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Effects of a Secretin Receptor Antagonist on Cerebellar Learning

Josefina Kather

Advisor: John Green

EFFECTS OF SECRETIN RECEPTOR ANTAGONIST ON EBC

Abstract

Eyeblink conditioning (EBC) is an important procedure used to understand the neuronal plasticity that occurs with learning and memory. Delay EBC requires a brainstem-cerebellar circuit while the role of the cerebellum in trace EBC is not as well understood because it requires a more complex neural circuitry involving regions of the medial prefrontal cortex and hippocampus. Secretin is a neuropeptide that is found in high concentrations within the cerebellum. Previous work has shown that blocking secretin's effects in the cerebellum with intra-cerebellar infusion of relatively large volume of a secretin receptor antagonist impairs delay EBC (Fuchs et al. 2014). Here we study the effect that intra-cerebellar infusion of 0.5 μ L secretin receptor antagonist (5-27 secretin) or vehicle prior to training sessions 1 and 2 has on delay and trace EBC in rats. A 600-ms tone CS was used for the delay EBC paradigm and a 300-ms tone CS followed by a 300-ms trace interval was used for the trace EBC paradigm. For delay EBC, the delay vehicle and antagonist groups displayed similar acquisition of conditioned responses (CRs). There was a trend for the trace antagonist group to underperform compared to the trace vehicle group though not quite at a significant level. One explanation for why the results for the delay EBC do not support previous work is that slow learning occurred in the delay vehicle group that may have prevented the effects of secretin receptor antagonist from reaching significance. The trend for the trace antagonist group to display decreased acquisition of CRs suggests that the cerebellum does play an important role in trace EBC. However, in order to better understand the neural circuitry involved in trace EBC, future work should analyze the role that cerebellar secretin itself has on trace EBC.

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Introduction

Eyeblink conditioning (EBC) is a form of classical conditioning that can be used to study the neural mechanisms involved in learning and memory. EBC occurs when a conditioned stimulus (CS) such as a tone or the flashing of a light precedes an unconditioned stimulus (US) such as an electrical shock to the periorbital region of the eye. The US causes the animal to reflexively blink its eye. This response to the US is known as the unconditioned response (UR). When the animal undergoes many pairings of the CS and US, it learns to blink when the CS is presented, in anticipation of the US. This response to the CS is called the conditioned response (CR). The acquisition of the CR when the CS is presented is considered a learned response. EBC can be divided into delay conditioning and trace conditioning. Delay EBC is when the CS and US overlap each other with the CS beginning slightly (usually about half a second) before the US. Trace EBC is when the CS and US do not overlap with the CS occurring first, followed by a short period (usually about half a second) with no stimuli, and then the US. Delay EBC has been found to be dependent on one of the deep cerebellar nuclei (the interpositus nucleus) and the region of the cerebellar cortex that is ipsilateral to the eye that is receiving the US (as reviewed by Freeman and Steinmetz, 2011). On the other hand, the role of the cerebellum in trace EBC is still debated (Woodruff-Pak & Disterhoft, 2008) given that it also requires forebrain brain regions (Kalmbach et al. 2009; Kalmbach et al. 2010; Siegel et al. 2015).

The cerebellar cortex is composed of three layers: granule layer, Purkinje cell layer, and molecular layer. The Purkinje cell layer is located between the granule and molecular layers (Figure 1). Purkinje cells (PC) provide the sole output from the cerebellar cortex. These cells are GABAergic and most PC axons terminate in the deep cerebellar nuclei (DCN) located in the center of the cerebellum. The rodent DCN is composed of three nuclei: dentate,

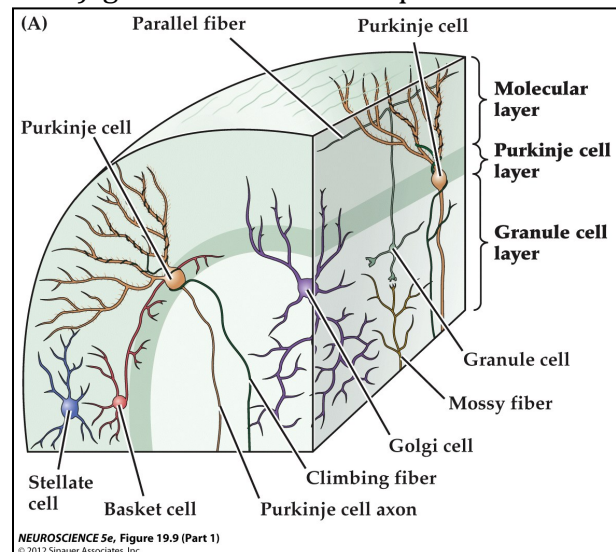


Figure 1. Cell layers of cerebellar cortex, inputs (mossy fibers, climbing fibers) and outputs (Purkinje cell axons). Adapted from Purves et al. (2012).

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interpositus and fastigial. Located in the granule layer, granule cells are a type of glutamatergic interneuron that receive input from other brain regions via mossy fibers (Figure 2). Basket cells (BC) are a type of GABAergic neuron in the molecular layer that synapses on axons of PCs providing feed forward inhibition of these cells. When granule cells become activated by mossy fibers, they excite BCs (not shown) allowing for BCs to inhibit PCs (as reviewed by Voogd and Glickstein, 1998).

Neural Substrates of Delay Eyeblink Conditioning

Delay EBC utilizes a specific neural circuit in the cerebellum and brain stem (Figure 3). Mossy fibers originating in the pontine nuclei deliver the auditory CS to granule cells, which then relay it to PCs (and BCs) via parallel fibers. Mossy fibers also project

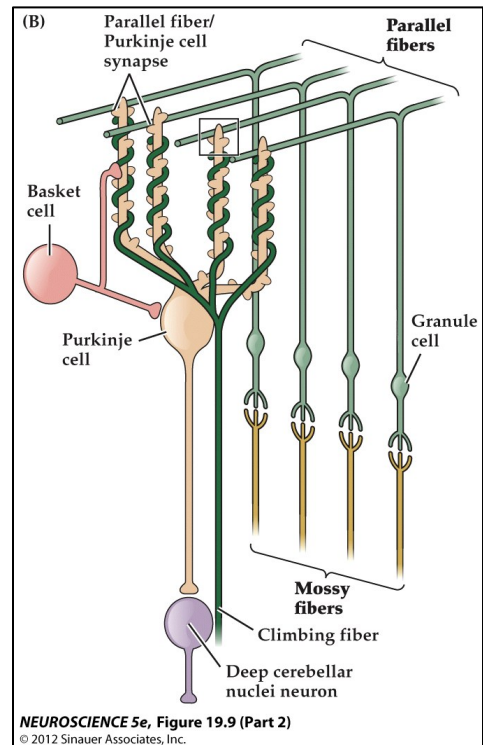


Figure 2. Circuitry of cerebellar cortex and the deep cerebellar nuclei. Adapted from Purves et al. (2012).

to the IPN located in the DCN. Neurons in the IPN project to the red nucleus located in the midbrain. Activation of the red nucleus by the IPN is what allows for a learned motor response to occur during EBC (as reviewed by Christian and Thompson, 2003). The US is relayed to Purkinje cells via climbing fibers originating from the inferior olive (as reviewed by Freeman and Steinmetz, 2011). Usually, PCs tonically inhibit neurons in the IPN of the DCN. The plasticity that occurs in PCs and in the IPN is what allows for the acquisition of CRs (Garcia and Mauk, 1998; Rasmussen et al, 2014). Garcia and Mauk (1998) found that infusion of muscimol, a GABA receptor agonist, into the IPN of albino rabbits prior to training completely abolished CRs in delay EBC. In the same study, infusion of picrotoxin, a GABA receptor antagonist, into the IPN after EBC training altered the response timing of CR. Picrotoxin would prevent GABA released from PCs from binding onto neurons in the IPN. These findings suggest that the CS can induce neural activity in trained animals via the increased synaptic activity between mossy fibers and the IPN. Furthermore, the disruption of CR timing with picrotoxin infusions supports the idea that the cerebellar cortex in

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trained animals provides temporal coding signals in the form of decreased PC activity to the IPN that allow for the coordinated execution of CRs. Ablation lesions of the cerebellar cortex by Garcia et al (1999) prior to training produced a decrease in acquisition.

Neural Substrates of Trace Eyeblink Conditioning

Some studies have suggested that the neural circuitry in the cerebellum for trace EBC is similar to the cerebellar circuitry for delay EBC (Takehara et al.

2003; Woodruff-Pak and Disterhoft 2008). For example, Woodruff-Pak et al. (1985) conducted an experiment in which the ipsilateral IPN was lesioned in albino rabbits that then underwent trace EBC. The CRs were fully prevented in animals with IPN lesions suggesting that acquisition in trace EBC also requires the IPN. Siegel et al. (2015) first trained mice to asymptotic performance and then infused muscimol into the IPN 15-minutes prior to the next training session and found that CRs were abolished. The findings of this study suggest that the expression of trace eyeblink CRs is cerebellar dependent. Finally, IPN single-unit activity is similar in delay and trace EBC (Green & Arenos, 2007).

However, the role of the cerebellum in trace EBC is still a controversial topic. Some studies have found that removal of PCs does not affect acquisition of trace EBC but does slow acquisition of delay EBC (Brown et al., 2009, Woodruff-Pak et al. 2006). Brown et al (2009) used 4-5 month old, homozygous *pcd* mutant mice and wildtype mice of the same age to study the role of the cerebellum in trace EBC. The *pcd* mutant mice lose all of their PCs by the fourth week of age. It was found that the *pcd* mutant group did not differ from

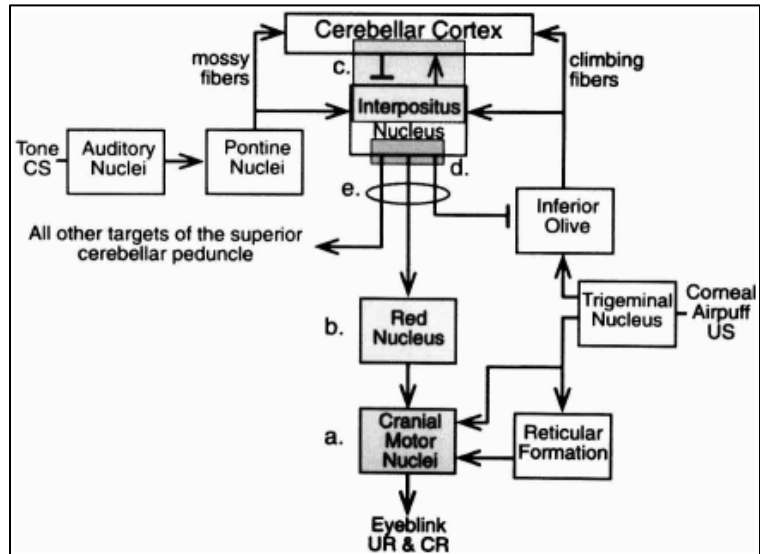


Figure 3. Simplified cerebellar learning circuit. Information about the CS enters the cerebellum from the pontine nuclei while information about the US enters from the inferior olive. The cerebellar cortex contains PCs that tonically inhibit the IPN. The removal of this inhibition allows the IPN to signal the red nucleus in the midbrain to produce a conditioned eye blink via the facial nucleus. From Christian and Thompson (2003).

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the wildtype group in CR frequency or CR amplitude. These findings suggest that the cerebellar cortex is not necessary for trace EBC. Woodruff-Pak et al (2006) used Purkinje Scn8a knockout mice and wildtype mice and trained them either with a delay paradigm consisting of trials in which a 600-ms tone CS co-terminated with a 100-ms US stimulus (500-ms delay) or with a trace paradigm consisting of a 250-ms CS tone followed by a 250-ms trace interval followed by a 100-ms US stimulation. The Scn8a gene codes for a sodium channel subunit that when removed causes a decrease in firing rate of PCs. The Purkinje Scn8a knockout displayed an impairment of acquisition in delay EBC but not trace EBC compared to the control group.

Other studies have found that trace EBC requires interaction between the hippocampus and cerebellum (Solomon et al.1986; Weiss et al. 1999; Tseng et al. 2004) and regions of the medial prefrontal cortex (mPFC) and the cerebellum (Kalmbach et al. 2009; Kalmbach et al. 2010; Siegel et al. 2015; Takehara et al. 2005; Siegel and Mauk, 2013; Siegel et al. 2015). Bilateral aspiration lesions to the hippocampi of three month old F1 hybrid rats significantly impaired CR frequency and CR amplitude in trace EBC compared to the control group that did not receive the lesions (Weiss et al. 1999). In the experiment conducted by Siegel et al (2015) with mice, bilateral aspiration lesions to the caudal regions (between bregma +1.75 and +0.75) of the mPFC prior to training prevented acquisition of CRs in EBC with 250-ms trace intervals. This evidence supports the view that trace EBC requires specific forebrain regions in addition to the cerebellum for acquisition to occur. Due to the stimulus-free period between the CS and the US, trace conditioning incorporates forebrain regions because associating the CS with the US requires “bridging the stimulus gap” (as reviewed by Woodruff-Pak and Disterhoft, 2008).

Cerebellar Cortex, Secretin, and Delay Eyeblink Conditioning

Secretin is a neuropeptide expressed in the somatodendritic region of PCs whereas secretin receptors are expressed on BCs, PCs, and neurons in the DCN (as reviewed by Yung et al., 2006). Purkinje cells endogenously release secretin when they become depolarized (Lee et al., 2005). The released secretin acts as a retrograde messenger that binds to secretin receptors on BCs and PCs. The use of in situ hybridization for the secretin receptor mRNA found that the secretin receptor was expressed in both PCs and BCs. To confirm that

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the secretin receptors were located on BCs, an immunostaining technique using parvalbumin antibodies was conducted. Since BCs are GABAergic, parvalbumin antibodies were used because they mark for GABA cells. It was found that there was a coexpression of parvalbumin and secretin receptor in the molecular layer of the cerebellum (Yung et al. 2001). This suggests that secretin acts on secretin receptors on both PCs and BCs.

Secretin has been found to enhance delay EBC when infused into the cerebellar cortex. Williams et al (2012) infused 1.0 μ L of secretin or vehicle into the cerebellar cortex of rats prior to the first three sessions of delay EBC. They found that secretin enhanced the expression of eyeblink CRs compared to vehicle infused rats. One possible explanation for this enhancement is that secretin reduced expression of Kv1.2 on BCs (Williams et al., 2012), which would increase BC release of GABA on PCs (Southan and Robertson, 1998). Yung et al (2001) utilized whole-cell patch-clamp recording of PCs of the rat cerebellum and found that PC exposure to secretin increased PC inhibitory postsynaptic currents (IPSC). IPSCs indicate that PCs are being inhibited. Inhibition of PCs would disinhibit the IPN allowing for a CR to be produced once the animal has learned to associate the CS to the US. In support of this model, intra-cerebellar infusion of tityustoxin-K α (TsTx) that directly blocked Kv1.2 function in cerebellar cortex also enhanced acquisition in EBC (Williams et al., 2012).

In a study by Fuchs et al (2014), the lobulus simplex, a region in the cerebellar cortex, of male Wistar rats was pharmacologically inhibited with a secretin receptor antagonist (5-27 secretin). Each animal received 1.0 μ L of 5-27 secretin or 1.0 μ L of phosphate-buffered saline vehicle prior to the first three training sessions of 280-ms delay EBC. There was an impairment of acquisition in the animals that received 5-27 secretin when tested with the delay EBC paradigm that had 100 CS-US consecutive trials per session (which promotes strong delay EBC in control rats). These findings support previous findings by Williams et al (2012) that secretin in the cerebellar cortex plays an essential role in acquisition of eyeblink CRs.

The present experiment attempted to replicate the findings of Fuchs et al (2014) by infusing a smaller volume (0.5 μ L) of 5-27 secretin into the lobulus simplex near the primary fissure prior to the first two training sessions of delay EBC. Decreasing the amount

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of 5-27 secretin infused helped to determine if the findings of Fuchs et al (2014) were a result of 5-27 secretin spreading to the IPN and to localize secretin involvement to a more specific area of cerebellar cortex. The lobulus simplex near the primary fissure was chosen as the site of infusion because it has been found to be important in EBC across a number of species (as reviewed by Freeman and Steinmetz, 2011). In a study conducted by Steinmetz and Freeman (2014) with rats, inactivation with muscimol of the ipsilateral hemispheric lobule VI (HVI)(lobulus simplex) near the lower part of the primary fissure prior to delay EBC impaired acquisition. Furthermore, the current study attempted to gather additional evidence on the role of the cerebellum in trace EBC. It was hypothesized that infusion of 5-27 secretin into the cerebellar cortex would decrease acquisition of CRs in both delay EBC and trace EBC. Secretin enhances delay EBC when infused into the lobulus simplex (Williams et al, 2012) whereas 5-27 secretin impairs delay EBC when infused into the same region of the cerebellar cortex (Fuchs et al, 2014). The effects of 5-27 secretin on trace EBC are not yet known. Given that inhibition of the cerebellar cortex near the lower part of the primary fissure with muscimol impairs trace EBC in mice (Siegel et al, 2015) and rats (Steinmetz & Freeman, 2014), it was predicted that 5-27 secretin would also impair trace EBC.

Methods

The basic experiment was a 2 x 2 factorial design: Secretin receptor antagonist infusion v. vehicle infusion and delay conditioning v. trace conditioning. There were four rats per cohort (tested at the same time) with each rat representing a different factor in the experiment. For instance, one rat received secretin receptor antagonist and delay conditioning where another rat received vehicle and delay conditioning. There were 14 cohorts of 4 rats each (56 rats) and 41 of these rats were included in data analysis (see below). The drug was administered via cannula; a needle-like tube that had one end in the left cerebellar cortex and the other end exposed for drug infusion. All testing procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Vermont.

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Surgery

In order for the cannula tip to come into direct contact with the brain, each rat must undergo surgery. Surgeries were done under aseptic conditions. Rats were completely anesthetized with 3% isoflurane in oxygen. Once the guide cannula was implanted (-11.0 anterior-posterior relative to bregma; -3.0 medial-lateral; -3.2 to -3.5 dorsal-ventral from the skull), it was secured to the skull with dental cement. A bipolar electrode was placed subdermally near the corner of the left eye for eye stimulation. The bipolar electrode is for delivering the periorbital shock. Two electromyography (EMG) wires that recorded muscle movement, in this case eye blinking, were placed subdermally above the animal's left eyelid. The wires were connected to a pedestal connector that can then be plugged into a tether/commutator that carries wires for recording eye blinks and for delivering the periorbital shock. The cannula, bipolar electrode, and pedestal connector were secured on the skull with dental cement. The animals received local injections of 0.15 mL bupivacaine around the wound as soon as the surgeries were completed. Subcutaneous injection of saline and analgesic (Carprofen) were also administered immediately after surgery. Animals were given 5-6 days to recover prior to eyeblink conditioning.

Eyeblink Conditioning

Each EBC session consisted of 100 trials. Conditioning occurred in a soundproof room. The room contained four identical chambers where each animal was individually tested. Each chamber contained a speaker in the top left corner where the tone was presented. The top of each chamber contained a tether and the pedestal connector and bipolar electrode of the animal were connected to this tether prior to each session of EBC. The tethers were also connected to the laboratory's computer system. Tone delivery and stimulation to the eye via the bipolar electrode was controlled by a computer running Spike2 software. Eyelid EMG activity was also recorded by this software.

The first day of conditioning was an adaptation day. Rats were plugged into the tethers but they received no stimulation. The following day was the first acquisition day. Immediately prior to sessions 1 and 2 of acquisition, rats received an intracerebellar infusion via the cannula of 0.5 μ L of 2.0 μ g/ μ L secretin receptor antagonist (5-27 secretin) or vehicle. This was done by inserting an internal cannula into the guide cannula, with the

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internal cannula protruding from the tip of the guide cannula by approximately 1.0 mm. Infusions were made with a 10 μ l Hamilton syringe loaded onto an infusion pump (KD Scientific, Holliston MA) set to deliver 0.5 μ L of solution over 2 minutes. At the end of the infusion period, the internal cannula remained in place for an additional 1 min to allow diffusion of the infused solution away from the cannula tip. As soon as the infusion was completed, rats were placed into their chambers and underwent either delay or trace conditioning. Each session consisted of 100 CS-US trials. For each trial of delay conditioning, a 615-ms tone CS co-terminated with a 15-ms periorbital stimulation US (600-ms delay EBC). For each trial of trace conditioning, a 300-ms tone CS was followed by a 300-ms trace period, which was followed by a 15-ms periorbital stimulation US (600-ms trace EBC). Following session 2, rats underwent four more sessions of EBC (3d – 6d) without infusions.

Data Analysis

The raw data from each trial of each session for each animal was analyzed using MATLAB®. Each trial was subdivided into four time periods: (1) a “baseline” period, 280-ms prior to CS onset; (2) a non-associative “startle” period, 0-80 ms after CS onset; (3) a “CR” period, 81-600 ms after CS onset; and (4) a “UR period,” 65-165 ms after US onset (the first 65 ms is obscured by the stimulation artifact). In order for a response to be scored as a CR, an eyeblink had to exceed the mean baseline activity for that trial by 0.5 arbitrary units (where these units had a range of 0.0-5.0) during the CR period. Eyeblinks that met this threshold during the startle period were scored as startle responses and were analyzed separately. Trials in which eyeblinks exceeded 1.0 arbitrary unit during the baseline period were discarded. Comparable scoring intervals and criteria were used to evaluate spontaneous blink rate during the initial adaptation day when no stimuli were administered. The primary dependent measure for all experiments was the percentage of CRs. Data were analyzed using repeated measures ANOVAs. Separate ANOVAs were conducted on data from infusion sessions (Sessions 1 and 2) and non-infusion sessions (Sessions 3-6). We computed all statistical analyses using SPSS 23.0. An alpha level of 0.05 was set as the rejection criterion for all statistical tests.

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Histology

Following conditioning, rats were euthanized with sodium pentobarbital. A stainless steel electrode, insulated except for its tip, was inserted into the guide cannula so that it protruded from the cannula tip by about 1.0 mm (i.e., the infusion location) and 0.1 mA dc current was passed through the electrode for approximately 10 seconds. The rat was then perfused with 0.9% saline followed by 10% buffered formalin. Once the brains were harvested, they were placed in 10% buffered formalin. Around five days prior to tissue analysis, the brains were transferred to a 30% sucrose/ 10% buffered formalin solution. Then brains were embedded in albumin and placed in a freezer. A cryostat was used to section the cerebellum at a 60 μ m thickness. The sections of cerebellum were then placed on glass slides and stained with cresyl violet and Prussian blue. Staining with cresyl violet was used to visualize cell bodies and staining with Prussian blue was used to visualize the electrode-produced lesions. If the cannula could not be located then the data from that animal were not used.

Results

Prior to data analysis the cannula placement for each animal was verified (Figure 4). Cannula placements were also compared to the cannula placements of Steinmetz and Freeman (2014). If the cannula placement could not be located in the lobulus simplex of the cerebellar cortex, the animal was removed.

The first day of training consisted of an adaptation session where rats were placed in experimental chambers for 100 “trials” but received no CS or US. Rats were then trained for six days receiving 100 CS-US paired trials each day (600-msec delay paradigm or 600-msec



Figure 4. Sample cannula placement (arrow) on side ipsilateral to the conditioned eye.

trace paradigm). Prior to training sessions 1-2, 5-27 secretin (0.5 μ L; 2.0 μ g/ μ L; Ant) or

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vehicle (0.5 μ L; phosphate-buffered saline; Veh) was infused into the lobulus simplex ipsilateral to the conditioned eye via cannula. A total of 41 animals were included in the data analysis (10 Delay Ant; 11 Delay Veh; 10 Trace Ant; 10 Trace Veh). A total of 15 animals were removed due to poor electromyographic (EMG) signals ($n=5$), problems with bipolar placement ($n=2$), died during surgery (2), euthanized due to upper respiratory infection ($n=1$), or not able to locate cannula placement ($n=5$).

Delay Eyeblink Conditioning

Both the Delay Vehicle and Delay Antagonist groups learned with a slight trend for Delay Vehicle to outperform Delay Antagonist though there was no statistically significant difference between the groups (Figure 5A). Data for sessions 1-2 was analyzed separately from sessions 3-6 in order to determine if infusions impaired CR expression without impairing learning. A 2 (group: Ant; Veh) by 2 (infusion sessions: 1-2) repeated-measures ANOVA on the percentage of CRs for infusion days yielded a non-significant group main effect ($F(1,19) = 0.51, p > 0.05$), a significant session main effect ($F(1,19) = 20.96, p < 0.05$), and a non-significant interaction effect ($F(1,19) = 1.22, p > 0.05$). A 2 (group: Ant; Veh) by 4 (noninfusion sessions: 3-6) repeated measures ANOVA on percentage of CRs yielded a non-significant group main effect

($F(1,19) = 0.72, p > 0.05$), a significant session effect ($F(3,57) = 7.95, p < 0.05$) and a non-significant interaction effect ($F(3,57) = 0.46, p > 0.05$).

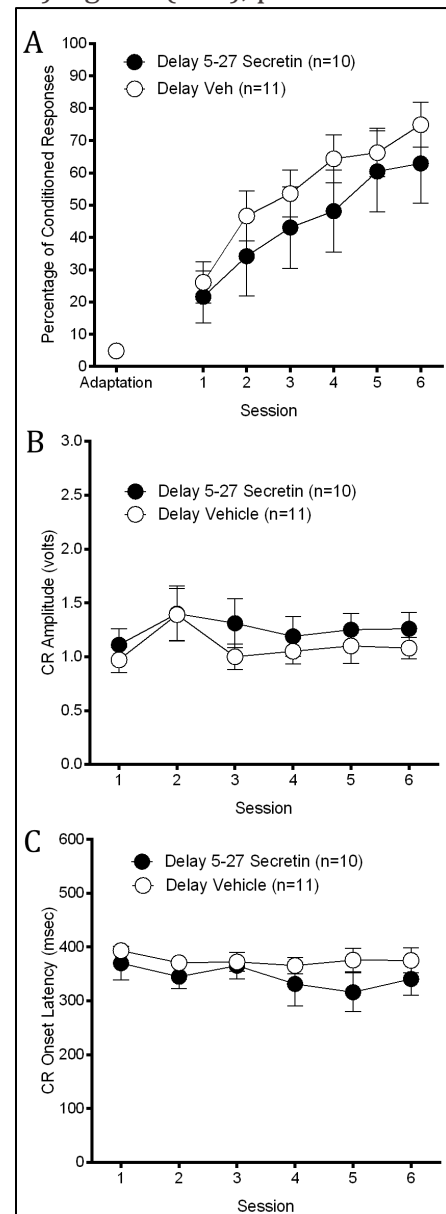


Figure 5. (A) Percentage of CRs over adaptation and sessions 1-6 for the delay paradigm for both groups (100 CS-US trials per session). Sessions 1-2 were when rats received infusion of either 0.5 μ L of 5-27 secretin (2.0 μ g/ μ L) or 0.5 μ L vehicle prior to training. (B) CR amplitude for sessions 1-6 for both groups. (C) CR onset latency for both groups.

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The CR amplitude measures the strength of each CR. Figure 5B shows the average amplitude for each session. It is independent from the percentage of CRs that measures CR frequency over the 100 trials. The CR onset latency measures the time it takes from the CS onset to when the rat begins a CR and is shown in Figure 5C. The repeated measures ANOVA for CR amplitude and CR latency for sessions 1-2 and sessions 3-6 were also not significant (Figures 5B and 5C). For sessions 1-2, both Delay Vehicle and Delay Antagonist groups displayed similar CR amplitude ($F(1,18) = 0.12, p > 0.05$) and CR latencies ($F(1,18) = 0.94, p > 0.05$). The same was found for sessions 3-6 with CR amplitude ($F(1,18) = 1.21, p > 0.05$) and CR latency ($F(1,18) = 1.42, p < 0.05$).

Trace Eyeblink Conditioning

While Trace Vehicle and Trace Antagonist both learned, there was a trend for Trace Vehicle to outperform Trace Antagonist by sessions 3-6; this approached but did not attain statistical significance at the $p < 0.05$ level (Figure 6A). A 2 (group: Ant; Veh) by 2 (infusion sessions: 1-2) repeated-measures ANOVA on the percentage of CRs for infusion days yielded a non significant main group effect ($F(1,18) = 0.48, p > 0.05$), a significant session effect ($F(1,18) = 32.53, p < 0.05$) and a non-significant interaction effect ($F(1,18) = 2.17, p > 0.05$). A 2 (group: Ant; Veh) by 4 (noninfusion sessions: 3-6) repeated measures

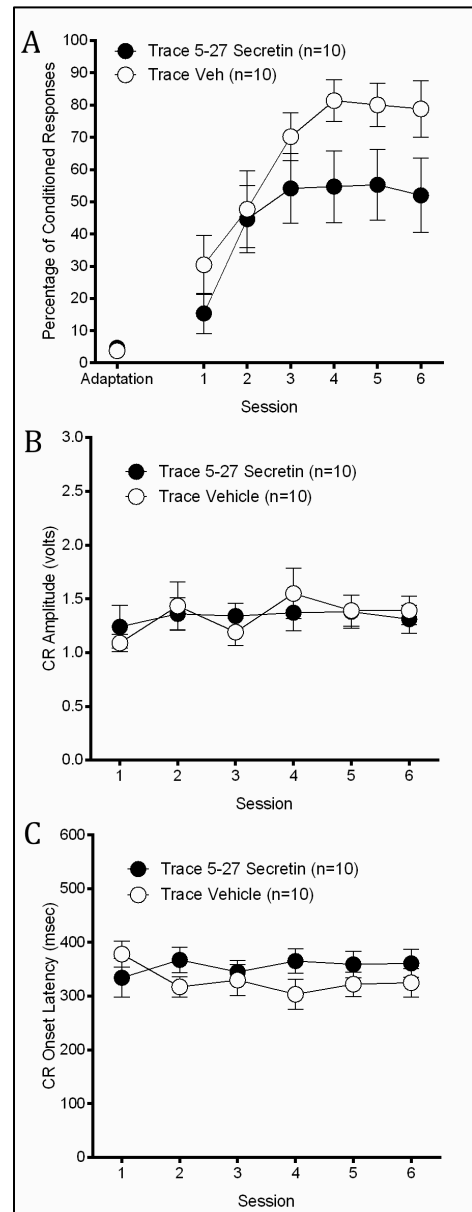


Figure 6. (A) Percentage of CRs over adaptation and sessions 1-6 for the trace paradigm for both groups (100 CS-US trials per session). Sessions 1-2 were when rats received infusion of either 0.5 μ L of 5-27 secretin (2.0 μ g/ μ L) or 0.5 μ L vehicle prior to training. (B) CR amplitude for sessions 1-6 for both groups. (C) CR onset latency for both groups.

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ANOVA on percentage of CRs a non-significant main group effect ($F(1,18) = 3.50, p = 0.078$), a significant session effect ($F(3,54) = 1.14, p < 0.05$) and a non-significant interaction effect ($F(3,54) = 1.01, p > 0.05$).

The repeated measures ANOVA for CR amplitude and CR latency for sessions 1-2 and sessions 3-6 were also not significant (Figures 6B and 6C). For sessions 1-2, both Trace Vehicle and Trace Antagonist groups displayed similar CR amplitude ($F(1,17) = 0.03, p > 0.05$) and CR latency ($F(1,18) = 1.28, p > 0.05$).

Delay vs. Trace Eyeblink Conditioning

The Trace Vehicle group outperformed the Delay Vehicle group, although this was not statistically significant (Figure 7A). A 2 (group: Ant; Veh) by 2 (infusion sessions: 1-2) repeated-measures ANOVA on the percentage of CRs for infusion days yielded a non significant main group effect ($F(1,19) = 0.05, p > 0.05$), a significant session effect ($F(1,19) = 33.24, p < 0.05$), and a non-significant group x session interaction effect ($F(1,19) = 0.26, p > 0.05$). A 2 (group: Ant; Veh) by 4 (noninfusion sessions: 3-6) repeated-measures ANOVA on the percentage of CRs for infusion days yielded a non significant group main effect ($F(1,19) = 1.92, p > 0.05$), a

significant session effect ($F(3,57) = 5.75, p < 0.05$), and a non-significant group x session interaction effect ($F(3,57) = 1.29, p > 0.05$). Similar results were found for comparisons of CR amplitude and CR onset latency.

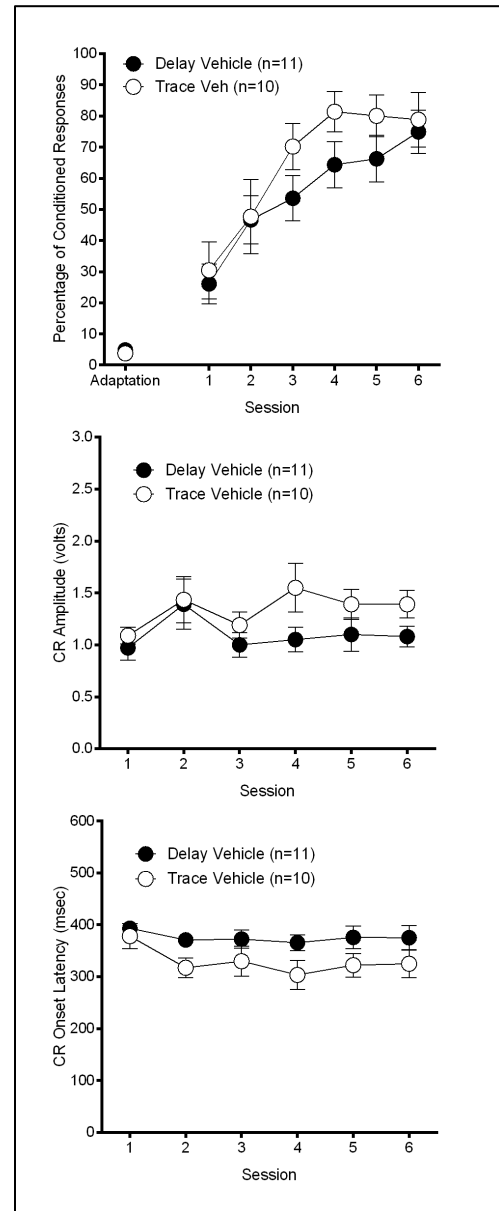


Figure 7. (A) Percentage of CRs over adaptation and sessions 1-6 for the delay vs. trace paradigm for both vehicle groups (100 CS-US trials per session). Sessions 1-2 were when rats received infusion of $0.5\mu\text{L}$ vehicle prior to training. (B) CR amplitude for sessions 1-6 for both groups. (C) CR onset latency for both groups.

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Discussion

Summary of Results

The effect of 5-27 secretin infusions into cerebellar cortex on both delay and trace EBC was studied in the present experiment. Given the results of Fuchs et al (2014), this study was aimed at determining if a smaller volume of 5-27 secretin could replicate an impairment in delay EBC. Instead of 1 μ L only 0.5 μ L of 5-27 secretin was administered over the course of two infusion days instead of three. This experiment also sought to better understand the role of the cerebellum in trace EBC because there is disagreement in the literature regarding whether or not trace EBC requires cerebellar cortex. While no statistically significant difference was found, there was a trend for the Delay Antagonist group and Trace Antagonist group to underperform in comparison to their respective control groups. This was particularly true for the Trace Antagonist group over non-infusion sessions 3-6 (Figure 6A). Finally, the Trace Vehicle group slightly outperformed the Delay Vehicle group (Figure 7). It was predicted that the Delay Vehicle group would outperform the Trace Vehicle or that at least the groups would perform the same.

Effects of 5-27 Secretin on Delay Eyeblink Conditioning

Acquisition of CRs during delay EBC has been found to be a cerebellar dependent process by many studies; however, the methods used by these studies differ. For example, Garcia et al. (1999) inactivated the anterior lobe of the cerebellar cortex of rabbits via aspiration lesions and electrolytic lesions after subjects had been trained to asymptotic performance in a 500-ms delay paradigm. It was found that the timing of CRs was disrupted and that the acquisition of CRs to a new CS was abolished. These results suggest that the cerebellar cortex is required for both retention of a previously established CR as well as for acquisition of a new CR. In addition, Woodruff-Pak et al, (2006) used pcd mutant mice lacking Purkinje cells and found that delay EBC was impaired.

It is important to note that the only experiment that has used 5-27 secretin to study the role of the cerebellum in rat delay EBC is the Fuchs et al. (2014) study. Thus it is possible that the type of delay paradigm used, the change of infusion procedures, and the type of species used prevented significant results from being found in the current experiment. The findings for the delay EBC group were different from our previous study

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(Fuchs et al. 2014). Specifically, there was no difference in performance between the Delay Vehicle and the Delay Antagonist groups with both groups displaying an equal percentage of CRs, CR amplitude, and CR onset latency across all six sessions. One plausible explanation for this is that the volume of 5-27 secretin infused into the lobulus simplex was not sufficient to produce an impairment in learning. Fuchs et al. (2014) infused 1 μL (1 $\mu\text{g}/\mu\text{L}$) of 5-27 secretin before training sessions 1-3 and found that delay EBC was impaired in the experimental group in post-infusion sessions 4-6. The present study infused 0.5 μL (2 $\mu\text{g}/\mu\text{L}$) of 5-27 secretin before training sessions 1-2. It is possible that this change in infusion procedure may have reduced the effects of 5-27 secretin on cerebellar learning. The cannula placement in the present study was slightly more anterior and lateral than Fuchs et al (2014) (11.3 mm posterior to bregma and 2.5 mm lateral to the midline in Fuchs et al. compared to 11.0 mm posterior to bregma and 3.0 mm lateral to the midline) in order to match the coordinates of Steinmetz and Freeman (2014), who found this to be the most effective location for inactivation-induced deficits in EBC. This change in infusion location may also have contributed to the difference in results. In addition, Fuchs et al. (2014) utilized a delay paradigm where the tone CS lasted 295-ms and co-terminated with a 15-ms periorbital stimulation as the US. The present study used a tone CS that lasted 615-ms that co-terminated with a 15-ms periorbital stimulation as the US. It is possible that the 295-ms paradigm allowed the Delay Vehicle group to learn more efficiently than the 600-ms paradigm. If so, the difference between the Delay Vehicle and Delay Antagonist groups in Fuchs et al. (2014) could have been made more pronounced. Furthermore, while Fuchs et al. (2014) found an impairment in learning with intra-cerebellar 5-27 secretin during the 100 CS-US per session delay paradigm, they found no difference between Delay Vehicle and Delay Antagonist groups when utilizing a delay paradigm consisting of 80 CS-US per session interspersed with CS or US probes. It was suggested that since the Delay Vehicle group learned more slowly in the 80 CS-US per session interspersed with CS or US probes paradigm compared to the Delay Vehicle 100 CS-US paradigm, the effects of 5-27 secretin on the Delay Antagonist group may have been more difficult to observe. These differences in results suggest that the effects of 5-27 secretin may only be revealed when EBC is strong in the control group.

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Effects of 5-27 Secretin on Trace Eyeblick Conditioning

Interestingly, in the trace EBC groups, the Trace Antagonist group showed a noticeable reduction in percent CRs compared to the Trace Vehicle group, unlike the Delay Antagonist group compared to the Delay Vehicle group. The difference between the Trace Antagonist and Trace Vehicle groups approached statistical significance in post-infusion sessions 3-6 ($p = 0.078$). The current findings suggest that trace EBC does require the cerebellar cortex. This would agree with the findings of Woodruff-Pak et al. (1985), Kalmbach et al (2009), and Siegel et al. (2015). However, it is important to note that while secretin in the cerebellar cortex has been shown to play a role in delay EBC (Fuchs et al., 2014; Williams et al., 2012), nothing is known about a possible role in trace EBC. Given our findings, it is possible that secretin plays a greater role in trace EBC. Secretin is endogenously released by PCs upon depolarization (Lee et al. 2005) and has been found to enhance cerebellar learning (Williams et al. 2012). The mechanism by which cerebellar cortical secretin facilitates EBC is not fully understood. One view is that secretin decreases the expression of the potassium channel subunit Kv1.2 at BC-PC synapses (Williams et al. 2012) that in turn increases the release of GABA from BCs onto PCs (Southan and Robertson, 1998). This in turn allows for the disinhibition of the IPN resulting in a CR. It is possible that secretin in the cerebellum plays a similar role in trace EBC as it does in delay EBC. This would support the finding that the Trace Antagonist group was impaired compared to the Trace Vehicle group. The reason why the Delay Antagonist did not display as great an impairment may have been because of the type of delay paradigm used as described in the previous section.

Comparison of Delay and Trace Paradigms

The difference in acquisition between the Delay Vehicle and the Trace Vehicle, although relatively small, was not expected. If anything, the Delay Vehicle group should have displayed a greater percentage of CRs than the Trace Vehicle group because delay EBC utilizes a simpler circuit compared to trace EBC (as reviewed by Woodruff-Pak and Disterhoft, 2008). One possible explanation for the slower learning of delay EBC compared to trace EBC that we observed is that the trace paradigm used required minimal forebrain activity and thus resembled the neural circuitry of delay EBC. This idea is supported by a

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Kalmbach et al. (2009) study that found that inactivation of the anterior cingulate region of the mPFC of rabbits after asymptotic training abolished CRs in a trace procedure that used a 500-ms trace interval but not in a trace procedure that used a 300-ms trace interval. This suggests that the role of forebrain regions such as the mPFC in trace EBC is dependent on the length of the trace interval. Since the current study utilized a 300-ms trace interval it is possible that mPFC activity was minimal and that trace EBC utilized more cerebellar dependent neural pathways. However, not all studies support this. Weiss et al. (1999) found that aspiration lesions to the hippocampi of rats impaired CR acquisition in a trace EBC paradigm consisting of 250-ms trace interval. These findings indicate that a 300-ms trace interval is sufficient to require forebrain regions in rats; yet they may be specific to aspiration lesions. A future study could compare the effects of aspiration lesions versus pharmacological inactivation of the hippocampus on different trace-interval EBC paradigms.

A Raybuck and Lattal (2014) review provides another possibility as to why Trace Vehicle acquired CRs more efficiently than Delay Vehicle. It addresses the idea that in trace conditioning there is a gradual increase in excitatory response upon CS offset rather than to CS onset. The animal learns that the US is presented some time after the CS. This causes the CS offset to become a better indicator of US onset because there is less time lapse between the two than CS onset and US onset. Applying this idea to the present experiment, the time interval between the CS offset and US onset was 300-ms for trace EBC as opposed to the 600-ms time interval between the CS onset and US onset for delay EBC. In support of this idea, the CR onset latency for the Trace Vehicle was around 300-ms for sessions 2-6, or right around the CS offset (Figure 7C). For sessions 2-6, the CR onset latencies for the Delay Vehicle group were always greater than that of the Trace Vehicle group. This method of behavioral learning is different from delay EBC because in delay EBC the animal acquires CRs at a rate dependent upon the interval between CS onset and US onset. This difference may explain why the Trace Vehicle group learned more efficiently than the Delay Vehicle group.

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Concluding Remarks

While the results of this study are inconclusive, they do suggest that trace EBC requires the cerebellar cortex, in addition to the IPN. It is possible that the subject size for each group was too small to reveal a significant difference between the groups. Increasing the number of animals in each group while using the same methods may help attain more conclusive results. Furthermore, the effects of intra-cerebellar 5-27 secretin on delay EBC may have been prevented from becoming significant because of a slow learning Delay Vehicle group. This is a possibility given that the first experiment in Fuchs et al. (2014) did not reveal a significant difference between the Delay Vehicle and Delay Antagonist groups because of the slow learning Delay Vehicle group. This is also supported by the differences between the Delay Vehicle and Trace Vehicle groups in the current study. While it was expected that the Delay Vehicle group would acquire CRs at the same rate or more quickly than the Trace Vehicle group, this was not the case. The slow learning Delay Vehicle group may have reduced detection of the effects that 5-27 secretin had on the Delay Antagonist group.

Also important is the cannula placement in the cerebellar cortex. While cannula placements were located using a microscope, a more in-depth analysis would be required to determine whether the exact location of the cannula tip was related to performance. Differences in cannula tip location may have had an impact on how drug infusion affected conditioning. Steinmetz and Freeman (2014) found that acquisition of CRs was severely impaired in rats that received infusion of muscimol prior to EBC via cannula tips located in the eyeblink conditioning microzone (EMC) in the anterior lobe or lobulus simplex of the cerebellar cortex. Rats that had cannula tip placements located outside of the EMC (usually a bit more dorsal) did not display as great of an impairment. The EMC consists of a specific group of PCs that are believed to play a direct role in CR acquisition. Given these results, the present study could analyze cannula placements in greater detail to determine their location relative to the EMC.

Future work should focus on understanding the role of cerebellar secretin in trace EBC. An experimental procedure similar to that of Williams et al. (2012) could be conducted to analyze how secretin modulates trace EBC. This would add to our knowledge of how the cerebellum treats trace EBC and how it compares to delay EBC. Another experiment could be set up to analyze how different paradigms affect both delay and trace

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EBC. For instance, delay conditioning could be analyzed with 295-ms, 400-ms, and 600-ms delay paradigms to determine if the rate of acquisition varies. Trace conditioning could be tested under 600-ms (300-ms tone followed by 300-ms interval), 700-ms (350-ms tone followed by 350-ms interval), and 800-ms (400-ms tone followed by 400-ms interval) trace paradigms to determine if the idea postulated by Raybuck and Lattal (2014) on the mechanism of trace conditioning is supported or not. These types of studies will help further elucidate the role the cerebellum has in trace EBC.

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