-related modulation of all three phases of the postburst afterhyperpolarization (AHP: fast, medium and slow) in principle neurons has been demonstrated to be a biomarker of successful learning in numerous tasks (Disterhoft and Oh 2006; Matthews et al. 2008; Saar and Barkai 2003; Santini et al. 2008). Hippocampus-dependent trace eyeblink conditioning has been the most extensively used learning paradigm to demonstrate the intrinsic excitability change (via reductions in the postburst AHP) in both CA1 and CA3 pyramidal neurons following successful learning (Moyer et al. 1996; Thompson et al. 1996). Furthermore, compounds that reduce the postburst AHP in CA1 pyramidal neurons have been shown to facilitate acquisition of various hippocampus-dependent tasks. One such compound is apamin, a specific blocker of small conductance potassium (SK2) channels that underlies the medium AHP in CA1 pyramidal neurons (Bond et al. 2004; Faber and Sah 2007; Kaczorowski et al. 2007; Kramar et al. 2004; Oh et al. 2000; Stocker et al. 1999) (but see Gu et al. 2008; Gu et al. 2005)). However, it has yet to be demonstrated that a specific pharmacologically-induced increase in any phase of the postburst AHP localized to the dorsal CA1 region, shown to be the primary subregion of the hippocampus to change during trace eyeblink conditioning (Weible et al. 2006), can prevent or retard successful acquisition of a hippocampus-dependent task. Therefore, we used NS309, a SK channel activator that has been demonstrated to specifically enhance the apamin-sensitive postburst AHP (Pedarzani et al. 2005), to carefully examine the impact of a focally increased medium AHP in the dorsal hippocampal CA1 region on acquisition of hippocampus-dependent, trace eyeblink conditioning.
Materials and Methods

Subjects used in the study were young adult (3-4 mo) male F1 F344XBN rats (Harlan, Indianapolis, IN) that were housed in groups of 2-3 with unrestricted access to food and water on a 14/10 hour light/dark cycle. Rats were handled and housed in accordance with standards established by the Institutional Animal Care and Use Committee of Northwestern University and the USDA.

In vitro hippocampal brain slice recordings

Behaviorally naïve rats were anesthetized with isoflurane and decapitated. Dorsal hippocampi placed in ice-cold artificial cerebro-spinal fluid (aCSF: consisting of (in mM) 124 NaCl, 1.25 NaH2PO4, 2.5 KCl, 26 NaHCO3, 25 glucose, 2.4 CaCl2, and 2.0 MgSO4, saturated with 95% O2/5% CO2) were sliced into 300µm thick sections using a Leica VT1000s vibratome. Afterwards, the slices were allowed to equilibrate at room temperature for at least one hour before being used for recordings.

CA1 pyramidal neurons were visually identified using video DIC-IR optics on an upright Axioskop microscope and patched with pipettes filled with an internal solution containing (in mM): 120 KMeSO4, 10 KCl, 10 HEPES, 10 phosphocreatine sodium salt, 4 ATP magnesium salt, 0.4 GTP sodium salt and 0.05% neurobiotin with pH corrected to 7.4 with KOH and osmolarity of 285 ± 5 mOsm. Whole cell recordings were collected at 10 kHz for the AHP and accommodation and 20 kHz for all other measures using a Dagan BVC-700 amplifier, digitized and interfaced to a PC with an Axon Digidata 1322A analog to digital converter, and acquired and analyzed using pCLAMP 9.2. The current-
clamp recordings were acquired with the neuron held at -65mV and with the perfusate maintained at 32°C. Series resistance was monitored throughout recording and neurons with more than a 20% change were discarded. The AHP was elicited with five 2 ms, 2.0 nA current pulses at 50 Hz via the recording electrode. Accommodation was elicited with a 1 s current pulse that evoked 5 action potentials in the first 100 ms. Synaptic responses were elicited with a bipolar stimulating electrode (FHC, Bowdoin ME) placed in the stratum radiatum, powered with a Digitimer DS2 isolated stimulator.

In vivo CA1 hippocampal recordings

Surgeries to implant a cannula for drug infusion and, in close spatial proximity, a four tetrode array for unit recordings in the left dorsal CA1 region were performed using previously published methods (Matthews and Disterhoft, 2009) and after the rats were anesthetized with a ketamine and xylazine (0.87 mg/kg and 0.13 mg/kg, respectively: i.p.) mixture, supplemented as necessary during the surgery. The cannula was implanted at 4.8 mm posterior, 3.0 mm lateral from bregma and 1.9 mm ventral from pia. The injection needle, when positioned in the cannula, protruded 0.1 mm below the tip of the cannula. The depth of penetration for the custom-made four tetrode array (3.3 mm posterior, 1.7 mm lateral from bregma) was determined with auditory monitoring of neuronal activity that insured placement in the CA1 pyramidal neuron layer. A tetrode was made with 4 formvar-coated nichrome wires (25 μm diameter bare; 37 μm diameter coated) that were bonded at one end with epoxylite and exhibited an average impedance of 0.5-1.5 MΩ. After appropriate positioning, the electrode array and cannula
were fixed in place with dental acrylic. Rats were given one week to recover from surgery before experimentation.

*NS309 concentration to be used for the behavioral training study* was identified by finding the concentration that significantly reduced the spontaneous firing rate of CA1 pyramidal neurons. Prior to the drug infusion and unit recordings, the rats were given one 60 min habituation session to the sound and light attenuated recording chamber (30.5 cm wide, 38.1 cm long) where they were allowed free movement in the chamber while being attached to a tether that connected the electrode array to the unit recording apparatus. After habituation, once daily unit recording sessions were conducted in the following manner: 10 min baseline, 5 min drug infusion, and 55 min post-infusion period. The rats were given 1.0 µl of one of 4 different drug concentrations (10 µM NS309, 100 µM NS309, 1 mM NS309 or DMSO) administered at a rate of 0.2 µL/min. Multiple drug concentrations were tested on the same rat in a blind pseudo random counterbalanced manner in recording sessions separated by at least 24 hours. Although it is not precisely known how long NS309 remains in the system in vivo, baseline neuronal firing rate did not reveal any significant difference across recording days (data not shown).

*Single neuron recording* data were recorded using the Cheetah-32 system (Neuralynx, Bozeman, MT). Neuronal activity was passed to a headstage amplifier (HS-27) via a customized adapter, buffered with the HS-27, filtered (600-6000Hz) and amplified (20,000X). Following each recording session, individual neurons were isolated offline using Neuralynx spike sort software based on firing rate and waveform characteristics. Criteria for inclusion in the analysis were 1) exhibit a mean firing rate of greater than zero during any of the 5 min collection periods over the 70 min recording
session, 2) exhibit a signal to noise ratio greater than 2.5:1, and 3) have an average firing rate less than 6Hz and a mean spike width (peak to valley) greater than 0.3 ms to be characterized as a CA1 pyramidal neuron (McEchron and Disterhoft 1997; Ranck 1973). 234 neurons met our criteria for the final analysis (1 mM n=50; 100 µM n=61; 10 µM n=42; DMSO n=81). At the conclusion of the recording sessions, a DC current (10 µA, 5 s duration) was passed through a single channel of the tetrode to verify the tetrode placement using standard histological methods (McEchron and Disterhoft 1997).

**Behavioral pharmacology**

**Surgeries** to implant the guide cannulae for drug infusion and a plastic connector strip for eyeblink conditioning were performed after the rats were anesthetized with isoflurane and placed in a stereotaxic apparatus. Guide cannulae, made of 26-gauge stainless steel tubing, were bilaterally implanted in the dorsal hippocampus (3.6 mm posterior and 2.0 mm lateral from bregma, 1.9 mm below the dura) and cemented in place with dental acrylic. A previous report using the same stereotaxic coordinates demonstrated that the diffusion of 1 µl of ibotenic acid injected into the dorsal CA1 region via guide cannulae was limited to the dorsal hippocampus (Matthews and Disterhoft, 2009). While not identical in structure, the spread of NS309 should be similar to that observed with ibotenic acid and be limited to the dorsal hippocampus. A plastic connector strip, which contained a wire to ground to the skull and two additional wires passed subcutaneously through the upper eyelid of the right eye to record the electromyographic activity of the orbicularis oculi muscle, was cemented between the cannulae. Rats were allowed a week to recover before training began.
Trace eyeblink conditioning was conducted once a day for ten consecutive days after the rats were habituated to the light- and sound-attenuated conditioning chamber and to the tether attaching the plastic connector strip and the cannulae that allows free movement in the training chamber. One µL of 100 µM NS309 or DMSO was infused over 5 min bilaterally into each hippocampus using two 2 µL Hamilton syringes with 32-gauge stainless steel injection needles placed in the guide cannula with its tip terminating 0.1 mm below the end of the cannula and a Stoelting dual infusion syringe pump (Stoelting, Wood Dale, IL). Trace eyeblink conditioning began approximately 30 min after drug infusion. During the training session, rats were presented with two stimuli: an 8 kHz, 85 dB, 250 ms tone and a 100 ms, 4 psi air puff to the cornea. Conditioned animals received 30 pairs of the tone and the airpuff separated by a 250 ms stimulus-free trace interval, a trace duration which has been demonstrated to make this task hippocampus dependent (Weiss et al. 1999). Pseudoconditioned animals also received 30 airpuffs and 30 tones per session, but the two were explicitly unpaired. The data acquisition, analysis and storage were all performed using custom software written in LabView. Learning was measured as the percentage of correct responses, which are eyelid closures during the last 200 ms of the trace interval.

Cannula placement was verified at the conclusion of the training sessions. Anesthetized rats were transcardially perfused with 0.9% saline and 10% formalin. The brains were removed, frozen, and sliced into 80 µm coronal slices. Slices were stained with cresyl violet. Animals with incorrect cannula placement or excessive tissue damage were excluded from the study (n=2) (see Figure 3).
**Molecular analyses of SK2 channel**

Microarray analysis was conducted using previously published methods (Kroes et al. 2007). The genes comprising the in-house prepared rat CNS microarray (1178 cloned rat CNS genes, representing 90% of the major gene ontological categories) were spotted in quadruplicate onto slides. Microarrays were performed in triplicate using hippocampal mRNA isolated 24 hours after the last training session from the left hippocampi of individual animals. Normalized microarray data were analyzed by Significant Analysis of Microarray (SAM) with a stringent false discovery rate cutoff of 1%, followed by ontological data mining using GoMiner (Zeeberg et al. 2003) and $\chi^2$ analysis to identify enriched pathways, as described in Burgdorf et al. (2011).

Quantitative real-time PCR analysis (qRT-PCR) was conducted as previously described (Burgdorf et al. 2011; Kroes et al. 2006) in a blind manner on dorsal hippocampus of individual rats that were trace eyeblink conditioned (n = 7), pseudoconditioned (n = 6), or naive controls (n = 6). The sequences of the qRT-PCR primers used in the study were as follows: KCNN2 (NM_019314), forward 5'-AGTAAGGAAGCATCAACGG -3' and reverse 5'-GTATTCGCTTGGTCAATTAC -3'; B2M (NM_012512), forward 5'-CCGTGATCTTTCTGGTCTT -3' and reverse 5'-AAGTTGGGCTTCCATTCTC -3'.

Western blot analysis was conducted using hippocampal tissue from another group of trained rats (trace conditioned, n = 6; pseudoconditioned, n = 3; naïve, n = 4). Membranes were prepared as described elsewhere (N’Gouemo et al. 2009). In brief, tissue was Dounce homogenized in ice cold TE buffer (10 mM Tris-HCl (7.4), 1 mM EDTA, 0.5% protease inhibitor cocktail (P8340, Sigma), 1% phosphatase inhibitor...
cocktail 2 & 3 (P0044 & P5726, Sigma). Samples were centrifuged at 25,000 x g for 15 min at 4°C, the supernatant decanted, and pellets resuspended in boiling lysis buffer (10 mM tris-HCl (7.4), 1% SDS, 0.5% protease inhibitor cocktail (Sigma), 1% phosphatase inhibitor cocktail 2 & 3 (P0044 & P5726, Sigma)). Samples were boiled for 10 min, centrifuged at 25,000 x g for 10 min, and the supernatant aliquoted and stored at -80 °C until assay. Total protein content was determined by the BCA assay (Pierce, USA), and samples were analyzed by SDS-PAGE. Samples (45 µg) were electrophoresed through 8% gels (Hoeffer, USA), transferred onto PVDF membranes (Millipore, USA) and blocked in 0.2% nonfat dry milk (NFDM), 1% BSA in TBS containing 0.05% Tween-20 (TBS-T) for 1 hr at 25°C. Membranes were probed with one of two SK2 antibodies raised against two independent regions of the full length SK2 protein (APC-028, 1:500; Alomone labs, Israel, aa 542-559; AV35094, Sigma, 1:500, aa 444-493) in 0.2% NFDM, 1% BSA TBS overnight at 4°C, followed by a 1 hr incubation at 25°C with a horseradish peroxidase (HRP) conjugated secondary antibody (sc-2313, 1:5000, Santa Cruz Biotechnology, USA) in 0.2% NFDM, 1% BSA TBS-T. Western blots were visualized by enhanced chemiluminescence (Immun-Star HRP, Bio-Rad, USA) and developed on film (BioMax, Kodak, USA). SK2-immunoreactive bands were quantified by ImageJ (NIH, USA). The blots used for SK2 protein quantification were stripped and reprobed with beta-actin as a protein loading control. Beta-actin protein levels were not altered in the naïve, trace or pseudoconditioned animals (F2,9=0.16, p>0.05). SK2 antibody from both Sigma and Alomone recognized a 64 kD band corresponding to the full length SK2 protein in rat and mouse whole brain membrane preparations. And both antibodies revealed that there was no difference observed in
SK2 protein levels in the control tissue (both antibodies: Fisher PLSD p’s > 0.05 pseudoconditioned vs. naïve). Thus, the results of both antibodies were combined and used for analysis.

Statistics

Repeated measure ANOVAs, ANOVAs, and t-tests were performed, where appropriate, using StatView to analyze the in vitro and in vivo electrophysiology, Western blots, and behavior data.

Results

NS309 reduces the excitability of CA1 pyramidal neurons and reduces the width of the EPSP in vitro

Before conducting the behavioral experiments, we first replicated the observations reported by Pedarzani and colleagues (Pedarzani et al. 2005) that bath application of NS309 increased the apamin-sensitive medium postburst AHP of CA1 pyramidal neurons in vitro. NS309 significantly enhanced the peak of the postburst AHP at 5 and 10 \( \mu M \) (Fig 1A: paired t-test, p’s < 0.05) without affecting the slow component of the postburst AHP (p = 0.94) or the basic membrane properties of the neuron (Table 1). Consistent with previous reports (Pedarzani et al. 2005), 10\( \mu M \) NS309 also increased spike frequency accommodation (Fig 1B: paired t-test, p<0.01).

In addition to increasing the medium postburst AHP, NS309 could also impact the synaptically evoked excitatory postsynaptic potentials (EPSPs), since SK channel activation reduces the EPSP width by shunting current near the synapse (Ngo-Anh et
al. 2005). Hence, we also tested whether 10 µM NS309 affected the EPSP. For this test, the isolated stimulator output was controlled to evoke an EPSP with a peak height of 7 mV before and after drug application so that NS309's impact on the EPSP could be examined independent of its potential action on the EPSP amplitude. NS309 significantly reduced the EPSP area, halfwidth, and duration (Fig 1C: paired t-tests, area p=0.005, duration p<0.01, half width p<0.05).

The intrinsic neuronal excitability measures were assessed after 100 µM NS309 bath application in two neurons to determine if further reduction in intrinsic neuronal excitability could be obtained with a higher concentration of NS309,. The postburst AHP measures after 100 µM NS309 application were 197 ± 15% (n = 2) of the baseline measurements. The number of action potentials evoked during accommodation measures was reduced by 5 action potentials (pre-NS309: 29 ± 2; post-NS309: 24 ± 2; n = 2). The EPSP duration was reduced by 33.5 ± 5.5% (n = 2). These results with 100 µM NS309 indicate that a ceiling effect on the intrinsic excitability was achieved with 10 µM NS309. Thus, the SK channel activator NS309 reduced the excitability of CA1 pyramidal neurons by increasing the medium postburst AHP and by increasing the calcium-dependent outward current at the synapses.

NS309 reduces the spontaneous firing rate of CA1 principal cells in vivo

Given that the detailed kinetics of NS309 action may differ in the in vivo from the in vitro situation, we wanted to find the most effective dose to use in the awake rat for subsequent behavioral experiments. Thus, a guide cannula and an electrode array were implanted unilaterally into dorsal CA1, approximately 2.0 mm apart, and cemented
into place (see Fig 2C). After recovery, conscious, freely moving rats received once
daily infusion of 10 µM, 100 µM, or 1 mM of NS309 or vehicle control (DMSO).
Neuronal activity was monitored with a 4 tetrode extracellular microelectrode array for a
10 min baseline period, a 5 min infusion of 1 µL of the drug, and a 55 min observation
period. No statistical differences were observed in the baseline periods between the
drugs or between days (F_{2,156} = 0.26, p>0.75).

Statistical analysis of the effect of the different drug concentrations on pyramidal
cell firing rate demonstrated a significant interaction of drug concentration by time
(F_{8,77}=2.46; p<0.05). Post-hoc analyses demonstrated that compared to DMSO
controls, 100 µM NS309 significantly reduced CA1 spontaneous firing 20 minutes
following drug infusion (see Figure 2). 10 µM NS309 also reduced firing rate for a few
time points; however not as robustly as the 100 µM dose. Post-hoc analyses
demonstrated that 1 mM NS309 significantly elevated spontaneous firing rate after drug
infusion compared to DMSO. This suggests a classic U-shaped dose-response curve,
and that at higher doses NS309 may have nonspecific effects. DMSO infusion had no
significant effect on spontaneous firing rate. It is important to note that the
concentration of NS309 seen by the recorded hippocampal neurons is lower than the 1
µl of a given concentration of NS309 infused directly into the dorsal CA1 hippocampus
after local diffusion. A previous report using the same stereotaxic coordinates
demonstrated that the diffusion of 1 µl of ibotenic acid injected into the dorsal CA1
region via guide cannulae resulted in a spread of ~0.17cm in radius which was limited to
the dorsal hippocampus (Matthews and Disterhoft, 2009). If a spherical volume of
diffusion is assumed, then a compound will diffuse and occupy ~0.015cm^3 (or ~15µl) of
space. Hence 1μl of 100μM NS309 delivered into 15μl of space would result in a net of 6-7μM (100μM in 16μl volume) of NS309 for a period of time before NS309 is removed (by clearance or breakdown) from the space. This calculation assumes that the ~15μl of brain space is similar to water and NS309 would have free movement to equilibrate; but brain space is not the same as water, so the actual NS309 concentration after ~30min of diffusion is considerably less than the original 100μM but somewhat greater than 6-7μM. Regardless, 100 μM produced the largest and most uniform decrease in firing rate when injected in vivo, so this dose of NS309 was selected for subsequent behavioral studies.

**NS309 impairs learning of trace eyeblink conditioning**

Rats were habituated to the injection and training paradigm for one session. For the next ten consecutive days, rats were once daily given an infusion of NS309 through cannulae bilaterally implanted into CA1 approximately 30 min prior to conditioning (see Figure 3D) then trace eyeblink conditioned. Trace conditioned rats received thirty trials of paired tones and airpuffs separated by a 250 ms stimulus-free trace interval, while pseudoconditioned rats received the same stimuli but tones and airpuffs were explicitly unpaired. Eyelid closures during the last 200 ms of the trace interval were conditioned responses.

Daily infusion of NS309 significantly impaired trace eyeblink conditioning as compared to controls (see Figure 3A). Repeated measures ANOVA demonstrated a main effect of training ($F_{1,26}=58.2$, $p<0.001$) and drug ($F_{1,26}=4.9$, $p<0.05$). NS309 had a significant effect on trace eyeblink conditioning ($F_{1,17}=6.8$, $p<0.05$), but had no effect on
pseudoconditioning (F1,9=2.6, p>0.10). Because there was a significant effect of NS309 on the level of acquisition in the trace conditioned rats, planned comparisons were done using unpaired t-tests on the individual days for the trained animals. NS309 treated rats performed at a significantly lower rate than the DMSO control treated rats on days 3-6 and 10 (p<0.05). Although NS309 treated rats were significantly impaired, they were able to learn the task (F1,9=20.3, p<0.0001), albeit at a slower rate and to a lower level. It is important to emphasize that NS309 had no noticeable effect on the amplitude of unconditioned eyeblinks from pseudoconditioned animals (rmANOVA p=0.43, see Figure 3C), indicating that NS309 was acting on associative ability and not the sensorimotor capacity of the rats to produce eyeblinks per se.

SK2 mRNA and protein levels are reduced after learning

The behavioral data strongly suggested that modulation of apamin-sensitive SK2 channels is an essential component for successful learning and for the learning-related postburst AHP reduction in hippocampal pyramidal neurons. Thus, we conducted molecular assays to verify that learning changes the apamin-sensitive SK2 channels.

Rats were trace eyeblink conditioned, pseudoconditioned, or left naïve, as described above. One day after training, the hippocampi were extracted and used for microarray, qRT-PCR, and/or Western blot analyses to measure mRNA and protein levels. The microarray analysis using SAM and GoMiner revealed that 102 total genes were significantly differentially expressed by learning the trace eyeblink conditioning task (see Supplemental Table 1). Of particular interest in our current study, the KCNN2 gene was significantly downregulated in trace conditioned rats. This latter result was
verified by qRT-PCR analysis that showed a significant reduction of KCNN2 mRNA levels in the hippocampus from trained as compared to pseudoconditioned and naïve animals (Fig 4B: F_{2,15} = 9.4, p < 0.005; Fisher PLSD post hoc, p’s < 0.01). Finally, the western blot analysis revealed a significant 30% reduction in SK2 levels in trace eyeblink conditioned rats (Fig 4A: F_{2,9}=9.5 p<0.01; Fisher PLSD post hoc test, p’s <0.01), and no significant difference in the control rats (p >0.05 pseudoconditioned vs. naïve). Thus, the molecular analyses clearly demonstrate that the apamin-sensitive SK2 expression is reduced following successful learning.

**Discussion**

The results from the present study are the first to demonstrate that direct modulation of the medium postburst AHP restricted to the dorsal CA1 region can impact acquisition of a hippocampus-dependent task. Direct infusion of the SK channel activator NS309 into the dorsal CA1 region caused a significant reduction in spontaneous firing rate of the pyramidal neurons and also significantly slowed the acquisition rate and magnitude on the hippocampus-dependent trace eyeblink conditioning task. Single neuron recording studies have shown that the dorsal CA1 region of the hippocampus shows the most substantial functional changes during trace eyeblink conditioning (Weible et al. 2006). The importance of learning-related reductions of apamin-sensitive SK2 channels was verified through the use of microarray, qRT-PCR and western blotting techniques. More importantly, these findings strengthen our hypothesis that the increase in intrinsic hippocampal pyramidal neuronal
excitability, via reductions in the postburst AHP, is a key cellular biomarker of successful learning on a hippocampus-dependent task.

The theory that intrinsic excitability must first be altered for learning to take place (Disterhoft and Oh 2006) is supported by the literature on aging animals. Aging is accompanied by a decrease in intrinsic excitability as measured by an increase in the AHP (Landfield and Pitler 1984). More specifically, many aged animals are learning impaired, and those that are impaired have larger medium and slow postburst AHPs than those that are able to learn associative (Moyer et al. 2000) and spatial (Tombaugh et al. 2005) hippocampus-dependent tasks. If the AHP in aged animals is pharmacologically manipulated to be equal to that from young animals, their learning also improves to levels comparable to that from young animals. This has been demonstrated with different compounds acting through several independent pathways. The cholinesterase inhibitors metrifonate and galantamine, as well as the M1 muscarinic agonist CI-1017 and the L-type calcium channel antagonist nimodipine, all act to increase intrinsic excitability in CA1 pyramidal neurons from aged animals by reducing the AHP and accommodation (Moyer et al. 1992; Norris et al. 1998; Oh et al. 1999; Oh et al. 2006; Weiss et al. 2000). In addition, all of these compounds improved the performance of aged rabbits in trace eyeblink conditioning (Deyo et al. 1989; Kronforst-Collins et al. 1997; Weible et al. 2004; Weiss et al. 2000). Importantly, the effect of NS309 on learning shows that the opposite of these aging studies is also true: decreasing intrinsic excitability in young animals causes them to be learning impaired. Since NS309 held the hippocampal neurons of young animals in a less excitable state
and made them “aged” as regards the size of their medium AHP, this study supports the theory that intrinsic excitability must be altered for learning to take place.

Increasing SK channel activity with NS309 increased the medium (not slow) postburst AHP, reduced in vivo CA1 neuron firing rate and significantly impaired learning, but did not block it all together. NS309’s effect was largest on the acquisition phase, days 3-6 (see Figure 4). This supports the view that SK channels are important for the encoding phase of learning (Vick et al. 2010). NS309 had less effect once the animals began to learn the task, as seen by the fact that there was no difference between NS309 and vehicle control groups on days 7-9 (see Figure 4). Based on our data, animals receiving NS309 likely would have eventually reached the same level of performance as control animals had we trained the rats for more days. However, if we were able to truly mimic normal aging by increasing both the medium and the slow postburst AHP, as observed in CA1 pyramidal neurons from aged animals (Moyer et al., 2000; Matthews et al., 2009), then learning may have been prevented in these cognitively-intact young adult rats just as that observed in normal aging-impaired subjects. Thus, the present findings also highlight how the slow postburst AHP, in combination with the medium AHP, might serve as the potential cellular mechanism that determines successful learning.

Changes in synaptic transmission, in addition to the intrinsic excitability changes, in the CA1 region may also underlie successful learning. The apamin-sensitive SK channel conductance in dendritic spines of CA1 pyramidal neurons has been shown to modulate excitatory synaptic transmission via their close association with NMDA receptors (Lin et al., 2008; Bloodgood and Sabatini, 2007; Ngo-Anh et al., 2005).
Blockade of SK channels with apamin enhances the EPSPs (Lin et al., 2008; Ngo-Anh et al., 2005) and facilitates long-term potentiation of excitatory synaptic transmission (Behnisch and Reymann, 1998; Stackman et al., 2002); whereas, the SK channel activator NS309 reduced the EPSP width and area (Figure 1C). Increases in multisynaptic boutons (Geinisman et al., 2001) and a modest increase in excitatory postsynaptic potential (EPSP) measured in vivo (Gruart et al., 2006) have been observed in the CA1 region following trace eyeblink conditioning. Ex vivo, a modest EPSP increase in CA1 region was observed immediately (one hour), but not 24-hour, after successful learning the trace eyeblink conditioning task (Power et al., 1997). This lack of a sustained learning-related EPSP increase ex vivo in the CA1 region is due in part to a corresponding increase in inhibitory transmission that may mask the EPSP enhancement following successful learning (McKay and Disterhoft, 2010). Hence, the present learning impairment observed with NS309 treatment may also be due, in part, to the reduced NMDA-dependent synaptic plasticity following an increase in the apamin-sensitive SK conductance in dendritic spines.

Previous reports using the SK channel agonists 1-EBIO and CyPPA, applied systemically, demonstrated impaired encoding of object memory, but no effect on fear conditioning (Vick et al. 2010). However, the systemically applied 1-EBIO and CyPPA caused a decline in motor behavior and may have confounded the results. To prevent activation of SK and IK channels in the periphery, we injected NS309 directly into the hippocampus. NS309 had no noticeable effect on eyeblinks from pseudoconditioned animals and did not impact the rats’ reflexive eyeblink response (the unconditioned
response) (Figure 3C), suggesting potential motor impairments were not an issue in the current experiments.

The current experiments were designed to test our hypothesis that modulation of the postburst AHP in the hippocampus is a key factor for successful learning. This does not preclude the potential negative impact of any other pharmacological compounds that may dampen the neural activity of hippocampal pyramidal neurons; such as the M-channel activator, retigabine. Our previous work clearly suggests that learning changes basal firing rates of CA1 pyramidal neurons in vivo and increases intrinsic excitability of hippocampal pyramidal neurons by reducing the fast, medium and slow postburst afterhyperpolarization ex vivo (reviewed in Disterhoft and Oh, 2006, 2007). However, learning the trace eyeblink conditioning task did not change the KCNQ mediated M-current in CA1 pyramidal neurons (Kuo et al., 2008). In addition, while there are numerous pharmacological compounds that would reduce the slow postburst AHP, normal aging is the only source for enlarging the slow AHP in hippocampal pyramidal neurons (Disterhoft and Oh, 2007). Thus, we have focused and designed our present experiments to systematically evaluate the potential impact of modulation of the medium, SK channel mediated postburst AHP in young adult rats.

Our present findings also add to the growing body of work that demonstrates the importance of learning-related SK channel alteration in other brain regions and after learning other associative tasks. In the olfactory cortex, SK2 but not SK3 mRNA levels are reduced after learning an odor discrimination task (Brosh et al. 2007). In situ hybridization using radiolabeled apamin and SK2 mRNA showed a decrease in binding sites in CA1 and CA3 following spatial learning (Mpari et al. 2010). Together, these
studies provide convincing evidence that fewer SK channels are made and inserted in
the membrane after successful learning. The diversity of tasks after which this
observation is made suggests SKs are universally involved in the molecular basis for
learning.

Calcium-dependent potassium channels play an important role in learning and
memory (Disterhoft and Oh 2006; Hammond et al. 2006). We focused on SK channels
as one member of this family with well-characterized and specific agonists, antagonists,
and antibodies. NS309 reduced in vivo CA1 pyramidal neuron firing rate and the
medium postburst AHP and EPSPs recorded in vitro, and impaired acquisition of the
early phase of trace eyeblink conditioning by nearly 50%. The present findings provide
strong support for the hypothesis that proper function and modulation of SK channels is
important for acquiring associative learning tasks and further strengthens our
hypothesis that postburst AHP reductions in hippocampal pyramidal neurons are a
biomarker of successful learning.

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Disclosures
References


Kuo AG, Lee G, McKay BM, and Disterhoft JF. Enhanced neuronal excitability in rat CA1 pyramidal neurons following trace eyeblink conditioning acquisition is not due to alterations in I_m. *Neurobiol Learn Mem* 89: 125-133, 2008.


Oh MM, Power JM, Thompson LT, Moriearty PL, and Disterhoft JF. Metrifonate increases neuronal excitability in CA1 pyramidal neurons from both young and aging rabbit hippocampus. *J Neurosci* 19: 1814-1823, 1999.


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**Figure Legends**

**Figure 1.** NS309 reduces the excitability of CA1 pyramidal neurons and decreases the width of the EPSP

**A.** Repeated ANOVA revealed a significant impact of NS309 bath application on the postburst AHP measures ($F_{1,8}=3.78$, $p<0.005$). Planned post-hoc analysis further revealed that the peak amplitude of the postburst AHP was significantly enlarged by the bath application of 5 µM (n=6) and 10 µM (n=9) NS309 while 1 µM NS309 had no effect (n=5). The AHP was evoked with five action potentials at 50 Hz. Action potentials are truncated for clarity. Scale bar: 2 mV, 100 ms. **B.** 10 µM NS309 significantly increased accommodation (n=6). Accommodation was measured as the number of action potentials elicited in 1 s by a depolarizing current step which evokes 5 action potentials in the first 100 ms. Scale bar: 20 mV, 100 ms. **C.** In addition to its effect on the AHP and accommodation, 10µM NS309 significantly reduced the area, duration, and halfwidth of the EPSP (n=6). EPSPs were evoked with a stimulating electrode was placed in the stratum radiatum with the stimulus intensity adjusted so that the amplitude of the EPSP was the same before and after bath application of NS309 or DMSO. Scale bar: 2mV, 100 ms. Data are presented as mean ± SEM. *$p<0.05$*

**Figure 2.** 100µM NS309 maximally suppresses spontaneous firing rate in vivo. **A.** The activity of individual neurons was isolated. Activity was monitored during a 10 min baseline, 5 min infusion (black bar), and 55 min post infusion period. DMSO vehicle injections had no effect on firing rate. The gray lines indicate the average firing rate
during the time period when behavioral training was done for the 100 μM concentration of NS309 and the DMSO control injected in the subsequent behavioral experiments. A total of 234 neurons from 3 rats reached criterion for final analysis (1 mM n=50; 100 μM n=61; 10 μM n=42; DMSO n=81). **B.** The 100μM dose robustly and significantly suppressed firing rate for the duration of the recordings, and thus was used for the behavioral pharmacological studies. **C.** Tetrode bundle placement was identified by passing a current (DC, 10μA, 5 s duration) through a single channel of one tetrode. Tetrode placement is indicated by a square, cannula placement is indicated by a circle.

**Figure 3. 100μM NS309 significantly impaired trace eyeblink conditioning.**

**A.** Rats were divided into four groups: vehicle trained (n=9), NS309 trained (n=10), vehicle pseudo (n=5), NS309 pseudo (n=6). Both control and NS309 animals learned the paradigm, but compared to controls, animals receiving NS309 infusions were significantly impaired on days 3-6 and 10 (*, p<0.05). NS309 had no effect on pseudoconditioning. **B.** *Top* Conditioned animals received a 250 ms tone followed by a 250 ms stimulus free trace interval and then a 100 ms puff of air to the eye. *Bottom* Late conditioned responses (CRs) were measured as eyelid closures during after the tone (two vertical grey lines) in the 200 msec before the airpuff (two vertical black lines). **C.** Eyeblinks from pseudoconditioned animals in response to the airpuff alone were not affected by NS309 (*top, vehicle, bottom, 100 μM NS309, rmANOVA p=0.43), indicating that hippocampally-infused NS309 had no effect on the sensory input or motor ability of the animal to blink. **D.** Cannula placement was verified in cresyl violet stained slices.
Those rats in which the cannula tracks did not terminate at or closely above the pyramidal band of CA1 were excluded from the study.

**Figure 4. Trace eyeblink conditioning decreases KCNN2 mRNA and protein levels in the hippocampus.**

A. KCNN2 protein levels (arbitrary units) as measured by Western analysis or B. KCNN2 mRNA levels normalized to beta 2-microglobulin as measured by qRT-PCR in the hippocampus of rats receiving trace eyeblink conditioning, pseudo eyeblink conditioning, or no training (naïve). N = 3-7 per group. *P < 0.01* Fisher PLSD Post hoc test comparing trace vs. pseudo or trace vs. naïve. Data are presented as mean ± SEM.
Table 1. Bath application of NS309 did not significantly affect the basic membrane properties of CA1 pyramidal neurons ex vivo.

<table>
<thead>
<tr>
<th></th>
<th>aCSF</th>
<th>NS309</th>
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<tbody>
<tr>
<td>Vrest (mV)</td>
<td>-65.1 ± 0.9</td>
<td>-64.8 ± 1.2</td>
</tr>
<tr>
<td>Input Resistance (MΩ)</td>
<td>88.6 ± 2.3</td>
<td>83.5 ± 4.3</td>
</tr>
<tr>
<td>Sag (mV)</td>
<td>3.22 ± 0.29</td>
<td>3.46 ± 0.42</td>
</tr>
<tr>
<td>sAHP (mV)</td>
<td>-1.77 ± 0.25</td>
<td>-1.61 ± 0.39</td>
</tr>
<tr>
<td>AP thresh (mV)</td>
<td>-50.1 ± 1.5</td>
<td>-51.5 ± 1.9</td>
</tr>
<tr>
<td>AP height (mV)</td>
<td>94.2 ± 4.3</td>
<td>94.1 ± 2.7</td>
</tr>
<tr>
<td>AP half width (ms)</td>
<td>1.14 ± 0.06</td>
<td>1.05 ± 0.06</td>
</tr>
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Vrest is the resting membrane potential immediately after breaking into the cell. Input resistance is calculated as the slope of the IV curve. Sag is the difference between the peak and steady state hyperpolarization in response to a -250 pA, 800 ms current injection. The sAHP was measured 1 s after the end of the last action potential in a five spike train. Action potentials were elicited by a long depolarizing pulse sufficient to evoke a single action potential in 100 ms. Threshold was measured where the first derivative of the upslope of the trace equals 20 mV/ms. Height is the difference between the baseline and maximal depolarization. Half width is width of the action potential at the midpoint between the maximal depolarization and the threshold. Values are means ± SEM. N=9.