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Cerebellar Structure and Function in Male Wistar-Kyoto Hyperactive Rats

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Abstract

Previous research has suggested that the Wistar-Kyoto Hyperactive (WKHA) rat strain may model some of the behavioral features associated with attention-deficit/hyperactivity disorder (ADHD). We have shown that, in cerebellar-dependent eyeblink conditioning, WKHA emit eyeblink CRs with shortened onset latencies. To further characterize the shortened CR onset latencies seen in WKHA rats, we examined 750-ms delay conditioning with either a tone CS or a light CS, we extended acquisition training, and we included Wistar rats as an additional, outbred control strain. Our results indicated that WKHAs learned more quickly and showed a shortened CR onset latency to a tone CS compared to both Wistar-Kyoto Hypertensive (WKHT) and Wistars. WKHAs and Wistars show a lengthening of CR onset latency over conditioning with a tone CS and an increasing confinement of CRs to the later part of the tone CS (inhibition of delay). WKHAs learned more quickly to a light CS only in comparison to WKHTs and showed a shortened CR onset latency only in comparison to Wistars. Wistars showed an increasing confinement of CRs to the late part of the light CS over conditioning. We used unbiased stereology to estimate the number of Purkinje and granule cells in the cerebellar cortex of the three strains. Our results indicated that WKHAs have more granule cells than Wistars and WKHTs and more Purkinje cells than Wistars. Results are discussed in terms of CS processing and cerebellar cortical contributions to EBC.

Keywords

WKHA; eyeblink classical conditioning; Purkinje cells; granule cells; timing; inhibition of delay; stereology

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder behaviorally characterized by inattention, hyperactivity, and impulsivity (American Psychiatric Association; 2000, DSM-IV TR, 2000). Such symptoms are usually expressed in the form of distractibility, difficulty in sustaining attention, and failure to appropriately control motor responses, which leads to significant impairments in social and academic functioning.

A number of studies using magnetic resonance imaging (MRI) techniques have found abnormalities in cerebellar structure in individuals diagnosed with ADHD (Ashtari, Kumra,

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Bhaskar, Clarke, Thaden, Cervellione et al., 2005; Berquin, Giedd, Jacobsen, Hamburger, Krain, Rapoport, & Castellanos, 1998; Bledsoe, Semrud-Clikeman, & Pliszka, 2009, 2011; Castellanos, Giedd, Berquin, Walter, Sharp, Tran et al., 2001; Castellanos, Lee, Sharp, Jeffries, Greenstein, Clasen et al., 2002; Mackie, Shaw, Lenroot, Pierson, Greenstein, Nugent et al., 2007; Montes, Ricardo-Garcell, De la Torre, Alcantara, Garcia, Acosta, & Bouzas, 2011). A meta-analysis of published MRI studies on ADHD revealed that the brain regions that showed the largest differences between participants diagnosed with ADHD and controls were cerebellar regions, the splenium of the corpus callosum, total and right cerebral volume, the right caudate nucleus, and several frontal regions (Valera, Faraone, Murray, & Seidman, 2007). A longitudinal assessment of cerebellar volume in ADHD concluded that individuals diagnosed with ADHD with a worse clinical outcome showed a progressive bilateral loss of volume of the inferior-posterior cerebellar lobules compared to both controls and individuals diagnosed with ADHD with a better clinical outcome (Mackie et al., 2007).

People diagnosed with ADHD are impaired in a range of neuropsychological procedures that examine cerebellar-dependent time perception skills. Among the most consistent findings are deficits in time discrimination of intervals that range from milliseconds to seconds (Smith, Taylor, Rogers, Newman, & Rubia, 2002). Although less consistently reported, overestimation (Rubia, Noorloos, Smith, Gunning, & Sergeant, 2003) and under-reproduction of time intervals (Barkley, Edwards, Laneri, Fletcher, & Metevia, 2001; McInerney & Kerns, 2003) have also been associated with ADHD. Such empirical observations correlate with behavioral impulsiveness in several studies (Toplak, Dockstader, & Tannock, 2006), suggesting that such individuals experience time faster than healthy controls.

Another procedure that requires the cerebellum and involves precisely timed motor responses is eyeblink conditioning (EBC). In this form of Pavlovian conditioning, organisms learn to blink (the conditioned response; CR) to a conditioned stimulus (CS) such as a tone in anticipation of an unconditioned stimulus (US) such as a mild electrical stimulation to the eyelid or an airpuff to the cornea. Alterations (e.g., shortening of CR latency) of eyeblink CR timing have been reported in children with ADHD (Coffin, Baroody, Schneider, & O'Neill, 2005; Frings, Gaertner, Buderath, Gerwig, Christiansen, Schoch et al., 2010). Our laboratory has reported similar alterations in eyeblink CR timing in two proposed animal models of ADHD, the spontaneously hypertensive rat (SHR; Chess & Green, 2008) and the Wistar-Kyoto Hyperactive (WKHA) rat (Thanellou, Schachinger, & Green, 2009).

One criticism that has been posed regarding the validity of the SHR strain as an animal model of ADHD is that SHRs are not only hyperactive but also hypertensive. It is thus possible that the behaviors of SHRs that seem to parallel the behavioral symptoms of ADHD are not due solely to hyperactivity, but instead are the net outcome of the interaction between hyperactivity and hypertension. WKHA and Wistar-Kyoto Hypertensive (WKHT) rats have been derived by crossing the SHR strain and its progenitor Wistar-Kyoto (WKY) strain and recombinant selective inbreeding (Hendley & Ohlsson, 1991) in an effort to isolate the hyperactive (WKHA) from the hypertensive (WKHT) phenotype in SHRs, since these two phenotypes are not genetically linked (Hendley, Atwater, Myers, & Whitehorn, 1983). Since the diagnosis of ADHD is largely behavioral, the face validity of a proposed animal model for the study of the particular clinical condition becomes an essential goal.

The purpose of the present experiments was to further analyze the behavioral properties of the abnormal eyeblink CR timing in WKHA rats, as well as to begin to investigate the anatomical underpinnings of this abnormal timing by using unbiased stereology to estimate the number of Purkinje cells (PCs) and granule cells (GCs) in cerebellar cortex.

Experiment 1

We have previously demonstrated that male, but not female, WKHA rats exhibit shortened CR onset latencies in comparison to WKHT rats when trained with 750-ms delay EBC (Thanellou et al., 2009). In the current experiment, we sought to replicate and expand on our previous work with male WKHAs in three ways. First, we compared the performance of male WKHAs and WKHTs to normoactive, normotensive Wistar rats. Second, we gave rats extended EBC. This allowed us to test for inhibition of delay (IOD). Inhibition of delay is a progressive shift of CR latency closer to US onset across sessions (Pavlov, 1927; Rescorla, 1967; Vogel, Brandon, & Wagner, 2003). Data from our laboratory has suggested that Wistar rats exhibit this phenomenon in long-delay conditioning when given extended training (Thanellou & Green, unpublished observations). If WKHA rats express eyeblink CRs with shorter latencies and exhibit IOD across training, it would suggest that EBC-related cerebellar cortical plasticity that shapes CR timing over training is normal in WKHA rats (Medina, Garcia, Nores, Taylor, & Mauk, 2000; Schonewille, Belmeguenai, Koekkoek, Houtman, Boele, van Beugen et al., 2010) and their timing abnormalities might lie elsewhere, such as in having fewer GCs or PCs. For example, Medina et al. (2000) proposed that IOD is governed by the gradual development of long-term potentiation at GC-to-PC synapses active early after CS onset, which suppresses interpositus nucleus activity and the generation of CRs early in the CS-US interval. Third, we compared performance of WKHAs, WKHTs, and Wistars to a tone CS versus a light CS to determine whether or not the abnormal response timing of WKHAs is modality-specific. We chose a tone and a light since these are the two most common types of CSs used in EBC and the cerebellum is critical for EBC to either a tone CS or a light CS (Freeman & Steinmetz, 2011). If the shortened eyeblink CR latencies that we have previously observed in male WKHAs to a tone CS (Thanellou et al., 2009) are due exclusively to alterations in cerebellar processing, we would expect to observe shortened eyeblink CR latencies regardless of CS modality.

Methods and Materials

Subjects—Wistar rats were obtained from Harlan (Indianapolis, IN). WKHA and WKHT rats were obtained from Dr. Edith Hendley at the University of Vermont. All animals were 90–100 days old at the time of surgery. Wistars weighed 330–370 g, WKHAs weighed 250–320 g and WKHTs weighed 320–380 g. Rats were housed in groups of 2 to 4 per cage prior to surgery with ad libitum chow and water. After surgery, rats were single-housed. The colony was maintained on a 12 hour light-dark cycle (lights on at 7 am).

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 eyeblinks 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. Rats were given 6–7 days to recover prior to EBC.

Apparatus—EBC took place in one of four identical testing chambers (30.5 × 24.1 × 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 × 91.4 × 50.8 cm; BRS-LVE, Laurel, MD). A fan in each sound-attenuating chamber provided background noise of approximately 60–65 dB sound pressure level (SPL). A speaker was mounted in each corner of the rear wall and a light 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.

A 765 ms, 2800 Hz, 80 dB tone, delivered through the left speaker of the sound-attenuating chamber, served as the tone CS. A 765 ms, 25 lux houselight served as the light CS. 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. 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 (Version 5, 2003). The eyelid EMG signals 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. 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.

Procedure—WKHA, WKHT and Wistar male rats were used in this experiment. Half of the rats of each strain were conditioned using a tone CS (Delay Tone) and the other half of the rats of each strain were conditioned using a light CS (Delay Light). Rats from within a strain were randomly assigned to Group Delay Tone or Group Delay Light.

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). During acquisition, rats were trained using 750 ms delay EBC task for 15 sessions (Days 2–16). We administered 100 trials per session at an average intertrial interval (ITI) of 30 sec (range = 20–40 sec). Trials consisted of a 765 ms tone (Delay Tone groups) or light (Delay Light groups) (CS) which coterminated with a 15 ms, 4.0 mA eyelid stimulation (US), with the exception of the first trial of each 10 trial block, which was a CS-alone trial (for examination of long latency blinks).

Behavioral Data Analysis—For conditioning sessions, paired 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; and (4) a US period, 65–165 ms after US onset. Eyeblinks 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. 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. CR acquisition was measured by percentage of CRs during the CR period across all trials within each session. The latency of the CR was measured by CR onset latency (the timepoint after the startle period at which the CR first crossed the 0.5 unit threshold) during the CR period across paired trials in which a CR was emitted, and by CR peak latency during CS-alone probe trials. CR peak latency was somewhat variable due to the small sample size but generally agreed with the results from CR onset latency measured on paired trials. In the interest of space, we describe only the CR onset latency results.

We computed all statistical analyses using SPSS 20.0. An alpha level of 0.05 was set as the rejection criterion for all statistical tests, except where indicated.

Results

Of all animals trained in this experiment, two animals were excluded from data analysis due to questionable EMG signals (1 Delay Tone WKHT, 1 Delay Light Wistar) and 1 was excluded due to an equipment failure (Delay Light WKHA). The final numbers of animals included were: Delay Tone groups: WKHA [$n=9$], WKHT [$n=8$], Wistar [$n=10$]; Delay Light groups: WKHA [$n=9$], WKHT [$n=10$], Wistar [$n=9$]. Figure 1 shows an example trial from Session 5 and Session 15 for each strain for the tone CS.

Measures of Nonassociative Responding—The amplitude of the UR was analyzed during Session 1 to address the possibility that strains differed in terms of sensitivity to the periorbital stimulation US. UR amplitude was measured 65–165 ms after the onset of the 15 ms US (the first 65 ms after US onset was obscured by the US artifact). A one-way ANOVA did not reveal any significant differences between strains trained either with a tone or a light CS (p 's > 0.05).

To investigate whether strains differed in terms of startle responses to the tone or light CS, we analyzed the percentage of eyeblink responses exceeding 0.5 in amplitude above baseline that occurred during the first 80 ms following CS onset across all sessions. Analyses revealed no significant effects (p 's > 0.05), indicating that all strains showed similar levels of startle responses to the tone or the light CS throughout training.

Adaptation—Analyses were conducted to evaluate the rates of spontaneous blinking across strains during the initial adaptation day (Figure 2, “adapt”). Analyses showed no significant effects between strains in percentage of spontaneous blinks (p 's > 0.05).

Acquisition: Tone CS—In tone CS EBC, WKHA rats outperformed WKHT and Wistar rats (Figure 2A). Percentages of CRs were analyzed with a 3 (strain) $\times$ 15 (session) repeated-measures ANOVA. This analysis revealed a significant effect for session, $F(4, 336) = 7.546$, $p < 0.001$, a significant effect for strain, $F(2, 24) = 7.118$, $p < 0.01$, and a non-significant interaction effect ($p > 0.05$). To further analyze the significant main effect of strain we used a series of independent-samples t -tests to compare pairs of strains. This analysis revealed significant differences in percentage of CRs to the tone CS between Wistar and WKHA rats ($p < 0.001$), and between WKHA and WKHT rats ($p = 0.01$) but not between Wistar and WKHT rats.

Additional analyses of within-session performance revealed that WKHAs showed a within-session increase in percentage of CRs to the tone CS (trials 1–20 vs. trials 81–100) from the beginning to the end of session 1 (Figure 2A). In subsequent sessions, WKHAs exhibited the same high percentage of CRs at the beginning and the end of the session. In contrast, WKHTs tended to show either no change or a within-session decrease in percentage of CRs and Wistars showed no change in within-session percentage of CRs (i.e., acquisition was strictly between sessions).

These observations were confirmed by a series of three (one per strain) 2 (first vs. last block) $\times$ 15 (session) repeated-measures ANOVAs on percentage of CRs. For WKHA rats, there was a significant effect for session, $F(14, 112) = 7.268$, $p < 0.01$, and a significant interaction effect, $F(14, 112) = 4.611$, $p < 0.001$. To further analyze the interaction effect, we conducted paired samples t -tests that revealed significant differences between first and last blocks only in session 1 ($p < 0.01$). For WKHT rats, there was a significant effect for session, $F(14, 98) = 5.549$, $p < 0.001$, and a significant interaction effect, $F(14, 98) = 5.949$, $p < 0.001$. Paired samples t -tests revealed significant differences between first and last blocks in sessions 1, 5–8, 14, and 15 (p 's < 0.05). For Wistar rats, there was a significant effect for session, $F(14, 126) = 3.904$, $p < 0.001$, and a significant interaction effect, $F(14,$

126) = 2.185, $p < 0.05$; however, paired samples t-tests failed to reveal a significant difference between first and last blocks in any session (p 's > 0.05).

Acquisition: Light CS—In light CS EBC, WKHA rats outperformed WKHT but not Wistar rats (Figure 2B). Percentages of CRs were analyzed with a 3 (strain) $\times$ 15 (session) repeated-measures ANOVA. Analysis revealed a significant effect for session, $F(14, 350) = 10.953$, $p < 0.001$, a significant effect for strain, $F(2, 25) = 3.749$, $p < 0.05$, and a non-significant interaction effect ($p > 0.05$). To further analyze the significant main effect of strain we used a series of independent-samples t-tests to compare pairs of strains. This analysis revealed significant differences in percentage of CRs to the light CS between WKHA and WKHT rats ($p = 0.01$) but not between WKHA and Wistar rats, or Wistar and WKHT rats (p 's > 0.05).

Additional analyses of within-session performance revealed that WKHAs, in contrast to EBC with the tone CS, showed a within-session *decrease* in percentage of CRs (trials 1–20 vs. trials 81–100) to the light CS across all sessions (Figure 2B). As with the tone CS, WKHTs tended to show either no change or a within-session decrease in percentage of CRs to the light CS and Wistars showed no change in within-session percentage of CRs (i.e., acquisition was strictly between sessions).

These observations were confirmed by a series of three (one per strain) 2 (first vs. last block) $\times$ 15 (session) repeated-measures ANOVAs on percentage of CRs. For WKHA rats, there was a significant effect for session, $F(14, 112) = 7.403$, $p < 0.01$, and a significant effect for block, $F(1, 8) = 22.306$, $p < 0.001$ but the interaction effect was not significant. For WKHT rats, there was a significant effect for session, $F(14, 126) = 6.358$, $p < 0.001$, a significant effect for block, $F(1, 14) = 79.383$, $p < 0.001$, and a significant interaction effect, $F(14, 126) = 1.962$, $p < 0.05$. Paired samples t-tests revealed significant differences between first and last blocks in sessions 3, 4, 6–11, and 13–15 (p 's < 0.05). For Wistar rats, there was a significant effect only for session, $F(14, 112) = 2.358$, $p < 0.001$, indicating acquisition was strictly between sessions.

CR Onset Latency: Tone CS—Six animals (across strains) did not emit CRs in one or more sessions across sessions 1–4. In addition, all strains had reached asymptotic percentage of CRs by session 5. Thus, we analyzed the CR onset latency data in sessions 5–15. WKHA rats showed shorter CR onset latencies to a tone CS compared to WKHT and Wistar rats (Figure 3A). WKHT rats showed shorter CR onset latencies compared to Wistar rats.

The overall ANOVA for CR onset latency in the Delay Tone groups revealed a significant effect for session, $F(10, 240) = 7.296$, $p < 0.001$, a significant effect for strain, $F(2, 21) = 24.220$, $p < 0.001$, and a non-significant interaction effect ($p > 0.05$). To further analyze the significant main effect of strain we used a series of independent-samples t-tests to compare pairs of strains. This analysis revealed significant differences in CR onset latency to the tone CS between Wistar and WKHA rats ($p < 0.001$), WKHA and WKHT rats ($p = 0.01$), and Wistar and WKHT rats ($p = 0.022$).

We performed additional analyses of within-session performance in sessions 5–15. For these analyses, some animals from each strain were excluded due to an absence of CRs in some blocks of some sessions. The final numbers of Delay Tone animals used in the statistical analysis were: WKHA [$n=9$], WKHT [$n=5$], Wistar [$n=3$]. Within-session analyses of CR onset latency to the tone CS revealed that WKHA rats showed a shorter CR onset latency at the beginning of sessions compared to the end. WKHT and Wistar rats did not show this difference.

These observations were confirmed by a series of three (one per strain) 2 (first vs. last block) x 11 (session) repeated-measures ANOVA on CR onset latency to the tone CS. For WKHA rats, there was a significant effect for session, $F(10, 80) = 7.820$, $p < 0.001$, and a significant effect for block, $F(1, 8) = 39.431$, $p < 0.001$ but the interaction effect was not significant. For WKHT and Wistar rats, there were no significant effects.

CR Onset Latency: Light CS—Three animals (across strains) did not show CRs during sessions 1–4 and, as for the tone CS, all strains had reached asymptotic percentage of CRs by session 5; thus, we analyzed CR onset latency only in sessions 5–15. Both WKHA and WKHT rats showed shorter CR onset latencies to a light CS compared to Wistar rats (Figure 3B). WKHAs and WKHTs did not differ.

The overall ANOVA for CR onset latency in the Delay Light groups revealed a significant effect for session, $F(10, 250) = 3.967$, $p < 0.01$, a significant effect for strain, $F(2, 25) = 7.087$, $p < 0.01$, and a non-significant interaction effect ($p > 0.05$). To further analyze the significant main effect of strain we used a series of independent-samples t-tests to compare pairs of strains. This analysis revealed significant differences in CR onset latency to the light CS between Wistar and WKHA rats ($p = 0.029$), and WKHT and Wistar rats ($p = 0.001$), but not between WKHA and WKHT rats ($p > 0.05$).

We performed additional analyses of within-session performance in sessions 5–15. For these analyses, some animals from each strain were excluded due to an absence of CRs in some blocks of some sessions. The final numbers of animals used in the statistical analysis were: WKHA [$n=5$], WKHT [$n=6$], Wistar [$n=3$]. Within-session analyses of CR onset latency to the light CS revealed that both WKHA and WKHT rats showed a shorter CR onset latency at the beginning of sessions compared to the end. Wistar rats did not show this difference.

These observations were confirmed by a series of three (one per strain) 2 (first vs. last block) x 11 (session) repeated-measures ANOVA on CR onset latency to the light CS. For WKHA rats, there was a significant effect for block, $F(1, 4) = 54.588$, $p < 0.01$. For WKHT rats, there was a significant effect for session, $F(10, 50) = 4.619$, $p < 0.001$ and block, $F(1, 5) = 8.491$, $p < 0.05$. For Wistar rats, there were no significant effects.

Inhibition of Delay Analysis: Tone CS—We also examined IOD, defined here as the gradual increase in eyeblink CR onset latency across acquisition (Vogel et al., 2003). For the IOD analysis, we excluded animals (1 WKHT rat, 2 Wistar rats) that exhibited less than 20% CRs across all acquisition days. The final numbers of animals used in the IOD analysis for the tone CS were: WKHA [$n=9$], WKHT [$n=7$], Wistar [$n=8$]. We used two measures of IOD. First, a longer CR onset latency in session 15 compared to session 5 was used to indicate IOD. Session 5 was chosen because it was the earliest session where all rats exhibited CRs and all strains had achieved an asymptotic percentage of CRs. Second, a greater percentage of CRs in the late part of the CS compared to the early part of CS as training progressed was used to indicate IOD. For the tone CS, we defined the early CS Period as 81–350 ms after CS onset and the late CS Period as 481–750 ms after CS onset. Both of these measures revealed IOD to the tone CS in WKHA and Wistar rats, but not in WKHT rats.

For the first IOD measure, WKHA and Wistar rats, but not WKHT rats, exhibited a longer CR onset latency in session 15 compared to session 5 (Figure 4). This was confirmed statistically. Differences in CR onset latencies in Delay Tone groups between acquisition days 5 and 15 were significant in WKHA rats, $t(8) = 5.845$, $p < 0.001$, and Wistar rats, $t(7) = 2.448$, $p < 0.05$, but were not significant in WKHT rats ($p > 0.05$).

We investigated the percentage of CRs elicited in the late vs. the early part of the CS as a second measure of IOD. These analyses again revealed that WKHAs and Wistars, but not WKHTs, exhibited IOD to the tone CS (Figure 5A). Separate 2 (early CS Period vs. late CS period) $\times$ 15 (session) repeated-measures ANOVAs were conducted on each strain. For WKHAs, a significant period $\times$ session interaction effect was revealed, $F(14, 112) = 4.58$, $p < 0.05$. Further investigation with a series of paired t-tests and a Bonferroni-corrected alpha level of 0.0033 (0.05/15) revealed that there were significantly more CRs in the late CS period compared to the early CS period in Sessions 8–15 (p 's < 0.0033), with no differences in Sessions 1–7. This is strongly indicative of IOD in WKHAs. For WKHTs, there was only a main effect of CS period, $F(1, 84) = 43.27$, $p < 0.05$ with significantly more CRs in the late CS period across sessions. For Wistars, a significant period $\times$ session interaction effect was revealed, $F(14, 98) = 6.23$, $p < 0.05$. Further investigation with a series of paired t-tests revealed that there were significantly more CRs in the late CS period compared to the early CS period in Sessions 5–15 (p 's < 0.0033), with no differences in Sessions 1–4.

Inhibition of Delay Analysis: Light CS—For the IOD analysis of the light CS, we again excluded animals (1 Wistar rat) that exhibited less than 20% CRs across all acquisition days. For the light CS we modified the time interval for the early part of the CS (i.e., 210–480 ms instead of 81–350 ms) since CRs to the light CS tended to have longer latency. The reason for this difference is unclear. One possibility is that the tone CS was more salient, and therefore perceived as more intense, than the light CS. Greater tone CS intensity produces shorter latency CRs (Kehoe, Schreurs, Macrae, & Gormezano, 1995; Leonard & Monteau, 1971). Another possibility is that the light CS pathway to the cerebellum is longer than the tone CS pathway (see Freeman & Steinmetz, 2011 for a review of these pathways).

We did not see evidence for IOD to the light CS in WKHAs or WKHTs in either of our two measures of IOD. In contrast, we did see evidence for IOD to the light CS in Wistars in our second measure of IOD. For the first IOD measure, none of the 3 strains exhibited a longer CR onset latency in session 15 compared to session 5 (Figure 4). This was confirmed statistically. Differences in CR onset latencies in Delay Light groups between acquisition days 5 and 15 were not significant in any strain (p 's > 0.05). For the second IOD measure, there was a significant period $\times$ session interaction effect for WKHAs, $F(14, 112) = 3.06$, $p < 0.05$ but follow-up paired t-tests revealed a significantly greater percentage of CRs in the late CS period only in sessions 6, 10, and 12 (p 's < 0.0033). For WKHTs, there was a significant period $\times$ session interaction effect, $F(14, 126) = 3.29$, $p < 0.05$ but follow-up paired t-tests revealed a significantly greater percentage of CRs in the late CS period only in session 6 ($p < 0.0033$). For Wistars, there was a significant period $\times$ session interaction effect, $F(14, 112) = 3.14$, $p < 0.05$ and follow-up paired t-tests revealed a significantly greater percentage of CRs in the late CS period in sessions 10–15 (p 's < 0.0033), suggesting IOD to the light CS (Figure 5B).

Experiment 2

In Experiment 2 we used unbiased stereology to estimate the numbers of cerebellar GCs and PCs in lobules IV–VII of cerebellar cortex in WKHA, WKHT and Wistar rats. These areas of cerebellar cortex have been shown to modulate acquisition and timing in EBC (Freeman & Steinmetz, 2011; Thompson & Steinmetz, 2009).

Methods and Materials

Subjects—Cerebella from a total of 21 male rats (WKHA, WKHT, Wistar; $n=7$ /strain) were used. These represented a separate group of rats from Experiment 1.

Histology—Rats were overdosed with sodium pentobarbital (150 mg/kg, i.p.) and transcardially perfused with 0.9% saline followed by 10% formalin. The brain was removed and stored in 10% formalin until preparation for cell counting.

Prior to cell counting, each cerebellum was carefully separated from the rest of the brain, and then bisected mid-sagittally. Over the course of 9 days, each left half of the cerebellum was gradually dehydrated and then infiltrated with increasing concentrations of embedding solution (glycol methacrylate). On the ninth day, the tissue block was hardened in a small mold. Each block was sectioned exhaustively at 30 μm in the sagittal plane using a rotary microtome and glass knives. Beginning with a random start (within an interval of 1–8), every eighth section was saved for counting. Sections were mounted on gelatin-subbed slides, dried overnight, and stained with cresyl violet.

Procedure—All aspects of cell counting were done with the counter (AT) blind to strain condition. Counting was accomplished using the Stereo Investigator system (MicroBrightField Bioscience, Williston, VT) controlling a computer-driven microscope fitted with a 60x oil objective (n.a. = 1.4) and a microcator for z-axis measurements. Our sampling area comprised cerebellar lobules IV–VII in the left cerebellum (Figure 6A).

The methodology that we employed to estimate numbers of cerebellar GCs and PCs in WKHAs WKHT, and Wistar males was unbiased stereology. Both GCs and PCs were counted with three-dimensional probes (optical disectors). An unbiased estimate of total neuron number was calculated using the following formula:

$$N = \sum Q^- * 1/\text{ssf} * 1/\text{asf} * 1/\text{tsf}$$

where $\sum Q^-$ is the total number of neurons counted over all sections sampled, ssf is the section sampling fraction (i.e., number of sections saved / total number of sections through the structure), asf is the area sampling fraction (i.e., counting frame area / (x step * y step)), and tsf is the thickness sampling fraction (i.e., disector height / average measured section thickness). For the estimation of the number of PCs, the counting frame area was 3446 μm^2 (58.7 $\mu\text{m} \times 58.7 \mu\text{m}$) and x and y steps were both 587 μm . Disector height was 13 μm . The guard height at the top of the disector was 3 μm . For the estimation of GCs, the counting frame area was 144 μm^2 (12 $\mu\text{m} \times 12 \mu\text{m}$) and x and y steps were both 120 μm . Disector height was 13 μm . The guard height at the top of the disector was 3 μm . Nominal section thickness was 30 μm . Figure 6 shows examples of GCs and PCs counted in this experiment.

Cells in the very well delineated Purkinje cell layer were classified as countable objects if a distinct nucleolus, cytoplasmic border and Nissl bodies were visible, and were counted if the cells were within the height of the disector and met criteria for the counting frame (Andersen, Gundersen, & Pakkenberg, 2003). The morphology of cells in the GC layer is quite uniform in terms of cell size, the presence of a distinct cytoplasmic border and Nissl bodies, and the observation that nuclei form the major constituent of the cell body (Andersen, et al., 2003). To this effect, GCs were classified as countable objects if a distinct cytoplasmic border and Nissl bodies were visible, and were counted if the cells were within the height of the disector and met criteria for the counting frame. Sporadic encounters of other cell types in the cerebellar GC layer that appeared significantly larger than typical GCs were excluded from counting. Such larger cells could either be Golgi cells or glial cells (glial cells appear to have lighter staining and the presence of a larger and ellipsoidal nucleus; Andersen et al., 2003). The possibility that unipolar brush cells were included in our counts is an unlikely one since this cell type is mostly concentrated in lobule IX, the flocculus, the nodulus, and the ventromedial zone of the paraflocculus whereas our

stereological counting was conducted in lobules IV–VII (Kalinchichenko & Okhotin, 2005). Nevertheless, since their somata are larger than GCs (but smaller than Golgi cells) (Kalinchichenko & Okhotin, 2005) they would still not meet the morphological criteria and would have been excluded. Last but not least, it is unlikely that our GCs counts were inflated by counting stellate or basket cells since these cell types are mainly found in the molecular layer. The distinct morphology of PCs left not doubt that countable objects were only PCs and not smaller stellate or basket cells.

Results

Surprisingly, WKHA rats had more, rather than fewer, GCs than WKHT and Wistar rats (Figure 7A) and more, rather than fewer, PCs than Wistar rats (Figure 7B). A one-way ANOVA was conducted comparing groups on the estimated total number of neurons for each cerebellar neuronal population. The analysis for PCs revealed a difference that approached significance, $F(2, 18) = 2.746$, $p = 0.091$. A series of three *a priori* contrasts revealed significant differences between WKHA and Wistar rats, $t(18) = 2.252$, $p < 0.05$. There were no significant differences between WKHA and WKHT rats or between WKHT and Wistar rats (p 's > 0.05). The analysis for the cerebellar GCs revealed a significant group difference, $F(2, 18) = 3.883$, $p < 0.05$. A series of three *a priori* contrasts revealed significant differences between WKHA and Wistar males, $t(18) = 2.614$, $p < 0.05$, as well as between WKHA and WKHT males, $t(18) = 2.143$, $p < 0.05$. There were no significant differences between Wistar and WKHT males ($p > 0.05$).

We also compared the number of GCs per PC across strains. A one-way ANOVA revealed that differences in the ratio of GCs to PCs approached significance, $F(2, 18) = 2.791$, $p = 0.088$. *A priori* contrasts revealed that ratios were not significantly different between WKHA rats (241.75 ± 12.76 GCs per PC) and WKHT rats (217.41 ± 6.62 GCs per PC) or between WKHA and Wistar rats (259.36 ± 19.73 GCs per PC), but there were significantly fewer GCs per PC in WKHT rats compared to Wistar rats, $t(18) = 2.362$, $p < 0.05$.

General Discussion

Male WKHAs learned more quickly and showed a shortened CR onset latency to a tone CS compared to both WKHTs and Wistars. These shortened CR onset latencies were not the result of an increase in startle responses to the tone, which is consistent with both our previous study (Thanellou et al., 2009) and a previous study in which WKHA rats showed normal prepulse inhibition (Drolet, Proulx, Pearson, Rochford, & Deschepper, 2002). Male WKHA rats' shortened CR onset latency to a tone CS compared to male WKHT rats replicates our previous study (Thanellou et al., 2009). The shorter CR onset latencies to the tone CS in the WKHA rats were especially prominent in the first 20 trials of each acquisition session. Furthermore, WKHAs and Wistars showed IOD to a tone CS in the form of a lengthening of CR latency over EBC and the development of differential CR expression between the early and late periods of the tone CS as training progressed.

In contrast to the tone CS, conditioning with a light CS was not systematically different in WKHAs; WKHAs exhibited a greater percentage of CRs compared to WKHTs but not compared to Wistars and a shortened CR onset latency compared to Wistars but not compared to WKHTs. In addition, WKHAs did not exhibit differential CR expression between the early and late periods of the light CS (in contrast to the tone CS data). Wistars showed evidence of IOD defined as the development of differential CR expression between the early and late periods of the tone CS as training progressed, but not in terms of a progressive shift of CR onset latency. It is possible that the development of IOD defined as a gradual shift of CR onset latency closer to the US as a function of acquisition training is slower with a light CS, and that Wistars in particular would benefit from further training.

It appears that the shorter CR latencies to a tone CS exhibited by WKHA rats throughout training may be due to deficits in eyeblink CR performance rather than to a deficit in learning the CS-US interval, since WKHA rats showed normal IOD. WKHA rats apparently acquire information regarding the duration of the long CS-US interval as evidenced by a shift of their CR onset latency closer to the US as a function of training (i.e., learned timing). However, WKHAs appear to have difficulty making full use of this learned timing. Two additional things are important to note here. First, we observed IOD (a shift in CR timing) after asymptotic performance had already been achieved. This suggests that EBC involves learning not to respond at “incorrect” times (cf. Drew, Zupan, Cooke, Couvillon, & Balsam, 2005). Second, WKHAs are not more responsive to the onset of the auditory CS since they show no more startle responses than other strains. Instead, it is their eyeblink CRs to the auditory CS that are rapid.

To our knowledge, this is the first demonstration of IOD in rat EBC. IOD in EBC has only recently been demonstrated in rabbits (Vogel et al., 2003) although it was demonstrated in humans a number of years ago (e.g., Boneau, 1958). In contrast, IOD is a well-established phenomenon in conditioned suppression (Hendry & Van Toller, 1965; Rosas & Alonso, 1996, 1997; Schachtman, Channell, & Hall, 1987) and appetitive conditioning (Bouton et al., 2008; Kirkpatrick & Church, 2000). In theory, there are two types of behavioral inhibition that may subserve the development of IOD; either conditioned inhibition develops to the early part of the CS (i.e., the early portion of the CS becomes a conditioned inhibitor; cf. Rescorla, 1967), or extinction occurs to the early part of the CS (i.e., the early part of the CS has both excitatory and inhibitory properties; cf. Medina et al., 2000). In a series of experiments (Thanellou & Green, unpublished observations) we explored the behavioral mechanisms that underlie the development of IOD in normoactive and normotensive Wistar rats. In those experiments, rats were trained for 15 sessions in a 750-ms delay paradigm using either a tone CS or a light CS. As in the current study, responding during the early part of either CS declined to low levels by the end of training. Subsequently, all rats were transferred to a 250-ms delay paradigm using a tone CS (retardation test). Our results suggested that the early part of the 750-ms tone CS acted as an extinguished CS and not as a conditioned inhibitor; rats that had been trained with the 750-ms tone CS showed a higher (rather than a lower) percentage of CRs to the 250-ms tone CS compared to rats trained with the 750-ms light CS, suggesting the lack of conditioned inhibition and, possibly, a rapid reacquisition type of effect. This would suggest that the IOD we observed in WKHAs means that they undergo normal extinction of early CRs to a tone CS (although they continue to respond more in the early part of the CS than Wistars) and it is possible that, with enough training, their CR onset latencies to a tone CS would become comparable to WKHT and Wistar rats.

The finding that WKHAs have more PCs and GCs than Wistars is consistent with studies that have shown that the number or functionality of PCs and GCs predicts the rate of EBC. For example, selective elimination of PCs (Chen, Bao, Lockard, Kim, & Thompson, 1996; Nolan & Freeman, 2006) or deletion of Nav1.6 in PCs (Woodruff-Pak, Green, Levin, & Meisler, 2006) slows EBC. Blockade of PC input to the interpositus nucleus with picrotoxin slows EBC (Bao et al., 2002; Sakamoto & Endo, 2008; Vogel, Amundson, Lindquist, & Steinmetz, 2009). In addition, selective and reversible blockade of GC input to PCs impairs the expression of the eyeblink CR (Wada, Kishimoto, Watanabe, Kano, Hirano, Funabiki, & Nakanishi, 2007). In none of these cases, except for a small deficit reported in Chen et al. (1996), was CR latency affected. However, this does not explain why WKHAs did not show facilitated learning to a light CS compared to Wistar rats.

The more profound and consistent abnormalities in WKHAs in EBC with a tone CS compared to a light CS suggest that there may be abnormalities in auditory CS input to the

cerebellum in WKHAs. Freeman and colleagues have recently delineated the auditory and visual CS pathways for EBC (see Freeman & Steinmetz, 2011 for a review). The auditory CS pathway is hypothesized to consist of the cochlear nucleus, superior olive, nucleus of the lateral lemniscus, inferior colliculus, and medial auditory thalamic nuclei, with the lateral pontine nuclei serving as the final relay to the cerebellum. Our behavioral results suggest that WKHA rats may have greater tone CS input into the cerebellum than WKHTs and Wistars. Greater tone input to the cerebellum would explain the facilitated acquisition rate, earlier CR onset, and the difference between the tone and light data observed in WKHA rats. For example, increasing the intensity of a tone CS decreases CR onset latency and increases percentage of CRs in EBC (Frey, 1969; Kehoe et al., 1995; Leonard & Monteau, 1971; Scavio & Gormezano, 1974). Increasing the intensity of a forelimb stimulation CS or a mossy fiber stimulation CS also decreases CR onset latency in EBC (Svensson et al., 2010) and decreases the latency of the PC response to CS input (Svensson, Ivarsson, & Hesslow, 1997). Our protocol of extended training (15 acquisition sessions) may have allowed the cerebellum of WKHAs to partially correct response timing to this potentiated tone input (in the form of IOD development).

Rubia and colleagues have defined impulsivity as premature, poorly timed actions (Rubia, Halari, Christakou, & Taylor, 2009) and others have noted a strong association between response timing difficulties and ADHD (e.g., Toplak et al., 2006). Children with ADHD consistently show abnormalities in timing and temporal information processing that are hypothesized to rely on the cerebellum (Frings et al., 2010; Zelaznik, Vaughn, Green, Smith, Hoza, & Linnea, 2012; for a review see Toplak et al., 2006). Yet, the role of the cerebellum in producing the behavioral symptoms of ADHD is poorly understood. The timing of the eyeblink CR may provide an index of the cerebellar component of impulsivity. The WKHA rat may model some aspects of this cerebellum-related motor impulsiveness by virtue of a combination of abnormal auditory inputs to the cerebellum and an overabundance of cerebellar neurons.

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Figure 1.

Amplified raw eyelid EMG and full-wave rectified, smoothed (10-ms time constant) and timed-shifted (10-ms to compensate for smoothing) eyelid EMG of an eyeblink CR to the tone CS from a WKHA rat (A), a WKHT rat (B), and a Wistar rat (C).

Figure 2. Percentage of eyeblink conditioned responses out of 90 CS-US trials in WKHA, WKHT, and Wistar rats. Rats received 100 trials per session, with a CS-alone every tenth trial: (A) Trials consisted of a 765 ms, 2800 Hz, 80 dB tone that coterminated with a 15 ms, 4 mA periorbital stimulation. *Panel 1:* There was a significantly greater percentage of CRs in WKHA rats compared to WKHT and Wistar rats; *Panel 2:* Most of the CR acquisition occurred in session 1 in WKHA rats. Thereafter, percentage of CRs in WKHA rats was consistently high within each session; (B) Trials consisted of a 765 ms, 25 lux light that coterminated with a 15 ms 4 mA periorbital stimulation. *Panel 1:* There was a significantly greater percentage of CRs in WKHA rats compared to WKHT rats, but not compared to Wistar rats; *Panel 2:* Percentage of CRs in WKHA rats tended to decline from the beginning to the end of a session. Error bars are standard error of the mean (SEM).

Figure 3. Conditioned response onset latency (milliseconds from CS onset) in WKHA, WKHT, and Wistar rats: (A) Conditioning with a tone CS. *Panel 1:* There was a significantly shorter CR onset latency in WKHA rats compared to WKHT or Wistar rats; *Panel 2:* CR onset latency tended to grow longer over the course of a session in WKHA rats. *Panel 3:* CR onset latencies distributed in fifteen 50-ms bins across the 750-ms CS-US interval for Session 1, Session 5, and Session 15. Each bin is a sum of CRs with particular onset latency for rats of a strain across an entire conditioning session. (B) Conditioning with a light CS. *Panel 1:*

There was a significantly shorter CR onset latency in WKHA rats compared to Wistar rats, but not compared to WKHT rats. *Panel 2*: CR onset latency tended to remain fixed over the course of a session in WKHA rats. *Panel 3*: CR onset latencies distributed in fifteen 50-ms bins across the 750-ms CS-US interval for Session 1, Session 5, and Session 15. Each bin is a sum of CRs with a particular onset latency for rats of a strain across an entire conditioning session. Error bars are SEM.

Figure 4. Conditioned response onset latency (milliseconds from CS onset) in acquisition day 5 and acquisition day 15 in WKHA, WKHT, and Wistar rats: (A) Conditioning with a tone CS. There was a significantly longer onset latency in session 15 compared to session 5 in WKHA and Wistar rats (indicating inhibition of delay), but not in WKHT rats; (B) Conditioning with a light CS. There were no significant differences in onset latency in session 15 compared to session 5 in any strain. Error bars are SEM.

Figure 5.

Percentage of eyeblink conditioned responses as a function of conditioning session and early vs. late period during the CS: (A) Conditioning with a tone CS. There was a significantly higher percentage of CRs in the late period (481–750 ms after tone CS onset) of the tone CS compared to the early period (81–350 ms after tone CS onset) beginning in Session 8 for WKHAs and Session 5 for Wistars (indicating inhibition of delay). There were no significant differences between periods in WKHT rats; (B) Conditioning with a light CS. There was a significantly higher percentage of CRs in the late period (481–750 ms after light CS onset) of the light CS compared to the early period (210–480 ms after light CS onset) beginning in Session 10 for Wistar rats (indicating inhibition of delay). There were no systematic differences between periods in WKHA and WKHT rats. Error bars are SEM.

Figure 6.

Digital photomicrographs of a cresyl violet stained sagittal cerebellar section showing an example of the defined counting area for the left (ipsilateral to the eye that received the US) lobules IV–VII of the cerebellar cortex (yellow outlined areas; total magnification = 4x; scale bar = 1000 μm), and examples of the two different types of neurons included in the counts (black arrows = Purkinje cells; white arrows = granule cells; total magnification = 2000x; scale bar = 20 μm).

Figure 7. Mean estimated total number of neurons in the left (ipsilateral to the eye that received the US) cerebellar cortex of lobules IV–VII: (A) Estimated total number of Purkinje cells as a function of strain; (B) Estimated total number of granule cells as a function of strain. Estimates were made using the optical fractionator.