Cold Block of *In Vitro* Eyeblink Reflexes: Evidence Supporting the Use of Hypothermia as an Anesthetic in Pond Turtles

Joyce Keifer* and Zhaoqing Zheng

Neuroscience Group, Division of Basic Biomedical Sciences, University of South Dakota Sanford School of Medicine, Vermillion, SD 57069

Key words: turtles, hypothermia, reflex pathways, MS222, anesthesia, animal welfare

*Correspondence: Joyce Keifer, Ph.D., Neuroscience Group, Division of Basic Biomedical Sciences, University of South Dakota Sanford School of Medicine, 414 E. Clark St., Vermillion, SD 57069; tel (605) 658-6355, fax (605) 677-6381, jkeifer@usd.edu

Summary Statement

Veterinary guidelines prohibit hypothermic anesthesia in amphibians and reptiles. Reflexes to noxious stimuli recorded *in vitro* are inhibited at temperatures above those during hypothermia induction, blocking transmission of painful stimuli.

ABSTRACT

Use of hypothermia as a means of anesthesia for amphibians and reptiles is prohibited by agencies that establish veterinary guidelines. This has recently been called into question by members of the scientific community based on reviews of published literature. Using pond turtles (*Trachemys scripta elegans*), hypothermia as a method for anesthesia to precede euthanasia by decapitation was assessed. Turtles were subjected to hypothermia using a cooling followed by freezing protocol. Body temperature measurements ranged between -1 to -2°C while core body temperature was -1°C. Ice crystal formation was never observed. A protective reflex to noxious stimuli, the eyeblink response, was recorded from *in vitro* brainstem preparations subjected to cold. At 5-6°C, reflex responses were suppressed, demonstrating minimal synaptic transmission in brain circuits above temperatures used for hypothermia induction. These and previous data indicate that a re-evaluation of the use of hypothermia as an anesthetic in amphibians and reptiles is warranted.

INTRODUCTION

In 2013, new guidelines for the euthanasia of animals by the American Veterinary Medical Association (AVMA) were published and remain the current edition (https://www.avma.org/KB/Policies/Documents/euthanasia.pdf). These guidelines define acceptable and unacceptable methods for the humane analgesia, anesthesia and euthanasia of animals to assist veterinarians in the proper care of animals. This document is also used to inform governmental agencies (National Research Council), funding agencies (National Institutes of Health, NIH), and institutional animal care and use committees (IACUC) in acceptable methods for the anesthesia and euthanasia of laboratory animals whether they are bred for research or wild-caught. In the most recent edition of the AVMA Guidelines, it was concluded that hypothermia was an "unacceptable" method for restraint or euthanasia of amphibians and reptiles (section S7.3.7). It was stated that this decision was based on available scientific literature; however, it was conceded that an understanding of nociception in these animals is incomplete and therefore recommendations for minimizing pain were "extrapolated from information available about mammals". The panel advised further consultation of multiple references for methods of euthanasia appropriate for the species and specific circumstance.

Recent publications have called for a re-evaluation of the data on which the decision by the AVMA was based. Much of this argument was presented in Shine et al. (2015) and Lillywhite et al. (2017). The decision of the AVMA has become an important issue not only for the ethical treatment of animals, but also because the NIH, the largest scientific funding agency in the United States, requires justification from researchers for unapproved procedures on animals regardless of approval from institutional review committees. Our laboratory has used pond turtles (*Trachemys scripta elegans*, red-eared sliders) as research subjects for over 25 years

for electrophysiological and molecular studies of learning and memory using an *in vitro* brainstem preparation (Keifer and Zheng, 2015; Zheng et al., 2012; 2017). One advantage of this preparation is that behaviorally relevant nerve-specific stimulation can be used, rather than non-specific stimuli, for studies of activity-dependent epigenetic regulation of gene expression in mature neurons. The sensitivity of these mechanisms to anesthetic procedures and stress imposed on the animal subjects is an important consideration for obtaining reliable data. Prompted by the AVMA, we turned to a chemical method of anesthesia to precede euthanasia by decapitation. After further investigation, we have concluded that hypothermia is currently the least stressful and most effective procedure to minimize discomfort to turtles preceding euthanasia. We document this with data showing blockade of a protective reflex pathway, the eyeblink reflex, recorded *in vitro* in response to cold temperatures and supporting data from previous literature.

MATERIALS AND METHODS

Animals and electrophysiological procedures

Freshwater pond turtles, *Trachemys scripta elegans*, of either sex and 5-6 inches in carapace length were purchased from commercial suppliers. All experiments involving the use of animals were approved by the USD Institutional Animal Care and Use Committee. Animals were pre-cooled by placing them in a covered box in a refrigerator for 45 min followed by placing them in a freezer for 1 h (Shine et al., 2015). Temperature of the turtle shell (carapace) and skin (measured near the tail) was recorded periodically while they were maintained in the freezer using a Raytek MiniTemp laser device. After 1 h, turtles exhibited no behavioral response (startle response, withdrawal of the limbs or head, or eyeblinks to touch) and were decapitated

using a small animal guillotine. The brain was rapidly dissected while the head was mounted on ice and periodically immersed in ice-cold physiological saline, the frontal cortex was minced, and the brainstem was removed for *in vitro* electrophysiological experiments.

Brainstems were transected at the levels of the trochlear and glossopharyngeal nerves and the cerebellum was removed as described previously leaving an isolated preparation of the pons (Keifer and Zheng, 2015; Zheng et al., 2012; 2017). The preparation was continuously bathed (2–4 ml/min) in physiological saline containing (in mM): 100 NaCl, 6 KCl, 40 $NaHCO_3$, 2.6 $CaCl_2$, 1.6 $MgCl_2$, and 20 glucose, which was oxygenated with 95% O_2/5% CO_2 and maintained at room temperature (22–24°C) at pH 7.6. Suction electrodes were used for stimulation and recording of cranial nerves. A twofold threshold single shock stimulus was applied to the trigeminal sensory nerve. Neural responses characteristic of a neural correlate of an eyeblink reflex were recorded from the ipsilateral abducens nerve that innervates the extraocular muscles controlling movements of the eye, nictitating membrane and eyelid (Keifer, 1993).

To record the responsiveness of eyeblink reflexes during application of cold temperatures, the recording chamber containing the brainstem preparation was immersed in crushed ice and the temperature was monitored using a thermometer placed in the physiological chamber. The chamber gradually became cooled from room temperature to 5°C after approximately 40 min. Once reflexes were recorded during cooling, the ice was removed and the chamber allowed to warm up in order to record responses after recovery from the cold. Cooling and warming could be applied to the preparation several times during the recording session to confirm the results.

Administration of MS222

Tricaine methanesulfonate (MS222; Western Chemical, Ferndale, WA, USA) was delivered to turtles using intraperitoneal (IP) injections into the coelomic sac anterior to the hindlimb. Other details of the procedures can be found in the Results.

Data and Statistical Analysis

The amplitude of reflex responses at different temperatures was analyzed by measuring the voltage envelope surrounding the neural discharge taken from images of the electrophysiological recordings. Using the histogram feature in Photoshop software, reflex responsiveness was quantified as total pixel density within the voltage envelope to compare records taken at each temperature (Zheng et al., 2012). Data were analyzed using a one-way ANOVA followed by a Tukey's *post-hoc* test for multiple comparisons.

RESULTS AND DISCUSSION

Issues related to MS222 and other chemical anesthetics in pond turtles

MS222 is commonly used to anesthetize fish and amphibians (administered by soaking in MS222-containing water) and is approved by the AVMA, but it is not widely used in reptiles. We examined the use of MS222 as an anesthetic in adult pond turtles (*Trachemys scripta elegans*, 5-6 inch carapace length, ~ 300 g, $n = 3$) by using IP injection of 600 mg/kg of buffered MS222 (Conroy et al., 2009). We encountered several problems related to using MS222 in turtles. First, the high concentrations of MS222 required for use as an anesthetic in reptiles renders it highly acidic (pH 2.0) and likely painful upon injection into tissues (Conroy et al.,

2009). Buffering MS222 to an acceptable pH can be achieved using a 1% solution but requires high volumes of injectate to achieve the concentrations required for anesthesia (18 ml for a 300 g turtle at 600 mg/kg MS222 for an initial dose). Instead, we used a higher concentration of buffered MS222 (20%, 600 mg/kg) injected IP which resulted in a highly variable onset of anesthesia and required at least two injections to achieve loss of consciousness (judged by protrusion of the turtle's head out of the shell and lack of eyeblink or flexion reflexes). One subject showed no loss of consciousness and complete unconsciousness was achieved after 2 h in the remaining two animals. Second, MS222 is a poor choice for anesthesia of turtles to be used for *in vitro* electrophysiological experiments. Once turtles were anesthetized, euthanized and the brainstem surgically isolated for electrophysiological experiments, the recordings showed strong depression of tissue responsiveness to nerve stimulation. After 3-5 h wash *in vitro*, electrical stimulation of the trigeminal nerve to evoke a neural correlate of an eyeblink reflex, which is monosynaptic and very robust, resulted in very little spiking activity. Brainstems were placed in physiological saline in the refrigerator overnight and tested the next day, and reflex responses again could not be evoked or were very weak. Typically, strong reflex responses can be recorded from this preparation on the second day *in vitro*. In one study of vestibulo-ocular reflexes in *Xenopus* tadpoles anesthetized with MS222 (0.05%) followed by euthanasia and isolation of a semi-intact *in vitro* preparation, investigators did not record from their preparations until 24 h after the dissection (Ramlochansingh et al., 2014), suggesting that strong depression of brain activity occurs after MS222 treatment. The detrimental effects of MS222 on electrophysiological experiments of anuran auditory evoked potentials (AEPs) have also been recently reported (Hall et al., 2016).

While intravenous injection of chemical anesthetic is recommended over intraperitoneal as being far more effective, this is very difficult to perform in turtles and is generally not used. Sodium pentobarbital (5 mg/kg) administered IP to anesthetize turtles to surgically prepare them for electrophysiological recordings of the intact brain was used previously by us (Sarrafizadeh et al., 1996). This procedure had a highly variable range of 2 ± 6 h to achieve a surgical plane of anesthesia. Given the well-known depressant action of barbiturate anesthesia on the brain, this method was not considered for our current *in vitro* electrophysiological studies. We have also used isoflurane gas on turtles (Sarrafizadeh et al., 1996), but they need to be intubated which requires an initial anesthetic. Our findings here using MS222 show that its use on turtles also results in variable anesthetic effects and unduly stresses the animals as shown by behavioral agitation during the long time period it takes to acquire anesthesia. Its negative impact on brain physiology eliminates its usefulness as an anesthetic for the type of *in vitro* experiments on turtles described here.

Effect of hypothermia on *in vitro* eyeblink reflexes

Considering the data above, we examined the status of turtles during induction of hypothermia. It was recommended by Shine et al. (2015) that animals are pre-cooled before exposing them to freezing to allow for more rapid cooling of brain and core body together. Pond turtles were placed in a covered box in a refrigerator (4°C) for 45 min and then placed in a freezer (-20°C) for 1 h. After 1 h, turtles exhibited no response (startle response, withdrawal of the limbs or head, or eyeblinks to touch) and were decapitated using a small animal guillotine. The brain was rapidly dissected, the frontal cortex minced, and the brainstem removed for *in vitro* electrophysiological experiments. The isolated brainstem preparation consists of the pons from the trigeminal cranial

nerve (V) extending through and including the auditory nerve (Zheng et al., 2012; see Fig. 2A) and contains the pontine eyeblink reflex circuitry involving the abducens motor nerve (VI). Data showing the temperature of the skin and shell, and the core body temperature (measured with a thermometer placed into the body cavity immediately after decapitation), are plotted in Fig. 1 (*n* = 5). During the period in the freezer, body temperature fell rapidly from a range of 8 to 15°C at the beginning of freezing to below 0°C after 1 h (Fig. 1). At this time, the skin and shell temperature ranged between -1 to -2°C. The animals were rapidly decapitated and core body temperature was recorded to be approximately -1°C (Fig. 1; one subject was in the freezer for 45 min). Ice crystal formation was never observed either on the body (skin or shell) or on tissues within the body cavity.

After a period of about 1 h for the brainstem preparation to warm to room temperature, recording of a neural correlate of the eyeblink response was performed (Keifer, 1993). Stimulation of the trigeminal sensory nerve was used to evoke burst discharge characteristic of eyeblinks recorded from the abducens motor nerve that innervates the muscles controlling movements of the eye (Fig. 2A). An example of a typical burst discharge is shown in Fig. 2Ba. The eyeblink reflex occurs in response to noxious stimuli such as an airpuff striking the cornea or eye. It is monosynaptic and very robust. We then determined the effect of temperature on neural transmission of this response. After cooling the preparation to between 8°C and 12°C, strong reflex responses were still maintained compared to responses from control conditions obtained at room temperature (Fig. 2C; data from two different preparations are shown). After cooling to 5-6°C, the reflex response was nearly completely blocked (Fig. 2C) as shown by an exemplar recording (Fig. 2Bb) in which nearly all of the evoked discharge activity was suppressed. As expected, the reduction in reflex responsiveness was reversible when the

preparation was again warmed to 12°C and above (Figs. 2Bc and 2C). The results of these experiments demonstrate that when turtle brain tissue is cooled to about 5-6°C, reflex responsiveness to noxious stimuli is significantly suppressed.

Freshwater aquatic turtles are ubiquitous in the United States ranging as far north as the Canadian border (Carr, 1952). The species used here, red-eared sliders, extend north to Illinois, and southern regions of Wisconsin and Minnesota. As a consequence, turtles are adapted to the cold to survive the winter months. These adaptations include: hibernation to lower body metabolism by diving deep into cold lakes and streams for days or weeks, often near stream inflows in the case of lakes where oxygen tension is higher; anaerobic metabolism of body tissues when oxygen tension is low; and body fluids that demonstrate supercooling to resist tissue damage to freezing and thawing (Lillywhite et al., 2017; Lowe et al., 1971; Lutz et al., 1985). It is difficult to determine pain or distress in reptiles and it is typically assessed behaviorally (aggressiveness, agitation, freezing) or by examining withdrawal reflexes to noxious stimuli (head withdrawal in the case of turtles). It is unclear if turtles experience cold thermal pain, either from a physiological (receptors) or cognitive standpoint. Exposure to cold and freezing conditions is part of the natural habitat of fresh-water turtles and therefore, it is considered unlikely that they experience pain or distress in such conditions (Lillywhite et al., 2017).

The rationale for using hypothermia as a method to induce anesthesia in turtles is based on their natural history. They normally experience the cold in their natural habitat and cold temperatures induce a behaviorally torpid state in turtles. A “torpid” state is defined by sluggishness, lethargy, inactivity, and as being mentally unalert (reduced brain activity). Data indicating that turtles are unlikely to experience pain or significant distress during induction of

hypothermia has been discussed extensively by Shine et al. (2015) and Lillywhite et al. (2017). Key points in relation to the current data are highlighted here. First, that formation of ice crystals during induction of hypothermia may cause pain was cited by the AVMA and is one of their leading arguments for the unacceptability of the use of hypothermia. However, the data indicate that this is highly unlikely. Many reptiles demonstrate supercooling of the body fluids and tissues in which freezing and formation of ice crystals does not begin until well below 0°C. In the case of the pond turtle, *Trachemys scripta elegans* (the species used here), the point at which ice crystals begin to form is -5.09°C (Lowe et al., 1971). This limit is not attained in our hypothermia procedure (Fig. 1) and formation of ice crystals either on the body or within the body cavity was never observed.

Second, it is well-known that the velocity of nerve conduction decreases with temperature until it completely fails. In tortoises, nerve blockade occurs at 1 to 3.5°C, and in bullfrogs at 0 to 2°C (Lillywhite et al., 2017; Roberts and Blackburn, 1975; Rosenberg, 1978). The conduction of peripheral C fibers, which are unmyelinated, small diameter, and carry nociceptive signals, are blocked at higher temperatures than A fibers in bullfrogs (Roberts and Blackburn, 1975). The effect of hypothermia on the *in vitro* eyeblink reflex shown here indicates that synaptic transmission in this robust monosynaptic pathway is largely blocked at 5 to 6°C. After induction of hypothermia, the skin and shell temperatures of turtles range from -1°C to -2°C whereas the core body temperature is about -1°C (Fig. 1). Therefore, there is minimal synaptic transmission within brain circuits above the temperature that animals are decapitated making them physically unable to detect sensory input. Furthermore, it has been shown by others that global brain activity, measured by the electroencephalogram (EEG), is negatively affected by cold temperatures. In amphibians and reptiles, brain activity as measured

by the EEG shows near-zero electrical activity (isoelectric signal) at body temperatures close to 0°C (2-4°C, lizards; ~0°C, cane toads; Hunsaker and Lansing, 1962; Parsons and Huggins, 1965; Shine et al., 2015). Therefore, even if painful stimuli could be conducted to the brain, perception of the stimuli as noxious would not occur.

Taken together, these data show that before the point that ice crystals would form, noxious stimuli fail to be conducted to the brain and there is minimal overall brain activity making it impossible for the perception of painful stimuli after induction of hypothermia. In an effort to include appropriate methods for anesthesia and euthanasia across a broad range of species, the AVMA has overlooked or not carefully considered the scientific literature and the ecological and ethological natural history of each species. The data indicate a re-evaluation of the use of hypothermia as an anesthetic in amphibians and reptiles.

Acknowledgements

We thank Dr. Linda J. Larson-Prior for valuable comments on the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author Contributions

Conceptualization: J.K.; Methodology: J.K., Z.Z.; Data Acquisition: J.K., Z.Z.; Manuscript preparation: J.K.

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Figures

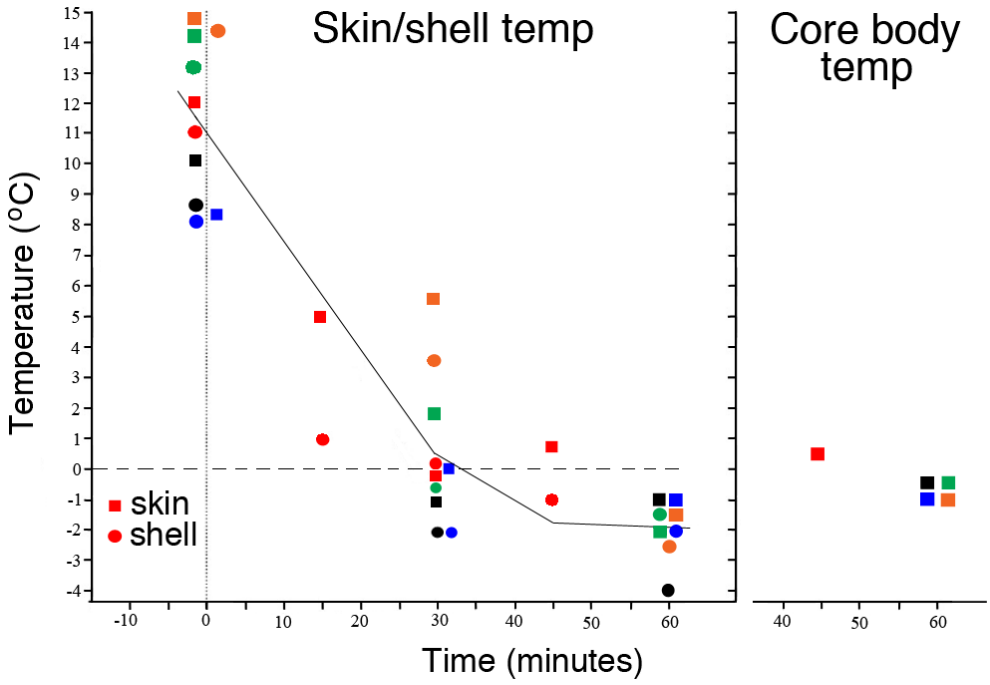

Fig. 1. Decrease in temperature of the skin and shell of turtles measured at different time points after transfer from a refrigerator to a freezer (0 time point). Different colored symbols represent individual subjects (*n* = 5). Core body temperature of the four animals examined after 1 h in the freezer was observed to be about -1°C.

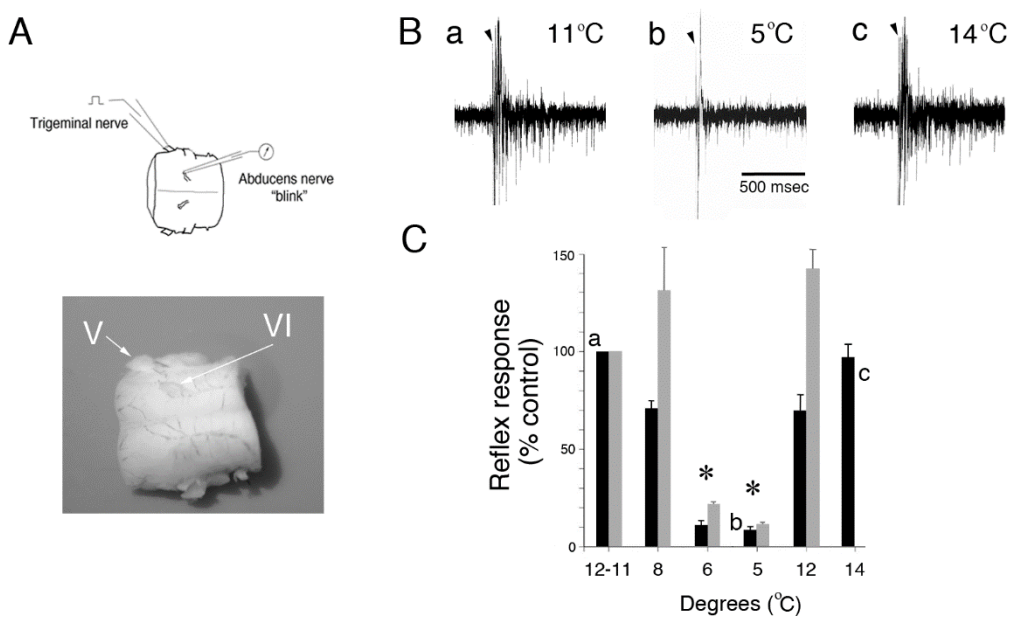

Fig. 2. Blockade of eyeblink reflex activity recorded from an *in vitro* brainstem preparation in cold temperatures. (A) Schematic illustration of the *in vitro* preparation and placement of stimulating and recording electrodes. A photograph of the preparation is shown which measures 0.5 cm in total length. The sensory trigeminal cranial nerve (V) is electrically stimulated while reflex activity is recorded in the abducens motor nerve (VI). (B) Electrophysiological recordings from the abducens nerve showing burst discharges characteristic of a neural correlate of an eyeblink reflex evoked by single shock trigeminal nerve stimulation (indicated by the arrowhead). Recordings were taken at the temperatures indicated. (C) Data (means ± SEM) from two different preparations showing the reduction of reflex responses with decreasing temperatures and recovery upon warming. *$p < 0.0001$ compared to 12°C for both experiments.