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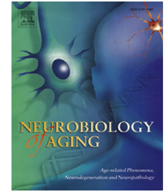

A switch in the mode of the sodium/calcium exchanger underlies an age-related increase in the slow afterhyperpolarization

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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 article, 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 in the CGCs during aging, a finding that is compatible with an increase in the sAHP current. We go on to show that age-related changes in the kinetics of spike frequency adaptation 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. These experiments suggest that the sodium calcium exchanger may be switching to a reverse-mode configuration in the CGCs during aging.
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1. Introduction

Over the last few 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 are keys to reducing this burden. However, research on 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 because of their simpler nervous system and relatively short life span. Notably, a number of 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 comprises 3 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 and 100 ms post spike, and is produced by small conductance 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 50 ms 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 on 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 Ca^{2+} channels have also been shown to provide a source of Ca^{2+} for SK; however, there is little evidence that low-voltage-activated Ca^{2+} channel derived Ca^{2+} activates the channel responsible for the I_{sAHP}, and no evidence for BK. Ca^{2+} derived from intracellular stores is also known to signal the K_{Ca}, and BK and SK are also both

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additionally sensitive to Ca^{2+} derived from *N*-methyl-D-aspartate receptors and SK from Ca^{2+} derived through the nicotinic acetylcholine receptor (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 (SFA), 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 in the duration and amplitude of the AHP of the cerebral giant cells (CGCs) from the pond snail *L. stagnalis*, which are a key pair of serotonergic neurons that 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 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} current in the CGCs is responsible for the age-related changes in the AHP and firing behavior.

In this article, 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 HVA Ca^{2+} channels. 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

L. 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 ultraviolet light. The temperature of the water was monitored and maintained at 21 °C. All animals were fed every other day, alternatively either English round lettuce or TetraPond (UK's best-selling pond food) fish sticks that are ground to form a powder. Animals were kept for 12 hours under light/dark cycle all year round. Animal numbers were counted every 2 weeks and survival curves were constructed to determine when animals had entered adulthood (95% survival) or old age (25% survival) (Hermann et al., 2007).

Before electrophysiological recording, the central nervous system (CNS) was prepared in the following way: shells were carefully removed from each snail before pinning to a Sylgard-lined Petri dish (Sylgard, Corning, UK). The snail was then immersed in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline at room temperature (see Section 2.2 for composition) and the CNS was removed using fine forceps and microscissors. The CNS was then transferred, with 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, UK) 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. Before 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; HEPES 10; pH adjusted to 7.9 with 10 M NaOH. In voltage clamp experiments to measure Ca^{2+} currents, the following zero Na^+ saline with K^+ channel blockers was used (in mM): tetraethylammonium chloride 50; 4-aminopyridine (4-AP) 4; KCl 1.6; $CaCl_2$ 3.5; $MgCl_2$ 2.0; HEPES 10; and pH adjusted to 7.9 with tetraethylammonium hydroxide. For experiments measuring the contribution of NCX, 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 experimental measurement of the contribution of the NCX and the $I_{Na(p)}$ to the resting membrane potential (RMP), the LiCl in the previous solution was substituted with *N*-methyl-D-glucamine (50 mM). $CdCl_2$ (100 μM) was used to inhibit HVA Ca^{2+} channels in the CGCs. All chemicals were 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, USA). Analog signals were converted to digital by a Digidata 1400A, an analog-to-digital converter (Axon Instruments, USA). For current clamp, data were sampled at ≥2 kHz and for voltage clamp at 20 kHz. Both were recorded on a PC using pCLAMP software (pCLAMP, version 10.0; Axoscope or Clampex, respectively) (Molecular Devices, USA). Micropipette electrodes were made using borosilicate glass and pulled using an Intracel P-30 vertical electrode puller. Each micropipette was filled with 4 M potassium acetate (Sigma, UK), and the tip dipped in black ink (Winsor and Newton, UK) to

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