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Beyond Motor Function: The Role of the Cerebellum in Rodent
Cognitive Flexibility

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4/16/18

Abstract:

Individuals diagnosed with certain disease states, such as schizophrenia and autism, sometimes present with impaired behavioral flexibility. Such individuals also sometimes present with cerebellar abnormality, suggesting a possible role of the cerebellum in disease states characterized by deficits in cognitive flexibility. To further understand the role the cerebellum may play in such disease states and determine the role of the cerebellum in behavioral flexibility, we compared rats that underwent pharmacological inactivation of the cerebellum with control rats in their completion of a set-shifting T-maze task (Stefani et al., 2003). The T-maze task required the rats to learn to discriminate along one sensory dimension (e.g., color) on day 1 and then, on day 2, to be able to switch to discriminating along another sensory dimension (e.g., texture) and ignore the day 1 dimension (“set-shift”). Infusions were made into a region of cerebellar cortex prior to day 2 training. Comparison between vehicle infused rats and rats with cerebellar pharmacological inactivation showed that pharmacological inactivation did not significantly impact rodent ability to set-shift. The finding that pharmacological inactivation of a small region of the cerebellar cortex did not significantly impact rodent set-shifting suggests the possibility that either the rodent cerebellar cortex is not involved in set-shifting or that a different or larger region of the rodent cerebellum must be inactivated to affect set-shifting ability.

Introduction:

Automatic responses serve many important functions in everyday life; however, certain instances require individuals to deliberately direct attention and mental effort to taking in, processing, and adapting to available information. In such instances, individuals employ executive functions in order to adapt their behavior. There are three main executive functions:

inhibition, working memory, and cognitive flexibility (Diamond, 2013). It is these three main executive functions and the sub-processes related to them that allow an individual to repress impulse, adapt behavior, learn in response to relevant feedback, and, ultimately, to make a goal-oriented decision. Previous literature looking at executive functions strongly links these processes with the prefrontal cortex. While the prefrontal cortex has often been associated with executive function, it is possible that the cerebellum may also contribute to such functions.

Anatomy of the cerebellum

The cerebellum is found tucked underneath the posterior lobes of the cerebrum, lying posterior to the pons, and, like the cerebrum, can be divided into left and right hemispheres. Finely spaced, parallel grooves on the cerebellum's surface distinctly demarcate the cerebellum as separate from the cerebrum. These parallel grooves are produced from the accordion-style folding of the single, continuous layer of tissue that makes up the entirety of the structure (Vanderan & Gould, 2016).

The cerebellum can be divided into three cortical layers consisting of two layers of gray matter between which white matter lies. The most superficial layer of the cerebellum is the molecular layer, a band of gray matter consisting of cell bodies and unmyelinated axons. The main neuron types of this molecular layer are stellate/basket cells (Voogd & Glickstein, 1998). Purkinje cell bodies make up the next layer and Purkinje cells are the sole output of the cerebellar cortex. They project to deeper areas of the cerebellum and areas of the brainstem where they then synapse on cerebellar nuclei and certain brainstem nuclei (e.g., vestibular nuclei) respectively (Voogd & Glickstein, 1998). Below the Purkinje cell body layer lies the granule cell layer, which also contains Golgi cells. Below the granule cell layer of the cerebellar cortex lies the white matter of the cerebellum which is mainly made up of myelinated axons connecting the

neurons of the cerebellar cortex to the deep gray layer in which cerebellar nuclei—dentate, fastigial, globose, and emboliform—are found (Vanderah & Gould, 2016).

The cerebellum can be divided into three lobes— the flocculonodular (below the posterior fissure), anterior (in front of the primary fissure), and posterior (behind the primary fissure)—based on fissures (deep groves of tissue) seen on the surface. These three lobes can then be further subdivided into 10 lobules. Lobule X is found in the flocculonodular lobe; lobules I through V are grouped into the anterior lobe; the posterior lobe contains lobules VI through IX (D’Mello & Stoodley, 2015).

The deep cerebellar nuclei play an essential role in connecting the cerebellar cortex to the cerebrum. Connecting the cerebellum and the cerebrum is an afferent pathway and an efferent pathway. The afferent pathway from the cerebrum consists of the cortico-pontine and the ponto-cerebellar tracts, which project cerebral cortical information through the pontine nuclei on to the cerebellum through the middle cerebellar peduncle via mossy fibers. The efferent system from the cerebellum is made up of the cerebello-thalamic and the thalamo-cortical pathways, which project from the deep cerebellar nuclei to the thalamus through the superior cerebellar peduncle; the thalamus then projects on to the cerebral cortex. Projections from the cerebellum to the cerebrum terminate in both motor and non-motor areas of the cerebral cortex including regions of the prefrontal cortex (O’Halloran et al., 2011).

Cerebellum and Cognition

Previous studies have found activation of the cerebellar posterior lobe during executive function tasks including random number generation and the Tower of London tasks where executive processes such as response inhibition, information integration, and cognitive flexibility are tested (Stoodley & Schmahmann., 2010). Further expanding on the role of the cerebellar

posterior lobe in more than motor function, Stoodley et al. (2010) found that while motor impairments are observed in subjects with lesions in the anterior lobe of the cerebellum and lobule VI, cognitive impairments are observed in subjects with posterior lobe lesions affecting lobules VI and VII (including Crus I and Crus II). Such studies demonstrating increased activation of cerebellar regions during cognitive tasks and a dichotomy in cerebellar regions affecting motor vs. cognitive abilities suggest an important role of the cerebellum in far more than motor function (Stoodley et al., 2010).

The Wisconsin Card Sorting Task (WCST) is often used as a way to test higher level processes that fall under executive functions including flexibility, decision-making and feedback utilization (Lie et al., 2006). The WCST consists of stimulus cards that can be categorized by number, color, and shape (Nyhus et al., 2009). Through trial and error, subjects must correctly identify the relevant card-sorting category (e.g., by number) and ignore irrelevant categories (e.g., color; shape). After a number of consecutive matches, the category used for classification is suddenly changed and the subject must identify the shift in classification and identify the new sorting category. In identifying the new sorting category, subjects must also ignore the first sorting category, thus ignoring distracting card grouping possibilities. For example, subjects may be presented with cards including a green square, a red circle, two orange squares, and five green triangles. The first relevant sorting category might be color, so subjects proceed to sort the cards by color, placing the red circle card in one pile, the two orange squares card in a separate pile, and the green square card and the five green triangles card together in a third pile. Over many trials of different cards being presented, the subject must continue sorting cards by color. However, at some point, the examiner will change the relevant sorting category — say the examiner changes the sorting category to number. The subject must then come to recognize that

color is no longer the correct category to use in sorting the cards and that cards are now sorted based on the shapes on the card (triangles, circles, squares, etc.). This process of identifying a shift in sorting category and following the new category while ignoring the old is defined as set-shifting, the changing of one “set” or category, such as color, to a new “set” or a different category such as shape. This task requires subjects to demonstrate many executive functions, such as cognitive flexibility in shifting from sorting based on one category to sorting based on another, and inhibition in no longer sorting cards based on the first category.

In a meta-analysis conducted by Demakis (2003) that looked at effects of frontal lobe damage on performance in the WCST, it was concluded that frontal lobe damage has a negative effect on performance. Through subtracting the mean performance on the WCST in patients with non-frontal lobe damage, from the mean performance of patients with frontal damage, and weighing the effect size of each study by its standard error, Demakis found that patients with frontal lobe damage were able to learn fewer WCST sorting categories and their performance was worse in that they demonstrated greater perseverative errors (after the sorting category was changed, they continued to sort cards based on the first category). However, subjects with frontal lobe damage did not demonstrate any greater non-perseverative errors, indicating that frontal lobe damage negatively affects the ability of subjects to shift between sets in the WCST (Demakis, 2003).

Further experiments using multiple forms of the Wisconsin Card Sorting Task combined with fMRI have demonstrated that the WCST activates many brain regions outside the frontal lobe, including the cerebellum. Lie et al. (2006) administered three versions of the WCST to twelve volunteers. WCST versions differed in that in version A, participants received only feedback of correct or incorrect choices and thus had to deduce the relevant sorting category on

their own and set-shift independently. In the second version, version B, participants were told when to set-shift. In version C, participants were told the sorting category prior to having to sort the cards. fMRI during all three versions of the task demonstrated differential brain activations for set shifting, working memory, attention control and error detection. Lie et al found increased cerebellar activation when comparing brain activation between version A, where subjects had to set-shift independently, and version B and C, where subjects were instructed to set-shift. This suggests that the cerebellum plays a role in identifying that one sorting category is no longer correct, and recognizing that a new sorting category has to be identified—effectively participating in set-shifting (Lie et al., 2006). Furthermore, Le, Pardo, and Hu (1998) found that fMRI measuring activation in the cerebellum during sustained attention tasks compared to shifting attention tasks showed greater activation of the posterior lateral cerebellar hemispheres during shifting attention tasks (specifically Crus I anterior and Crus I posterior) (Le, Pardo, and Hu, 1998).

Further evidence pointing to the cerebellum's potential role in set-shifting ability can be seen in that subjects diagnosed with disease states linked with cerebellar abnormalities, such as schizophrenia and autism, demonstrate a lesser ability to ignore previously learned stimulus dimensions (set 1) that were no longer relevant during set 2, deemed perseverative errors, on tasks such as the WCST and Tower of London task (Hughes et al., 1994; Pantelis et al., 1999). In individuals with autism, the cerebellum has been found to be the most consistent site of neural abnormality and patients with schizophrenia demonstrate abnormal cerebellar size (Allen, 2005; James et al., 2004; Yeganeh-Doost et al., 2011; Andreasen et al., 2008). Thus, while set-shifting is known to be heavily dependent on the prefrontal cortex, the cerebellum may also play a

significant role in this ability and contribute to the greater amount of perseverative errors seen in individuals with certain disease states, as seen in the WCST.

Rat studies support the idea that the cerebellum may be involved in what are usually thought of as pre-frontal functions including switching attention sets and switching strategies. Bartolo et al. (2009) studied hemi-cerebellectomized (ablation of the right cerebellar hemisphere and right vermis) and intact rats in a four-choice serial task. Rats were trained to enter a series of doors in a specific pattern such as right, left, right, right with the correct pattern changing each day of training. This task required rats to continuously identify that the sequence they had previously learned was no longer correct and then to change their response, effectively demonstrating cognitive flexibility in switching strategies and shifting between “dimensions” (dimensions being the specific pattern to follow). Results demonstrated that hemi-cerebellectomized rats had more errors when having to learn and apply a new pattern that differed from the previously learned pattern compared to control rats (De Bartolo et al., 2009). De Bartolo et al. were further able to demonstrate that the greater amount of errors made by hemi-cerebellectomized was not due to motor or motivation deficits as hemi-cerebellectomized rats demonstrated proficient locomotor function and equal motivation compared to intact rats. De Bartolo et al. concluded that the cerebellum is involved in the processing and utilization of sensory information and is strongly linked to flexibility, thus explaining why hemi-cerebellectomized rats demonstrated difficulty changing responses as the correct sequences changed (De Bartolo et al., 2009).

Further supporting the cerebellum’s role in cognitive flexibility, Dickson et al. (2017) studied the ability of chimeric mice with low Purkinje cell numbers to discriminate between pairs of visual stimuli on a touchscreen in 10 stages of cognitive flexibility tests. The mice used by

Dickson et al. demonstrated variable Purkinje cell loss ranging from 0%-100%. As Purkinje cells are the sole output of the cerebellar cortex and it is believed that higher cognitive functions take place within the cerebrum, Dickson et al., analyzed how various numbers (Low: 0-371; Medium: 7,306-49,618; and High: 52,302-108,390) of Purkinje cells affected cognitive function. Mice were trained on a total of 10 stages that included simple discrimination (recognize white lines as the correct stimulus dimension), intradimensional shift (recognize that white lines are still the relevant dimension but white lines making a plus sign are now correct whereas vertical white lines were correct before), and extradimensional shift (white lines are no longer the relevant dimension, gray images are now the relevant dimension). A touchscreen presented two options for choosing. In the simple discrimination stage the options were of the same dimension (white horizontal lines or white vertical lines). In the intradimensional and extradimensional shift, the options consisted of two dimensions and the mouse had to choose the option based on the relevant dimension (options were white horizontal lines and gray circle or white vertical lines and gray square—identify lines as correct dimension and vertical lines as correct within that dimension—recognize that the correct option contains white vertical lines). Mice were required to nosepoke the correct stimulus to receive a reward. Chimeric mice with low Purkinje cell numbers ($\geq 95\%$ loss of Purkinje cells) demonstrated impaired flexibility, as impaired reversal learning performance and impaired extradimensional set-shifting was observed (Dickson et al. 2017).

To further study the role of the cerebellum in extradimensional set-shifting ability, the current study compared set-shifting performance of control rats with rats that underwent inactivation of the cerebellar regions Crus I and Crus II— regions that have previously been demonstrated to show greater activation during set-shifting tasks and in which lesions produce

cognitive impairments (Le et al., 1998; Stoodley et al., 2010). The maze task, created by Stefani et al. (2003) and used extensively in our lab (Chess et al., 2011; Eddy et al., 2013; Eddy et al., 2014; Lipatova et al., 2014; Lipatova et al. 2016) trains rats to establish an association between one of two stimuli within a “dimension”, color (black vs white) or texture (rough vs. smooth), and a response, traveling down the rewarded path. The rats are trained on a T-maze apparatus. This maze task is formally similar to the WCST in that the stimulus dimension of arm color and arm texture are analogous to the WCST card dimensions of number of shapes, color of shapes, and type of shapes. This shift between stimulus dimensions requires the integration of feedback, the inhibition of previously learned behavior, and the performance of a desired action, thus testing the role of cerebellar cortex regions in executive function attributed to frontal lobe regions.

Stefani et al.’s experiments using the set-shifting maze task demonstrated the role of NMDA and AMPA receptors in the medial prefrontal cortex in set-shifting ability. Blockade of glutamatergic neurotransmission in the mPFC prior to the shift from one “set” to another “set” (for example, from a black vs. white discrimination to a rough vs. smooth discrimination) revealed that receptors within the mPFC are involved in the modification of previously learned information and the recall of previously learned information (Stefani et al., 2003). NMDA and AMPA receptors within the mPFC were important in the ability to shift between dimensions, and AMPA receptors were also important in remembering the set 1 discrimination (Stefani et al., 2005).

While Stefani et al. (2003, 2005) demonstrated that set-shifting processes in rats are heavily dependent on the medial prefrontal cortex, the cerebellum may also play a role in this ability. Thus, we hypothesized that the inactivation of a small region of the cerebellum would

lead to an increase in perseverative behavior, indicated by rats continuing to follow the relevant dimension learned on Day 1 during Day 2, when the relevant dimension has changed.

Methods:

Subjects:

32 male Wistar rats (between 59 and 63 days old at the start of the experiment) were purchased from Charles River Laboratories. Rats were kept on a 12-hour light-dark cycle and handling, surgery, and experimentation occurred during the light period of the cycle. Rats had unrestricted access to food for one week before undergoing surgery. After surgery, rats were placed in individual cages and underwent another week with unrestricted food access. One week after surgery, rats were placed on a food-restricted diet in order to reach 90% of free-feed body weight and were maintained on this diet over the course of the experiment.

Surgery:

Rats were placed under isoflurane anesthesia and implanted with bilateral 22-gauge stainless steel guide cannulas. The cannulas were targeted at the coordinates -12.5 posterior to bregma, +/- 3.5 lateral, -4.0 ventral, in the Crus I/II area of the hemispheric portion of lobule VII of cerebellar cortex. These coordinates were verified in a preliminary experiment. Cannulas were affixed to the skull using jeweler's screws and dental acrylic. During surgery, an electric heating pad was used to maintain the rat's body temperature at approximately 37 °C. After surgery, rats received 5.0 mg/kg of the analgesic Carprofen, .15 ml bupivacaine as a local anesthetic, and 1 ml of lactated Ringer's solution for hydration. The rats received a second dose of Carprofen 24 hours post-surgery.

Handling:

One week after surgery, the rats were weighed to obtain a baseline weight and then placed on a food restricted diet in order to reach target weight (90% of baseline weight), and handling began. This included petting, holding, and maneuvering the rat. Handling lasted for four to five days.

Apparatus

The set-shift apparatus used was that described by Eddy et al. (2013). The maze was constructed of painted polycarbonate and set up so that four arms extended from each side of a center square platform (sides measuring 14.0 cm). Each arm was 14.0 cm wide, 40.6 cm long, and 20.3 cm high. A food well was located 2.5 cm from the end of the arm. Food wells measured 1.9 cm in diameter and .63 cm in depth so that food pellets placed in the food wells could not be seen by rats positioned at the entrance of the arms. Two of the arms were painted light gray and two were painted dark gray with two of the arms being smooth and two of the arms being rough (achieved through mixing sand into the paint). Thus the arms of the maze were light-smooth, light-rough, dark-smooth, and dark-rough. The center square platform was painted an intermediate gray. An intermediate gray polycarbonate insert could be placed in the maze to section off one arm of the maze from the center square platform in order to create a T configuration. The holding chamber in which rats were held between maze runs was made up of polycarbonate painted intermediate gray and measuring 35.6 cm × 35.6 cm × 35.6 cm.

Open Arm Habituation:

After all rats had achieved their target weight, and at least four days of handling had passed, open arm habituation began. On the first day of open arm habituation, the rat was handled for one minute before being placed into an open cross-maze in which four sucrose pellets were placed in every maze arm. The rat was allowed to explore for ten minutes or until

all sucrose pellets (16) had been eaten, whichever came first. The rat was then removed from the maze and placed in the holding chamber for two minutes before being returned to its cage. After day one, this process was repeated with only one pellet in each arm of the maze (4 pellets total). Open arm habituation with four pellets was performed for two days. If by day two of the four pellet habituation the rat did not eat all pellets in under five minutes, the rat could receive up to another two days of four pellet open arm habituation. If after four days of open arm habituation with four pellets the rat had still not eaten all pellets in under five minutes, the rat was removed from the study.

Blocked Arm Habituation:

After open arm habituation had been completed, the rat was moved on to blocked arm habituation. The rat was handled for one minute before being placed in the holding chamber for two minutes. During this time, the arm of the maze opposite the starting arm was blocked off and one arm was baited with two sucrose pellets. After two minutes in the holding chamber, the rat was placed at the base of the starting arm and allowed to run down one arm. Once the rat had chosen an arm to travel down and had explored the well and eaten any available sucrose pellets, the rat was removed from the maze and returned to the holding chamber. It was noted if the rat had chosen the correct arm and eaten the sucrose pellets. The maze was then rotated and cleaned using Nok-Out odor eliminator and the arm in the opposite direction of the arm the rat traveled down on the first run was baited. After run two, a random arm was baited for each run and the maze was cleaned between each rotation. If three correct arms were travelled down in a row, the fourth trial was not baited with sucrose pellets so that the rat could get used to not being rewarded for a choice. After two days of blocked arm habituation, experimental testing began.

Set-Shifting Task:

The set-shifting task consisted of two days. On day one (“set 1”), each rat was assigned a dimension for which he was specifically rewarded for a correct discrimination. Each rat was put into the holding chamber for two minutes prior to beginning the maze task. The arm opposite the start arm was blocked off so that the maze was in a T configuration. The one arm of the maze that corresponded to the dimension (color or texture) and stimulus within that dimension (black or white; rough or smooth) assigned to the rat (that was not the starting arm) was baited with one to two sucrose pellets. The rat was placed into the maze at the base of the starting arm when the two minutes were up, and allowed to make a choice. It was noted if the rat made the correct or wrong choice. After exploring the well and eating any available sucrose pellets, the rat was returned to the holding chamber, and the maze was rotated, cleaned, and re-baited. This procedure was repeated until the rat made 8 correct choices in a row. The same process was repeated for day two (“set 2”) with the opposite dimension. On day two, the rat received 80 trials. Set 2 was recorded using an overhead digital camera.

Prior to day two of the set-shifting task, the rats were split into two groups to undergo infusion via Hamilton syringes. Twenty minutes prior to testing on day 2 of set shifting, one group was bilaterally infused (into Crus I/II of cerebellar cortex) with vehicle solution (0.9% saline vehicle) and one group was bilaterally infused with muscimol (2 mM) (Muscimol is a GABA_A receptor agonist that inactivates neural tissue for several hours). Internal cannulas were bilaterally inserted into guide cannulas and the internal cannulas extended 1 mm beyond the end of the guide cannulas inside the rat brain. Infusion volume for each cannula was 0.5 μ L and was delivered at a rate of 0.25 μ L per minute using a microinfusion pump (volumes were determined in a preliminary experiment). The internal cannulas were left in place for one minute following completion of infusion to allow vehicle or muscimol to diffuse away from the internal cannula

tips. After one minute, the internal cannulas were removed and replaced with the dummy cannulas. Rats were then allowed to sit in a transportation container for twenty minutes prior to the start of testing.

Perfusions and Histology:

After the rats had finished both days of set shifting, they were overdosed with sodium pentobarbital and trans-cardially perfused with 4% paraformaldehyde. A marking lesion corresponding to cannula tip location was made by inserting a stainless steel insect pin through the guide cannula so that it protruded 1 mm below the guide cannula tip. Then, 100 microamperes of current was delivered to the insect pin for 10 seconds. Their brains were harvested and stored in 4% paraformaldehyde. In order to determine if the cannulas were placed in the correct location of the cerebellar cortex, the brains were embedded in gelatin-albumin. The brains were then frozen and sliced (60-80 μm thickness) using a cryostat at approximately -26 degrees Celsius. The slices were mounted on chrome alum subbed glass slides and stained using Cresyl violet (for cell bodies) and Prussian blue (for iron deposits from the marking lesion). The stained sections were evaluated (blind to group membership) for correct cannula placement.

Video Analysis:

Video analysis of set 2 performance was made blind to the identity of the rat in each video through a third-party re-coding the video names. Video analysis consisted of using a timer application to time how long it took for each rat to reach the food well in whichever arm the rat traveled fully down after it had been placed in the start arm. Furthermore, any significant shift of head or body of the rat, at the choice point, towards the arm opposite that of the arm the rat ultimately choose to travel down was marked.

Results:

We began the study with 32 rats. Six rats were removed prior to data analysis as a result of fatality from surgery, loss of head cap during study, or failure to eat food reward pellets within time limit (5 minutes) after undergoing four days of open arm habituation. An additional six rats were removed post-data analysis due to infusion sites located too posterior, inability to locate infusion sites, infusion sites located too inferior, infusion sites located too superior, and cannula related damage. Removal of 12 rats in total left us with 10 rats per group and infusion sites of the rats that made it through data analysis are depicted in Figure 4.

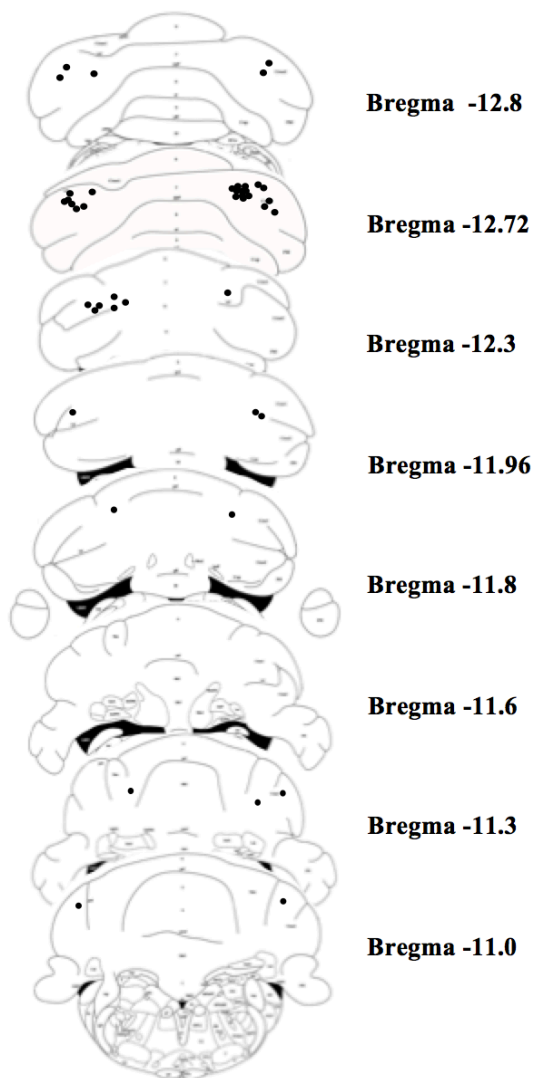


Figure 4- Infusion sites of rats used in study

Set 1: Trials to criterion. Figure 5A depicts the number of trials to a criterion of 8 correct choices in a row in Set 1. Rats that would be infused with muscimol prior to Set 2 and rats that would be infused with vehicle prior to Set 2 did not differ. This was supported by a one-way ANOVA, $F < 1$.

Set 1: Time per trial. Figure 5B depicts the average time to complete each trial in Set 1. The time per trial for Set 1 was used to ensure that rats in the two groups were performing equally in motor abilities. Rats that would be infused with muscimol prior to Set 2 and rats that would be infused with vehicle prior to Set 2 did not differ. This was supported by a one-way ANOVA, $F < 1$.

Set 2: Trials to criterion. Figure 5A depicts the number of trials to a criterion of 8 correct choices in a row in Set 2. Inactivation of the Crus I/Crus II region of cerebellar cortex did not affect the number of trials to a criterion of 8 correct choices in a row in Set 2 (i.e., extradimensional set-shifting). This was supported by a one-way ANOVA, $F(1,19) = 1.72$, $p = 0.21$.

Set 2: Time per trial. Figure 5B depicts the average time to complete each trial in Set 2. Muscimol inactivation of the Crus I/Crus II region of cerebellar cortex slowed performance. This was supported by a one-way ANOVA, $F(1,19) = 4.66, p < 0.05$.

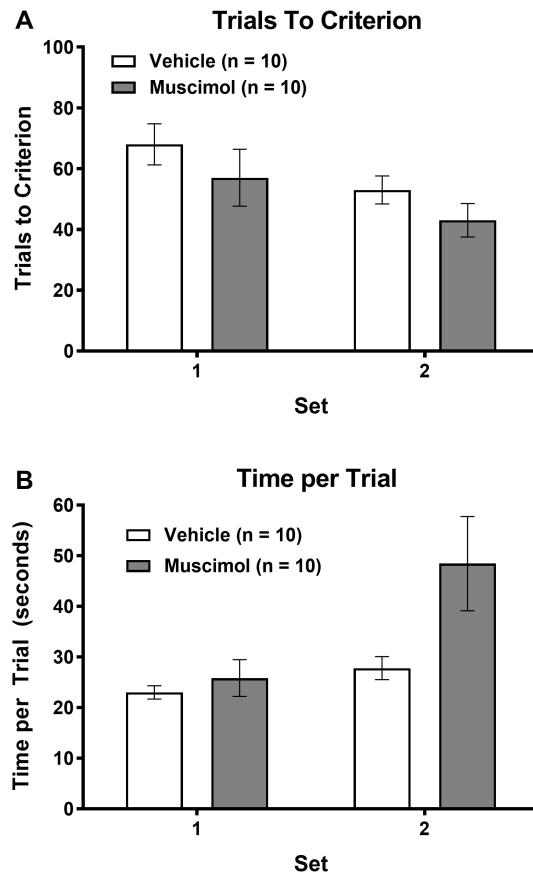


Figure 5- Trials to criterion and time per trial

Set 2: Performance across trial blocks. The 80 trials in Set 2 were grouped into 10 blocks of 8 trials each and separate repeated-measure ANOVAs were conducted on the percentage of perseverative arm choices (arm choices that were correct in Set 1 but not in Set 2) (Figure 6A) and percentage of reinforcement arm choices (arm choices that were correct in both Set 1 and Set 2) (Figure 6B). Fewer perseverative arm choices were made across blocks (i.e., an increase in correct responses when the correct arm was one that had not been reinforced in Set 1) and inactivation of the Crus I/Crus II region of cerebellar cortex had no effect. This was

supported by a 2 (Group: Muscimol, Vehicle) x 10 (Set 2 Block of Trials) repeated-measures ANOVA, which revealed a significant effect of Block, $F(9,162) = 22.00, p < 0.01$ but no effect of Group and no Group x Block interaction, largest $F = 2.81$. More reinforcement arm choices were made across blocks (i.e., an increase in correct responses when the correct arm was one that also had been reinforced in Set 1) and inactivation of the Crus I/Crus II region of cerebellar cortex had no effect. This was supported by a 2 (Group: Muscimol, Vehicle) x 10 (Set 2 Block of Trials) repeated-measures ANOVA, which revealed a significant effect of Block, $F(9,162) = 6.96, p < 0.01$ but no effect of Group and no Group x Block interaction, F 's < 1 .

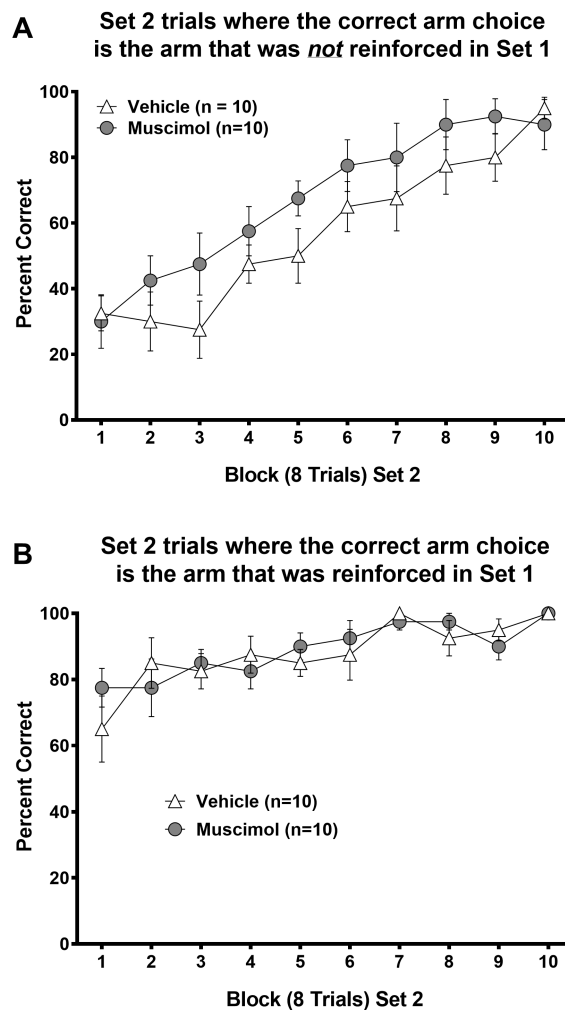


Figure 6- Set 2 trials by block

Set 2: Video analysis. The time required to complete each of the 80 trials in Set 2 was calculated from video by measuring the seconds it took from placement in the stem of the T to reaching the food well (Figure 7). This allowed a finer-grained examination of any motor effects of Crus I/II inactivation. Crus I/II inactivation did not have any effect on this measure, and latency to complete each trial remained steady throughout the 80 trials of Set 2. These observations were supported by a 2 (Group: Muscimol, Vehicle) x 80 (Set 2 Trial) repeated-measures ANOVA. Neither the Trial effect nor the Trial x Group interaction effect was significant, F 's < 1 . Unlike our gross measure of Set 2 time per trial, which simply divided the total time to complete Set 2 by 80 trials, this finer-grained analysis of Set 2 time per trial failed to reveal a significant difference between groups, $F(1,17) = 2.69, p = 0.12$.

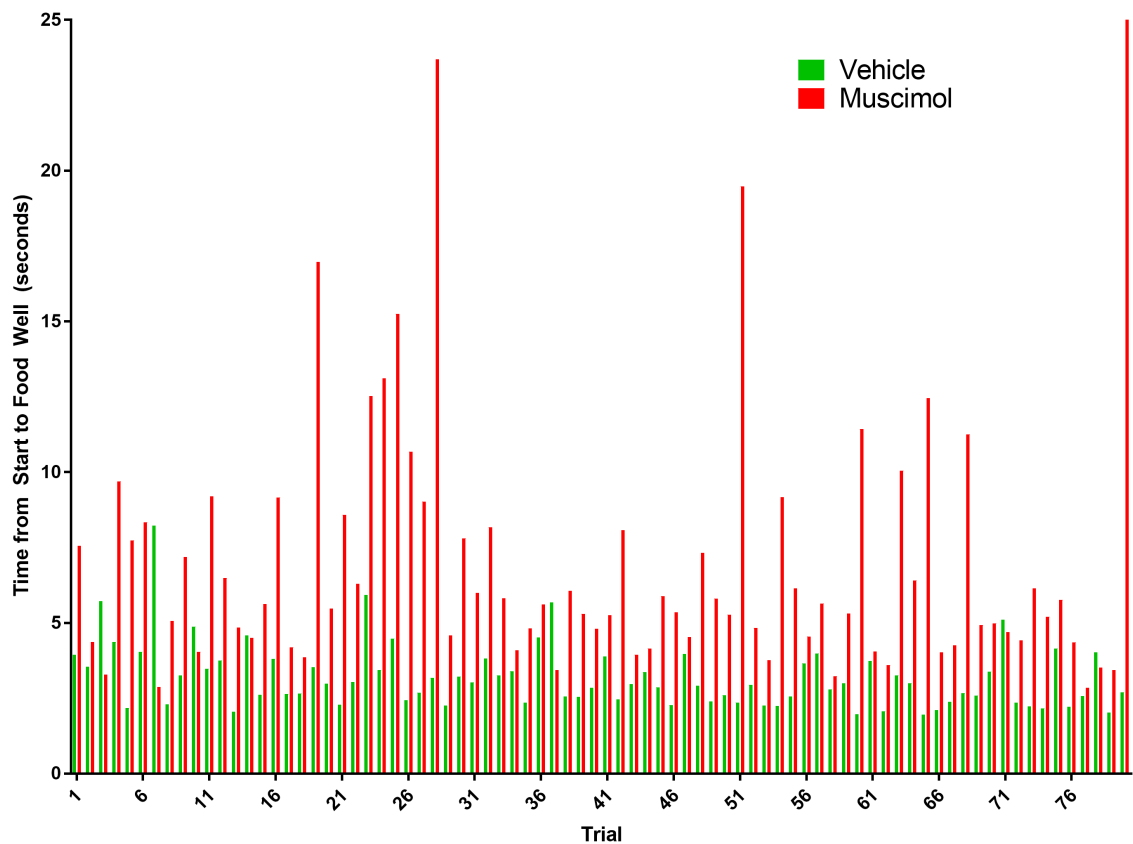


Figure 7- Set 2 trial-by-trial latencies

Discussion:

To gain a deeper understanding of the role of the cerebellum in cognitive flexibility, we have tested the set shifting ability of rats with pharmacological inactivation of Crus I and Crus II— areas previously linked with cognitive functions in humans such as language processing, working memory, and shifting attention (Stoodley & Schmahmann, 2010; Igloi et al., 2015; Le et al., 1998)—on the set-shifting T-maze task (Stefani et al., 2003). We found several results that may help to guide future research on the cerebellum in cognitive flexibility. First, the rat groups (vehicle infusion and muscimol infusion groups) learned set-shifting at a statistically equal rate. On day 1 of the task, with no infusions, rats were trained until they had completed eight trials correctly in a row. Rats in the two to-be-infused groups learned at a statistically equal rate on day 1. On day 2 (set-shifting), rats underwent intra-cerebellar infusion of either muscimol or vehicle and were tested for a total of 80 trials on the opposite dimension discrimination from day 1. For example, if a rat had a color discrimination on day 1 (black vs. white), he was tested on a texture discrimination on day 2 (rough vs. smooth). The two groups learned at a statistically equal rate across the 80 trials on day 2. On day 2, both the muscimol and vehicle infused groups demonstrated decreasing perseverative errors (errors in which the rats followed the discrimination from day 1) across trial blocks (trial blocks consisted of eight trials). Additionally, in one of our measures— time per trial— rats who received muscimol inactivation performed slower during Set 2 than rats who received vehicle infusion, but in our more detailed measure— video analysis of time per trial— there was no statistically significant difference, suggesting that motor deficits were relatively mild. In conclusion, we found no impairment in extradimensional set-shifting ability in rats with pharmacological inactivation of the Crus I and Crus II regions of

cerebellar cortex. Our results fail to support a role of these regions of cerebellar cortex in extradimensional set-shifting in rodents.

For a long time, literature on the cerebellum mainly analyzed the cerebellum's contributions to motor functions. However, more recently, a greater amount of attention has been focused on possible contributions of the cerebellum to non-motor functions. It has previously been found that cerebellar lesions involving the anterior lobe and parts of lobule VI may result in classic motor symptoms such as ataxia and tremors, yet cerebellar lesions of the posterior lobe may also produce cerebellar cognitive affective syndrome (CCAS) characterized by impairments in non-motor functions including executive and language impairments (Stoodley & Schmahmann, 2010). Furthermore, neuroimaging studies of humans during tasks such as sensory processing, mental imagery, and shifting attention demonstrate activation of the cerebellum when subtracting for activation by motor components. Activated cerebellar regions include the dentate nucleus, inferolateral regions (hemispheric portions of lobules VIII-X) on both sides, and the left posterior quadrangular lobule (hemispheric portions of lobules IV and V) and left superior semilunar lobule (hemispheric portion of lobule VII, also known as Crus I and II) (Gao et al., 1996; Ryding et al., 1993; Allen et al., 1997; Stoodley & Schahmann, 2010).

Although we found no significant impairment from inactivation of the Crus I and Crus II regions of cerebellar cortex in ability to perform on an extra-dimensional set-shift task, several other studies have demonstrated a role for the cerebellum in cognitive flexibility. Dickson et al. demonstrated that, in a chimeric mouse model, extensive loss of Purkinje cells (the sole connection between the cerebellar cortex and the deep cerebellar nuclei) resulted in cognitive deficits in reversal learning and extradimensional set-shifting (Dickson et al., 2017). De Bartolo et al. found that right unilateral hemi-cerebellar lesions in rats severely impaired rodents'

behavior in adapting to changing sequences in a four-choice learning task (De Bartolo et al., 2009). Finally, Lie et al., using fMRI, found increased cerebellar activation in uninstructed set-shifting (version A) compared to instructed set-shifting (version C) in human subjects completing the Wisconsin Card Sorting Task (WCST). Thus, there is evidence that the cerebellum does, indeed, play a role in cognitive flexibility, but, as demonstrated by our study, it is not Crus I and Crus II alone that account for the contribution of the cerebellum to cognitive flexibility functions in rodents.

Dickson et al's 2017 study on *Lurcher* chimeric mice in a 10-stage Intra-Extra Dimensional Set-Shifting (IED) task is the most comparable to our set-shifting T-maze task and a detailed comparison of their subjects, procedures, and results to our study may be informative. Their study looked at discrimination and cognitive flexibility using *Lurcher* chimeric mice. *Lurcher* is a semi-dominant mouse mutation that leads to near complete loss of Purkinje cells, the sole output of the cerebellar cortex, during the first four postnatal weeks in response to increased activity of $\delta 2$ glutamate receptors causing excitotoxicity (Doughty et al., 2000; Wetts & Herrup, 1982). Purkinje cells, being the sole output of the cerebellar cortex, are a requisite link between the cerebellar cortex and the cerebrum. These neurons project from the cerebellar cortex to deep nuclei of the cerebellum where they synapse on neurons of cerebellar efferent pathways. Some neurons of the efferent pathways then carry cerebellar cortex information to the thalamus to be relayed on to cerebral cortices, including the pre-frontal cortex, while other deep nuclei neurons project to brainstem regions including the red nucleus (involved in mediating flexion of the upper limbs) and the inferior olive (involved in a motor-learning loop) (O'Halloran et al., 2011).

Dickson et al. (2017) used a 10 stage IED task where mice performed on each stage of the task for 60 minutes or until the mouse had completed 64 trials. The set-up of the task consisted of a mouse in a conditioning chamber with a touch screen and a food receptacle. The touch screen presented visual stimuli on the left and right sides of the screen with one side of the screen presenting a correct stimulus and the other side presenting an incorrect stimulus. The mouse was required to nose poke the correct side of the screen to receive a food reward. Stage 1 was a simple discrimination test where mice were presented with one stimulus of one dimension (white lines or gray shapes) and had to nose poke the correct stimulus (e.g. vertical lines). Stage 2 reversed the relevant stimulus so that the same dimension was presented as that of stage 1 (if white lines were presented in stage 1, white lines were presented in stage 2) but the opposite stimulus was now correct (if vertical lines were correct in stage 1, horizontal lines were correct in stage 2). Stage 3 was a compound discrimination task so that mice were presented with stimuli consisting of two dimensions (white lines on top of gray shapes). Mice had to identify the correct stimulus by first identifying the correct dimension—the dimension that was tested in stage 1 and stage 2 (if white lines were tested in stage 1 and 2, then the relevant dimension in stage 3 would still be white lines) and then by identifying the correct stimulus within that dimension (horizontal or vertical lines). Stage 4 was an intradimensional shift test where mice were presented with compound stimuli that were novel from the compound discrimination task and had to identify the correct stimulus based on the same dimension that was correct in stage 3 (if lines were relevant in stage 3, lines were also the relevant dimension in stage 4). Stages 5 through 7 were the same as stage 4 with novel stimuli presented in each stage but with the relevant dimension remaining constant (lines continued to be the correct dimension). Stage 8 was an intradimensional shift reversal of stage 7 so that whichever dimension was relevant remained the relevant dimension

but the correct stimulus was opposite of the correct stimulus in stage 7 (if lines were the relevant dimension in stage 7, lines were the relevant dimension in stage 8 but if horizontal lines were the correct stimulus in stage 7, vertical lines were the correct stimulus in stage 8). Stage 9 was an extradimensional shift test where mice were presented, again, with compound stimuli but now had to recognize that the relevant dimension had switched (if lines had been the relevant dimension for the intradimensional shift stages, then gray images were now the relevant dimension) and identify the correct stimulus (gray teardrop was correct vs the gray leaf). Finally, stage 10 was an extradimensional shift reversal where the dimension from stage 9 remained relevant but the correct stimulus in stage 9 was now opposite (if gray teardrop was correct in stage 9, the gray leaf was now the correct stimulus) (Dickson et al., 2017).

Dickson et al. (2017) reported finding significant impairments in extra-dimensional set-shifting performance and reversal learning performance for mice with greater than or equal to 95% Purkinje cell loss (0-3711 remaining Purkinje cells) with no impairments for the medium (7,306-49,618 Purkinje cells) and high (52,302-108,390 Purkinje cells) groups. Such large loss of these cerebellar neurons, as observed in the 95% Purkinje cell loss group, would affect a much larger region of the cerebellar cortex than the muscimol inactivation of Crus I and Crus II as performed in our study. In mammals, Purkinje cells can be grouped into a pattern of parallel longitudinal zones representing areas of the cerebellar cortex that project to different cerebellar or vestibular target nuclei (Voogd & Glickstein, 1998). Of these zones, cerebellar cortex regions Crus I and Crus II have been found to be associated with up to nine stripes and seven interstripes (stripes being zonal areas and interstripes being borders of zones found to have little to no labeling with tracer [H-leu, WGA,HRP, and BDA]) (Serapide et al., 2001). While Crus I and Crus II are associated with nine stripes, Purkinje cells, as the sole output of these zones, are

associated with all of the cerebellar zones. Thus it is clear that massive Purkinje cell loss would affect a much larger region of the cerebellar cortex than inactivation of Crus I and Crus II alone. Impairments in extradimensional set-shifting, as well as reversal, as observed by Dickson et al. may then be due to a much larger region of cerebellar cortex dysfunction (essentially, disconnection of a large portion of cerebellar cortex from the deep cerebellar nuclei) and may indicate that a region/regions other than Crus I and Crus II are responsible for the role of the cerebellum in rodent cognitive flexibility.

Dickson et al.'s IED task, although similar to our T-maze set-shifting task, differs from our T-maze set-shifting task in several ways. First, and perhaps most importantly, Dickson et al.'s set-shifting task consists of 10 test stages whereas our task consists of only 2 test stages. Mice in Dickson et al.'s study learn to complete one simple discrimination, one simple discrimination reversal, one compound discrimination, four intradimensional shifts, one intradimensional shift reversal, one extradimensional shift, and one extradimensional shift reversal. Rats in our study only had to complete one compound discrimination and one extradimensional shift. Mice in Dickson et al.'s study learned to identify the same stimulus dimension (i.e. lines) for eight test stages in a row before performing an extradimensional shift (having to recognize the opposite dimension, i.e. gray images). The mice in Dickson et al.'s study, due to learning to identify the same stimulus dimension for 8 tests in a row, likely experienced a greater level of interference of the original dimension (lines) when it came time to identify a new dimension (gray images) in the extradimensional shift task. Dickson et al.'s extradimensional shift test thus may be more difficult than the extradimensional shift test in our study where rats had only learned and been tested on an original dimension (rough) for one test before having to perform an extradimensional shift and recognize the opposite dimension (light).

The level of difficulty of the extradimensional shift in Dickson et al.'s study compared to that of the extradimensional shift task in our study may help to explain why Dickson et al. found impaired performance for mice with greater than or equal to 95% Purkinje cell loss on the extradimensional set shift while our study found no significant difference between pharmacologically inactivated rats and vehicle infused rats. Perhaps a greater level of cognitive challenge, requiring greater executive function involvement, is needed for the cerebellum to play a role.

Although Dickson et al.'s IED task may build interference over the course of the first 8 test stages leading up to the extradimensional shift, their IED task does have the advantage of more specifically measuring "set formation" compared to our T-maze task. Set formation is the idea that intradimensional shifts are easier than extradimensional shifts. Intradimensional shifts are easier because recognition of stimuli within a dimension you already are attending to (e.g., after learning to recognize horizontal lines, the shift requires recognition of vertical lines; lines are the "set") is simpler than having to learn to attend to a new dimension (e.g., after learning that lines are the "set" the shift requires recognition of a new "set" such as gray images) because you are shifting within a "set," or dimension.

Dickson et al.'s *Lurcher* mouse model has some shortcomings for examining cerebellar cortex function. Two of the main issues with such a model for studying cerebellar contributions to non-motor functions are that subjects demonstrate significant ataxia and that Purkinje cell loss affects structures and connections of the brain outside of just Purkinje cells, including granule cells in cerebellar cortex and inferior olivary neurons (Wetts & Herrup, 1982; Doughty et al., 2000).

Dickson et al.'s finding that mice with greater than or equal to 95% Purkinje cell loss took significantly longer to generate their IED response and to collect food rewards across all stages was in mice who were also reported to be ataxic, or lacking in coordination of voluntary muscle movement. The motor effects reported must be considered in the interpretation of collected data, as motor deficits may impact the ability of the mice to complete Dickson et al.'s 10-stage IED task. The IED task required mice to nose poke visual stimuli that matched a learned discrimination and then to retrieve a food reward from a food receptacle. The ability of ataxic mice to coordinate movement is greatly disrupted and may interfere with the mouse's ability to nose-poke and to collect the food reward, thus producing the greater time period it took for mice with greater than or equal to 95% Purkinje cell loss to both generate an IED response and collect food rewards.

Although the effects of such motor deficits must be considered as a possible explanation for the time difference observed in *Lurcher* mice compared to control mice on completion of the IED task and retrieval of food reward, the impairment in reversal and extradimensional set-shifting found by Dickson et al. is not likely due to such motor deficits. Dickson et al. tested the same mice in several discrimination and set-shifting problems, including an intradimensional set-shift task. Intradimensional set-shifting in Dickson et al.'s study required mice to perform the same motor components of nose poking a visual stimulus that corresponded to a learned discrimination and retrieving a food reward. However, no significant impairment was observed in *Lurcher* mice in the intradimensional set-shift stage. As Dickson et al. used a within-subject design, and all mice completed each stage, it is unlikely that motor deficits are responsible for the time difference observed in the extradimensional set-shift stage (Dickson et al., 2017).

The second main issue with the *Lurcher* model used by Dickson et al. revolves around the anatomical effects induced by the *Lc* mutation. Mice used by Dickson et al. were heterozygous for the *Lc* mutations. Within the first 4 weeks of postnatal life, Purkinje cells of *Lurcher* mice die due to the *Lc* gene inducing increased activity of $\delta 2$ glutamate receptors (Wetts & Herrup, 1982). However, the *Lc* gene and Purkinje cell death also leads to the secondary loss of cerebellar cortical granule cells and inferior olivary neurons (Wetts & Herrup, 1982; Doughty et al., 2000). Wetts and Herrup (1982) estimate that 75% of inferior olivary neurons are lost and 90% of granule cells are lost due to indirect actions of the *Lc* mutation in *Lurcher* mice. Whereas Purkinje cell axons are the sole output of the cerebellar cortex, inferior olivary neurons serve as a major input source for the cerebellum, projecting from the inferior olivary nucleus within the brainstem to the contralateral cerebellum (Watson et al., 2012). Inferior olivary neurons serve as the sole source of climbing fibers to the cerebellum and function in forming a motor network with the cerebellum concerned with coordination of eye movements, control of axial (non-trunk) muscles, control and timing of automatic movements, control of appendicular movement, and sequencing of goal-directed complex movements (Azizi, 2007). Inferior olivary neurons' axons climb to the cerebellum where they synapse on Purkinje cells of the cerebellar cortex and have an excitatory effect on the Purkinje cells, helping to set the rhythm of neuronal firing within the cerebellar cortex innervated by the Purkinje cell (Watson et al., 2012; Azizi, 2007).

Loss of approximately 75% of inferior olivary neurons in the *Lurcher* chimeric mouse model used by Dickson et al., complicates interpretation of Dickson et al.'s results as being due solely to Purkinje cell loss. Loss of inferior olivary neurons serves to weaken, if not eliminate, the connection between the inferior olivary nucleus of the brainstem with the cerebellar cortex. This loss in signaling makes it so that the inferior olivary nucleus's function in helping to

facilitate and coordinate motor movement through coordination of signaling is severely diminished and the subject experiences loss of motor control. Motor control deficits, such as diminished control of appendicular and axial muscles, and diminished ability to sequence goal-directed complex movements may reduce the capacity of *Lurcher* mice to nose-press stimuli presented to them in the 10 stage IED task used by Dickson et al. as well as diminish the mice's capability of retrieving food rewards. However, as discussed previously, it is unlikely that these motor deficits caused by loss of inferior olivary neurons account for Dickson et al.'s findings that *Lurcher* mouse models took significantly longer to generate their IED response and to collect food rewards across all stages as all mice were tested across all stages but impairments were only found for the reversal and extradimensional set shift stages (Dickson et al., 2017).

In addition to the loss of inferior olivary neurons, about 90% of granule cells are lost in *Lurcher* mice (Watson et al., 2012). 90% granule cell loss is very significant, especially when considering that cerebellar granule cells account for the largest percentage of neurons in the brain, outnumbering all other neurons combined, and that cerebellar granule cells are the only excitatory neurons within the cerebellar cortex (Wagner et al., 2017; Giovannucci et al., 2017; D'Angelo, 2013). Granule cells of the cerebellar cortex have been found to play a role in encoding sensory and motor context, as well as in signaling reward expectation (Wagner et al., 2017). Their role in such functions is believed to come from modulating input of mossy fiber inputs on Purkinje cells. Mossy fibers are inputs to the cerebellum from precerebellar nuclei and information from these fibers is transmitted to Purkinje cells through relay by granule cells (Fujita, 2016). Thus, loss of granule cells may lead to loss of information input to remaining Purkinje cells from mossy fibers in *Lurcher* mice, effectively eliminating the ability of Purkinje cells to properly function in *Lurcher* mice and producing a highly dysfunctional cerebellum.

However, it is important to keep in mind that our muscimol infusions not only produce inhibition of Purkinje cells but also produce inhibition of granule cells, since both neuron types express GABA_A receptors.

Looking beyond the Dickson et al. study, our findings may differ from our expected results due to differences in cerebellar anatomy between species. The deep cerebellar nuclei play an essential role in connecting the cerebellar cortex to the cerebrum. The largest cerebellar nucleus is the dentate nucleus and contributes, in humans, to functions including organizing and initiating voluntary movements. The dentate nucleus may also contribute to cognition (Bond et al., 2017). The dentate nucleus of humans and apes contains a phylogenetically older part and a phylogenetically newer part. While lower species, including rodents, have the “older” part, they lack the “newer” part found in humans and apes. The “newer” part in humans and apes is located ventrolaterally and the “older” part homologous with the dentate nucleus of lower species is located dorsomedially (Leiner, Leiner, & Dow, 1989). Neurosurgical reports on post-surgical effects of stereotaxic lesions of the dentate nucleus in order to reduce spasticity in humans with cerebral palsy suggest that lesions localized to the ventrolateral portion produce no motor symptoms (Siegfried et al., 1970). However, lesions in rhesus monkeys that were placed more medially, overlapping the dorsomedial portion, produced motor dysfunction symptoms, such as tremors and ataxia (Zervas, 1970).

The dentate nucleus serves as an important connection between the cerebellum and “executive” areas of the cerebrum, such as the prefrontal cortex. Dum and Strick created an unfolded map of the dentate nucleus in juvenile *Cebus apella* monkeys. They then used retrograde transneuronal transport of neurotropic viruses to study the organization of projections from the dentate nucleus to cerebral cortex areas and plotted their results on the unfolded map.

Dum and Strick found that dorsal portions of the dentate nucleus projected to the motor areas, including the primary motor and premotor areas, of the cerebral cortex while the ventral dentate nucleus projected to prefrontal and posterior parietal regions of the cerebral cortex—areas associated with higher cognition (Dum & Strick, 2003). Testing for this apparent motor/cognition divide demonstrated in *Cebus paella* monkeys, Kuper et al. used functional imaging of the dentate nucleus in humans during motor tasks involving finger tapping and cognition tasks including a verbal working memory task and a visuospatial task. Kuper et al. found that in humans, activation of the dorso-rostral dentate nucleus was linked with motor function while cognitive tasks produced activation of the caudal dentate nucleus (Kuper et al., 2011).

Combining Leiner, Leiner, and Dow's findings with those of Dum and Strick and Kuper et al., we may make some inferences. Leiner, Leiner, and Dow state that "lower species," such as rodents, lack the "new" portions of the dentate nucleus found in humans which is the ventrolateral portion of the human and ape dentate nucleus. Dum and Strick, and Kuper et al., then found that in monkeys and humans, dorsal portions of the dentate nucleus are linked with motor functions while ventral regions of the dentate are associated with cognitive functions. Together, these data suggest that rodents lack the ventral portion of the dentate nucleus found in humans and monkeys, meaning that rodents lack the portion of the dentate nucleus involved in non-motor functions. If this is the case, it suggests that the link between the cerebellum of rodents and the non-motor functional regions of the cerebrum may be much less prominent than those connections in higher species such as humans and suggests that the cerebellum plays a much smaller role in non-motor tasks than the cerebellum of higher species. A less significant connection of the cerebellum to non-motor areas of the cerebrum in rats may have prevented

inactivation of such a small region of the cerebellum from producing significant cognitive effects in our study. It may be necessary to inactivate a much larger region of the cerebellum in rodents to produce a large enough non-motor effect as to be observed in a set-shifting T-maze task. Furthermore, projections from the cerebellum to the prefrontal cortex, via the thalamus, have not been verified in rodents although projections from the prefrontal cortex to the cerebellum via the pontine nuclei are known to exist. Thus rodents may lack the functional connection from cerebellum to prefrontal cortex that is necessary for a role of the cerebellum in cognitive flexibility.

Despite several studies involving humans and mice that have demonstrated cerebellar contribution to non-motor tasks, our study found that Crus I and Crus II of the cerebellar cortex do not contribute to set-shifting ability in rats. However, our results, in combination with Dickson et al.'s 2017 study, may help to guide future research. Future studies should look at inactivation of a larger region of the cerebellum in extradimensional set-shifting or a different region of the cerebellum, such as the dentate nucleus. Additionally, studies looking at inactivation of Crus I and Crus II in set-shifting may be implemented using “higher order” species, such as apes and monkeys, whose cerebellum is more anatomically similar to that of humans. Finally, our study could be replicated with additional test days so that rats perform several intradimensional set-shifts on the T-maze leading up to an extradimensional shift so that greater interference causes greater cognitive demand during the extradimensional shift. Perhaps inactivation of Crus I and Crus II in rodents may produce cognitive deficits in set-shifting ability in times of greater cognitive demand.

Acknowledgements

This research was supported by funding from the University of Vermont Honors College and University of Vermont Office of Undergraduate Research. I would like to thank Dr. John Green for his all of his support, advice, and time and for giving me the opportunity to complete this study. I would like to thank Megan Shipman and all other members of the Green Laboratory for their tremendous efforts in helping me to collect data.

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