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RESEARCH ARTICLE

Exposure to low temperature prepares the turtle brain to withstand anoxic environments during overwintering

Ebrahim Lari[1,2,*] and Leslie T. Buck[1,3,*]

ABSTRACT

In most vertebrates, anoxia drastically reduces the production of the essential adenosine triphosphate (ATP) to power its many necessary functions, and, consequently, cell death occurs within minutes. However, some vertebrates, such as the painted turtle (*Chrysemys picta bellii*), have evolved the ability to survive months without oxygen by simultaneously decreasing ATP supply and demand, surviving the anoxic period without any apparent cellular damage. The impact of anoxia on the metabolic function of painted turtles has received a lot of attention. However, the impact of low temperature has received less attention and the interactive effect of anoxia and temperature even less. In the present study, we investigated the interactive impacts of reduced temperature and severe hypoxia on the electrophysiological properties of pyramidal neurons in painted turtle cerebral cortex. Our results show that an acute reduction in temperature from 20 to 5°C decreases membrane potential, action potential width and amplitude, and whole-cell conductance. Importantly, acute exposure to 5°C considerably slows membrane repolarization by voltage-gated K^+ channels. Exposing pyramidal cells to severe hypoxia in addition to an acute temperature change slightly depolarized membrane potential but did not alter action potential amplitude or width and whole-cell conductance. These results suggest that acclimation to low temperatures, preceding severe environmental hypoxia, induces cellular responses in pyramidal neurons that facilitate survival under low oxygen concentrations. In particular, our results show that temperature acclimation invokes a change in voltage-gated K^+ channel kinetics that overcomes the acute inhibition of the channel.

KEY WORDS: Anoxia tolerance, Voltage-gated channels, Thermal adaptation, Membrane properties, Action potential

INTRODUCTION

For most animals, oxygen is essential for life as severe O_2 deprivation for only a few minutes is detrimental or even fatal (Selvan et al., 2017). If blood O_2 concentration decreases further than the compensatory capacity of the cardiovascular system (e.g. hyperventilation, tachycardia), aerobic metabolism is reduced or ceases, and anaerobic energy production is insufficient to support ion regulation (Buck and Pamenter, 2018). Thus, when deprived of sufficient oxygen, ion gradients in the brain of anoxia-intolerant organisms destabilize, followed by an excessive release of excitatory neurotransmitters (Rego et al., 1996). This results in neuronal hyperactivity and compromises cellular integrity by causing a massive efflux of K^+ and an influx of Na^+ and Ca^+, further depolarizing the membrane potential and activating enzymes such as proteases, lipases, phosphatases and endonucleases (Syntichaki and Tavernarakis, 2003).

However, some vertebrates, such as painted turtles (*Chrysemys picta bellii*), red-eared slider turtles (*Trachemys scripta elegans*) and crucian carp (*Carassius carassius*), can survive extended periods, up to 5 months, without oxygen at 3–4°C while overwintering (Bickler and Buck, 2007). These anoxia tolerance champions have developed astonishing adaptations to withstand extremely low levels or the complete absence of oxygen. For instance, painted turtles possess one of the largest internal stores of glycogen to fuel anaerobic glycolysis (Jackson, 2000; Leander et al., 2000) and a complex buffering and detoxifying system to manage the toxic accumulation of protons and lactate (Ultsch et al., 1984; Jackson, 1997; Jackson et al., 1999). They are also able to drastically reduce their metabolic rate by 88–90% (Else and Hulbert, 1981; Herbert and Jackson, 1985a; Buck et al., 1993; Land et al., 1993) and stabilize protein structures (Ramaglia and Buck, 2004) during anoxia.

Like anoxia, lower winter temperatures have a profound effect on painted turtles. For example, whole-animal metabolic rate is reduced by 94% with acclimation from 20 to 3°C (Herbert and Jackson, 1985a, 1985b), and this is reflected in a 5- to 15-fold decrease in systemic cardiac power output, which reflects reduced cardiac ATP demand (Hicks and Farrell, 2000; Stecyk et al., 2004a; 2004b). With anoxia and low temperature exposure, metabolic rate is further reduced to lower than 10% of the low temperature, normoxic rate (Herbert and Jackson, 1985a, 1985b) and the systemic cardiac power output of 5°C-acclimated turtles is 7- to 20-fold less in anoxia than in normoxia at 5°C (Hicks and Farrell, 2000; Stecyk et al., 2004a).

The brain is the most oxygen-demanding and O_2-sensitive organ and in anoxia-tolerant species, the response to hypoxia or anoxia is 'synaptic arrest' (Buck and Pamenter, 2018). That is, anoxia-tolerant organisms minimize ionic conductance across membranes by either reducing ion channel density or reducing channel open times to maintain cellular membrane potential in the wake of reduced pump activity as a result of reduced ATP supply (Ultsch et al., 1984; Pék-Scott and Lutz, 1998; Bickler and Buck, 2007; Rodgers-Garlick et al., 2013). Interestingly, in anoxia-tolerant painted turtle brain, γ-aminobutyric acid (GABA) release by stellate neurons increases, resulting in increased GABA-A receptor currents in pyramidal neurons and clamping of the membrane potential at the reversal potential for the GABA-A receptor. This prevents depolarization to the threshold potential and suppresses overall electrical activity, preventing the devastating effects of low oxygen stress (Tišler and Zagorc-Končan, 1999; Pamenter et al., 2011;

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Received 29 April 2021; Accepted 27 August 2021

Journal of Experimental Biology (2021) 224, jeb242793. doi:10.1242/jeb.242793

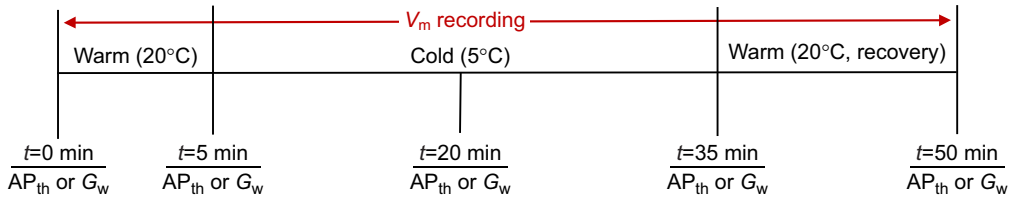

Fig. 1. Experimental time line for the effect of an acute temperature change to 5°C on pyramidal neurons of painted turtles acclimated to 20°C. Membrane potential (V_m) was recorded constantly throughout the experiment. Action potential threshold (AP_{th}) and whole-cell conductance (G_w) were recorded at *t*=0, 5, 20, 35 and 50 min from the start of the experiment. Only one of the three aforementioned parameters was recorded in each neuron.

2012; Hawrysh and Buck, 2019). Thus far, the GABA-mediated increase in Cl^- conductance is the only ion conductance known to increase with anoxia (Buck and Pamenter, 2018).

Little is known about the interactive impacts of low temperature and anoxia on the turtle brain's cellular functions. To the best of our knowledge, the only study on this subject investigated Na^+/K^+-ATPase activity in response to low temperature and anoxia (Stecyk et al., 2017). Brain Na^+/K^+-ATPase activity and density of 21 and 5°C-acclimated animals were similar under normoxic conditions; however, when low temperature and anoxia were combined, it resulted in a 55% decrease in Na^+/K^+-ATPase activity, largely due to a reduction in the number of functional Na^+/K^+-ATPase proteins (Stecyk et al., 2017). In another study, low temperature itself resulted in a substantial decrease in the transcripts of neuroexcitatory genes (glutamate), while there was only a mild suppression of inhibitory transcripts (GABA) in red-eared slider turtles, *T. scripta* (Couturier et al., 2019).

As almost all studies of anoxia tolerance at the cellular level have been conducted at room temperature (20–23°C), our knowledge of the turtle brain's response to overwintering temperatures (1–5°C) and anoxia under those conditions is limited. In the present study, we first investigated the changes in turtle pyramidal neuron electrophysiological properties, such as membrane potential, action potential characteristics and whole-cell conductance in response to reduced temperature (from 20°C to 5°C). Then, in the second part of the study, we investigated the response of pyramidal cells to anoxia at 5°C in animals acclimated to 20 or 5°C.

MATERIALS AND METHODS

Animal husbandry and chemical preparations

This study was approved by the University of Toronto Animal Care Committee and conformed to the care and handling of animals as outlined in the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals, Vol. 2. Adult, female (5–7 years old) western painted turtles, *Chrysemys picta bellii* (Gray 1831), were obtained from Niles Biological (Sacramento, CA, USA). Animals were housed in 6000 l round tanks with a rock island located under a heat lamp for basking. A constant flow-through of 20°C dechlorinated freshwater kept the temperature steady and removed the biological waste. Turtles were kept on a continuous 12 h:12 h light:dark cycle and were fed daily.

Artificial cerebrospinal fluid (aCSF), osmolarity 285–290 mOsM, was produced by adding (in mmol l^{-1}): 107 NaCl, 2.6 KCl, 1.2 $CaCl_2$, 1.0 $MgCl_2$, 2.0 NaH_2PO_4, 26.5 $NaHCO_3$, 10.0 glucose and 5.0 imidazole to ddH_2O and adjusting the pH to 7.4. Pipette solution, simulating intracellular ionic composition, osmolarity 285–290 mOsM, was produced by adding (in mmol l^{-1}): 8 NaCl, 0.0001 $CaCl_2$, 10 Na-Hepes, 20 KCl, 110 potassium gluconate, 1 $MgCl_2$, 0.3 NaGTP, and 2 NaATP to ddH_2O and adjusting the pH to 7.4.

Cortical slice preparation

Following the decapitation of an animal, the whole brain was rapidly dissected out of the cranium and submerged in 3–5°C aCSF. From each cerebral hemisphere, 3–4 cortical sheets were cut (total 6–8 pieces) and stored at 4°C until use. One cortical sheet was used per experiment, and a single neuron was recorded per sheet.

Whole-cell electrophysiological technique

A cortical sheet was placed on the bottom of a perfusion chamber system (RC-26 open bath chamber with a P1 platform; Harvard Apparatus, Saint-Laurent, QC, Canada), and held in place using a horseshoe-shaped anchor with spandex threads stretched across it. The chamber was gravity perfused from one of two 1 liter glass bottles that contained aCSF, gassed with either 95% O_2–5% CO_2 or 95% N_2–5% CO_2 to achieve normoxic or extreme hypoxic conditions, respectively. Viton tubing (Cole Parmer, Burlington, ON, Canada), which has a low gas permeability, was used to minimize gas exchange with air and maintain P_{O_2} conditions throughout saline perfusion. A plastic cover with a hole for the recording electrode was placed over the chamber. The space between the cover and solution surface was gently gassed with 95% N_2–5% CO_2 during anoxic conditions. The aCSF passed through an LCS-1 Liquid Cooling System (CL-100) connected to a Single Channel Bipolar Temperature Controller (Warner instruments L.L.C., Hamden, CT, USA), before entering the chamber, to control the temperature in the chamber.

Whole-cell recordings of neurons were performed using an Ag/AgCl electrode, dipped in an electrode solution, inside a fire-polished micropipette (5–8 MΩ). Micropipettes were produced from borosilicate filament glass capillary tubes (Harvard, Montreal, QC, Canada) using a P-97 micropipette puller model (Sutter

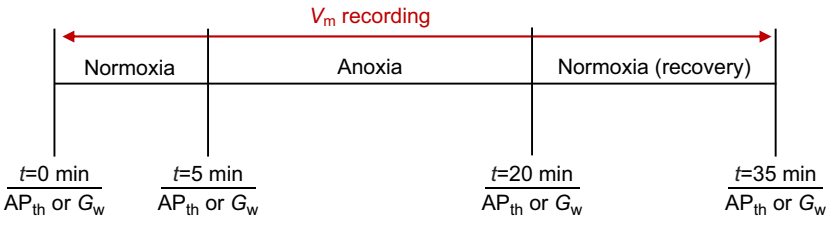

Fig. 2. Experimental time line for the effect of anoxia at 5°C on pyramidal neurons from turtles acclimated to 20 or 5°C. Turtles acclimated to 20°C were exposed to 5°C for at least 10 min before recording started. The temperature was maintained at 5°C throughout this experiment. V_m was recorded constantly throughout the experiment. AP_{th} and G_w were recorded at *t*=0, 5, 20, 35 and 50 min from the start of the experiment. Only one of the three aforementioned parameters was recorded in each neuron.

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Instruments, Novato, CA, USA). A CV-7B head stage connected the electrode to a MultiClamp 700B digital amplifier and a Digidata 1440A interface (Molecular Devices, Sunnyvale, CA, USA). The data were collected at 10 kHz, visualized, and stored on a computer via Clampex 10 software (Molecular Devices, San Jose, CA, USA). Cell-attached seals were achieved using the blind-patch technique described by Blanton et al. (1989), followed by compensating for the membrane capacitance. Then, a whole-cell patch configuration was established by voltage clamping the recording potential to −80 or −70 mV at 20 or 5°C, respectively, and applying negative pressure after a 1–20 GΩ seal was established. The patched cells were allowed a minimum of 5 min to acclimate to experimental conditions before measurements were conducted. After the acclimation period, access resistance was measured, and if it varied by >25% throughout an experiment, the patch was discarded. The liquid junction potential (LJP) between the turtle aCSF and the pipette solution at both 20 and 5°C and differences in LJP between the two temperatures were measured. All data were corrected for these values (for example, Fig. 3D).

Electrophysiological identification

Under current-clamp configuration, a constant current was injected into the cell for 450 ms to depolarize the membrane by −30 mV, surpassing its action potential threshold (AP_{th}), to identify the patched neuron. Neurons were identified as either pyramidal or stellate, following action potential characterization described by Connors and Kriegstein (1986). Pyramidal cells displayed a gradual reduction in the frequency and the amplitude of action potentials, whereas stellate cells sustained activity throughout the stimulation period.

Action potential properties

AP_{th} was determined under current-clamp configuration by injecting a series of currents for 500 ms in 5 pA increments until at least one action potential was elicited. The spike amplitude was calculated by adding the absolute value of the non-excited membrane potential to the spike peak. Spike width was measured as the time elapsed during upstroke from and downstroke to the half-amplitude point of the spike. Upstroke (dV/dt_{up}) and downstroke (dV/dt_{down}) rates were calculated by dividing the half-spike amplitude by the duration of the upward and downward movement between the half-spike point and spike peak, respectively.

Whole-cell conductance

Whole-cell conductance (G_w) was measured under a voltage-clamp configuration. A series of 150 ms, stepwise increasing voltages in 5 mV increments were applied, and at 100–110 ms from the start of the step, the current amplitude associated with that voltage was measured (Ghai and Buck, 1999). The line graph of $\Delta A/\Delta V$ was produced, and the change in G_w was determined by comparing the slopes of the line associated with each treatment.

Experimental design

The goal of the first experiment was to investigate the effects of an acute temperature change on the electrophysiological features of pyramidal cells. Cortical sheets from turtles maintained at room temperature (the 20°C acclimation group) were used in this experiment. At the end of the 20°C acclimation period, the patched cell was recorded for 50 min. During the first 5 min control period (t=0–5 min), no temperature changes were made. Then, the temperature was reduced to 5°C for 30 min (t=5–35 min).

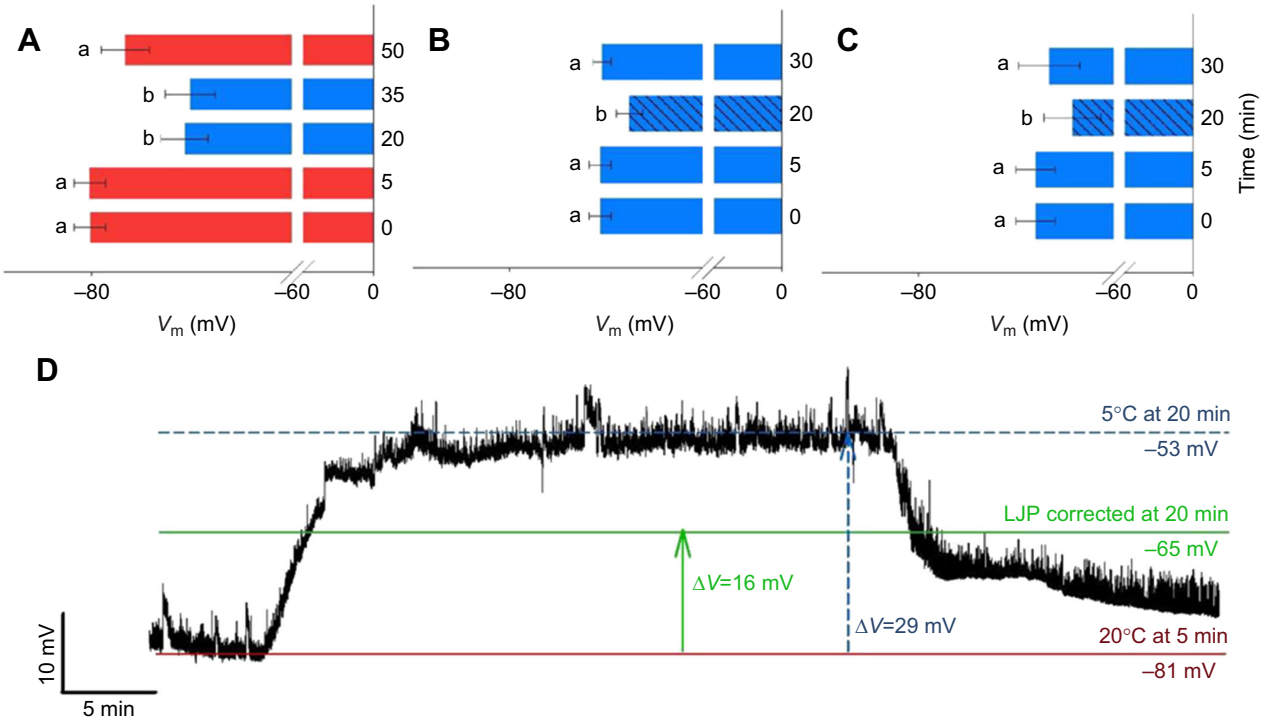

Fig. 3. Effect of temperature and severe hypoxia on V_m of pyramidal neurons. (A) The impact of an acute temperature change from 20°C to 5°C on V_m. (B,C) The effect of anoxia at 5°C on V_m in animals acclimated to 20°C (B) and 5°C (C). Red and blue bars represent recordings from turtles at 20 and 5°C, respectively. Cross-hatched bars represent anoxia treatment. Bars (means±s.e.m.) that share a letter are not significantly different from each other (P≥0.05). (D) A typical V_m recording from the acute temperature change experiment. The red line represents V_m at 20°C after 5 min, the blue line represents V_m after 30 min at 5°C, and the green line represents the actual voltage change (ΔV) after liquid junction potential (LJP) correction.

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Finally, for the last 15 min (t=35–50 min) of the experiment, the temperature was increased to 20°C. One of the following three recordings was done on each patched neuron. In some neurons, the membrane potential (V_m) was passively recorded throughout the experiment. In the rest of the neurons, either AP_{th} or G_w was recorded at t=0, 5, 20, 35 and 50 min (Fig. 1).

The second set of experiments aimed to investigate the impact of severe hypoxia (anoxia), in a low temperature environment (5°C), on the pyramidal neurons of animals acclimated to 20 or 5°C. This also consisted of brain sheets from 20°C-acclimated animals undergoing an acute temperature change to 5°C before anoxic exposure to determine whether prior acclimation to low temperature is beneficial. For acclimation to 5°C, 20 turtles from the 20°C acclimation group were transferred to a walk-in environmental chamber, and the temperature was reduced at the rate of 2°C per day until it reached 5°C. Turtles in this group were held at 5°C for at least 15 days before being killed for use in experiments. The animals had access to both a dry basking area and water throughout the acclimation and housing period. To limit the variables to temperature only, a 12 h:12 h light:dark cycle was maintained in low temperature chambers. Cortical sheets from each group (20 or 5°C acclimated) were stored at the corresponding temperature until used (1–4 h). For the sheets from 20°C-acclimated animals, the temperature of the patching chamber was gradually reduced to 5°C, and they were given at least 15 min to acclimate to the new temperature before attempting to patch them. In each trial, neurons (from both groups) that were patched at 5°C, were perfused with a normoxic aCSF at t=0–5 min, severely hypoxic aCSF at t=5–20 min, and normoxic aCSF (recovery) at t=20–30 min. In some neurons, V_m was recorded,

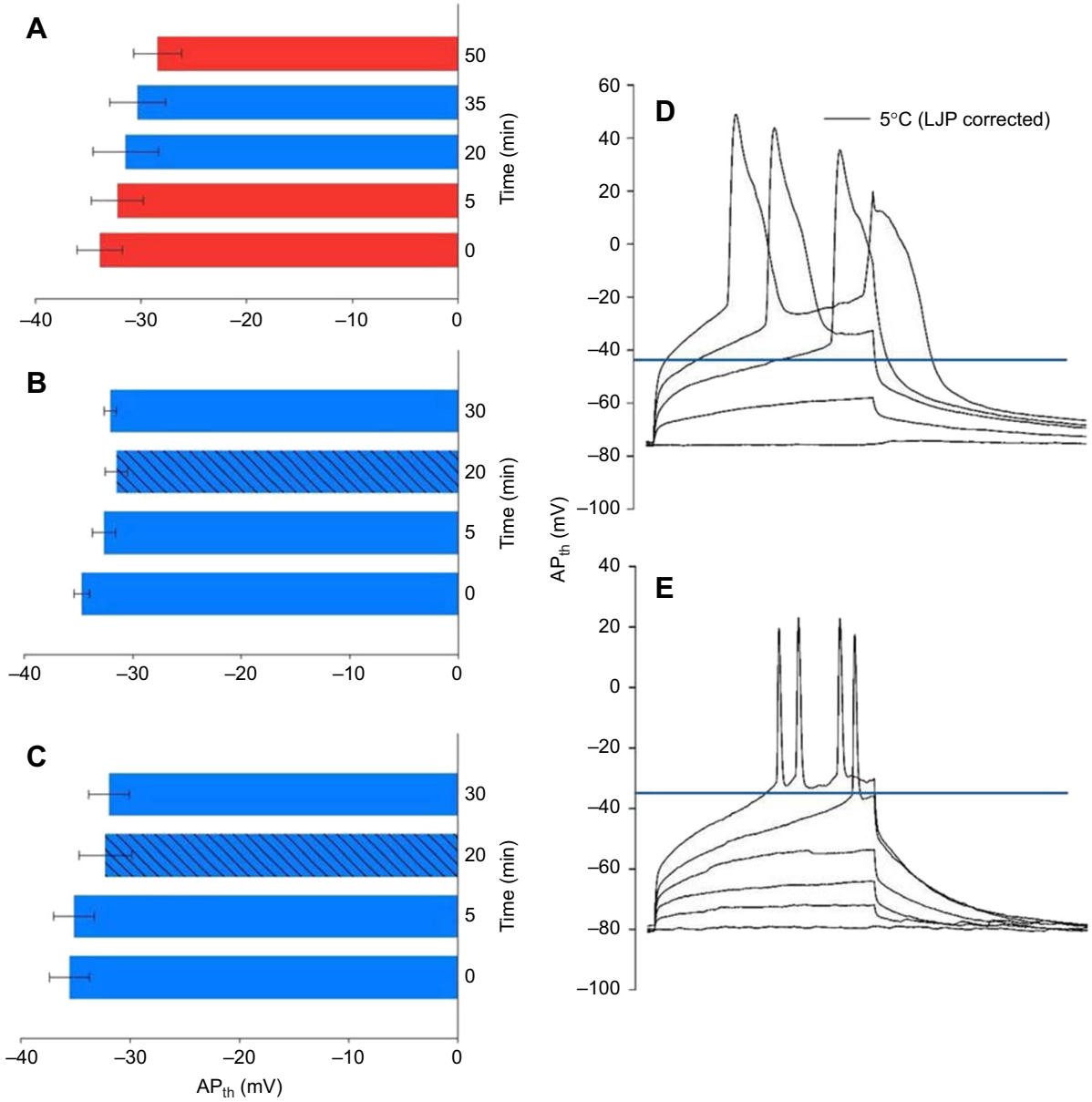

Fig. 4. Effect of temperature and severe hypoxia on AP_{th} of pyramidal neurons. (A) The impact of changes in temperature. (B,C) The effect of anoxia at 5°C in animals acclimated to 20°C (B) and 5°C (C). Red and blue bars represent recordings from turtles at 20 and 5°C, respectively. Cross-hatched bars represent anoxia treatment. Bars (means±s.e.m.) show no significant differences (P>0.05). (D,E) Typical AP_{th} recordings from the temperature experiment at 5°C (D) and 20°C (E). The blue lines in D and E show APth.

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and in others, either AP_{th} or G_w was recorded at t=0, 5, 20 and 30 min (Fig. 2).

Statistical analysis

All experimental data were analyzed for parametric assumptions of normality and homogeneity of variance using Shapiro–Wilk's and Mauchly's test of sphericity, respectively. Where the assumption of homogeneity of variances was violated, the outcome of ANOVA analysis with a Greenhouse–Geisser correction was reported. A one-way repeated-measures ANOVA was used to compare differences in AP_{th}, spike height, spike width and V_m, whereas the impacts of treatments on the upstroke and downstroke rate were analyzed using a two-way repeated-measures ANOVA. A Bonferroni *post hoc* test followed a significant difference among groups.

RESULTS

Membrane potential (V_m)

In brain sheets from animals acclimated to 20°C, an acute decline in temperature from 20°C to 5°C depolarized the membrane potential of pyramidal neurons by 11 mV at both 15 and 30 min post-exposure (t=20 and 35; $F_{1.6,9.4}$=24.15, P<0.001; Fig. 3A,D).

When brain sheets from animals acclimated to either 20 or 5°C were exposed to an anoxic period at 5°C, membrane potential depolarized in pyramidal neurons from both groups to 70 and 68 mV, respectively ($F_{1.4,5.5}$=31.61, P=0.001; $F_{1.3,5.3}$=8.08, P=0.029, respectively) (Fig. 3B,C). However, the acclimation temperature itself appeared to have no impact on the level of depolarization under anoxia as the ΔV_m at 3 and 4 mV was not significantly different between the 20 and 5°C-acclimated groups (P>0.05). Notably, the anoxia-mediated ΔV_m at 20°C was

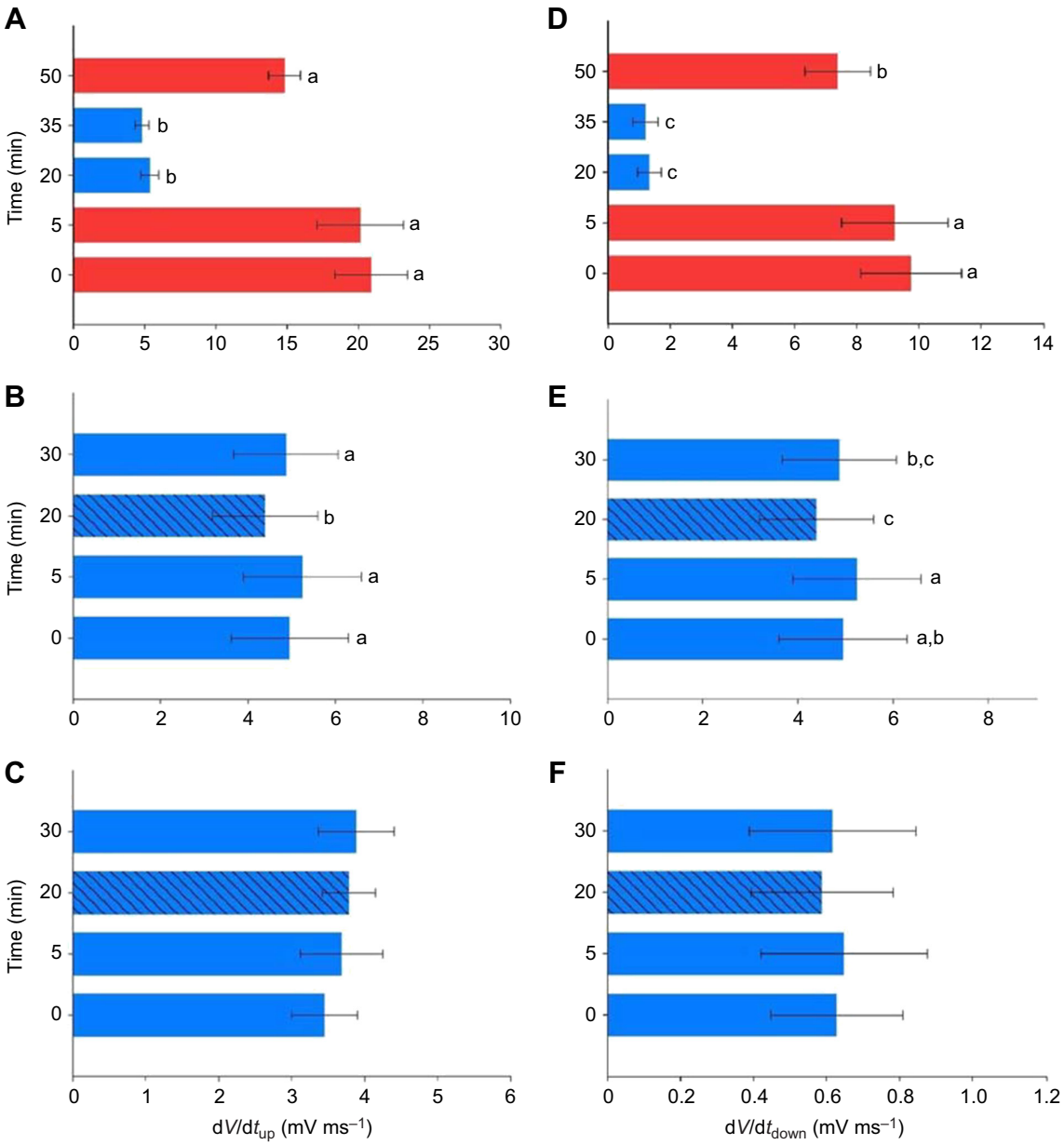

Fig. 5. Effect of temperature and severe hypoxia on depolarization (dV/dt_{up}) and repolarization (dV/dt_{down}) rate of pyramidal neurons. (A,D) Impact of an acute temperature change from 20°C to 5°C. (B,E) The effect of anoxia at 5°C in animals acclimated to 20°C. (C,F) The effect of anoxia at 5°C in animals acclimated to 5°C. Red and blue bars represent recordings from turtles at 20 and 5°C, respectively. Cross-hatched bars represent anoxia treatment. Bars (means ±s.e.m.) that share a letter are not significantly different from each other (P≥0.05). No significant difference was observed in figures where the bars have no letter.

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noticeably more than 5°C at 10 mV (historical data from our lab; Hogg et al., 2015).

Action potential properties

Acute reduction of temperature from 20°C to 5°C did not affect AP_{th} of the pyramidal neurons (33.9 and 32 mV, respectively; $F_{1.5,7.67}$=1.91, P=0.21) (Fig. 4A); neither did it significantly change with anoxic exposure at 5°C in pyramidal neurons from 20 and 5°C-acclimated animals (ΔV: 1.1 and 2.8 mV; respectively; $F_{3,12}$=3.0, P=0.07; $F_{1.0,4.0}$=2.89, P=0.16) (Fig. 4B,C). However, it generated a trend towards higher AP_{th} in 5°C-acclimated animals, which could potentially become statistically significant with a bigger sample size. This is contrary to our previous results that found anoxia at room temperature (20°C) increases AP_{th} in pyramidal neurons (Pamenter et al., 2011). At this point, it is not clear why the results are different.

Acute exposure of brain sheets from 20°C-acclimated turtles to 5°C drastically reduced both upstroke (dV/dt_{up}) and downstroke (dV/dt_{down}) rates of the action potentials from pyramidal neurons ($F_{1.27,633}$=36.93, P=0.001; $F_{1.21,6.07}$=31.90, P=0.001, respectively). The impact on dV/dt_{down} was more severe than on dV/dt_{up}. Under low temperature conditions, dV/dt_{up} and dV/dt_{down} declined by 76% and 87% compared with that at 20°C, respectively (Fig. 5A,D).

In contrast, compared with the effect of temperature change, the impact of anoxia on dV/dt_{up} and dV/dt_{down} at 5°C was mild or not significant in 20 or 5°C-acclimated animals. In 20°C-acclimated animals, anoxia at 5°C caused a reduction in dV/dt_{up} and dV/dt_{down} of 16% and 29%, respectively ($F_{3,12}$=10.71, P=0.001; $F_{3,12}$=71.35, P<0.001, respectively) (Fig. 5B,E). The impact on dV/dt_{down} was higher than that on dV/dt_{up}. In 5°C-acclimated animals, anoxia did not result in significant changes in dV/dt_{up} and dV/dt_{down} (Fig. 5C,F).

In corroboration with effects on dV/dt_{up} and dV/dt_{down}, acute exposure to 5°C increased the width of action potentials in the pyramidal neurons by 834% ($F_{1.03,5.13}$=13.0, P=0.01) (Fig. 6A,B). When 20°C-acclimated animals were exposed to anoxia at 5°C, spike width increased ($F_{3,12}$=23.8, P<0.001) (Fig. 6C) compared with control. However, anoxia at 5°C did not cause significant changes in 5°C-acclimated turtles ($F_{1.1,4.2}$=3.09, P=0.15) (Fig. 6D).

An acute decline in temperature increased the spike height in pyramidal neurons ($F_{4,20}$=11.43, P<0.001). The increase in spike height was approximately 15% of the t=5 min control (Fig. 7A). Also, we observed a statistically significant difference between the groups in both anoxia experiments. However, the difference was not attributed to anoxia as it was not significantly different from the control group (Fig. 7B,C). The duration of exposure to 5°C (15 or 30 min) did not have a significant effect on AP_{th}, dV/dt_{up}, dV/dt_{down} and spike height and width in the acute temperature change experiment.

Whole-cell conductance (G_w)

G_w of the neurons was altered under exposure to acute low temperature ($F_{2,176}$=42.63, P<0.001). When acutely exposed to 5°C, the slope of $\Delta I/\Delta V$ of the pyramidal neurons was reduced compared with the control, but no difference was observed between

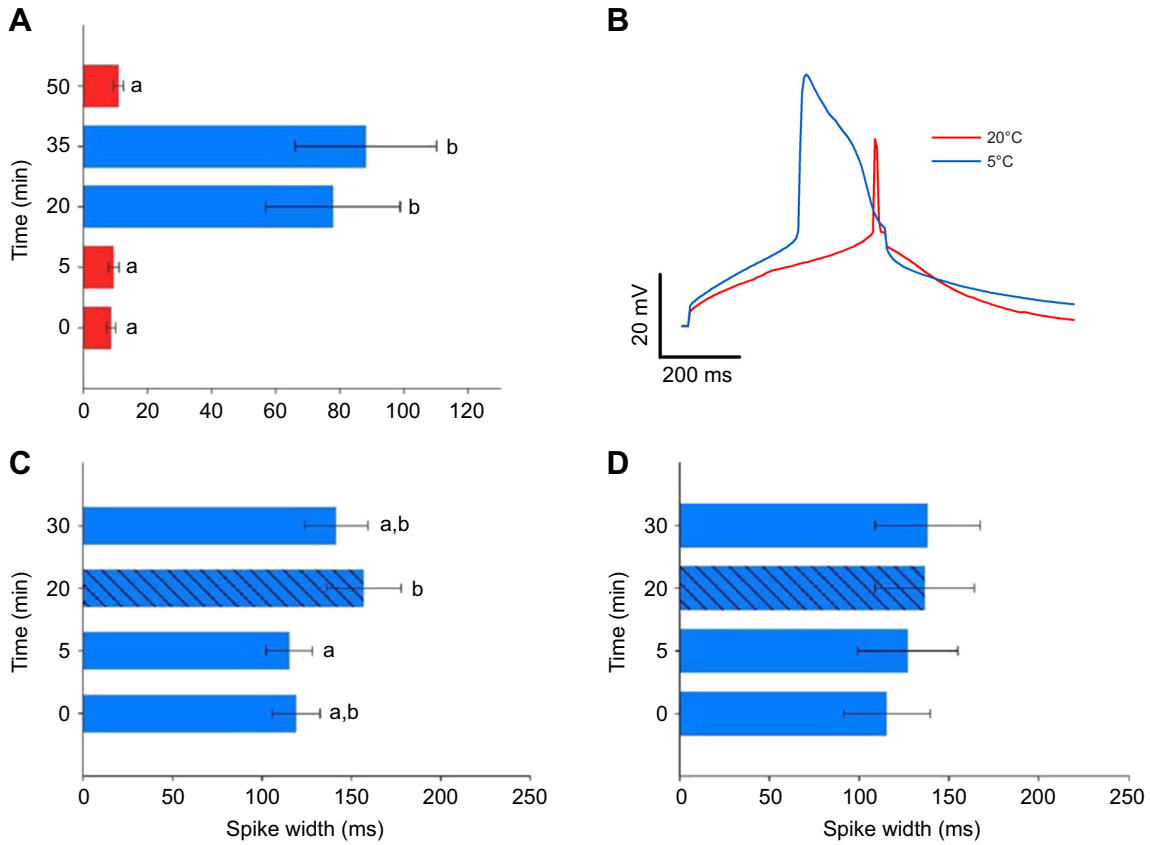

Fig. 6. Effect of temperature and severe hypoxia on spike width of action potentials from pyramidal neurons. (A) The impact of changes in temperature. (B) Typical action potential recordings from the temperature experiment at 20 and 5°C. The blue arrow indicates the hump in the repolarization stage. (C,D) The effect of anoxia at 5°C in animals acclimated to 20°C (C) and 5°C (D). Red and blue bars represent recordings from turtles at 20 and 5°C, respectively. Cross-hatched bars represent anoxia treatment. Bars (means±s.e.m.) that share a letter are not significantly different from each other (P≥0.05).

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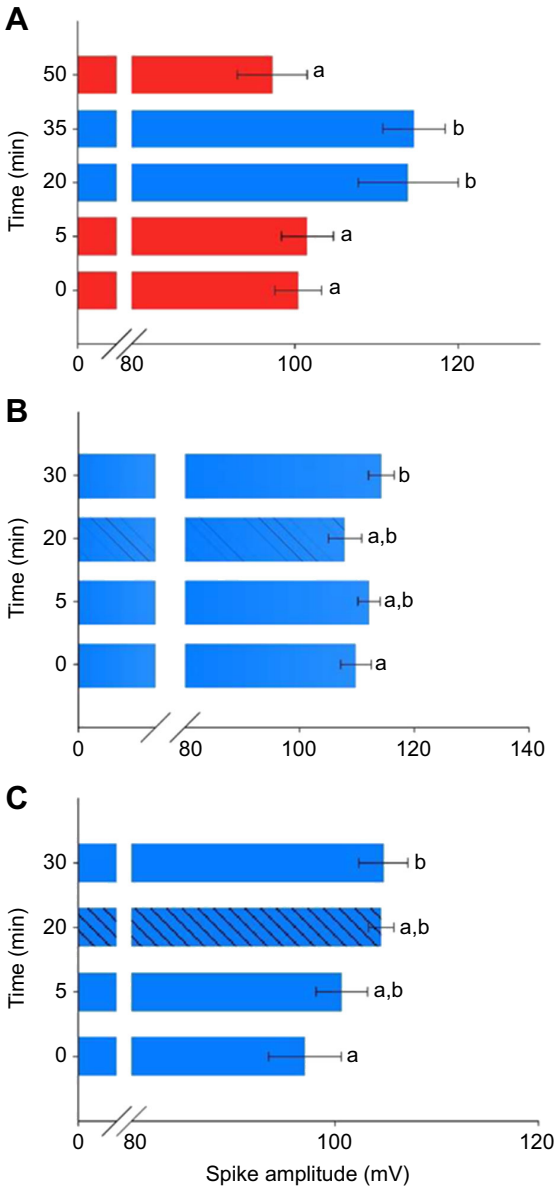

Fig. 7. Effect of temperature and severe hypoxia on spike amplitude of evoked action potentials. (A) The impact of changes in temperature. (B,C) The effect of anoxia at 5°C in animals acclimated to 20°C (B) and 5°C (C). Red and blue bars represent recordings from turtles at 20 and 5°C, respectively. Cross-hatched bars represent anoxia treatment. Bars (means ±s.e.m.) that share a letter are not significantly different from each other ($P \geq 0.05$).

the two time points of the low temperature treatment (t=20 and 35 min). The average G_w was reduced from 3.8 pS at 20°C to 2.4 pS at 5°C (Fig. 8A).

Anoxia at 5°C did not influence G_w of the neurons from the 20 or 5°C acclimation groups. Interestingly, however, G_w of 5°C-acclimated neurons (~4.5 pS) was approximately double that of the 20°C-acclimated cells (2.1 pS) (Fig. 8B,C). At 20°C, anoxia increased G_w in pyramidal neurons, as per our earlier studies (Pamenter et al., 2011).

DISCUSSION

The western painted turtle is unique in that it is the most anoxia-tolerant air-breathing vertebrate known; and many studies have been conducted to understand the adaptations within this species to withstand overwintering O_2 scarcity (Bickler and Buck, 2007). Yet, few studies have investigated the impact of the other important variable associated with overwintering: low temperature (Stecyk et al., 2004a; 2007; 2017; Couturier et al., 2019). Therefore, we set out to perform low temperature experiments to investigate the interactive effects of low temperature and anoxia on the physiological function of turtle pyramidal neurons.

Our results support the ion channel arrest hypothesis proposed by Hochachka (1986). The hypothesis predicts that both anoxia and a decrease in temperature would elicit a decrease in ion channel conductance. This is because ion channels are less sensitive to changes in temperature than are other proteins, including membrane-bound and soluble proteins. Generally, Q_{10} values for ion channels are in the 1.5–2 range, and they can be temperature range specific (McLarnon et al., 1993; Milburn et al., 1995; Wu et al., 2001). If ion channel activity were not inhibited by low temperature, then cells would leak to death because ion pumps (such as Na^+/K^+-ATPase) would be significantly impacted by low temperature and ion channels would not be. We demonstrated that an acute 15°C reduction in temperature caused depolarization of the pyramidal neuron membrane potential, an increase in action potential amplitude, and a reduced rate of action potential upstroke and downstroke. All these changes indicate the arrest of channel activity.

Our results showed that both acute and chronic exposure to low temperature depolarizes the membrane to a new membrane potential. In 20°C-acclimated animals, acute (3–5 min) reduction of temperature to 5°C depolarized V_m from −81 mV to −65 mV, a change of 16 mV. Gradual membrane depolarization in response to reduced temperature has been reported in neurons of other ectothermic animals. For instance, a decline in the range 30–10°C depolarized the stretch receptor neurons in crayfish at a rate of 0.3 mV $°C^{-1}$ (Moser et al., 1979). Endothermic animals' neurons, however, show a different response pattern. In a study done on guinea pig hippocampal pyramidal neurons, a gradual decline in temperature from 35°C to 20°C only hyperpolarized the membrane, although this was not significantly different from V_m at 35°C, but another 5°C reduction (20°C to 15°C) provoked a sharp ~20 mV depolarization (Aihara et al., 2001). Also, a decrease in temperature (37°C to 25°C) significantly hyperpolarized cultured mice hippocampal neurons (Shibasaki et al., 2007).

The average V_m of pyramidal neurons at 5°C, whether acclimated to 20°C or 5°C, was depolarized to approximately −65 to −68 mV, respectively. When these cells were exposed to anoxia, they slightly depolarized further by approximately 3 mV. However, previous studies showed acute exposure to anoxia depolarizes the membrane of pyramidal cells by round 10 and 15 mV in painted turtle and goldfish, respectively (Hogg et al., 2015; Hossein-Javaheri et al., 2017). Comparing the impact of low temperature and anoxia suggests that reduced temperature has a more significant effect on V_m than anoxia. It also appears that pyramidal neurons, with already depolarized V_m as a result of reduced temperature, do not need to depolarize much further when exposed to reduced O_2 levels. This depolarization seems to be a part of GABAergic response of neurons to winter conditions, which has been studied for anoxia but also needs to be investigated for low temperature (Pamenter et al., 2011). Historical data show a depolarization of AP_{th} in response to anoxia at room temperature (Pamenter et al., 2011). However, we found that neither low temperature nor anoxia in cold waters affected AP_{th} in pyramidal neurons. Unlike AP_{th}, all other major properties of action potentials such as depolarization and repolarization rates and spike height and width were

Journal of Experimental Biology (2021) 224, jeb242793. doi:10.1242/jeb.242793

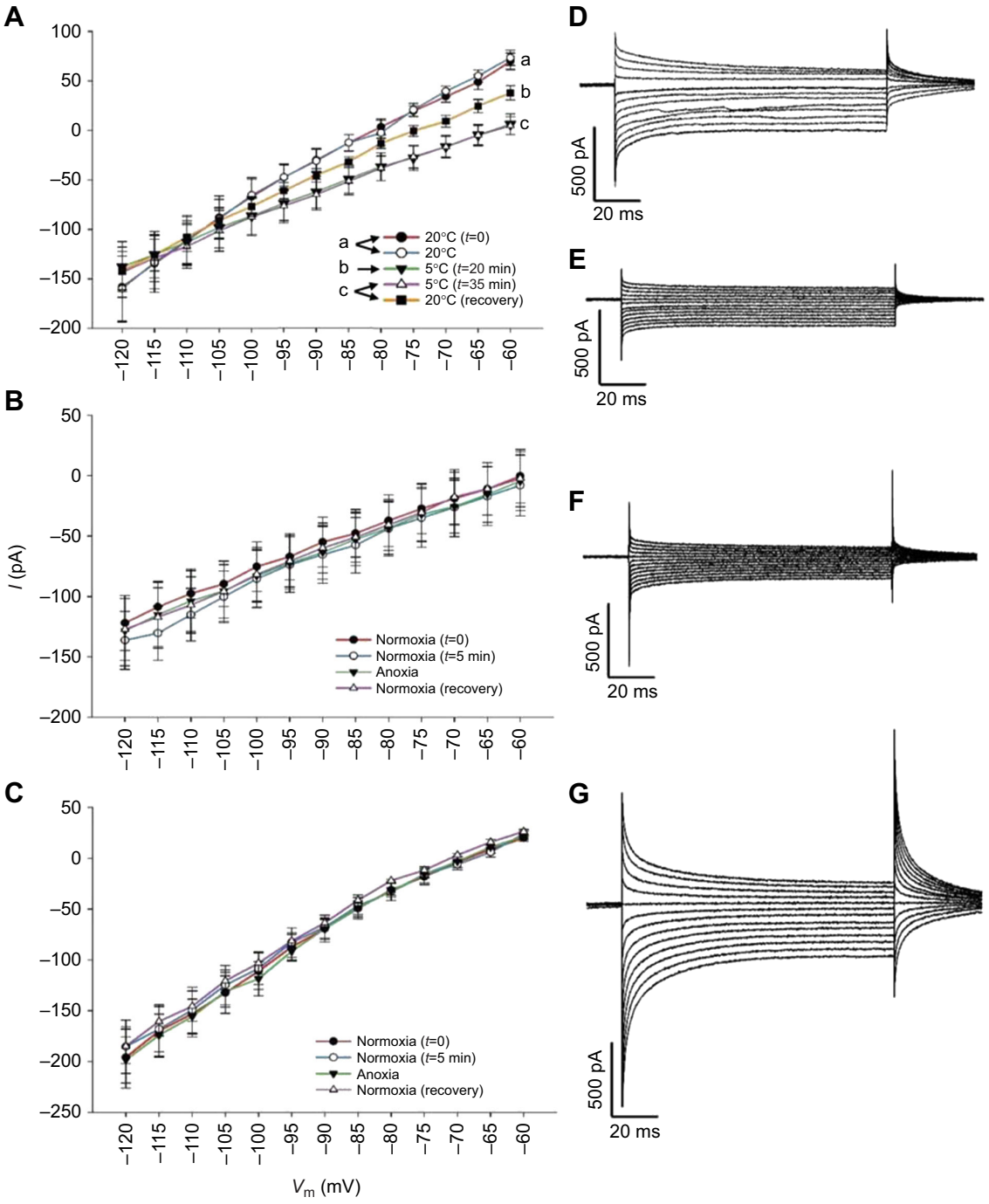

Fig. 8. Changes in G_w. (A) The impact of changes in temperature. (B,C) The effect of anoxia at 5°C in animals acclimated to 20°C (B) and 5°C (C). (D,E) G_w traces of a neuron from a 20°C-acclimated turtle at 20°C (D) and 5°C (E). (F,G) G_w traces of a neuron from a 20°C-acclimated (F) and a 5°C-acclimated turtle (G) at 5°C. Lines that share a letter are not significantly different from each other ($P \geq 0.05$, means±s.e.m.).

drastically altered by 5°C exposure, all indicating a channel arrest or slowdown.

Unraveling the response of membrane channels to temperature changes is complicated as temperature is an inherent thermodynamic property of every molecule (Arrigoni and Minor, 2018). Thus, temperature impacts the activity of every channel on the membrane to a degree, based on their thermal stability (Creighton, 2010). Therefore, at an acclimation temperature of 20°C, the most stable form of the channel is likely expressed. When the temperature is acutely changed to 5°C, a less stable form of the channel is likely expressed and is responsible for the altered action potential kinetics that we observed (Arrigoni and Minor, 2018).

The temperature coefficient Q_{10} is a common indicator of the impact of temperature on an enzyme or ion channel activity. It seems that reduced temperature affects K^+ channels, with a Q_{10} of 1.6 from 20 to 30°C (McLarnon et al., 1993), more than Na^+ channels, with a Q_{10} ranging from 1.4 to 1.5 across 4–36°C (Milburn et al., 1995). These Q_{10} differences could explain the more sluggish dV/dt_{down} compared with dV/dt_{up} when neurons are acutely exposed to 5°C. The same phenomenon was first reported in squid

giant axon recordings by Hodgkin and Katz (1949), and that the impact was due to the higher temperature sensitivity of potassium than Na^+ channel gating (Kukita, 1982). The hump in dV/dt_{down} (Fig. 6B) suggests that reduced temperature delayed the opening of voltage-dependent K^+ channels, as chemically blocking these channels creates a similar effect (Newland et al., 1992). Therefore, the spike immediately preceding the hump represents Na^+ channel closure.

In animals that were acclimated to 5°C, both dV/dt_{down} and dV/dt_{up} declined even further. This reduction is most likely the result of a decreased concentration of Na^+ and K^+ voltage-gated channels in the membrane. Although less likely, it is possible that changes in their response to colder temperature might be because of an RNA editing that changes the kinetics of the K^+ channels, as observed in polar octopuses (Garrett and Rosenthal, 2012). Overall, it seems that the change in action potential properties is largely due to temperature altering voltage-gated channel kinetics, for K^+ channels in particular, as part of the cell's subtle adaptations to survive the new conditions (Rodríguez et al., 1998; Garrett and Rosenthal, 2012).

Temperature changes altered neuronal action potential properties. These impacts could be different based on the direction and amplitude of the change and the neuron properties and animal physiology. The impacts on spike width seem to be universal as a reduction in temperature increases it in the neurons of ectothermic and endothermic animals such as locust, crayfish, painted turtle and guinea pig (Heitler et al., 1977; Rodríguez et al., 1998; Santin et al., 2013). However, the impact of temperature changes on spike height differs from one species to another. For instance, a 10°C decline in temperature (from 20°C to 10°C) did not significantly change action potential height of locus coeruleus neurons in bullfrog (Santin et al., 2013); but a decrease from 20°C to 15°C reduced the amplitude of action potentials in guinea pig hippocampal pyramidal neurons (Aihara et al., 2001). Heitler et al. (1977) observed an increase in action potential amplitude in two types of neurons from locusts when temperature was reduced from 30°C to 20°C, as we did in painted turtle pyramidal neurons.

Animals acclimated to 20°C that were exposed to anoxia at 5°C displayed a significant further change in all four properties (action potential height and width, dV/dt_{down} and dV/dt_{up}). In comparison, animals acclimated to 5°C showed no substantial change in any of these properties. These data suggest that pyramidal neurons enter a partial channel arrest when acutely exposed to a colder environment, and therefore adding anoxia would further suppress channel activity. However, when they are chronically exposed (acclimated) to low temperature, they enter complete channel arrest; thus, anoxia would not reduce channel activity further. For instance, depolarization and repolarization rate in 5°C-acclimated animals were approximately 20% and 90% slower than in 20°C-acclimated animals. The effects of low temperature on channels appear to be different from those on pumps as Stecyk et al. (2017) showed that acclimation to low temperature did not change the density or activity of Na^+/K^+-ATPase.

The effects of acute and chronic exposure to reduced temperature on G_w of the pyramidal cells were opposite to each other, as acute exposure reduced and chronic exposure increased G_w. Acute exposure to colder temperatures (35°C to 15°C and 37°C to 27°C, respectively) generated similar impacts on G_w of guinea pig hippocampal pyramidal and CA1 neurons to those we observed in painted turtle (Thompson et al., 1985; Aihara et al., 2001). These data suggest that pyramidal neurons apply different strategies to respond to acute versus chronic exposure to low temperature water. The chronic increase of G_w in response to low temperature was similar to that observed earlier in anoxia (Pamenter et al., 2011). The immediate response of the neurons is more reactive to the sudden change, whereas the chronic response is probably more active with a significant rearrangement of the membrane macromolecules.

A significant portion of the changes in pyramidal cell physiological properties at low temperature, especially in acute exposure, could be associated with the Q_{10} temperature coefficient effect, which is a measure of the rate of enzymatic activity change across a temperature difference of 10°C. The impact of thermal changes is different for different macromolecules. For instance, in 21°C- and 5°C-acclimated normoxic turtles, ventricular currents of voltage-gated Na^+, L-type Ca^{2+} and inward rectifier K^+ channel currents were decreased at different rates (Q_{10}=2.1, 5.0 and 1.4, respectively) (Stecyk et al., 2007).

In summary, it appears that acclimation to low temperature, which maintains rapid K^+ channel kinetics, and naturally occurs before a severe reduction in environmental O_2 concentration during hibernation, prepares turtle pyramidal neurons for enduring the upcoming low temperature anoxic period. Neurons also set a new V_m, which is likely due to activation of GABA-A receptors (Pamenter et al., 2011). Further investigation of the changes in macromolecular structure of neuronal membranes and ion channel proteins will clarify the adaptations that these cells undergo to withstand the low temperature and anoxic overwintering period.

Acknowledgements
The authors would like to thank the Natural Sciences and Engineering Research Council of Canada and the Banting Postdoctoral Fellowship program for support.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: E.L.; Methodology: E.L., L.T.B.; Validation: E.L.; Formal analysis: E.L.; Investigation: E.L.; Resources: L.T.B.; Writing - original draft: E.L.; Writing - review & editing: E.L., L.T.B.; Visualization: E.L.; Supervision: L.T.B.; Project administration: E.L.; Funding acquisition: L.T.B.

Funding
This work was supported by a Natural Sciences and Engineering Research Council of Canada for Discovery Grant and Accelerator Award to L.T.B. (458021 and 478124) and by a Banting Postdoctoral Fellowship to E.L. (411651).

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