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TIME-DEPENDENT BIDIRECTIONAL EFFECTS OF CHRONIC CAFFEINE ON FUNCTIONAL RECOVERY OF THE DORSAL LIGHT REFLEX AFTER HEMILABYRINTHECTOMY IN THE GOLDFISH *CARASSIUS AURATUS*

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Abstract—Caffeine works through a variety of complex mechanisms to exert an often bidirectional set of functional and structural neurological changes in vertebrates. We investigated the effects of chronic caffeine exposure on functional recovery of the dorsal light reflex (DLR) in hemilabyrinthectomized common goldfish, *Carassius auratus*. In this lesion model, the unilateral removal of the vestibular organs results in a temporary loss of gravitationally modulated postural control which is quantifiable via the DLR. We compared the functional recovery over 24 days of post-surgery goldfish continuously held in a caffeine solution of 2.5 mg/L ($n = 10$), 5.0 mg/L ($n = 10$), 10.0 mg/L ($n = 11$), or 0.0 mg/L control ($n = 9$). Comparison to a sham surgery group (n = 11) indicated statistically significant changes in the DLR of all hemilabyrinthectomized fish on day 1. The control group recovered over the study period and approached, but did not reach sham surgery DLR. Although the caffeine-treated fishes appeared to initiate some postural recovery within the first 2 weeks, beginning on day 10, all caffeine groups diverged from the control group with a deterioration of postural control. All three caffeine groups were significantly deficient in comparison with the control on days 10–24. These results suggest that caffeine exposure can at first be benign, but that high dosage or prolonged exposure hinders functional recovery.

Key words: caffeine, trauma, functional recovery, hemilabyrinthectomy, neurotrauma.

INTRODUCTION

Hemilabyrinthectomy, the unilateral removal of the vestibular organs, has been documented to cause numerous postural, psychosomatic, and neuromuscular changes in various vertebrates (Smith and Curthoys, 1989). Such changes include imbalance in falling cats (Chan et al., 2002), asymmetrical posture in multiple species (Vibert et al., 1997; Stewart et al., 2005) increases in acetylcholinesterase in rat brains (Torte-Hoba et al., 1996), and the loss of gravitationally modulated orientation in fish (Ott and Platt, 1988a). Remarkably, through the process of vestibular compensation, animals recover some of the postural changes, for example the righting-reflex, in a species-specific way (Ott and Platt, 1988b).

The common goldfish, *Carassius auratus*, demonstrates an acute response to a hemilabyrinthectomy as well as a chronic progressive recovery, resulting in a return to normative behavior over the course of 30 days. This response is quantifiable using the dorsal light reflex (DLR) (Ott and Platt, 1988a,b). Hemilabyrinthectomized goldfish will lean at an angle up to 90° toward a light source demonstrating their loss of gravitationally modulated postural control. As recovery occurs, the fish will gradually return to gravitationally modulated postural control and no longer demonstrate the DLR.

This model has been employed to assess the possible beneficial influence of various drugs on functional recovery from lesion through neural compensation. Chlorpheniramine, ACTH4-10, substance P, and dizocilpine have all been shown to accelerate recovery of a hemilabyrinthectomized goldfish (Mattioli et al., 2000; Piratello and Mattioli, 2004).

Caffeine is one of the most widely available and consistently consumed stimulants in the world (Nehlig and Boyet, 2000). It occurs in the water supply influenced by human runoff, both treated and untreated (Comeau et al., 2008) and is known to affect the nervous system of vertebrates. When goldfish are kept in a caffeine solution, it enters the blood stream and has been shown to drastically affect acetylcholine levels in the brain as well as other biomarkers in the liver (Li et al., 2012). Apparently caffeine exerts its primary action by competitively inhibiting adenosine A1 and A2a receptors (Johansson et al., 1997; Halldner et al., 2004; Higgins et al., 2007). Fishes, including zebrafish and goldfish, have adenosine receptors (Rosati, 1995; Beraudi et al., 2003; Maximino et al., 2011). There have been clear signs of dose-dependent neuroendocrine responses to caffeine levels in common goldfish (Li et al., 2012). Furthermore,

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Abbreviations: ANOVA, analysis of variance; DLR, dorsal light reflex; TBI, traumatic brain injured.

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the goldfish has been proposed as an excellent organism for neuroendocrine studies (Popesku et al., 2008). Thus, we hypothesized that hemilabyrinthectomized *C. auratus* would demonstrate a dose-dependent change in recovery as measured by the DLR as a result of chronic exposure to various levels of caffeine.

EXPERIMENTAL PROCEDURES

This study was approved by Nova Southeastern University Institutional Animal Care and Use Committee which follows the guidelines of the National Institutes of Health Office of Laboratory Animal Welfare on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Post-surgery goldfish were divided into five groups: A sham group (n = 11) given sham surgery and kept in tanks without caffeine and held in normal, dechlorinated tap water, a control group (n = 9) kept in normal water without caffeine, a low group (n = 10) kept in 2.5 mg/L caffeine solution, a mid group (n = 10) kept in 5.0 mg/L caffeine solution, and a high group (n = 11) kept in 10.0 mg/L caffeine solution. The dose levels were selected based on previous caffeine studies on goldfish (Li et al., 2012).

The DLR of each fish was determined on days 1, 3, 8, 10, 15, 17, 22, and 24 by measuring the angle the fish leaned toward light. As in other studies, as fish recovered from the lesion, the angle of lean decreased until they returned to nearly baseline DLR (Ott and Platt, 1988b; Mattioli et al., 2000). Differences in recovery among the treatments were used to assess the effects of caffeine on vestibular compensation and implied neural recovery.

Fish care

Unsexed goldfish, 5–9-cm total length weighing 6.04–12.81 g with a mean weight of 7.88 g were purchased from Ozark Fisheries, Stoutland, MO, USA. They were kept in continually aerated 60-L tanks with a maximum of six fish per tank and two tanks per treatment group for a total of 10 tanks. Tanks were maintained at 19 ± 1 °C on a 14:10 Light:Dark photoperiod. A 19-L water change was conducted daily and fish were fed 0.25 g of Goldfish Flakes every other day (United Pet Group, Cincinnati, OH, USA). All fish were acclimated for 4 weeks prior to surgery.

Surgery

Surgical methods followed Mattioli et al. (2000) with minor modifications: Individual specimens were caught with a net and placed in a Tricaine Methanesulfonate solution of 0.8 g/L concentration (Sigma–Aldrich, Saint Louis, MO, USA). After gill movements ceased, the fish was handled with gloved hands, covered in wet gauze, and placed in a surgical tray. A flow of 0.3 g/L TMS solution was perfused through the animal's mouth across the gills throughout the surgery. Under high-intensity light and a surgical microscope, the skin and slime coat were scraped away from the right side of the head. A small amount of the skull directly dorsal and slightly superior to the eye was perforated and removed with a sterilized No. 11 blade (X-Acto, Westerville, OH, USA). The vestibular organ and semi-circular canals were then removed with sterile surgical tweezers. The area was cleaned with fresh gauze and the wound was sealed using a quick drying dental acrylic polymer (Monster Makers, Cleveland, OH, USA). Immediately post-surgery, fish were orally perfused with fresh water across their gills until they began moving. At this point, they were placed in a holding tank and observed until definitive signs of acute recovery were present (Ott and Platt, 1988b). Fish were sorted at random into one of the four treatment groups. Fish undergoing the sham surgery received exactly the same treatment, however, after the skull was opened, no part of the fish was removed and the hole was immediately sealed. Care was taken to insure that sham surgery fish were unconscious for the same length of time as the hemilabyrinthectomized fish.

Treatment

Caffeine was purchased in the form of caffeine powder (Sigma–Aldrich, Saint Louis, MO, USA). A caffeine solution was produced for each group by mixing 2.0 L of distilled water with the correct amount of caffeine to achieve final tank concentration of 10.0, 5.0, 2.5, and 0 mg/L respectively. The concentrations were created so that 15.0 mL of concentrate could be added to the 19 L daily water change and maintain a consistent concentration in the tank. Throughout the experiment and subsequent data analysis double-blinded methods were maintained to prevent biases in treatment or data analysis.

Data collection

During experimental days 1, 3, 8, 10, 15, 17, 22, and 24, all of the operated fish were assessed for dorsal light reflex. The sham surgery fish were only assessed on day 1 to determine the effectiveness of the lesion.

A tank was designed with a cover and painted black except a portal on one side and a viewing window at the front. Graph paper was permanently placed around the portal on the front of the tank. The fish were placed in a stabilized 4-cm wide clear plastic tube facing forward with a SZ2-LGDI 12v22w dual-head surgical microscope light on the fish's right side (Olympus, Central Valley, PA, USA). Fish were recorded from the front with time-lapse photos taken every 10 s on a GoPro Hero 3 camera (GoPro, Riverside, CA, USA). Photos were sorted and labeled in Picasa 3 (Google, Mountain View, CA, USA). The angle of fish lean was assessed utilizing a digital screen protractor (Iconico, New York, NY, USA) with vertical being 0° and totally to the fish's right being 90°. Thus, a fish with the strongest possible dorsal light reflex could lean as much as 90°, or completely to its right, and a fish with the weakest possible dorsal light reflex would lean 0° or completely upright. As the angle approaches 0°, functional recovery is assumed to have taken place (Ott and Platt, 1988a). The fish had a limited ability to swim, maneuver, and in rare cases turn away

from directly facing the camera in the measurement tube. Thus, the best 10 photos were chosen for each fish each day based on clarity, consistency, and facing the correct direction. A fish's average dorsal light reflex on a test day was assessed as a numerical value equal to the average angle that fish was leaning across the 10 photos. A total of three fish were lost within the first 4 days post-surgery and were excluded from the dataset. Additionally, one fish never showed symptoms of a successful hemilabyrinthectomy and was excluded from the dataset.

Data analysis

Data were compiled in Excel (Microsoft, Redmond, WA, USA) and analyzed using SPSS version 22.0 (IBM, New York, NY, USA). A one-way repeated measures analysis of variance (ANOVA) was conducted for each group followed by a *t*-test to assess if there was any variance between the angle of lean on day 1 and subsequent days. For comparison between groups a two-way mixed ANOVA was run and followed by pairwise comparison of each treatment group with the other treatment groups each day. Statistical significance was accepted as $p < 0.05$.

RESULTS AND DISCUSSION

Hemilabyrinthectomized goldfish maintained in non-lethal caffeine solutions demonstrated a bidirectional functional recovery pattern of the DLR, at first improving slightly, and then returning to higher levels of dysfunction. These results are consistent with the literature suggesting that multiple responses to caffeine can occur, specifically in the case of traumatic brain damage (Khor et al., 2013; Rivera-Oliver and Díaz-Ríos, 2014; Rodriguez et al., 2014).

There was a significant difference among the treatments ($F(3,36) = 21.8$, $p < 0.001$) and all four study groups were significantly different from the sham group on day one, indicating successful surgery ($F(4,46) = 19.5$, $p < 0.001$) (Fig. 1). The general pattern of the graphed results indicates an initial recovery of the caffeine-treated groups followed by a return to higher DLR angles (Fig. 1). A one-way repeated measures ANOVA was conducted for each group followed by a *t*-test comparing day 1 values with subsequent days. Results indicated that within the first week of measurement, there were no significant changes in any group except the 5 mg/L caffeine group on day 3 (ANOVA; $F(7,63) = 3.0$, $p < 0.01$; $t(9) = 3.367$, $p < 0.01$). By day 24, there was no significant recovery in any group except the control group (Fig. 1). According to a one-way repeated measures ANOVA the control group experienced significant recovery throughout the study ($F(7,56) = 5.990$, $p < 0.001$). The *t*-tests of the control group revealed initial signs of functional recovery on day 8 ($t(8) = 3.210$, $p < 0.05$) and increased recovery on days 10–24 compared to day 1 values (Fig. 1).

While the one-way repeated measures ANOVA suggests that some recovery occurred in the 5.0 mg/L treatment group, a two-way mixed ANOVA ($F(3,36) = 21.779$, $p < 0.001$) followed by a pairwise comparison revealed no significant difference between any groups within the first week. When comparing differences between the groups, we see a divergence at

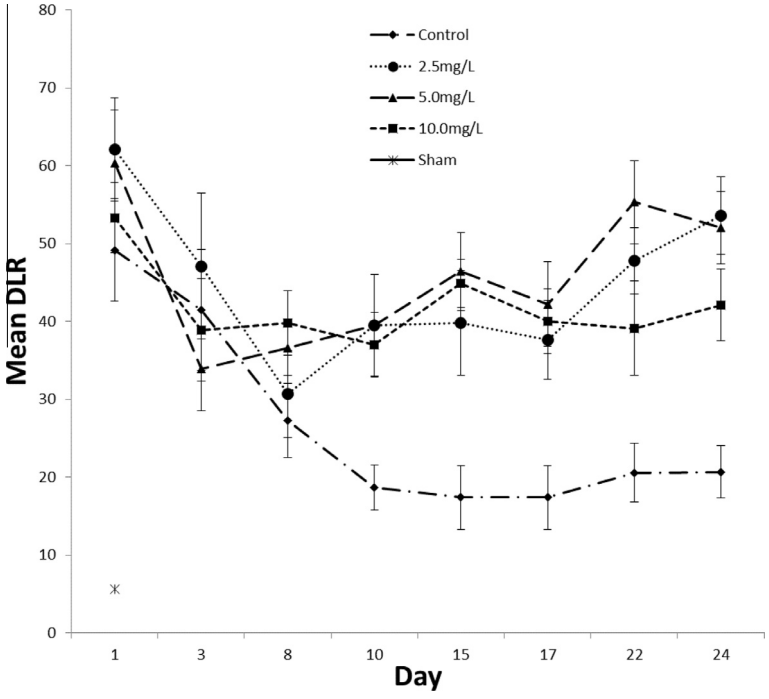

Fig. 1. The mean dorsal light reflex (DLR) of hemilabyrinthectomized goldfish under chronic exposure to caffeine and tested repeatedly on days post-surgery. The labyrinth organ was not removed from sham-operated fishes. Vertical bars represent standard error of the mean (SEM).

day 10 when all three caffeine groups become significantly different from the control group but not from each other (Fig. 1). The significant differences remained throughout the rest of the study ($F(3,36) = 21.779$, $p < 0.001$)(Fig. 1).

Taken together, these results indicate that some functional recovery of caffeine treatment groups was possible. Although there was significant change within the 5 mg/L group in the first week, the DLR was not significantly different from the control or treatment groups. These results are in-line with other studies reporting that there is a dose-related physiological response to caffeine in goldfish (Li et al., 2012). While little is known about the uptake of caffeine in goldfish, it is clear that with chronic exposure all three dosage levels exerted an inhibitory effect on the DLR. Based on the curves and the slight initial recovery in the 5.0 mg/L group it is plausible that dosage is a key factor in the action of caffeine. Caffeine may be totally benign or even helpful at specific levels in the early stages of recovery, but such effects were not clearly delineated in our study.

Caffeine is known to have bidirectional effects in multiple behavioral studies including with fishes (Khor et al., 2013; Rodriguez et al., 2014). Likewise, the effects of caffeine on multiple physiological and neurological processes are well documented, i.e. on spatial learning and long-term memory (Khor et al., 2013; Rivera-Oliver and Díaz-Ríos, 2014). Combining the overwhelming, and often bidirectional, evidence about caffeine's impact on diverse systems such as acetylcholine, calcium channels, dopamine, and adenosine, into a cogent picture about what may have happened to inhibit functional recovery in this lesion model is beyond the scope of this preliminary study. There are, however, a few salient points.

We hypothesize that the process of learning, locomotion, and synaptic rewiring was interrupted at a key juncture by the perpetual effects of caffeine on the adenosine–dopamine–glutamate systems. The cascade of adenosine-modulated neural protective functions was likely inhibited by the antagonistic action of caffeine. This may have resulted in simply stopping the learning process that produces functional recovery from occurring, but it may have also involved increasing secondary apoptosis due to brain trauma.

Chronic caffeine exposure can restructure the entire brain, lengthen dendrites (Vila-Luna et al., 2012), protect zebra fish larvae (Khor et al., 2013), and protect against age-related memory loss (Leite et al., 2011). Conversely, it was seen to inhibit neurogenesis in rats (Han et al., 2007), permanently damage zebra fish larvae (Rodriguez et al., 2014), and disrupt motor-parameters in mice (López-Cruz et al., 2014). It has been shown that chronic sub-lethal caffeine exposure can have an effect on goldfish. It was further demonstrated that chronic caffeine exposure has significant impacts on goldfish brain acetylcholine levels, as well as liver and blood serum biomarkers (Li et al., 2012). One of the primary effects of caffeine is to antagonistically compete with adenosine and prevent adenosine-modulated neural control. In addition to its role in rest, locomotion, learning, and memory, adenosine is thought to be a neural protector (Dunwiddie and Masino, 2001). However, the specific role of adenosine is not clearly understood and there are documented bidirectional effects.

Brain trauma causes massive increases in extracellular adenosine (Robertson et al., 2001; Frenguelli et al., 2007). Several studies have shown that, depending on when adenosine agonists and antagonists were administered to traumatic brain-injured (TBI) mice, the effects could help or hinder the recovery processes (Li et al., 2008, 2009; Dai and Zhou, 2011). These bidirectional effects are believed to come from the variegated ways that adenosine receptors modulate glutamate excitotoxicity and neuroinflammation (Dai and Zhou, 2011). It should also be noted that endogenous levels of adenosine in the brain vary widely with time of day (Huston et al., 1996). It has been proposed that there is an exceptionally abstruse interplay between adenosine receptors, dopamine receptor binding, and the release of glutamate (Rivera-Oliver and Díaz-Ríos, 2014).

The process of functional recovery involves a complex interaction among sensory systems and neural circuitry. One study showed that caffeine and other adenosine antagonists had a marked effect on dopamine antagonism in rat forebrains (Collins et al., 2010). Dopamine is involved in the process of locomotion, and is likely important when it comes to balance recovery in hemilabyrinthectomized goldfish. There is an understanding that significant interactions can occur between the dopamine and adenosine systems (Ferré, 2008). It is further believed that all these interactions have definitive behavioral effects. There are clear interactions between the adenosine and dopamine systems and the glutamate system (Xie et al., 2007). An increased release of glutamate can result in excitotoxicity which is believed to be one of the causes of secondary apoptosis in TBI (Dai and Zhou, 2011). Such a process would invariably inhibit functional recovery on multiple levels. Furthermore, the variegated interactions of these systems that may be both anti-inflammatory initially and inflammatory later is in accordance with our surprising bidirectional results.

Our study supports the utility of the hemilabyrinthectomized goldfish model in studies of neural recovery as well as indicating multiple directions for future study of caffeine and adenosine function. Further, because our results clearly show a negative effect of constant caffeine dosing on neural recovery, we advise caution when considering treatments that maintain high basal levels of caffeine or other adenosine antagonists.

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