Title

A switch in the mode of the sodium / calcium exchanger underlies an age-related increase in the slow afterhyperpolarization

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Keywords

Sodium Calcium Exchanger; NCX; *Lymnaea stagnalis*; aging; slow afterhyperpolarization; cerebral giant cells

Abbreviations

NCX Sodium Calcium Exchanger

RMP Resting Membrane Potential

SFA Spike Frequency Adaptation

Abstract

During aging, the Ca^{2+} sensitive slow afterhyperpolarization (sAHP) of hippocampal neurons is known to increase in duration. This change has also been observed in the serotonergic cerebral giant cells (CGCs) of the pond snail *Lymnaea stagnalis*, but has yet to be characterized. In this paper, we confirm that there is a reduction in firing rate, an increase in the duration of the sAHP, and an alteration in the strength and speed of spike frequency adaptation (SFA) in the CGCs during aging, a finding that is compatible with an increase in a sAHP current. We go on to show that age-related changes in the kinetics of SFA are consistent with a reduction in Ca^{2+} clearance from the cell, which we confirm with Ca^{2+} imaging and pharmacological manipulation of the sodium calcium exchanger (NCX). These experiments suggest that the NCX may be switching to a reverse-mode configuration in the CGCs during aging.

1. Introduction

Over recent decades, many western societies have seen an increase in the life-expectancy of their populations, and with this the burden of age-related diseases such as dementia and normal brain aging has grown. Understanding the molecular mechanisms that underlie normal brain aging, and identifying therapeutic interventions is key to reducing this burden. However, research into normal brain aging at a molecular level in humans is currently hampered by the complexity of the neural networks, and the invasive nature of the experimental procedures, including electrophysiology. Vertebrate models have provided considerable insight into the molecular and electrophysiological changes associated with normal brain aging, although invertebrate models, such as the pond snail *Lymnaea stagnalis* have also proven to be extremely useful for studying these mechanisms due to their simpler nervous system and relatively short life-span. Notably, a number of the age-related changes identified in these invertebrate systems are well conserved with vertebrate species (Yeoman, et al., 2012).

Neuronal firing rate and firing behavior are controlled in part by the afterhyperpolarization (AHP) of the action potential. The AHP itself is comprised of three temporally, and pharmacologically distinct components: the fast AHP (fAHP), the medium AHP (mAHP) and the slow AHP (sAHP) (Sah and Faber, 2002). The fAHP occurs in the first 10 ms after the action potential peak and is typically the result of voltage and Ca^{2+} activation of large conductance Ca^{2+}-activated K^+ channels (BK), although other currents are also known to contribute. The mAHP occurs between 10-100 ms post spike, and is produced by Small and Intermediate Conductance Ca^{2+} activated K^+ channels (SK / IK), which unlike BK channels are only sensitive to Ca^{2+} (Sah, 1996). The sAHP begins to activate at around 50ms post action potential spike, and lasts in some cases in excess of 1000 ms, to produce a prolonged hyperpolarization. The molecular identity of the channel responsible for I_{sAHP} is currently unknown, but like the SK current, it too is only sensitive to Ca^{2+} (Weatherall, et al., 2010).

In vertebrate models, the Ca^{2+} that activates Ca^{2+}-dependent potassium channels (K_{Ca}) is derived from a wide variety of intracellular and extracellular sources; the precise source being dependent upon the neuronal type (Fakler and Adelman, 2008, Stocker, 2004). Ca^{2+} that is derived through high-voltage

activated (HVA) Ca^{2+} channels, for example L-type (Ca_v1) and N-type (Ca_v2), most commonly activates BK, IK, and SK. Low-voltage activated (LVA) Ca^{2+} channels have also been shown to provide a source of Ca^{2+} for SK, however, there is little evidence of LVA Ca^{2+} channel derived Ca^{2+} activating the channel responsible for the I_{sAHP}, and no evidence for BK. Ca^{2+} derived from intracellular stores is also known to signal to the K_{Ca} , and BK and SK are also both additionally sensitive to Ca^{2+} derived from N-methyl-D aspartate receptors (NMDAR) and SK from Ca^{2+} derived through the nicotinic acetylcholine receptor (nAChR) (Isaacson and Murphy, 2001, Ngo-Anh, et al., 2005) .

Both K_{Ca} and HVA Ca^{2+} currents appear to be sensitive to the aging process. There is considerable evidence showing an age-related increase in both the mAHP and the sAHP in rat (Foster, 2007, Landfield and Pitler, 1984), mouse (Murphy, et al., 2006), and rabbit (Moyer, et al., 2000) hippocampal neurons, and also in neurons from the prefrontal cortex of monkeys (Kirischuk and Verkhratsky, 1996). This increase in the AHP in aged animals is associated with a reduction in excitability *in vivo* (Moyer, et al., 1992), and possibly a change in firing behavior (Driver, et al., 2007). It is also associated with an increase in spike frequency adaptation, which is defined as the intrinsic ability of a neuron to reduce its firing frequency in response to constant depolarization (Wang, 1998). Interestingly, in the case of hippocampal neurons, changes in their biophysical properties are associated with behavioral deficits, most notably impairment to learning and memory (Norris, et al., 1996). The causes of the increased mAHP and sAHP have been attributed to an increase in HVA Ca^{2+} currents (Campbell, et al., 1996, Moyer and Disterhoft, 1994, Murchison and Griffith, 1996, Pitler and Landfield, 1990, Thibault and Landfield, 1996), and increased Ca^{2+} release via ryanodine receptors (Gant, et al., 2011).

An age-related increase in the AHP is not simply limited to neurons from vertebrates (Yeoman, et al., 2012). Age-related changes have been observed in firing frequency, and both the duration, and amplitude of the AHP of the Cerebral Giant Cells (CGCs) from the pond snail *Lymnaea stagnalis*, a key pair of serotonergic neurons which are involved in learning and memory (Patel, et al., 2006, Watson, et al., 2012b, Yeoman, et al., 2008). In addition, work on another important interneuron,

RPeD1, which is responsible for driving ventilation in *Lymnaea*, shows a similar decrease in spontaneous firing rate, and firing rate during artificial depolarization (Klaassen, et al., 1998). Staras *et al* have provided some evidence that a Ca^{2+} sensitive K^+ current is present in the CGCs, and that it is linked to HVA-derived Ca^{2+} (Staras, et al., 2002). However, this current has not been characterized, and it is unclear whether an increase in a K_{Ca} in the CGCs is responsible for the age-related changes to the AHP and firing behavior.

In this paper we provide further evidence that the firing frequency of the CGCs declines with age, and confirm that this reduction is associated with an age-related increase in the duration of the sAHP. We go on to show that in young animals, spontaneous firing frequency, firing frequency during artificial depolarization, and the sAHP are sensitive to disruption of Ca^{2+} signaling through high-voltage activated Ca^{2+} channels (HVA). Interestingly however, we found that these biophysical parameters were markedly resistant to HVA Ca^{2+} current blockade in old animals, despite HVA Ca^{2+} currents being of equal amplitude and sensitivity to pharmacological block in both age-groups. Finally, we present data showing that the apparent resistance of the sAHP (and firing frequency) to HVA Ca^{2+} channel block in old animals is related to a disruption in Ca^{2+} buffering, specifically an age-related switch in the direction of the sodium calcium exchanger (NCX), which we believe to be driven by an increase in the $I_{Na(p)}$. We conclude that Ca^{2+} delivered through the reverse mode NCX provides a source of Ca^{2+} for the sAHP in old animals.

2. Materials and Methods

2.1 Experimental animals and preparation

Lymnaea stagnalis were bred at the University of Brighton. Batches of identically aged *Lymnaea* were kept in separate Plexiglass® tanks of continually perfused Cu-free tap water. The water was continually filtered, and sterilized using UV light. The temperature of the water was monitored and maintained at 21ºC. All animals were fed every other day, alternating between English round lettuce or Tetrapond (UK) fish sticks ground up to form a powder. Animals were kept on a 12 hour light:dark cycle all year round. Animal numbers were counted every 2 weeks and survival curves constructed to determine when animals had entered adulthood (95% survival) or old-age (25% survival) (Hermann, et al., 2007).

Prior to electrophysiological recording, the central nervous system was prepared in the following way: shells were carefully removed from each snail before pinning to a Sylgard (Corning, UK) lined Petri dish. The snail was then immersed in N-2-hydroxyethylpiperazine-N`-2-ethansulfonic acid (HEPES) HEPES-buffered saline at room temperature (see below for composition) and the central nervous system (CNS) removed using fine forceps and microscissors. The CNS was then transferred, dorsal side up, to a Sylgard lined Perspex perfusion bath and pinned down securely. The cerebral ganglia were inverted to reveal the CGCs, and the outer ganglion sheath removed with fine forceps. The inner sheaths were then softened by exposing the preparation locally to protease (Sigma Type XIV, Sigma, U.K.) for 2 minutes before washing thoroughly with HEPES-buffered saline.

CGCs from the right cerebral ganglion (rCGCs) were used for both current and voltage clamp experiments. Preparations were continually perfused with HEPES-buffered saline at a drip rate of ~0.05 mL/s via a gravity feed mechanism. Prior to conducting voltage clamp experiments, both cerebral buccal connectives, and the cerebral commissure were axotomized by crushing with fine forceps to improve the space clamp. rCGCs were initially impaled with a low resistance ‘current injecting’ microelectrode, before the higher resistance, voltage recording electrode was inserted.

2.2 Chemicals

The composition of HEPES-buffered saline was (in mM): NaCl 50; KCl 1.5; $CaCl_2$ 4; $MgCl_2$ 2; N-2-hydroxyethylpiperazine-N`-2-ethansulfonic acid (HEPES) 10; pH adjusted to 7.9 with 10 M NaOH. For voltage clamp experiments measuring Ca^{2+} currents the following zero Na^+ saline with K^+ channel blockers was used (in mM): Tetraethylammonium chloride (TEA) 50, 4-aminopyridine (4-AP) 4, KCl 1.6, $CaCl_2$ 3.5, $MgCl_2$ 2.0, HEPES 10, pH adjusted to 7.9 with TEA OH. For experiments measuring the contribution of the Na^+/Ca^{2+} exchanger, the following saline was used (in mM): LiCl 50; KCl 1.6; $CaCl_2$ 4; $MgCl_2$ 2; HEPES 10; pH adjusted to 7.9 with 10 M LiOH.

For experiments measuring the contribution that the Na^+/Ca^{2+} exchanger and the $I_{Na(p)}$ make to the resting membrane potential, the LiCl in the previous solution was substituted with N-methyl D-glucamine (50 mM). 100 μM $CdCl_2$ was used to inhibit HVA Ca^{2+} channels in the CGCs. All chemicals obtained from Sigma-Aldrich, UK.

2.3 Recording methods

Both current and voltage clamp experiments were carried out using an Axoclamp 2B amplifier (Molecular Devices, U.S.A.). Analogue signals were converted to digital by a Digidata 1400A analogue to digital converter (Axon Instruments, U.S.A.). For current clamp, data were sampled at $\geq$ 2 KHz, and for voltage clamp at 20 KHz. Both were recorded on a PC using pClamp software (v10.0, Axoscope or Clampex respectively) (Molecular Devices, U.S.A.). Micropipette electrodes were made using borosilicate glass and pulled using an Intracel® P-30 vertical electrode puller. Each micropipette was filled with 4M potassium acetate (Sigma, U.K.), and the tip dipped in black ink (Winsor and Newton, UK) to visualize the micropipette tip prior to neuronal impalement. Electrode tip resistances were recorded for each current clamp experiment and were in the range of 11-18 MΩ. For voltage clamp, electrode tip resistances were recorded in the range of 5-8 MΩ for the current passing electrode, and 11-18 MΩ for the voltage recording electrode.

2.4 Electrophysiology

2.4.2 Current injection during current clamp

To investigate spike frequency adaptation, CGCs were injected with 10, 20, or 30 nA of current sequentially in bridge balance mode for 5s. Current injection commenced approximately 3 minutes post electrode impalement, following stabilization of the firing frequency and resting membrane potential (RMP). Cells were allowed to recover to their basal firing rate between each current injection.

2.4.1 Two-electrode voltage clamp

Two-electrode voltage clamp (TEVC) was used to elicit Ca^{2+} currents in the CGCs of young and old *Lymnaea*. Cells were initially held at -60 mV and then stepped to -80 mV for 100 ms to remove all Ca^{2+} and voltage dependent inactivation. Cells were then subsequently stepped from -50 to +50 mV in 5 mV increments, each of 200 ms duration.

2.5 Ca^{2+} imaging

The calcium-sensitive dye Oregon Green 488 BAPTA 1 (Molecular Probes) was injected by applying repetitive, short pressure pulses to the microelectrode with a Picospritzer (General Valve Corp.). The injection protocol consisted of 60–120 single pulses, 8–18 ms long each, at a pressure of 15 psi with 15–30 s intervals between pulses. We recorded the fluorescence in the axonal branches of the CGCs in intact CNS preparations with a Hamamatsu Orca CCD camera (Hamamatsu Photonics) attached to a Leica MZFL III stereomicroscope (powered with a short-arc mercury lamp) at narrow bandpass (excitation 465–495 nm, emission 515–560 nm). We acquired the data at a frame rate of 6 fps. The measured optical signal reflected the change in fluorescence/light emission relative to its mean value (DF/F). The time courses were corrected for photobleaching by linear regression

2.5 Data analysis

Clampfit's® 'Event Detection' and 'Template Search' functionality were used to determine half-width, and Interspike Interval (1/instantaneous frequency) during artificial depolarization (Spike Frequency Adaptation). Measurement of the slow afterhyperpolarization (sAHP) of action potentials was also made using Clampfit's 'Event Detection' and 'Threshold Search' functionality by setting the thresholds as: 1) the peak of the fAHP and 2) the resting membrane potential (RMP). The RMP was taken as the stable period of membrane voltage prior to the initial slow deflection of the action potential. The Δt between these two thresholds was taken to be the sAHP duration, i.e. the time taken to reach RMP (Power, et al., 2002). For a given animal, the sAHP for all action potentials occurring in an isolated 30s section trace were first measured and then averaged. This value was taken forward to calculate the sample mean ±SEM. Figure 1Bi provides information about where the sAHP was measured on a typical young and old action potential.

Analysis of Spike Frequency Adaptation (SFA) was conducted in the following way: the reciprocal of each interspike interval (i.e. instantaneous frequency) during an episode of artificial depolarization was plotted against the time at which the spike occurred, and fitted with a monoexponential function (Equation 1 (Wang, 1998)). The first instantaneous frequency was taken to be f_0, and from the subsequent fit it was possible to determine the steady state firing frequency (f_{ss}) and the time constant of SFA, τ_{adap}. Both f_0 and f_{ss} were then used to calculate F_{adap} according to Equation 2, and τ_{Ca} calculated according Equation 3 (Wang, 1998).

Equation 1. $$\boldsymbol{f(t) = f_{ss} + (f_0 - f_{ss})e^{-t/\tau_{adap}}}$$

Equation 2. $$\boldsymbol{F_{adap} = (f_0 - f_{ss})/f_0}$$

Equation 3. $$\boldsymbol{\tau_{Ca} = \frac{\tau_{adap}}{1 - F_{adap}}}$$

The % increase in half-width during a period of current injection was taken as the % change in half-width duration between the first and last spike of each current injection period.

Voltage clamp recordings were filtered at 1/5 of the sampling frequency (4,000 Hz) using a low-pass Gaussian filter (Clampfit). Membrane resistances were measured in all cells by subjecting them to an initial hyperpolarizing voltage step to -80 mV from -60 mV, and measuring the current response (Clampfit). Leak current was then determined and subtracted from each recording using the leak subtraction function in Clampfit.

Tests for significance were performed using either the Student's t-test or repeated measures (RM) two-way analysis of variance (ANOVA). Comparisons between data were considered statistically significant if $p<0.05$. Statistical analysis, and figures were generated in GraphPad Prism® 6. In both figures, and figure legends: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Data are presented as mean ± SEM in all instances.

3. Results

3.1 Current clamp

3.1.1 The sAHP duration increases with age, and negatively correlates with firing frequency

To confirm that the sAHP increases with age in CGCs, and to determine whether any increase is related to a change in firing frequency, we conducted a series of basic current clamp experiments measuring electrical activities in these neurones in young and old *Lymnaea*. Changes to both firing frequency and the duration of the sAHP were observed in the CGCs during aging. A section of current clamp trace from a young (top) and old (bottom) CGC is shown in Figure 1Ai illustrating these changes. The mean CGC firing rate calculated from 17 young and 13 old *Lymnaea* reduced by approximately 48% with age (young 66.2 ± 4.0 spikes / min vs. old 36.3 ± 3.5 spikes / min, $p<0.001$ unpaired Student's t-test, Figure 1Aii). In addition, the duration of the sAHP was found to be significantly longer in rCGCs from old animals compared to young (Fig 1 Bi and Bii), with mean durations of 445 ± 43 and 1098 ± 182 ms respectively ($p<0.001$, unpaired Student's t-test). In young animals there was a particularly strong relationship between sAHP duration and firing rate ($R^2=0.54$, $p<0.001$; Pearson's correlation, Fig. 1C). However, in old animals, despite the relationship being significant, the association was less strong ($R^2=0.40$, $p<0.05$; Pearson's correlation; Figure 1C). As shown previously by Patel *et al*, we also showed that the half-width of the CGCs action potential was also found to be significantly shorter in old animals (Figure 1 Biii, $p<0.05$).

3.1.2 SFA in response to artificial current injection is altered during aging

To determine whether SFA is altered with age in the CGCs (a process controlled in part by the sAHP) a series of current clamp experiments were conducted measuring adaptation following injection of a range of current amplitudes. SFA was observed in response to a 5s artificial current injection (10, 20 and 30 nA) in all young and old CGCs (as indicated by a reduction in instantaneous firing frequency over the course of current injection; Figure 2Ai and Aii). There was no significant difference in the mean initial instantaneous firing frequency (f_0) between young and old CGCs over the range of

current injection amplitudes ($p>0.05$, two-way ANOVA; Figure 2Aiii). However, the mean steady state instantaneous firing frequency (f_{ss}) was found to be significantly slower in old CGCs ($p<0.05$, two-way RM ANOVA; Figure 2Aiv).

Over the course of each current injection period (at 10, 20 and 30 nA for 5 s), the mean action potential half-width increased significantly in both young and old CGCs ($p<0.0001$ in young and old, two-way RM ANOVA; Figure 2Bi and Bii; see methods), although the increase was significantly larger in CGCs from old animals compared to young ($p<0.05$, two-way RM ANOVA). In addition, we found that the prolongation of the action potential was dependent upon the amplitude of the current injected, with larger currents producing greater increases in half-width ($p<0.0001$, two-way RM ANOVA; Figure 2Bii). In both age-groups, the half-width duration of the first spike of a current injection pulse was independent of current amplitude, indicating that age-related changes to the half-width are a result of a process occurring during the burst, for example, a change in the inactivation properties of a K^+ channel.

3.1.3 The CGC sAHP duration is sensitive to Cd^{2+} in young, but not old *Lymnaea*

To determine the Ca^{2+} sensitivity of the CGC sAHP, current clamp recordings were made from young and old animals in the absence and presence of 100 μM $CdCl_2$. In young animals, the sAHP duration of the CGC reduced by approximately 40% during superfusion of Cd^{2+} (from 537.4 ± 72.52 ms in control, to 321.6 ± 45.17 ms in $CdCl_2$; $p<0.01$, paired Student's t-test; Figure 3A and B), supporting the hypothesis that young CGCs possess a K_{Ca}. In old animals however, the sAHP duration in the CGCs was unaffected by Cd^{2+} treatment, showing only a minor, non-significant reduction (Figure 3A and B).

3.1.4 The sensitivity of SFA to Cd^{2+} in young and old CGCs

$I_{K(Ca)}$, in particular the I_{sAHP}, is known to underlie SFA in a variety of neuronal types (Sah, 1996,Sah and Faber, 2002). It is therefore important to establish whether SFA in the CGCs is sensitive to HVA Ca^{2+} channel block, and whether its sensitivity changes with age. To test this, we subjected young

and old animals to the same artificial current injection protocol employed previously (3.1.2), however, in this instance the experiment was conducted in the absence and presence of 100 μM $CdCl_2$.

The application of $CdCl_2$ significantly increased the number of spikes per burst in both young and old animals over all current injection amplitudes ($p<0.01$ and $p<0.05$ young and old respectively, two-way RM ANOVA, data not shown). In young animals, both f_0 and f_{ss} increased significantly over the range of current amplitudes following treatment with $CdCl_2$ ($p<0.001$ and $p<0.01$ respectively; two-way RM ANOVA; Figure 4Aii and Aiii). In old animals, f_0 was increased over the range of current injection amplitudes following application of $CdCl_2$ ($p<0.01$, two-way RM ANOVA; Figure 4Bii), however, f_{ss} did not change significantly ($p>0.05$, two-way RM ANOVA; Figure 4Biii), i.e. neurons still demonstrated marked adaptation.

3.1.5 The dynamics of SFA during aging

The dynamics of SFA have previously been shown to closely follow the intracellular kinetic profile of Ca^{2+}. According to a recent model of SFA, a reduction in Ca^{2+} clearance can be observed as a reduction in the speed at which a neuron adapts (Wang, 1998). Because of the apparent lack of resilience of old CGCs to long depolarizations (perhaps indicating a problem with Ca^{2+} handling) we decided to investigate if the speed of SFA in the CGCs is reduced with age. Plots of instantaneous frequency vs. time of spike were constructed from the raw data of SFA generated in Figure 3, and non-linear regression used to determine the exponential time constant (τ_{adap}) according to Equation 1.

The mean τ_{adap} calculated across the range of current injection amplitudes was significantly longer in old CGCs compared to young ($p<0.001$, two-way RM ANOVA; Figure 5A). The strength of adaptation (F_{adap}, calculated using *Equation* 2) was found it to be slightly higher in CGCs from older animals, although it only reached borderline significance ($p=0.054$, two-way ANOVA; Figure 5B). An estimation of the time constant of Ca^{2+} clearance (τ_{Ca}) was also calculated by substituting values for F_{adap} and τ_{adap} obtained from individual animals described above into Equation 3 (see (Wang,

1998). Using this approach, mean τ_{Ca} values for old animals were found to be significantly longer than young counterparts ($p<0.05$, two-way RM ANOVA, Figure 5C).

3.1.6 Ca^{2+} clearance is altered in CGCs from old animals

To investigate age-related changes to Ca^{2+} clearance and handling in the CGCs further, we used a fluorescent Ca^{2+} probe (Oregon Green 488 BAPTA) and imaging to measure the kinetics of Ca^{2+} clearance in spontaneously firing young and old neurons (Figure 6). Individual spontaneous action potentials marked by the vertical lines at the top of Fig. 6A evoked rapidly rising increases in intracellular Ca^{2+} concentrations. In the young CGCs the increase in $[Ca^{2+}]_i$ decayed back to baseline levels with a time constant of approximately 200 ms. Ca^{2+} transients recorded in the old CCGs took significantly longer to reach baseline ($p<0.05$; Fig 8Bii). Overall, the time constant of decay and the area under the Ca^{2+} transients were significantly greater in the old CGCs compared to the young CGCs ($p<0.05$; Figure 6Bi).

We next probed the activity of the Na^+/Ca^{2+} exchanger (NCX), a major extruder of intracellular Ca^{2+}, to establish if a change in its activity is responsible for the age-related decrease in Ca^{2+} clearance. Under normal circumstances, the NCX has a depolarizing influence on the CGCs by transporting 3 Na^+ into the cell for every Ca^{2+} that leaves. Application of Li^+, which is an NCX blocker (Iwamoto, et al., 1999, Nikitin, et al., 2006, Ponce-Hornos and Langer, 1980, Szebenyi, et al., 2010), therefore causes the cell to hyperpolarize from its resting membrane potential (RMP). The amplitude of this hyperpolarization is proportional to the pump's activity (Nikitin, et al., 2006). Here, we determined the contribution of the NCX to the RMP in CGCs from 5 young and 5 old *Lymnaea* by substituting Na^+, with Li^+ HEPES-buffered saline, and measuring the change in membrane potential. The RMP of young CGCs hyperpolarized by approximately -13 mV following transition to Li^+ substituted saline (-60.2 ± 4.0 mV to -73.9 ± 3.6 mV; $p<0.05$, two-way RM ANOVA, n=5; Figure 7A (top trace) and B). In old animals however, Li^+ substituted saline produced a significant membrane *depolarization* (-56.8 ± 3.6 mV to -49.7 ± 3.4 mV; $p<0.05$, two-way RM ANOVA, n=5, Figure 7A (bottom trace) and Bii). The difference in membrane potential produced by the transition to Li^+ saline in both age groups were

consequently highly significant (Figure 7Bii): in young, the change in membrane potential was -13.7 ± 2.6 mV compared with old, which was +7.1 ± 0.8 mV ($p<0.0001$, unpaired Student's t-test). We also measured the sAHP duration before and after transition into Li^+ substituted saline in old animals. In this age group, we observed a significant reduction in the duration of approximately 20% ($p=0.01$, paired Student's t-test; Figure 7Ci and Cii). It was not possible to compare sAHP duration in young animals due to lack of action potential firing.

3.1.7 The combined contribution from the NCX and $I_{Na(p)}$ is similar in both young and old CGCs

Despite the marked difference in the contribution of the NCX to RMP in young and old CGCs (depolarizing influence in young, and a hyperpolarizing influence in old), no statistically significant age-related difference in the RMP in normal saline was noted. One possible reason for this may be an increase in the persistent Na^+ current ($I_{Na(p)}$) in old CGCs, which would act to drag the RMP back up to the potential seen in young animals. To date, all known blockers of the $I_{Na(p)}$, including riluzole have lacked sensitivity in *Lymnaea* (Nikitin, et al., 2006), therefore, we examined the contribution of this current to RMP in young and old CGCs by measuring the change in membrane potential following a switch from normal, to zero Na^+ saline. The transition to zero Na^+ saline effectively blocks the $I_{Na(p)}$, revealing its contribution to the RMP. It also blocks activity of the NCX however, meaning that any change in membrane potential is a combination of both currents. Nonetheless, if the activity of the $I_{Na(p)}$ is increased in old animals (in combination with a hyperpolarizing NCX), we can predict that a switch to zero sodium would cause the membrane potential to hyperpolarize to a similar potential in both age-groups.

Following transition to zero Na^+, the resting membrane potential of the CGCs from both young and old animals hyperpolarized ($p<0.0001$, two-way RM ANOVA; Figure 8A and Bi). In young, the RMP fell from -59.9 ± 1.6 to -86.0 ± 4.2 mV ($p<0.0001$, Bonferonni post-hoc test), and in old from -55.6 ± 2.9 to -75.1 ± 2.5 mV ($p<0.0001$, Bonferonni post-hoc test). The RMP for both the young and old CGCs in Na^+ saline were similar, as were the RMPs following transition into zero Na^+ saline ($p>0.05$ effect of age, $p>0.05$ interaction, two-way RM ANOVA; Figure 8Bi). There was therefore

no statistically significant difference between young and old CGCs in the overall change in membrane potential (ΔMP) ($p>0.05$, unpaired Student's t-test, Figure 8Bii), although ΔMP was slightly less in old CGCs.

The change in RMP following transition to zero Na^+ is caused by a combination of blocking both the NCX and $I_{Na(p)}$. Subtraction of the mean ΔMP value in zero Na^+ saline from that seen following transition to Li^+ saline (block of the NCX only) should therefore reveal the influence of the $I_{Na(p)}$. This indirect measurement of the $I_{Na(p)}$ revealed a depolarizing effect on RMP of approximately 13.3 mV in young, and approximately 26.7 mV in old, an increase of over 100%.

3.2 Voltage clamp

3.2.1 Cd^{2+} sensitivity of HVA-Ca^{2+} currents in the CGCs from young and old animals

To exclude the possibility that the altered sensitivity to Cd^{2+} observed in old animals during spontaneous firing and SFA was due to a reduction in a Cd^{2+} sensitive Ca^{2+} current (or a reduced sensitivity to $CdCl_2$), we measured peak Ca^{2+} currents in the absence and presence of 100 μM $CdCl_2$ in both age-groups.

Peak inward Ca^{2+} currents occurred between -25 and 10 mV in both young and old CGCs. The % block of the peak inward current with 100 μM Cd^{2+} was similar in both groups, and statistically indistinguishable (78% ± 10% and 81% ± 8% in young and old CGCs respectively; $p=0.823$, unpaired Student's t-test, $n=5$; Figure 9). IV plots of both young and old in control conditions were similar and statistically indistinguishable ($p=0.9$, 2-way RM ANOVA).

4 Discussion

In this paper we set out to determine the age-related changes to firing frequency, the sAHP duration, and excitability in the CGCs, which are an important pair of neurons that regulate feeding and learning and memory in *Lymnaea stagnalis*. We found that during aging there is a reduction in firing frequency, and an associated increase in the duration of the sAHP. We also found that during artificial depolarization, the strength, and speed of SFA are altered in a way that is consistent with a decrease in Ca^{2+} buffering. Evidence obtained from Ca^{2+} fluorophore decay rates from the CGCs of young and old animals supported this hypothesis. Finally we showed that an age-related switch in the direction of the Na^{+}/Ca^{2+} exchanger can explain these changes, and also why the sAHP and firing frequency appear to be resistant to voltage-gated Ca^{2+} channel block in old animals.

4.1 Changes to firing frequency and the sAHP duration

In the initial part of this paper we confirmed that there was an age-related reduction in the firing rate of the CGCs along with an age-related increase in the duration of the sAHP. The 48% reduction in mean firing rate observed here was slightly less than the 57% observed by Patel *et al* (Patel, et al., 2006), but was nonetheless a highly significant decrease in excitability. The mean values reported for the duration of the sAHP were larger than the mean time to RMP values previously reported by Patel *et al* for both the young and old age groups. The measurements of time to RMP and sAHP duration are essentially identical, with both measuring the period of time between the fast AHP and the RMP. One plausible explanation for this difference however may lie in how the two studies define the RMP. Defining the RMP at a more positive membrane potential would equate to a sAHP measurement that is longer in duration.

We went on to show that there was a significant relationship between the duration of the sAHP and CGC firing rate in both age groups. In young animals, there was a clear and highly significant negative correlation between firing rate and the duration of the sAHP, suggesting that this is a major determinant of firing frequency. In old animals however, the relationship was less robust, although

still significant. This observation may possibly be because the sAHP is no longer the dominant factor in determining firing frequency in old animals.

4.2 Change to SFA adaptation during aging

The $I_{sAHP,}$ which underlies sAHP in many vertebrate and invertebrate neurons, also contributes to the phenomenon of SFA. Therefore, an age-related increase in the sAHP duration should also enhance SFA. We found that both young and old animals undergo SFA in response to artificial depolarization, and that the reduction in instantaneous frequency of each spike with time followed a monoexponential time-course in line with Equation 1. In old animals however, the steady state firing frequencies (f_{ss}) were significantly slower compared to young, supporting the hypothesis that old animals possess an enhanced K_{Ca}. Also consistent with this hypothesis is the marginally significant increase in F_{adap} that we observed with age. F_{adap} is the % reduction in instantaneous frequency during SFA, and the larger values observed in old animals suggests that for a similar magnitude of depolarizing stimulus old CGCs typically respond with a stronger adaptation compared to young.

In addition to changes to SFA with age we also observed a lengthening of spike half width as the depolarization progressed. Interestingly, we found that the increase in spike width was dependent upon 1) the intensity of the current injection, and 2) the age of the CGC. One possible reason for spike width broadening could be inactivation of A-type K^+ channels, which are known to have fast inactivation kinetics (Hille, 2001). The inactivation of these channels is voltage dependent and so it certainly fits that more inactivation will occur at higher current amplitudes, leading to wider action potentials. Why this effect is more pronounced in old animals compared to young is not known, although one possibility could be smaller A-type currents in old animals, which has previously been shown in rat CA1 neurons (Alshuaib, et al., 2001). Alternatively, the inactivation kinetics of the channel could change with age. The consequences of increased spike broadening during a period of current injection are also yet to be tested. One potential effect of a broader spike is greater Ca^{2+} entry over the course of the action potential due to the prolonged period of depolarization. This may increase the release of 5-hydroxytryptamine (5-HT) from the CGCs, and in combination with

decreased reuptake of 5-HT from the synapse, may act to compensate for a reduced CGC firing frequency (Patel, et al., 2006, Yeoman, et al., 2008).

4.3 Changes to Cd^{2+} sensitivity of firing frequency, the sAHP, and SFA during aging

An age-related increase in the sAHP has been previously observed in rats, mice, and rabbit hippocampal neurons (Foster, 2007, Landfield and Pitler, 1984, Moyer, et al., 2000, Murphy, et al., 2006). The current that underlies the sAHP in these neurons is sensitive to Ca^{2+} with previous experiments showing that blockade of HVA Ca^{2+} currents can reverse the age-related sAHP increase (Landfield and Morgan, 1984, Moyer, et al., 1992). We therefore examined the effects of age on the Ca^{2+} sensitivity of the sAHP, action potential firing frequency and SFA in CGCs using the HVA-Ca^{2+} channel blocker $CdCl_2$. Our findings showed that $CdCl_2$ only affected the duration of the sAHP and firing frequency in young CGCs, with no significant effects in old CGCs. Furthermore, SFA was also less sensitive to $CdCl_2$ in old animals. One possible explanation for this could be a reduction in the sensitivity of HVA-Ca^{2+} channels to $CdCl_2$ with age, or insufficient block. However, voltage clamp experiments examining the sensitivity of HVA-Ca^{2+} currents to $CdCl_2$ failed to show any differences in young and old animals.

4.4 NCX mode reversal may contribute to the age-related changes to the CGCs

We produced several pieces of evidence that were consistent with an age-related change to Ca^{2+} buffering in the CGCs. First, Ca^{2+} fluorophore decay rates during spontaneous spike discharge in the axons of CGCs from old animals were slower than young. Second, we observed an age-related reduction in the speed of SFA, along with an increase in its strength which is consistent with an increased time constant of Ca^{2+} clearance (τ_{Ca}) in older CGCs (Liu and Wang, 2001, Wang, 1998). Finally, our experiments measuring the contribution of the electrogenic NCX to membrane potential in young and old animals showed a switch in the direction of the exchanger with increasing age, indicating an additional source of Ca^{2+} entry into the cell (and the I_{sAHP}; see Figure 11). The latter experiments used Li^{+} substituted saline (an NCX blocker) to measure both the activity, and direction of the NCX through its effect on CGC membrane potential. Consistent with blocking the reverse-

mode NCX in the old (and reducing Ca^{2+}entry to the cell), we saw a decrease in the Ca^{2+} sensitive sAHP in all animals, which further supports our argument that the NCX is providing Ca^{2+} to the cell. The addition of Li^{+} does not fully restore the sAHP to the mean values observed in the young however, which probably reflects the fact that $[Ca^{2+}]_i$ is being altered through additional mechanisms. Indeed, changes to plasma membrane and sarco-endoplasmic membrane ATPase activity (Jiang, et al., 2012, Michaelis, et al., 1992), voltage gated Ca^{2+} channel currents (Campbell, et al., 1996, Frolkis, et al., 1984, Moyer and Disterhoft, 1994), Ca^{2+} binding proteins (Verkhratsky and Toescu, 1998) and mitochondrial structure and function have been noted to change with age, some of which can lead to activation of K_{Ca} currents (Foster, 2007).

It should be noted here however that despite substantial evidence in the literature demonstrating the ability of Li^{+} to block the NCX, the ion does have a number of off-site targets, including the Na^{+}/K^{+}ATPase and IP_3 signaling. Chronic Li^{+} perfusion has been shown to inhibit IP_3 signaling, producing a reduction in endoplasmic reticulum mediated Ca^{2+} release and a decrease in $[Ca^{2+}]_i$. However, this decrease in $[Ca^{2+}]_i$ is not consistent with an increase in the Ca^{2+} sensitive sAHP seen in old CGCs. Li^{+} has also been shown to decrease Na^{+}/K^{+}ATPase activity in brain synaptosomes, which could lead to an increase in $[Na]_i$ and a potential block in the forward mode of the NCX. However, other studies in rat hippocampal neurons have shown Li^{+} to have no effect on the Na^{+}/K^{+}ATPase. An alteration in the chemical gradient for Na^{+} during the ion substitution experiments may also affect Na^{+}/K^{+} ATPase activity, potentially increasing its activity as it attempts to restore the Na^{+} gradient across the membrane. Although this is consistent with our observations in young CGCs, it does not explain the observations in old CGCs. The inconsistencies in the literature suggest that these off-site effects are unlikely to be the mechanism which underlies our observations, however, we cannot definitively exclude this possibility.

The values for the Ca^{2+} decay time constant, which we recorded during Ca^{2+} imaging, and those calculated from measurements of the speed and strength of SFA were both consistent with an age-related reduction in Ca^{2+} clearance. However, the actual values for each age group were not similar. There are a number of possible reasons for this observation. Firstly the mathematical model applied

here assumes a single kinetic compartment, in which Ca^{2+} enters, distributes, activates the I_{sAHP}, and is then cleared from the cell. Under-representation of the number of compartments has been shown to produce larger τ_{Ca} values than that those observed through imaging (Peron and Gabbiani, 2009). Secondly, the time constants derived from the imaging experiments described here were obtained during the discharge of a series of isolated, spontaneous action potentials, whereas those τ_{Ca} values derived from SFA data were obtained from CGCs that were undergoing current injection. Data in rat sub thalamic neurons suggests that the time constant for Ca^{2+} decay is larger during driven bursts, compared to spontaneous single spiking (Goldberg, et al., 2009).

Despite evidence that the activity of the NCX reduces with age in both the reverse and forward modes (Canzoniero, et al., 1992, Michaelis, et al., 1984), to our knowledge, this is the first report of an age-related NCX mode switch. There are a number of possible mechanisms through which the NCX may have switched to a reverse mode. First, the NCX protein complex may have undergone modulation by intracellular signaling molecules. The NCX contains an intracellular loop which incorporates peptide sequences that are responsive to several second messengers and enzymes, including phosphotidyl inositol diphosphate, protein kinases A and C, $[H^+]_i$, nitric oxide, and adenosine triphosphate (Annunziato, et al., 2004). Interestingly, this region is also responsive to reactive oxygen species (ROS), which as well as being implicated in neuronal aging of vertebrate species, may play a role in changes to neuronal excitability in certain neurons of the *Lymnaea* CNS (Watson, et al., 2012a). Indeed, lower levels of the antioxidant glutathione have been observed in the nervous system of older *Lymnaea* (Watson, et al., 2014). Second, there may have been a shift in the concentration of intracellular ionic species, for example a high $[Na^+]_i$ or low $[Ca^{2+}]_i$ (Annunziato, et al., 2004), driving the NCX to work in reverse mode. An increase in the $[Na^+]_i$ may occur as a result of an age-related reduction in the activity of Na^+/K^+ ATPase. Interestingly, the activity of the Na^+/K^+ ATPase is closely coupled with the activity and direction in which the NCX functions. Reducing the activity of Na^+/K^+ ATPase can increase $[Ca^{2+}]_i$ via effects on the NCX (Golovina, et al., 2003,Lynch, et al., 2008). There is also evidence that Na^+/K^+ ATPase alpha-2 isoform knockout mice have increased $[Ca^{2+}]$ stores (Lynch, et al., 2008). Furthermore, a reduction in the activity of this pump has been

observed in central neurons of rats during aging (Benzi, et al., 1994, Murali, et al., 2008,Torlinska and Grochowalska, 2004). It is possible that the $[Na^+]_i$ in old CGCs may be elevated to such an extent that the driving force for Na^+ entry is reversed, causing Ca^{2+} to be transported into the cell. An obvious source for the supply of extra intracellular Na^+ with age however is the persistent Na^+ current ($I_{Na(p)}$). Data from our zero Na^+ and Li^+ substitution experiments show indirectly that the depolarizing influence of $I_{Na(p)}$ increases by approximately 100% in old animals, perhaps in an attempt to maintain a constant RMP due to an age-related hyperpolarizing switch in the NCX. Unfortunately, there are no identified inhibitors of this current, and therefore it has not been possible to directly measure specific age-related changes.

In conclusion, we have shown that the firing frequency of the CGCs is reduced with age, in association with an increase in the sAHP duration. We have gone on to show that an age-related reversal of the NCX may not only impair Ca^{2+} buffering, but may also provide an additional source of Ca^{2+} to activate the I_{sAHP}. This research underlines the value of *Lymnaea stagnalis* as a model system to study the neuronal, biochemical, and molecular mechanisms of age-related behavioral changes, including learning and memory. Its well-characterized behaviors and relatively simple, yet defined nervous system provides researchers with a top-down, systems level model to understand the causes of numerous age-related behavioral changes that are conserved with vertebrates (Hermann, et al., 2014, Yeoman, et al., 2012).

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Figure 1. Age-related changes to the basic firing properties of the CGCs. During aging, an increase in the duration of the sAHP is associated with a reduction in the spontaneous firing rate of the CGCs. *Ai* 20s section of current clamp trace from the CGC of a young (top trace) and old (bottom trace) snail. *Aii* Firing frequency reduces with age (***$p<0.001$, unpaired Student's t-test). *B* Traces of a typical action potential from the CGCs of a young (black, *Bi*) and old (grey, *Bii*) snail. The sAHP duration increases with age (***$p<0.001$, unpaired Student's t-test); dotted line indicates the resting membrane potential. Measurement of the sAHP is indicated with dotted lines and arrows. *Biii* CGC half-width decreases with age. *C* Association between the sAHP duration and firing frequency in CGCs from young (black circles) and old (grey circles) snails. R^2 and p values are provided in the insets. For Aii, Bii, and C n=17 young and n=13 old.

Figure 2. Spike frequency adaptation is altered during aging in the CGCs. *Ai* Sample current clamp traces from a young (top) and old (bottom) CGC during a 5s, 20 nA current injection. *Aii* Plot of instantaneous frequency (IF) vs. time of spike for the traces shown in Ai. Plots have been fitted with a single exponential curve (Figure 5). *Aiii* Mean f_0 values for young and old CGCs. No significant effect of aging on f_0 was observed during 5s current injections at current amplitudes of 10, 20 and 30 nA. *Aiv* Mean f_{ss} values for young and old CGCs from the same current injections episodes detailed above. f_{ss} values were significantly slower in old CGCs compared to young ($p<0.05$ effect of age, $p<0.0001$ effect of current amplitude, two-way RM ANOVA; *$p<0.05$ Bonferonni post-hoc test). *Bi* Sample action potentials at the beginning of current injection (f_0, solid black line), and at the end of current injection (f_{ss}, dotted line) from young (top) and old (bottom) CGCs during a 5 s 20 nA artificial current injection. *Bii* The % increase in half-width during sustained current injection is significantly larger in CGCs from old animals compared to young ($p<0.05$ effect of age, $p<0.0001$ effect of current amplitude, two-way RM ANOVA; *$p<0.05$ Bonferonni post-hoc test). For Aiii, Aiv, and Bii, n=12 young, and n=15 old. f_0 = extrapolated firing frequency at time = 0 s; f_{ss} = steady state firing frequency.

Figure 3. Changes to the sAHP of the CGC action potential during aging, and response to HVA-Ca^{2+} channel block with Cd^{2+}. *A* Sample current clamp recordings of the CGC action potential from young (left) and old (right) animals, in the absence (black) and presence (grey) of 100 μM $CdCl_2$. *B* The duration of the sAHP is reduced following treatment with $CdCl_2$ in young, but not old CGCs (**p<0.01, Paired Students t-test). For B, n=7 for both groups.

Figure 4. Effect of Cd^{2+} on the basic parameters of SFA in young and old CGCs. Values of f_0 and f_{ss} are both increased in young animals following treatment with $CdCl_2$. In old animals however, only a small but significant increase in f_0 is observed. *Ai* Sample current clamp recordings from the CGC of a young animal in the absence (top) and presence (bottom) of 100 μM $CdCl_2$ during a 5s, 20 nA current injection. *Aii* and *Aiii* Changes to f_0 and f_{ss} in CGCs from young animals following treatment with $CdCl_2$. There is a significant increase in both values over the range of current injection amplitudes following treatment with Cd^{2+} ($p<0.001$ and $p<0.01$ effect of treatment on f_0 and f_{ss} respectively, $p<0.0001$ effect of current on both f_0 and f_{ss}, $p<0.01$ interaction for f_{ss} only, two-way RM ANOVA; *p<0.05, **p<0.01, ***p<0.001 Bonferonni post-hoc tests). *Bi* Sample current clamp recordings from the CGC of an old animal in the absence (top) and presence (bottom) of $CdCl_2$ during a 5s, 20 nA current injection. *Bii* Following treatment with $CdCl_2$, f_0 increases significantly in old animals ($p<0.01$ effect of treatment, $p<0.0001$ effect of current amplitude, two-way RM ANOVA). *Biii* In old animals, the steady state firing frequency (f_{ss}) is not sensitive to the effects of $CdCl_2$ ($p>0.05$ effect of treatment, $p<0.01$ effect of current amplitude, two-way RM ANOVA); n=6 for both groups.

Figure 5. Effect of age on the dynamics of SFA in the CGCs. Aging reduces the speed of adaptation (τ_{adap}), resulting in a larger calculated τ_{Ca}. *A* Age-related decrease in the speed of adaptation (increased τ_{adap}) over a range of 5s current injections (10, 20, and 30 nA; $p<0.001$ effect of age, $p<0.001$ effect of current amplitude, $p<0.05$ interaction, two-way RM ANOVA; ***p<0.001 Bonferonni post-hoc test). *B* Changes to the strength of adaptation (F_{adap}) over the 3 current injection amplitudes. There is a trend towards an increase in F_{adap} (strength of adaptation) during aging in the

CGCs (p=0.054 effect of age, two-way RM ANOVA). *C* The calculated τ_{Ca} (based on Wang's equations) is increased in CGCs from old animals indicating a reduction in Ca^{2+} clearance ($p<0.05$ effect of age, $p<0.01$ effect of current amplitude, $p<0.01$ interaction, two-way RM ANOVA; **$p<0.01$ Bonferonni post-hoc test). For A, B and C, n=12 young, and n=15 old.

Figure 6. Effects of age on the Ca^{2+} dynamics in spontaneously firing CGCs. A) Spontaneous action potentials evoked Ca^{2+} transients in both young (solid line) and old (dotted line) CGCs. The timing of the action potentials is shown above by the vertical lines. Bi) Mean peak areas for Ca^{2+} transients were significantly greater in old compared to young CGCs. Bii) These differences in the area were due to a slower time constant for decay. n=6 for both age groups.

Figure 7. Contribution of the NCX to RMP changes with age in the CGCs,. *A* Examples of current clamp traces pre, and post perfusion with Li^+ replacement saline in young (top) and old (bottom). The dotted line indicates the stable RMP prior, and post saline transition. *Bi* During transition to Li^+ saline, the resting membrane potential of CGCs from young animals hyperpolarizes, whilst to old depolarizes ($p<0.05$ effect of Li^+, $p<0.001$ interaction, two-way ANOVA; **$p<0.01$ Bonferonni post-hoc test between young and old Li^+). *Bii* The change in membrane potential (ΔMP) following Li^+ saline perfusion in CGCs from young and old animals is significantly different ($p<0.0001$, unpaired Student's t-test). *Ci* and *Cii* Li^+ substitution reduces the sAHP duration in CGCs from old animals ($p=0.01$, paired Student's t-test). For Bi, Bii, and Cii n=5 for both groups. NCX = Sodium Calcium Exchanger and RMP = Resting Membrane Potential.

Figure 8. Combined effect of blocking the NCX and the persistent Na^+ current ($I_{Na(p)}$) on CGC RMP. *A* Examples of current clamp traces pre, and post perfusion with zero Na^+ saline in young (top) and old (bottom). The dotted line indicates the stable RMP prior, and post saline transition. *Bi* In CGCs from both young and old animals the RMP hyperpolarized following transition into zero Na^+ saline ($p<.0.0001$, two-way RM AVOVA). *Bii* The change in membrane potential (ΔMP) following transition to zero Na^+ saline in CGCs from old animals compared is not statistically significant

($p>0.05$, unpaired Student's t-test). NCX = Sodium Calcium Exchanger and RMP = Resting Membrane Potential.

Figure 9. HVA-Ca^{2+} currents in the CGCs of young and old *Lymnaea* are both sensitive to $CdCl_2$. *A* Current / Voltage relationship for inward Ca^{2+} currents in young and old CGCs in the absence / presence of 100 μM $CdCl_2$. There is no statistically significant difference between traces corresponding traces in either age-group ($p>0.05$, two-way RM ANOVA). *B* The % block of HVA Ca^{2+} currents with 100 μM $CdCl_2$ is not significantly different with age. n=5 for both groups.

Figure 10. Illustration of the age-related changes to Ca^{2+} homeostasis in the CGCs. In young animals (top) the NCX is working in the forward mode and extrudes Ca^{2+} from the cell using the Na^+ gradient created in part by the Na^+/K^+ATPase. This process is electrogenic and depolarizes the cell. Application of Li^+ saline blocks the NCX and causes the neuron to hyperpolarize (middle). In old animals, the NCX work in reverse mode (possibly because of an increase in the $[Na^+]$ (increased I_{Nap}) activity), transporting Ca^{2+} into the cell in exchange for Na^+ (bottom). The reverse mode causes hyperpolarization of the neuron. NCX block with Li^+ saline in old CGCs causes the cell to depolarize.