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The Effects of Two Forms of Physical Activity on Eyeblick Classical Conditioning

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Abstract

Voluntary exercise, in the form of free access to a running wheel in the home cage, has been shown to improve several forms of learning and memory. Acrobatic training, in the form of learning to traverse an elevated obstacle course, has been shown to induce markers of neural plasticity in the cerebellar cortex in rodents. In three experiments, we examined the effects of these two forms of physical activity on delay eyeblink conditioning in rats. In Experiment 1, exercising rats were given 17 days of free access to a running wheel in their home cage prior to 10 days of delay eyeblink conditioning. Rats that exercised conditioned significantly better and showed a larger reflexive eyeblink unconditioned response to the periocular stimulation unconditioned stimulus than rats that did not exercise. In Experiment 2, exercising rats were given 17 days of free access to a running wheel in their home cage prior to 10 days of explicitly unpaired stimulus presentations. Rats that exercised responded the same to tone, light, and periocular stimulation as rats that did not exercise. In Experiment 3, acrobatic training rats were given 15 days of daily training on an elevated obstacle course prior to 10 days of eyeblink conditioning. Activity control rats underwent 15 days of yoked daily running in an open field. Rats that underwent acrobatic training did not differ in eyeblink conditioning from activity control rats. The ability to measure the learned response precisely, and the well-mapped neural circuitry of eyeblink conditioning offer some advantages for the study of exercise effects on learning and memory.

Keywords

eyeblink classical conditioning; voluntary exercise; wheel running; acrobatic training; cerebellum; amygdala

1. Introduction

Voluntary exercise in rodents, in the form of free access to a running wheel in the home cage, is most commonly associated with improvements in learning and memory and plasticity in the hippocampal formation, including enhanced long-term potentiation (LTP), neurogenesis, and neurotrophin expression [58]. Voluntary exercise also affects other brain

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areas, including the basolateral nucleus of the amygdala [33], the cerebellum [11,31,34], the locus coeruleus [78], and the frontal cortex [97], although exercise-associated effects on brain areas outside of the hippocampal formation are less studied.

Enhanced neurogenesis in the rodent dentate gyrus of the hippocampal formation after voluntary exercise, in terms of enhancements in cell proliferation and/or survival, has been observed repeatedly [55,60,75,76]. Voluntary exercise also lowers the induction threshold and enhances the expression of dentate gyrus LTP [29,75,79]. Many studies have shown that exercising rodents have increased hippocampal brain-derived neurotrophic factor (BDNF) mRNA and protein [1,22,33,37,81,97] and increased TrkB (high-affinity BDNF receptor) mRNA [56,81]. BDNF mRNA levels continue to increase significantly across 1-28 days of exercise [1] and elevated protein levels have been detected after up to 90 days of wheel running [8]. Voluntary exercise may increase levels of BDNF via uptake of peripheral insulin-like growth factor-1 (IGF-1) [23], the action of IGF-1 centrally [26], and/or the action of the noradrenergic (NE) system [30,36]. The increase in BDNF caused by voluntary exercise may lead to enhanced neurotransmitter release via effects on synapsin I [82,83] and synaptophysin [83], as well as activation of intracellular signalling systems, such as calcium calmodulin protein kinase II (CamKII) [80,84] and cAMP response-element binding protein (CREB) [81,84].

Improvements in hippocampus-dependent learning and memory have most often been assessed with one of two tasks, the Morris water maze or contextual fear conditioning. In terms of the Morris water maze, both rats [1,81,84] and mice [25,53,60,73,75] given free access to a running wheel have a significantly lower latency to find the hidden platform and spend more time swimming in the platform quadrant during probe trials. A handful of studies have shown exercise-associated improvements in other spatial learning and memory tasks in rodents, including the Y-maze [77], the appetitively-motivated, “land-based” radial arm maze [2], and the aversively-motivated, “water-based” radial arm maze [47]. Improvements in the radial maze suggest that voluntary exercise may improve working memory in addition to spatial memory. In terms of contextual fear, both rats [7,19,33,78; but see 93] and mice [25,40] given access to a running wheel spend a significantly higher percentage of time freezing in a context that has previously been paired with a foot shock, although at least one study suggests that exercise increases freezing even to a context that has not been paired with footshock [19].

In the current experiments, we hypothesized that voluntary exercise would facilitate eyeblink conditioning, a form of learning and memory with a well-understood neural circuit. Eyeblink conditioning offers several advantages for studying the effects of voluntary exercise on learning and memory. First, eyeblink conditioning is not affected by differences in muscle tone or body weight, both of which are affected by voluntary exercise. Second, eyeblink conditioning is not affected by differences in activity level. Third, both learned and reflexive eyeblinks can be precisely quantified so that changes in performance (e.g., latency or size of the eyeblink) can be more easily separated from changes in learning. Finally, and most importantly, the neural circuitry underlying eyeblink conditioning is well understood and stimulus input pathways (e.g., pontine nuclei; inferior olive), sites of plasticity (interpositus nucleus of the cerebellum; select areas of cerebellar cortex), response output pathways (e.g., red nucleus) and modulatory brain areas (e.g., hippocampal formation, basolateral and central nuclei of the amygdala, medial prefrontal cortex) have all been characterized [72].

We also examined a second form of physical activity, “acrobatic” training on an elevated obstacle course. This motor skill training has been shown to induce plasticity in cerebellar cortex, including an increase in the number of synapses per Purkinje cell [11,39,41,42], an

increase in the volume of astrocytes per Purkinje cell [45], an increase in dendritic spines of Purkinje cells [48], an increase in stellate cell dendritic arborization [42], increased expression of BDNF and TrkB [46], and an increased expression of nitric oxide synthase [74]. Although comparable changes may not occur in the deep cerebellar nuclei [44], we hypothesized that changes in cerebellar cortex with acrobatic training would also facilitate eyeblink conditioning.

2. Experiment 1: Voluntary exercise facilitates delay eyeblink conditioning

The purpose of Experiment 1 was to compare the effects of voluntary exercise (free access to a running wheel in the home cage) on delay eyeblink conditioning to a sedentary control condition. A longer, nonoptimal CS-US interval (750 ms) was used to slow the development of eyeblink CRs, such that any potential differences between the activity conditions in the learning and timing of CRs would be more easily revealed.

2.1. Method

2.1.1. Animals—Male Wistar rats ($n=22$) were obtained from Harlan (Indianapolis, IN). Rats were 59–63 days of age at the time of arrival. Rats were individually housed and maintained on a 12:12 light/dark cycle (lights on at 7 AM and off at 7 PM) and given free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

2.1.2. Voluntary Wheel Running—Eleven of the rats (Group Exercise) were placed in a cage with a running wheel (Model ENV-046; Med Associates, Georgia, VT) and the other 11 rats (Group No Exercise) remained in standard acrylic cages. The diameter of the running wheel was 36 cm. Rats housed with a running wheel were allowed 10–11 days of running prior to surgery and an additional 6–7 days of running between surgery and the beginning of eyeblink conditioning, for a total of 17 days of running prior to conditioning. Distance run over the previous 24 hours was recorded each morning at approximately 9 AM. Exercising rats continued to have access to the running wheel between daily conditioning sessions.

2.1.3. Surgery—Rats were anesthetized using 3% isoflurane in oxygen, and, using aseptic surgical procedures, each rat was surgically prepared with differential electromyographic (EMG) recording wires for recording eye blinks and a bipolar periocular stimulation electrode for delivering the stimulation US. In addition, a ground wire was connected to three stainless steel skull screws. The EMG wires for recording activity of the external muscles of the eyelid, the orbicularis oculi, were constructed of two strands of ultra-thin (75 μ m) Teflon-coated stainless steel wire soldered at one end to a mini-strip connector. The other end of each wire was passed subdermally to penetrate the skin of the upper eyelid of the left eye and a small amount of the insulation was removed. The bipolar stimulation electrode (Plastics One, Roanoke, VA) was positioned subdermally immediately dorsocaudal to the left eye. The mini-strip connector and the bipolar stimulation electrode were cemented to the skull with dental cement. The wound was salved with antibiotic ointment (Povidone), and an analgesic (buprenorphine) was administered (s.c.) immediately after surgery and twice the following day.

2.1.4. Eyeblink Conditioning Apparatus—Eyeblink conditioning took place in one of four identical testing chambers (30.5 $\times$ 24.1 $\times$ 29.2 cm; Med-Associates, St. Albans, VT), each with a grid floor. The top of each chamber was modified so that a 25-channel tether/commutator could be mounted to it. Each testing chamber was housed within an electrically-shielded, sound-attenuating chamber (45.7 $\times$ 91.4 $\times$ 50.8 cm; BRS-LVE, Laurel, MD). A fan in each sound-attenuating chamber provided background noise of approximately 60 dB

sound pressure level (SPL). A speaker was mounted in each corner of the rear wall and a light (off during testing, except when used as a stimulus) was mounted in the center of the rear wall of each sound-attenuating chamber. The sound-attenuating chambers were housed within a walk-in sound-proof chamber.

Stimulus delivery and recording of eyelid EMG activity were controlled by a computer interfaced with a Power 1401 high-speed data acquisition unit and running Spike2 software (CED, Cambridge, UK). A 765 ms, 2800 Hz, 80 dB tone, delivered through the left speaker of the sound-attenuating chamber, served as the CS in Experiments 1 and 3. The tone and the houselight were presented individually in Experiment 2. A 15 ms, 4.0 mA unipolar periorbital stimulation, delivered from a constant current stimulator (model A365D; World Precision Instruments, Sarasota, FL), served as the US in all experiments. The eyelid EMG signals (sampling rate = 2 kHz) were amplified (10k) and bandpass filtered (100-1000 Hz) prior to being passed to the Power 1401 and from there to a computer running Spike2 (Version 5, 2003). Spike2 was used to full-wave rectify, smooth (10 ms time constant), and time shift (10 ms, to compensate for smoothing) the amplified EMG signal.

2.1.5. Eyeblink Conditioning Procedure—All rats were given 8-9 days after surgery for recovery prior to eyblink conditioning. For the first two days after surgery, all rats were housed in standard acrylic cages. For the next 6-7 days after surgery, exercising rats were put back in a cage with a running wheel. For each day of conditioning, each rat was plugged in, via the connectors cemented to its head, to a 25-channel tether/commutator, which carried leads to and from peripheral equipment and allowed the rat to move freely within the testing box. On Day 1 (adaptation), rats were plugged in but no stimuli were delivered. They remained in the chamber for 60-min (the approximate length of a training session). On Days 2-11 (conditioning), 100 trials per day were delivered, at an average inter-trial interval (ITI) of 30-sec (range = 20-40 sec). Each trial consisted of an 80-dB, 765-ms tone conditioned stimulus (CS) which coterminated with a 15-ms, 4.0 mA periocular stimulation unconditioned stimulus (US) (750 ms delay procedure).

2.1.6. Eyeblink Conditioning Data Analysis—For conditioning sessions (Experiments 1 and 3), trials were subdivided into four time periods: (1) a “baseline” period, 280-ms prior to CS onset; (2) a “startle” period, 0-80 ms after CS onset; (3) a CR period, 81-750 ms after CS onset (i.e., CS-US interval); and (4) a US period, 65-165 ms after US onset (the first 65-ms was obscured by the shock artifact). Eye blinks that exceeded mean baseline activity by 0.5 arbitrary units (where these units had a range of 0.0-5.0) during the CR period were scored as CRs. This time point was scored as CR onset. The difference in time between CS onset and CR onset represents CR onset latency. Trials in which eyeblinks exceeded 1.0 arbitrary units 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 dependent measure of conditioning was the percentage of eyblink CRs during the CR period across sessions. The timing of the eyblink CR was measured by CR onset latency during the CR period across trials in which a CR was emitted. UR amplitude was the peak of the reflexive eyblink between 65-ms and 165-ms after US onset. UR amplitude was calculated only for Session 1 of conditioning (before a significant number of CRs had developed, which can alter UR amplitude) in Experiments 1 and 3.

For pseudo-conditioning sessions (Experiment 2), tone-alone and light-alone trials were subdivided into three time periods: (1) a “baseline” period, 280-ms prior to CS onset; (2) a “startle” period, 0-80 ms after CS onset; and (3) an eyblink period, 81-750 ms after CS onset (i.e., comparable to the CS-US interval in Experiments 1 and 3). Periocular stimulation-alone trials consisted of only a single time period: a US period, 65-165 ms after

US onset (the first 65-ms was obscured by the shock artifact). Scoring criteria were otherwise comparable to those used to score CRs and URs in Experiments 1 and 3.

We computed all statistical analyses using SPSS 17.0.2. An alpha level of 0.05 was set as the rejection criterion for all statistical tests.

2.2. Results

One rat (Group Exercise) died on the day after surgical preparation for eyeblink conditioning and his data were not included in the analyses (Group Exercise, $n=10$; Group No Exercise, $n=11$).

2.2.1. Voluntary Wheel Running—In the final 24-hr before the adaptation session (Day 17 of running wheel access), the average running distance was 3.47 km (± 1.30 SEM) for the rats with a running wheel in their home cage.

2.2.2. Adaptation Session—In the initial adaptation session without any stimuli, percentage of spontaneous eyeblinks was low (Figure 1) and did not differ between exercising and non-exercising rats, $t(19) = 1.29$, $p > 0.05$.

2.2.3. Conditioning—During conditioning sessions, both groups conditioned but exercising rats showed significantly better conditioning than non-exercising rats (Figure 1). A 2 (Group: Exercise vs. No Exercise) $\times$ 10 (Session) repeated-measures ANOVA on percentage of eyeblink CRs revealed significant main effects of Session, $F(9,171) = 10.51$, $p < 0.01$ and Group, $F(1,19) = 4.58$, $p < 0.05$. The Group $\times$ Session interaction effect was not significant, $p = 0.12$.

2.2.4. Conditioned Response Timing—For analysis of eyeblink CR onset latency, only Sessions 2-10 were analyzed, since 2 rats (one from each Group) produced no CRs in Session 1. There were no differences between exercising and non-exercising rats in eyeblink CR onset latency during Sessions 2-10 of conditioning. A 2 (Group: Exercise vs. No Exercise) $\times$ 10 (Session) repeated-measures ANOVA on CR onset latency revealed no significant effects, F 's < 1 .

2.2.5. Unconditioned Responses to the CS and the US—Percentage of startle responses to the CS (reflexive eyelid movements within 80-ms of tone onset) were low in all sessions ($< 6\%$), did not differ between exercising and non-exercising rats, and decreased across sessions. A 2 (Group: Exercise vs. No Exercise) $\times$ 10 (Session) repeated-measures ANOVA on percentage of startle responses revealed only a significant effect of Session, $F(9,171) = 2.46$, $p < 0.02$.

Unconditioned response amplitude was greater in exercising rats across 10-trial blocks of trials in Session 1 (i.e., prior to the development of eyeblink CRs that can modulate the amplitude of the UR; Figure 2A). This was confirmed by a 2 (Group: Exercise vs. No Exercise) $\times$ 10 (Block of 10 trials in Session 1) repeated-measures ANOVA on UR amplitude which revealed only a significant main effect of Group, $F(1,19) = 6.05$, $p < 0.03$. We also analyzed UR amplitude across trials of Block 1 of Session 1 (Figure 2B), and found a significant effect of Group, $F(1,19) = 6.22$, $p < 0.03$. However, a one-way ANOVA on UR amplitude on trial 1 of Block 1 of Session 1 (i.e., the first periocular stimulation) did not reveal a difference between groups, $F(1,19) = 2.04$, $p > 0.17$. This last finding suggests that the apparent enhancing effect of exercise on eyeblink UR amplitude may have been due to a very rapid associative process, such as fear conditioning, rather than an effect on the reflexive eyeblink per se.

2.3 Discussion

Exercising rats exhibited significantly better delay eyeblink conditioning than non-exercising rats. UR amplitude was also significantly greater in exercising rats in session 1 of conditioning. An auditory CS can enhance the rabbit nictitating membrane UR to eye stimulation from the very first pairing of the CS-US [35,96]. UR amplitude was higher in exercising rats from the very first block of conditioning, but not on the very first trial of conditioning. We examined this issue further in Experiment 2, which used an explicitly unpaired stimulus procedure in which we could measure the reflexive eyeblink to the periocular stimulation without the tone present.

3. Experiment 2: Voluntary exercise does not enhance sensitization or pseudoconditioning

The purpose of Experiment 2 was to compare the effects of voluntary exercise (free access to a running wheel in the home cage) on unpaired tone, light, and periocular stimulation presentations to a sedentary control condition. We included a light in this experiment, in addition to the tone used in Experiment 2, to extend the generality of our results on possible stimulus sensitization effects of exercise.

3.1 Method

3.1.1. Animals—Male Wistar rats ($n=16$) were obtained from Harlan (Indianapolis, IN). Rats were 59-63 days of age at the time of arrival. Rats were individually housed and maintained on a 12:12 light/dark cycle (lights on at 7 AM and off at 7 PM) and given free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

3.1.2. Voluntary Wheel Running—Eight of the rats were placed in a cage with a running wheel (Model ENV-046; Med Associates, Georgia, VT) and the other 8 rats remained in standard acrylic cages. The voluntary wheel running was the same as in Experiment 1.

3.1.3. Surgery—The surgeries were the same as in Experiment 1.

3.1.4. Eyeblink Conditioning Apparatus—The eyeblink conditioning apparatus was the same as in Experiment 1.

3.1.5. Eyeblink Pseudo-Conditioning Procedure—As in Experiment 1, all rats were given 8-9 days after surgery for recovery prior to eyeblink conditioning. On Day 1 (adaptation), rats were plugged in but no stimuli were delivered. They remained in the chamber for 60-min (the approximate length of a training session). On Days 2-11 (conditioning), 200 trials per day were delivered, at an average inter-trial interval (ITI) of 15-sec (range = 10-20 sec). Fifty of the trials consisted of a 80-dB, 765-ms tone, 50 of the trials consisted of a 765-ms light, and 100 of the trials consisted of a 15-ms, 4.0 mA periocular stimulation. Trials were intermixed such that no more than 3 trials of a particular type occurred in succession.

3.1.6. Data Analysis—The data analysis was the same as in Experiment 1, except that tone, light, and periocular stimulation trials were analyzed separately.

3.2. Results

Two rats (Group No Exercise) had poor EMG signals during pseudo-conditioning and their data were not included in the analyses (Group Exercise, $n=8$; Group No Exercise, $n=6$).

3.2.1. Voluntary Wheel Running—In the final 24-hr before the adaptation session (Day 17 of running wheel access), the average running distance was 2.47 km (± 0.97 SEM) for the rats with a running wheel in their home cage. A one-way ANOVA comparing the running distances in Experiments 1 and 2 showed that these distances did not differ significantly, $F < 0.35$.

3.2.2. Adaptation Session—In the initial adaptation session without any stimuli, percentage of spontaneous eyeblinks was low (Figure 3) and did not differ between exercising and non-exercising rats for trials that would be tone trials during pseudo-conditioning, $t(12) = 0.99$, $p > 0.05$ or for trials that would be light trials during pseudo-conditioning, $t(12) = 1.25$, $p > 0.05$.

3.2.3. Pseudo-conditioning—During unpaired tone, light, and periocular stimulation presentations, neither group showed any evidence of pseudo-conditioning to either the tone (Figure 3A) or the light (Figure 3B). A 2 (Group: Exercise vs. No Exercise) $\times$ 10 (Session) repeated-measures ANOVA on percentage of eyeblink responses to the tone revealed no significant effects, F 's < 1.4 . The same repeated-measures ANOVA on percentage of eyeblink responses to the light revealed only a significant effect of Session, $F(9, 108) = 3.49$, $p < 0.01$. However, this significant effect was due to a significant linear decrease ($p < 0.04$), rather than a linear increase, in percentage of eyeblink responses to the light across sessions.

3.2.4. Reflexive Responses to the Tone, the Light, and the Periocular Stimulation—Percentages of startle responses to the tone and to the light (reflexive eyelid movements within 80-ms of stimulus onset) were low in all sessions ($< 7\%$), did not differ between exercising and non-exercising rats, and did not change across sessions. A 2 (Group: Exercise vs. No Exercise) $\times$ 10 (Session) repeated-measures ANOVA on percentage of startle responses to the tone revealed no significant effects, F 's < 0.9 . The same repeated-measures ANOVA on percentage of startle responses to the light also revealed no significant effects, F 's < 1.7 .

Unconditioned response amplitude to the periocular stimulation (measured 65-165-ms after US onset; the first 65-ms was obscured by the stimulation artifact) did not change across sessions and, unlike Experiment 1, was not significantly different between groups (Figure 4). A 2 (Group: Exercise vs. No Exercise) $\times$ 10 (Session) repeated-measures ANOVA on UR amplitude failed to reveal any significant effects, F 's < 2.4 .

3.3. Discussion

Voluntary exercise does not enhance eyeblink responses to a tone, a light, or periocular stimulation. The enhancement in the UR that we observed in Experiment 1 may reflect an enhancement in fear conditioning to the CS, which can facilitate delay eyeblink conditioning through amygdala-cerebellum interactions (see General Discussion).

4. Experiment 3: Acrobatic training does not affect delay eyeblink conditioning

The purpose of Experiment 3 was to compare the effects of motor skill training (daily traversals of an elevated obstacle course) to an activity control condition. Some studies have focused on motor learning associated with acrobatic training, as opposed to voluntary

exercise. Acrobatic training consists of daily trials on an elevated obstacle course that requires coordinated forelimb and hindlimb movement and is designed to encourage problem solving and coordination [47]. Animals trained on the acrobatic task sequentially traverse a series of obstacles such as a grid platform, a rope, a ladder, barriers, and a chain to reach a series of platforms. Synaptogenesis, or the growth and formation of synapses, occurs in cerebellar cortex with this type of motor skill learning, compared to forced exercise, voluntary exercise, or an inactive control [11].

4.1 Method

4.1.1. Animals—Male Wistar rats (n=14) were obtained from Charles River (Wilmington, MA). Rats were 62-67 days of age at the time of arrival. Rats were individually housed and maintained on a 12:12 light/dark cycle (lights on at 7 AM and off at 7 PM) and given free access to food and water. One rat (Group AC) had an eyelid EMG of poor quality and was removed from the experiment. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

4.1.2. Elevated Obstacle Course (“Acrobatic”) Training—Following two days of handling, 7 of the rats (Group Acrobatic Training) were given daily training sessions in an elevated obstacle course adapted from those used in previous studies [11,46]. The other 7 of the rats (Group Activity Control) underwent an activity control condition [11,46]. Each activity control rat was yoked to an acrobatic training rat and allowed to run in an open container for the amount of time it took the acrobatic training rat to complete the acrobatic course. Group Acrobatic Training rats were trained before their yoked controls in order to determine the amount of time the controls were allowed to spend in the open container (while being handled as equivalently as possible to the acrobatic training rats). We chose not to include a second, inactive, control group in this experiment because the activity control condition does not affect cerebellar synaptogenesis relative to an inactive control group [11] and we were interested specifically in the effects on eyeblink conditioning of physical activity that induces cerebellar synaptogenesis.

The elevated obstacle course requires rats to learn a variety of skilled forelimb, hindlimb, and coordinated whole-body movements. Group Acrobatic Training rats were given five daily trials on the elevated obstacle course. For each trial, Group Acrobatic Training rats sequentially traversed across a grid platform (25 × 40 cm with 1 × 1 cm openings), two small parallel rods (1.2 cm diameter, 44 cm across), a rope (2 cm diameter, 48 cm across), a ladder with 4 cm spaced rungs, two barriers (13 cm & 17 cm high), and a heavy metal link chain (24 links) and then traversed back through the obstacles to end at the grid platform. The Group Acrobatic Training rats received 15 days of training (12 days prior to surgical preparation for eyeblink conditioning; 3 days immediately before eyeblink conditioning) on the elevated obstacle course. The first day consisted of a heavily aided (by the experimenter) traversal of the obstacles, while the next three days consisted of aided traversals of the obstacles (Days 2-4). Most rats required only occasional assistance after Day 4. Rats were guided through the task by the experimenter's hand (holding either the tail or lifting the abdomen) and by light prodding applied to the hindquarters. Motivation for rapid and skillful task completion was the return to the holding bin, and the avoidance of falling and postural instability.

4.1.3. Surgery—The surgeries were the same as in Experiment 1.

4.1.4. Eyeblink Conditioning Apparatus—The eyeblink conditioning apparatus was the same as in Experiment 1.

4.1.5. Eyeblink Conditioning Procedure—The eyeblink conditioning procedure was the same as in Experiment 1.

4.1.6. Data Analysis—The data analysis was the same as in Experiment 1.

4.2. Results

One rat (Group Activity Control) had a poor EMG signal during conditioning and his data were not included in analyses (Group Acrobatic Training, $n=7$; Group Activity Control, $n=6$).

4.2.1. Acrobatic Training—On the first day of acrobatic training, average time to complete the course was 193.8 sec (± 40.02 SEM). On the final day of acrobatic training (Day 15), average time to complete the course was 82.7 sec (± 5.09 SEM). A repeated-measures ANOVA across days revealed a significant decrease in latency to complete the course, $F(14,84) = 9.27$, $p < 0.01$.

4.2.2. Adaptation Session—In the initial adaptation session without any stimuli, percentage of spontaneous eyeblinks was low (Figure 5) and did not differ between exercising and non-exercising rats, $t(11) = 0.58$, $p > 0.05$.

4.2.3. Conditioning—During conditioning, both groups conditioned and there were no differences between groups (Figure 5). A 2 (Group: Acrobatic Training vs. Activity Control) $\times$ 10 (Session) repeated-measures ANOVA on percentage of eyeblink CRs revealed a significant main effect of Session, $F(9,99) = 16.05$, $p < 0.01$. Neither the Group main effect nor the Group $\times$ Session interaction effect was significant, F 's < 1.1 .

4.2.4. Conditioned Response Timing—Only Sessions 5-10 were analyzed, since one rat (from Group Activity Control) did not produce any eyeblink CRs until Session 5. There were no differences between groups or across sessions in eyeblink CR onset latency during conditioning. A 2 (Group: Acrobatic Training vs. Activity Control) $\times$ 10 (Session) repeated-measures ANOVA on CR onset latency revealed no significant main effects, F 's < 1 , although the Group $\times$ Session interaction effect approached significance, $F(5,55) = 2.16$, $p = 0.07$.

4.2.5. Unconditioned Responses to the CS and the US—Percentage of startle responses to the CS (reflexive eyelid movements within 80-ms of tone onset) was low in all sessions ($< 6\%$) but was higher in activity control rats, particularly in the later sessions of conditioning. A 2 (Group: Acrobatic Training vs. Activity Control) $\times$ 10 (Session) repeated-measures ANOVA on percentage of startle responses revealed a significant effect of Group, $F(1,11) = 7.56$, $p < 0.02$ and a significant Group $\times$ Session interaction effect, $F(9,99) = 9.60$, $p = 0.04$. Further investigation of the interaction effect with simple effects tests comparing groups within each session revealed a significantly greater percentage of startle responses in activity control rats in Sessions 7 ($p < 0.04$), 8 ($p < 0.02$), and 10 ($p < 0.04$).

Unconditioned response amplitude decreased in both groups across 10-trial blocks of trials in Session 1, and was not significantly different between groups (Figure 6). This was confirmed by a 2 (Group: Acrobatic Training vs. Activity Control) $\times$ 10 (Block of 10 trials in Session 1) repeated-measures ANOVA on UR amplitude which revealed only a significant main effect of Block, $F(9,99) = 2.52$, $p < 0.02$. We also analyzed UR amplitude across trials of block 1 of Session 1, and found no significant effects, F 's < 1.5 . Finally, a one-way ANOVA on UR amplitude on trial 1 of block 1 of Session 1 (i.e., the first periocular stimulation) did not reveal a difference between groups, $F < 0.1$.

4.3. Discussion

This experiment failed to find support for any effects of acrobatic, motor skill training on eyeblink conditioning over and above anything that might be produced by the forced activity component of the procedure, despite the fact that this type of motor skill training is known to produce changes in cerebellar cortical synapses.

5. General Discussion

The primary results of the three experiments reported here can be summarized as follows. In Experiment 1, 17 days of voluntary exercise in a running wheel facilitated delay eyeblink conditioning. Exercising rats also showed a larger UR early in conditioning, although not on the first trial. In Experiment 2, 17 days of voluntary exercise in a running wheel did not produce pseudoconditioning, nor did it affect reflexive responding to periocular stimulation. In Experiment 3, another form of physical activity was examined. In this experiment, 15 days of motor skill training in an elevated obstacle course did not affect delay eyeblink conditioning. It is possible that the forced activity engaged in by both groups in Experiment 3 may have facilitated eyeblink conditioning; we did not include a sedentary control condition in this experiment to evaluate this possibility. However, the important conclusion of Experiment 3 is that physical activity that produces cerebellar synaptogenesis does not facilitate eyeblink conditioning.

Exercise-associated changes in the cerebellum are the most obvious candidate for explaining the facilitated eyeblink conditioning we observed in Experiment 1. The effects of exercise on the cerebellum are not well-studied. Exercise has been shown to increase blood vessel density in cerebellar cortex [11] and the activity of cytochrome oxidase in cerebellum [31]. Exercise can also improve rotarod performance [59], a test of coordination that engages motor cortex and cerebellum. If exercise-associated changes in the cerebellum are to explain exercise-associated facilitation of eyeblink conditioning, we will have to reconcile this with the fact that acrobatic training, which induces synaptogenesis in cerebellar cortex, did not affect eyeblink conditioning. It may be the case that acrobatic training induces cerebellar cortical plasticity only in limb regions of cerebellar cortex, and projections from these regions do not converge on deep cerebellar nuclei neurons responsible for generation of eyeblink CRs.

Exercise-associated changes in the hippocampus are a second candidate for explaining the facilitated eyeblink conditioning we observed in Experiment 1. It is interesting to compare our exercise-associated facilitation of eyeblink conditioning in male rats to stress-associated facilitation of eyeblink conditioning in male rats, especially given the fact that voluntary exercise can affect corticosterone concentrations in the hippocampus [27]. Stress, in male rats, is the most well-studied facilitator of eyeblink conditioning. Shors and colleagues have shown that male rats exposed to an inescapable stressor show enhanced short-delay [62,63,65,67,68,70,94], long-delay [5,10,63] and trace [4,9,10,50,85,95] eyeblink conditioning. Corticosterone is both necessary and sufficient for enhancement of eyeblink conditioning in male rats; adrenalectomy before stressor exposure prevents stress-induced enhancement of conditioning and enhancement of conditioning can be produced by injection of corticosterone [10]. Enhancement of eyeblink conditioning can also be produced by intracerebroventricular injection of corticotropin-releasing hormone [64]. The facilitatory effect of stress on eyeblink conditioning is blocked by systemic [67] or intra-basolateral amygdala [69] injections of NMDA antagonists during stressor exposure. The effects of stress on eyeblink conditioning in males are dependent on an intact hippocampus [5] and an intact basolateral nucleus of the amygdala [85] during stress exposure, and an intact bed nucleus of the stria terminalis (BNST) during conditioning [4].

Since stress in male rats and voluntary exercise in male rats both enhance delay eyeblink conditioning (and contextual fear conditioning; see [6] for an example in the stress literature and [33] for an example in the exercise literature), it might be hypothesized that a similar plasticity mechanism underlies the enhancement of conditioning following stress and exercise interventions. With this in mind, it is interesting that stress and exercise often produce *opposite* effects on markers of hippocampal plasticity. For example, stress decreases BDNF in the dentate gyrus [13] while exercise increases BDNF in dentate gyrus [29,33,82]. A second difference between uncontrollable stress and voluntary exercise is that stress decreases neurogenesis in the dentate gyrus [12,71] while exercise increases it [55,60,75,76]. Finally, stress raises the induction threshold and decreases the expression of dentate gyrus LTP [66; but see 38], while voluntary exercise lowers the induction threshold and enhances the expression of dentate gyrus LTP [29,75,79]. An interesting possibility is that even mild stress, such as from voluntary exercise, can improve eyeblink conditioning while exerting opposite effects on hippocampal plasticity measures from more intense stress.

Exercise-associated changes in the amygdala are a third candidate for explaining the facilitated eyeblink conditioning we observed in Experiment 1. Amygdala-dependent processes can modulate delay eyeblink conditioning. For example, contextual fear conditioning prior to eyeblink conditioning can enhance the amplitude of subsequent eyeblink CRs [15-18,32] or the rate of eyeblink conditioning [57]. Amygdala lesions abolish this latter effect [57]. Both the central and basolateral nucleus of the amygdala are engaged during short-delay eyeblink conditioning in rats, as revealed by single-unit recording [61] and amygdala lesions have been shown to slow the rate of short-delay eyeblink conditioning [14,49].

Voluntary exercise may induce plasticity in the amygdala, leading to facilitated fear conditioning and it is this facilitated fear conditioning that increased the rate of eyeblink conditioning in our experiments. Consistent with this idea is the fact that we observed a greater amplitude eyeblink UR in exercising rats after a few pairings of the tone with the periocular stimulation (Experiment 1) but not when the periocular stimulation was presented by itself (Experiment 2), which may be an indicator of enhanced associative fear of the tone. In rabbits, eyeblink conditioning can enhance the amplitude of the eyeblink UR elicited in the presence of the CS, a phenomenon known as reflex facilitation [20,54,86-89,91,92]. Reflex facilitation can occur very rapidly (5-12 trials) in eyeblink conditioning [88]. Reflex facilitation, like potentiation of the whole-body startle response in the presence of a fear CS, is dependent on the amygdala [89] and may be thought of as an indicator of fear of the CS used in eyeblink conditioning. Further support for this hypothesis comes from Brown and colleagues who have shown, in rats, facilitation of the short-latency eyeblink reflex (R1) elicited by stimulation of the supraorbital branch of the trigeminal nerve during a fear CS. This is highly correlated with other measures of fear of the CS, including freezing, ultrasonic vocalization, and defecation [51], is greatest around the time that a footshock US had occurred during the fear CS [52], and is a basolateral amygdala-dependent process [21,24,51]. Importantly, reflex facilitation is not dependent on the interpositus nucleus, unlike the eyeblink CR [86,91,92]. The amygdala may influence the eyeblink UR independently of the cerebellum via projections from the central nucleus of the amygdala to the lateral tegmental field [90]. How exactly the amygdala and cerebellum may interact during eyeblink conditioning is still not completely clear.

Our results raise the issue of whether exercise primarily affects (short-term) performance of eyeblink CRs or whether exercise affects (longer-term) learning and memory of the CS-US association. One way that these issues can be separated in classical conditioning is by using a test phase in which the CS is presented by itself under the same conditions in all groups [cf. 32]. For example, exercising and non-exercising groups would undergo eyeblink

conditioning, exercise would be stopped, and groups would be tested several days later by presenting the CS alone. In the above example, more CRs observed in the exercise group in training might be due to a stronger CS-US association or just to short-term changes in the performance of the CR. However, if differences were also observed at test after all subjects have ceased exercise, then one can conclude that exercise had an effect on learning. Along this line of reasoning, Falls et al. [28] recently found that mice given two weeks of exercise prior to cued fear conditioning and then two weeks without exercise after cued fear conditioning showed stronger fear-potentiated startle than mice who had never exercised. This suggests that exercise facilitated cued fear learning. It will be interesting to see if the same holds true for eyeblink conditioning.

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Fig. 1. Percentage of eyeblink conditioned responses as a function of conditioning session in Experiment 1. Error bars are SEM.

Fig. 2. Unconditioned response amplitude (peak of the eyeblink 65-165 ms after periorbital stimulation onset; the first 65 ms is obscured by the stimulation artifact) in Experiment 1 as a function of: (A) 10 trial block of CS-US trials in Session 1; (B) Trial in Block 1 of Session 1. Error bars are SEM.

Fig. 3. Percentage of eyeblink responses as a function of pseudoconditioning session in Experiment 2 for (A) Tone-alone trials; (B) Light-alone trials. Error bars are SEM.

Fig. 4. Unconditioned response amplitude (peak of the eyeblink 65-165 ms after periorbital stimulation onset; the first 65 ms is obscured by the stimulation artifact) in Experiment 2 as a function of Session. Error bars are SEM.

Fig. 5. Percentage of eyeblink conditioned responses as a function of conditioning session in Experiment 3. Error bars are SEM.

Fig. 6. Unconditioned response amplitude (peak of the eyeblink 65-165 ms after periorbital stimulation onset; the first 65 ms is obscured by the stimulation artifact) in Experiment 3 as a function of: (A) 10 trial block of CS-US trials in Session 1; (B) Trial in Block 1 of Session 1. Error bars are SEM.