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Task-related activation of the non-human primate cerebellum during normal aging

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Thesis

TASK-RELATED ACTIVATION OF THE NON-HUMAN PRIMATE CEREBELLUM DURING NORMAL AGING

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ABSTRACT

The exact relationship between the cerebellum and cognitive processing is unknown. In recent years, evidence has shown there is a significant impact in the posterior cerebellum due to the progression of neurodegenerative conditions such as cerebellar cognitive affective syndrome and Alzheimer's Disease, indicating it could possibly serve a modulatory role in cognitive functioning. This has led to increasing interest in how the cerebellum and cognition are related and the impact it has on cognitive performance. As cognition is often affected in aging individuals and the cerebellum has been shown to change greatly with increasing age, there is also a growing interest in determining whether age has an impact on cognitive performance due to changes in the cerebellum. Using the Rhesus monkey as a model system, this study will examine whether there is a pattern of activation in the posterior cerebellum while performing a cognitive task. There are two objectives for this study:(a) determine the specific cells and regions that are active during such a task and (b) determine whether there are any age-related changes in the posterior cerebellum and whether cerebellar activation patterns relate to cognitive impairment indices in aged animals. We hypothesize that performance of a cognitive task will result in activation of the posterior

lobe in the cerebellar cortex and that this same region will show a reduced activation in aged animals who have high cognitive impairment indices (CIIs). We will do this by using immunohistochemistry (IHC) with c-Fos antibody. c-Fos is an immediate early gene upregulated in neurons when they are active. We will quantify neuronal activation in the posterior cerebellar lobes. Additionally, these data will be correlated with age and cognitive impairment index in these animals. We found that performance on a cognitive task was associated with regionally specific activation in the cerebellar cortex and in the deep cerebellar nuclei. Clusters of Purkinje cells and granule cells were activated during the task. Activation was not active throughout the entirety of lobule VIIA. In most of the monkeys tested in this study, there was more activation of Purkinje cells on the side of the lobule closest to Crus I, whereas on the other side there was only some activation. Additionally, granule cell activation appeared in clusters near activated Purkinje cells, however, these cells were not active in all regions where Purkinje cells were active. In monkeys who performed a behavioral task, it was apparent that there was greater activation in one hemisphere than the other, whereas those who did not perform a task showed little difference between the two hemispheres. This study provides further evidence that there is activation of the posterior lobe in the cerebellum during the execution of a cognitive task. As such, in future studies, the role the posterior cerebellum and, specifically, the activation of Purkinje cells play in cognitive function could be a key factor in understanding how they may affect the prognosis of neurodegenerative conditions.

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CCAS	Cerebellar Cognitive Affective Syndrome
CII	Cognitive Impairment Index
CSF	Cerebrospinal Fluid
DRST	Delayed Recognition Span Task
IHC	Immunohistochemistry
MCP	Middle Cerebellar Peduncle
mcRN	Magnocellular Red Nucleus
MRI	Magnetic Resonance Imaging
NGS	Normal Goat Serum
NIH	National Institutes of Health
pcRN	Parvocellular Red Nucleus

OVERVIEW

The cerebellum is a large folded region appended to the brainstem. The primary function of the cerebellum has long been thought to be the modulation of motor control. In the last twenty years, however, the role of the cerebellum has expanded to include a cognitive function. Brain activity in the cerebellum is elevated during cognitive tasks (Voogd, 2003; Schmahmann, 2019). As a result, there is increasing interest in what role this structure plays in cognitive function and to what extent. Schmahmann has been an advocate of the cerebellar involvement in cognition and has examined the possible cognitive role of the cerebellum in neurodegenerative conditions, specifically in the syndrome he described, Cerebellar Cognitive Affective Syndrome (CCAS). Several of his studies showed significant cognitive impairment (e.g., executive function, linguistic processing, etc.) when patients had damage confined to the cerebellum and specifically in the posterior lobe (Schmahmann, 2019). Individuals with damage to the posterior lobe exhibited greater cognitive deficits in comparison to those who had lesions in the anterior lobe and vermis (Schmahmann, 2019). These findings are consistent with other studies of the cerebellum that show activation in the anterior lobe and vermis in relation to motor functions, whereas activation in the posterior lobe is more common with cognitive functions (Diamond, 2000; Lara-Aparicio et al., 2022; Balsters et al., 2010; Koziol et al., 2014). Regardless, the studies of the specific role of the cerebellum in cognitive function have been limited. This limitation is due in part to the limited spatial resolution of non-invasive imaging in the human. While studies using functional MRI have implicated the cerebellum in cognitive tasks, the cells and circuits underlying this function cannot

currently be resolved in human cerebella using modern technology. This gap in knowledge can be addressed through the use of the non-human primate model, in which controlled behavioral experiments can be performed and neuronal activation measured at the single neuron level in post-mortem tissue. The non-human primate has a cerebellum of similar size to the human, and includes regions that are connected with the prefrontal cortex, the cerebral region involved in cognitive tasks such as working memory and executive function. The primary objective of this study is to evaluate the pattern of activation in the cerebellum during a cognitive task and to determine the specific cells and regions in the cerebellum that are active during such a task. This can be achieved by the use of cellular markers that show neuronal activity in regions of the cerebellum and by quantifying these markers. We hypothesize that the execution of a cognitive task will recruit the posterior lobe of the cerebellar cortex, a region that is interconnected in the primate frontal cortex (Schmahmann and Pandya, 1995, 1997; Middleton et al., 2001).

A second goal of this study is to determine whether the activation of the posterior lobe of the cerebellum is related to age, and to the cognitive ability of each subject. Previous studies have noted that the cerebellum may age at a slower rate than the rest of the brain and is often one of the last regions to be affected in neurodegenerative conditions such as Alzheimer's and Parkinson's Disease (Guevara et al., 2022; Liang et al., 2020; Lin et al., 2020). Several of these studies also determined that the progression and stage of cognitive decline in relation to these conditions heavily depended on cerebellar function in the posterior lobe. Thus, the cerebellum may compensate for age-related degeneration in the frontal lobe. Given this information, it is important to

determine how the posterior lobe of the cerebellum is affected during the normal aging process to help understand why it has such an impact on these conditions. Therefore, the second objective of this study is to determine any age-related alterations in activity in the posterior cerebellum and determine based on behavior in the same animal, whether cerebellar activation patterns relate to cognitive impairment indices (CIIs) in aged animals. We hypothesize that the cerebellum will show a reduced cellular activation in the posterior lobe in aged animals with high cognitive impairment.

THE CEREBELLUM

Overview and External Anatomy

The cerebellum is a symmetrical brain region located inferior to the cerebrum and connected to the brainstem by three symmetrical pairs of white matter bundles: the inferior, middle, and superior cerebellar peduncles. These peduncles contain axons conveying input or relaying output to the cerebellum. The cerebellum is bounded superiorly and separated from the overlying cerebral cortex by the tentorium cerebelli, an extension of the dura mater. The falx cerebelli is an inferior extension of the tentorium cerebelli that extends longitudinally between the left and right hemispheres posteriorly. The fourth ventricle is the anterior-inferior border and is interposed between the brainstem and the cerebellum. This ventricle is filled with cerebrospinal fluid (CSF) that is drained by three foramina between the brainstem and the cerebellum: the midline foramen of Magendie, and the paired foramina of Luschka. The CSF exits the fourth ventricle through these three channels and enters the cisterns of the subarachnoid space which surround the cerebellum.

Similar to the cerebrum, the cerebellum contains a folded laminated gray matter surface (cerebellar cortex) above underlying white matter. Unlike the cerebral cortex, the folds of the cerebellum, known as folia, and the intervening sulci are oriented orthogonally to the main longitudinal axis of the brainstem. The cerebellar cortex is divided into the medial, intermediate, and lateral zones. They are further divided into the vermis, paravermis, and two lateral hemispheres. The vermis is a midline ridge that separates the left and right hemispheres. There is no distinct division between the vermis

and the lateral hemispheres, but their point of separation can be distinguished by the presence of a small depression in the cortex that curves inward along the lateral border of the vermis on either side. This depression is classified as the paravermis and marks the intermediate zone. The three zones are further divided into ten smaller lobules whose borders are distinguished by fissures. One minor difference is how the lobules are labeled, which can vary between studies. These lobules are grouped into specific lobes – the anterior, posterior, and flocculonodular lobes. These lobes are determined by their position relative to the primary and posterolateral fissures. The anterior lobe consists of lobules I through V, the posterior lobe consists of lobules VI through IX, and the flocculonodular lobe consists of lobule X. The lobules of the anterior lobe lie superior to the primary fissure, those of the posterior lobe lie between the primary and posterolateral fissures, and the lobule of the flocculonodular lobe lies inferior to the posterolateral fissure. Although the orientation of the folia changes slightly from the anterior to the posterior direction, these markers that distinguish the borders between the lobes and lobules remain the same.

Deep within the white matter of the cerebellum lie four paired deep cerebellar nuclei: the fastigial, dentate, globose, and emboliform nuclei – the latter two comprise the interposed nuclei and are alternatively referred to as the posterior and anterior interposed nuclei. The fastigial nuclei are the smallest pair and lie in the most medial aspect of the cerebellum within the vermal region and superior to the roof of the fourth ventricle (Gray, 1995). The interposed nuclei lie within the intermediate zone adjacent to the vermis and are located lateral and inferior to the fastigial nuclei. Both the fastigial and

interposed nuclei have a similar ovoid shape. The dentate nuclei are the largest of the pairs and lie within the lateral hemispheres. As a whole, the dentate nucleus consists of an irregularly folded sheet of neurons enclosing a mass of white matter fibers, called the dentate hilum, that are primarily derived from dentate neurons (Chan-Palay, 1977). These fibers, in turn, comprise most of the superior cerebellar peduncle, in addition to efferent fibers originating from the emboliform and globose nuclei (Gray, 1995). Unlike the other deep cerebellar nuclei, the dentate nucleus has serrated edges and, according to Demole (1927), is split into two regions: the dorsomedial microgyric dentate and the ventrolateral macrogyric dentate. Demole (1927) also pointed out the distinguishing features of each region; the microgyric region has larger cells than the macrogyric region and the latter has a mix of both large and small cells. The larger cells of the macrogyric region are arranged in rows among scattered smaller cells (Demole, 1927). It should be noted that Jansen and Brodal (1940) and Dow (1942) determined that the extended ventrolateral part (pars paraflocculari*s*) of the dentate of animals, such as rabbits, does not correspond to the ventrolateral part of the human dentate nucleus. The human and anthropoid ape dentate nucleus, on the other hand, closely resemble each other as well as with the dentate nucleus of sub-anthropoid mammals, although the nucleus is much larger and more folded in the former (Jansen and Brodal, 1940).

As indicated above, the three cerebellar peduncles are the three white matter pathways that connect the cerebellum to the brain and spinal cord. The superior peduncle, also referred to as the brachium conjunctivum, contains the efferent fibers of the dentate and interposed nuclei, a fascicle of the fastigial nucleus, and some spinocerebellar fibers

(Gray, 1995; Knierim, 2020). These fibers ascend antero-posteriorly through the ipsilateral superior cerebellar peduncle into the dorsal pons, where they pass below the inferior colliculus, cross the midline in the decussation of the superior cerebellar peduncle, continue ascending until they reach the thalamus (Leitner et al., 2015). A related structure of note is the red nucleus. It is a structure on the ventral midbrain, encapsulated by the superior cerebellar peduncle, and located above the middle and inferior cerebellar peduncles. Similar to the cerebellum, there has been little research on its non-motor functions, especially in humans (Basile et al., 2021). In most species, the red nucleus is divided both functionally and anatomically into the magnocellular red nucleus (mcRN) and the parvocellular red nucleus (pcRN). The magnocellular and parvocellular red nucleus each have projections via different pathways, which may account for their differences in function. The mcRN is the most well understood of the two regions since it is more easily distinguished from its counterpart and, due to other studies primarily focusing on its motor functions, is often the most active region (Basile et al., 2021). In some species, such as the cat, the pcRN can be difficult to identify on its own and is grouped together with other structures as part of a nuclear complex (Ogawa, 1939; Onodera and Hicks, 2009). On the other hand, the segregation of the red nucleus varies with humans and non-human primates, as well as with quadrupedal and bipedal species, as their pcRN shows greater activity and is larger than the mcRN (Basile et al., 2021; Onodera and Hicks, 2009). It is understood that the mcRN in nearly all species serves primarily as a motor component and connects to the rubrospinal tract, sending projections to the medulla and spinal cord (Onodera and Hicks, 2009; Liu et al., 2000;

Basile et al., 2021). The pcRN has a complementary motor function to the mcRN, but sends projections through the rubro-olivo-cerebellar tract, with its main efferent to the inferior olive (Basile et al., 2021; Habas et al., 2010; Liu et al., 2000). Based on electrophysiological and MRI studies, this indicates that the pcRN may serve a role in sensory processing and planning (Liu et al., 2000; Habas et al., 2010; Basile et al., 2021). Some studies have pointed out that there is more neuronal activity of the pcRN in bipedal species compared to quadrupedal ones (Basile et al., 2021). It should also be noted that the pcRN is more clearly developed and identifiable in non-human primates and humans, indicating that this region may serve a role in higher-order functioning (Habas et al., 2010; Basile et al., 2021).

A second major efferent pathway is the uncinate tract of the fastigial nucleus. This is not considered as part of any of the three cerebellar peduncles, however. The fibers originating from this nucleus cross over the midline in the rostral cerebellar commissure and pass dorsal to the superior cerebellar peduncle to enter the vestibular nuclei laterally (Gray, 1995). The fibers then traverse the medial and spinal vestibular nuclei and distribute to the medial reticular formation where they terminate (Gray, 1995). The middle cerebellar peduncle (MCP), also known as the brachium pontis, is an afferent pathway that carries input fibers, primarily from the pontine nuclei of the basilar pons into the cerebellar cortex (Duvernoy et al., 2009). The inferior cerebellar peduncle has both an afferent and efferent pathway. It consists of an outer, compact fiber tract (restiform body) on the upper medulla and scattered fiber bundles (juxtarestiform body) that enter the cerebellum (Duvernoy et al., 2009). The former is the afferent pathway and

its fibers are carried from the spinal cord and medulla to the cerebellum via the spinocerebellar, trigeminocerebellar, cuneocerebellar, reticulocerebellar, and olivocerebellar tracts (Gray, 1995). The latter is the efferent pathway and consists of vestibulocerebellar fibers and Purkinje cell axons from the vermis. These fibers and axons travel from the cerebellum to the vestibular nuclei and uncrossed fibers of the fastigial nucleus (Gray, 1995). The fibers of the fastigial nucleus then rise past the superior cerebellar peduncle and cross over into the brainstem (Gray, 1995).

Cerebellar Structure Is Evolutionarily Conserved

In addition to humans, the cerebellum has been studied in a variety of species including rats, cats, and monkeys. It is, therefore, important to consider the similarities and differences in both structure and function between them. Although there have been many studies focused on the cerebellum, few have looked into how its structure compares between different species and humans. Of these studies includes Larsell's 1953 study, which looked extensively into the relationship between the organization of the cat and the monkey cerebellum. He based much of his observations on the embryological development of the cerebellum in both species, in addition to outer anatomical comparisons between them during the adult stage. This allowed him to determine how the cerebellum is organized before any significant changes due to function. Larsell studied how both the vermis and the cerebellar hemispheres developed from the fetal to adult stage, focusing particularly on the lobules. Based on his observations, the structural organization of the cerebellum during the development of the cat appears to be almost

identical to that of the monkey. There are, however, clear differences in the size of the lobules and hemispheres. It is important to note that Larsell observed the most significant differences when comparing the same species (e.g., just monkey species) than with different ones (e.g., monkey vs cat). For instance, he noted lobule I is a region that is functionally related to the tail in animals and is much larger in the spider monkey (Ateles) than it is in macaques (Larsell, 1953). The reason proposed for this was that spider monkeys use their tail almost like a fifth limb, whereas macaques have little use for theirs (Larsell, 1953). This is further confirmed in Sultan and Glickstein's 2007 study in which they observed that cerebellar enlargement it not uniform or simply an attribute of large brains, but rather behavior-based. When comparing avian cerebella and brain size, they determined that large brains have specialized cerebellar enlargement, which can be indicated by the association of different enlarged folia between species (Sultan and Glickstein, 2007). In the same study, it was noted that the increase in cerebellar size in some species of birds (e.g., woodpeckers, parrots) is due to an increase in lobuli VI – IX, whereas in others (e.g., owls) it is due to an enlargement of lobuli I, II, and X (Sultan and Glickstein, 2007). This, along with Larsell's findings, suggests that the differences in lobule sizes of the cerebellum may likely be due to functional uses, which may be a factor in why the posterior lobe of the cerebellum is much larger in humans.

When compared together, the atlases of the cerebellum created by Schmahmann, displaying the human cerebellum, and Madigan and Carpenter, displaying the Rhesus monkey cerebellum, show nearly identical organization. This indicates that the cerebellum of the monkey, although smaller, may be compared to that of humans at an

almost one-to-one basis. One of the main differences that can be seen between the human cerebellum and that of other animals is the size of the lobules and, most notably, the cerebellar hemispheres. In humans, the cerebellar hemispheres are the largest regions of the cerebellum, whereas in other species, excluding monkeys, they are often quite small. Based on the studies aforementioned, this enlargement could likely be due to the behaviors or functions utilized. Regardless, differences in size does not appear to affect the general organization of the cerebellum. In all specimens studied, the cerebellum consists of three distinct regions and coils inward from a superior to inferior aspect. The three regions are comprised of the vermis at the midline, the lateral cerebellar hemispheres, and the flocculonodular lobe, with each being further divided into lobules. Although the divisions of the anterior lobe (lobules I – V), vermis, and flocculonodular lobe (lobule X) are consistent between species, there is debate on how the divisions of the posterior lobe are connected. In his and Brodal's book *Aspects of Cerebellar Anatomy*, Jansen claimed there are bridges connecting certain lobules to each other and where they connect can vary between different species. For instance, in the rat, cat, and monkey, the folium-tuber vermis has bridges connecting to the ansiform lobule and the paramedian lobule, whereas the uvula has connections to the ventral paraflocculus and the nodulus to the flocculus (Larsell, 1952, 1953). Jansen observed in the fin whale that the connections between lobules were confined by narrow cortical bridges and where they connect is not the same as in Larsell's observations. In some cases, for instance, the ventral paraflocculus had a bridge connecting it to the caudal part of the pyramis in addition to the uvula (Larsell, 1953). These observations, in relation to the variation of connections

between lobules, appear to be confined to the posterior lobe as the anterior lobe seems to be fairly consistent with where its connections are located. Similar to the claims aforementioned, Jansen (1954) proposed that these observations could be due to morphological and functional differences, specifically in the posterior lobe of the vermis and cerebellar hemispheres.

Another factor that should be considered between species is whether the development of the cerebellar cortex follows that of the cerebral cortex. It has long been believed that the cerebellum and prefrontal cortex were not related as the former was thought to serve a role in motor functions and the latter in cognitive functions (Diamond, 2000). Recently, research has provided some support for the cerebellum's possible role in cognition, leading to increased interest in whether the cerebellum has followed a similar evolutionary track to the rest of the brain and if its development can be linked to the prefrontal cortex. Although not many studies have focused on this particular aspect of cerebellar development and evolution, there is some evidence that there may be a link between the development of both structures. In Balsters et al.'s 2010 study comparing non-human primates and humans, the authors observed that certain regions of the posterior cerebellum, notably Crus I and Crus II, increase in proportion to the prefrontal cortex. It was also noted that the volume of these regions in the human cerebellum were larger than in capuchins and chimpanzees, suggesting an evolutionary development in line with higher-order functioning similar to that seen with the enlargement of the frontal lobe in humans (Balsters et al., 2010). In a separate study, Guevara et al. (2022) found that the cerebellum is estimated to be epigenetically older than the dorsolateral prefrontal

cortex in young adult humans and chimpanzees. In humans, however, the age of the cerebellum is epigenetically younger in older individuals (Guevara et al., 2022). This could be a factor in understanding why the cerebellum is one of the last regions to be affected in neurodegenerative conditions and why in some people the progression of these afflictions is worse than in others with the same condition. In addition, this could also be a factor in why some older individuals cognitively age more successfully than others. By first understanding how the cerebellum is functionally impacted during normal aging, through the use of the non-human primate model, it would then be possible to factor in other aspects such as that stated above and translate these findings to the human cerebellum.

Structural and Functional Divisions of the Cerebellum

The physical structure of the cerebellum is clear in most animals and humans, allowing researchers to agree on where the divisions lie and which regions are connected. When classifying the divisions of the cerebellum, the lobes and lobules can be distinguished longitudinally and the overarching regions (i.e., the vermis, intermediate hemispheres, and lateral hemispheres) can be distinguished laterally via the fissures. Despite differences in size between species, the development and location of the subdivisions, fissures, and lobules of the cerebellum appear to be consistent (Larsell, 1953; Sultan and Glickstein, 2007). This was also seen upon review of Schmahmann's atlas of the human cerebellum and Madigan and Carpenter's atlas of the Rhesus monkey cerebellum, in which the divisions seen in both atlases correspond with each other on a

one-to-one basis. Upon simple observation of the cerebellum, the divisions are as follows (Fig. 1a). The anterior lobe lies most superior and includes lobules I through V, with the division determined to end at the posterior end of lobule V and demarcated by the primary fissure. The posterior lobe is located in the central region of the cerebellum and consists of the lateral hemisphere lobules VI through IX. This region is distinguished by the primary fissure at its superior end and by the posterolateral fissure at its inferior end. The flocculonodular lobe lies below the posterolateral fissure and consists of lobule X.

In contrast to the anatomical divisions, there is heavy debate on where the functional divisions of the cerebellum lie. It is understood that the cerebellum serves an important role in regulating movement and balance, but its role in cognition is only recently being recognized. This poses a problem when classifying the functional divisions as it is unknown how specific regions are involved and which types of cognitive functions trigger activation in those regions. Due to this, it has proven difficult to determine with certainty which regions and lobules are related to cognition. Given the current knowledge, there are three categories under which the functional activity of the cerebellum can be compartmentalized: the spinocerebellum, the cerebrocerebellum, and the vestibulocerebellum (Fig. 1b). This is just one way in which the regions of the cerebellum can be grouped in relation to their function. The grouping can vary as some researchers only identify the former and the latter in addition to another group (the pontocerebellum or cortico-ponto-cerebellum) (Haines and Mihailoff, 2018), while others consider the vestibulocerebellum as part of the spinocerebellum (Lara-Aparicio et al., 2022; Voogd and Ruigrok, 1997; Miall, 2013). It is clear, however, that the functional

grouping of the cerebellar lobes and lobules are classified primarily on either cognitive or motor function. Although the regions involved are not as straightforward as seen in the structural divisions, it is proposed that the spinocerebellum and vestibulocerebellum are related to motor functions and the cerebrocerebellum is related to cognitive functions (Lara-Aparicio et al., 2022; Voogd, 2003; Schmahmann, 2019). It is believed that the spinocerebellum consists of the vermis, the paravermis (intermediate hemispheres), and the anterior lobe (lobules I – V) (Vuillier et al., 2011). The vestibulocerebellum is believed to consist of the flocculonodular lobe. For those who do not consider the vestibulocerebellum under its own category, the flocculonodular lobe is compartmentalized under the spinocerebellum. The cerebrocerebellum is proposed to include nearly the entire posterior lobe (lateral cerebellar hemispheres) (Vuillier et al., 2011). In general, there seems to be some agreement on which lobules are involved in motor activity and which ones could be involved in cognitive function. The entirety of the vermis and paravermis, vermis lobules I – IX and the region immediately adjacent, lobule V, lobule VIII, and lobule X are all considered to serve motor functions in the cerebellum (Koziol et al., 2014). This leaves hemisphere lobules VI, VIIA (Crus I and Crus II), VIIB, and IX as being involved in cognition (Koziol et al., 2014). There is some debate on how involved hemisphere lobule IX is in cognition, but some studies have suggested it may have a role in language function (Zhang et al., 2023).

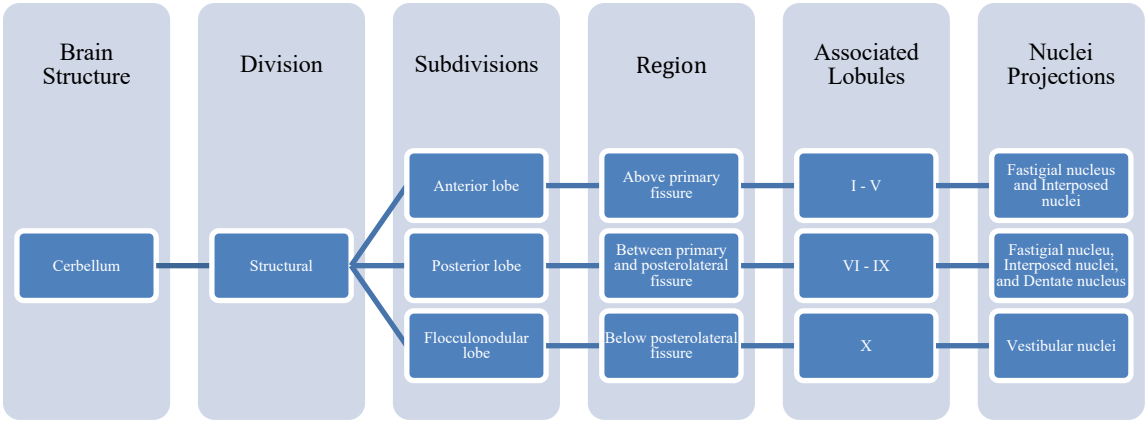


Figure 1a. Anatomical divisions of the cerebellum based on physical location.

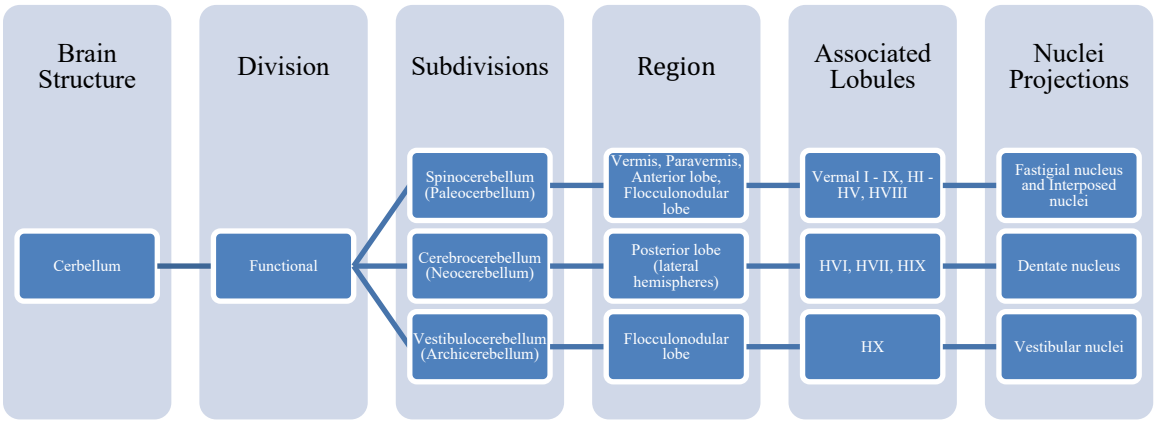


Figure 1b. Functional divisions of the cerebellum based on activation patterns

Morphology of the Cerebellar Cortex

The cerebellar cortex consists of three layers in the following order from deep (internal) to superficial (external): a region of gray matter called the granular layer, a Purkinje cell layer, and a region called the molecular layer. The granular layer is a highly cell-dense region that consists predominantly of granule cells, in addition to unipolar brush cells and Golgi cells. The Purkinje cell layer is a thin region that consists of a

discontinuous monolayer of Purkinje cells. The molecular layer is a cell sparse region that consists primarily of axons from granule cells (parallel fibers) and dendrites of Purkinje fibers, as well as the cell bodies and processes of interneurons called stellate and basket cells. Granule cells are the most abundant cell type within the cerebellar cortex, with about two to seven million cells in the human cerebellar cortex (Fox and Barnard, 1957; Braitenberg and Atwood, 1958). When evaluated in terms of Purkinje cells, it is calculated that there are approximately three thousand cells for each Purkinje cells (Lange, 1975). Granule cell axons extend into the molecular layer where they bifurcate into parallel fibers traveling orthogonally to the dendrites of Purkinje cells. Golgi cells are inhibitory interneurons whose axons extend parallel to Purkinje cell dendrites and climbing fibers into the molecular layer, where they receive input from parallel fibers. They can also extend deeper within the granular layer to form cerebellar glomeruli. Unipolar brush cells have a short, single dendrite that forms excitatory synapses on a granule cell. They are found in large numbers within the nodulus and flocculus, however their function in these regions is not well understood (Gray, 1995). Purkinje cells are fairly large with their soma measuring 50 to 70 microns vertically and 30 to 35 microns transversely (Gray, 1995). They have a fan-like appearance consisting of one axon that extends deep into the cerebellum towards the deep cerebellar nuclei and one or two dendrites that ascend into the molecular layer, forming a rich arborization. Stellate cells are located more superficially in the molecular layer. Their axons extend only to the terminals of Purkinje cell dendrites. Basket cells are located deeper in the molecular layer. They have thick axons with collaterals that ascend the length of Purkinje cell

dendrites and descend towards Purkinje cell soma (Gray, 1995). The terminal branches of basket cells can extend across several rows of Purkinje cells, allowing synapses up to 72 Purkinje cells for a single basket cell (Gray, 1995).

There are two functionally and anatomically separate types of input fibers to the cerebellum: the mossy fibers and the climbing fibers. Mossy fibers are derived from the brainstem and spinal cord nuclei. Their axons extend into the deep cerebellar white matter, where they send a projection to one or more deep cerebellar nuclei and terminate as specialized endings (rosettes) in the granular layer (Gray, 1995). These mossy fiber rosettes form complex synapses with the axons of Golgi cells and the terminals of granule cell dendrites; this complex is referred to as cerebellar glomeruli (Gray, 1995). Climbing fibers are the terminals of olivocerebellar fibers derived from a collection of brainstem nuclei called the inferior olivary nuclear complex (Angaut et al., 1985; Gray, 1995). Olivocerebellar fibers project their axons past the granular layer and into the molecular layer where they become climbing fibers and wrap around Purkinje cell dendrites (Angaut et al., 1985; Gray, 1995). Each climbing fiber interacts with only one Purkinje cell and sends a strong excitatory synapse, triggering the activation of the cell.

Function of the Cerebellar Cortex

Each cell and fiber of the cerebellum has a specific function and target through which their activity is acted upon. Granule cells can form cerebellar glomeruli in the granular layer through which they either form excitatory synapses via mossy fibers or inhibitory synapses via Golgi cells (Gray, 1995). This synapse complex is one way in

which parallel fibers can be modulated. If not inhibited by another cell, the function of these fibers is to excite Purkinje cells in the molecular layer. Due to their morphology and large quantity, it is estimated that close to 250,000 parallel fibers can cross a single Purkinje dendritic tree (Gray, 1995). They also serve as the main input source for Golgi cell dendrites. Golgi cells are an inhibitory GABAergic (inhibitory) interneuron with activity centralized within the cerebellar glomeruli (Gray, 1995). They send inhibitory feedback to granule cells, blocking them from synapsing on the Purkinje cell. The synaptic terminals of mossy fibers lie in the center of cerebellar glomeruli and, unlike Golgi cells, they send excitatory feedback to granule cells, deep cerebellar nuclei, and unipolar brush cells (Gray, 1995). Similar to mossy fibers, climbing fibers excite deep cerebellar nuclei, but their primary function is to excite Purkinje cells. Although there is only one climbing fiber per Purkinje cell, the terminal branches divide and span the length of Purkinje cell dendrites, allowing for multiple synapses on a single cell. For this reason, climbing fibers provide the strongest input on Purkinje cells compared to other input sources such as parallel fibers. Purkinje cells serve as the only output source of the cerebellar cortex (Voogd, 2003). Each has a single axon that extends deep into the cerebellum where it synapses with the deep cerebellar nuclei and sends an inhibitory signal (Gray, 1995). Stellate and basket cells are GABAergic interneurons that act on Purkinje cells (Gray, 1995). Both receive excitatory input from parallel fibers, although basket cells can receive synapses from Purkinje cell collaterals, climbing fibers, and mossy fibers as well. Stellate and basket cells act on different locations on Purkinje cells. The former synapses on the terminal branches and the latter primarily on the soma.

It is important to note that Purkinje cells have parallel, longitudinal zones through which they act. In most species, these zones can be distinguished through efferent (corticonuclear) and afferent (olivocerebellar) fiber connections (Voogd, 2003). In the rodent species, however, there is a substance in some Purkinje cells called zebrin, which is a group of proteins than can be observed through immunohistochemistry staining (Voogd, 2003). Zebrin-positive and zebrin-negative Purkinje cells form an alternating, band-like pattern identical to the zoning pattern seen in other species that do not have zebrin (Voogd, 2003). There are two sets of zones originating from the anterior vermis and hemisphere and each zone projects to a specific deep cerebellar nucleus (Voogd et al., 1987). In the anterior vermis, there are 3 zones: A, X, and B. The A zone is located most medially, extends the length of the vermis until it reaches the nodulus, and projects to the fastigial nucleus. The X and B zones are predominantly in the anterior portion of the vermis and terminate near the ansiform lobule. The X zone projects to the interstitial cell groups, located between the fastigial and globose nuclei, and the B zone projects to the lateral vestibular nucleus of Deiters (Voogd et al., 1987; Voogd, 2003). Although not one of the main Purkinje cell zones, the nodulus of the vermis projects to the vestibular nuclei and is organized into four more zones in the caudal aspect of the hemispheres (Voogd, 2003). In the anterior hemisphere, there are five zones directly following those of the anterior vermis: C_1, C_2, C_3, D_1, and D_2. C_1 and C_3 project to the emboliform nucleus (Voogd, 2003). They are present in the anterior and intermediate (paramedian lobule) regions of the cerebellar hemispheres, but not in the folial chain around the ansiform lobule and the paraflocculus (Voogd, 2003). C_2 projects to the globose nuclei

and is present throughout the cerebellar hemispheres and flocculus (Voogd, 2003). D_1, and D_2 project to the caudal and rostral portions of the dentate nucleus (Voogd, 2003). They span the length of the hemispheres and terminate near the beginning of the flocculus, where they are replaced by the four nodular zones.

Cognition and the Effect of Age

The exact relationship between the cerebellum and cognition is not fully understood, however there are some common patterns seen in the findings of previous studies. One of these is the activation in certain regions of the cerebellum depending on whether a motor or cognitive function is being performed. During motor tasks, the cerebellum often shows activity primarily in the anterior lobe, the vermis, and the flocculonodular lobe (Schmahmann, 2019); Lara-Aparicio et al., 2021). Additionally, activity has also been reported from hemisphere lobule VIII and the most anterior part of hemisphere lobule VI in the posterior cerebellum in relation to motor control functions (Sokolov et al., 2019; Lara-Aparicio et al., 2021). Cognitive-related function, on the other hand, are often associated with the lateral cerebellar hemispheres, specifically lobule VI, lobule VIIA (Crus I and Crus II), and lobule IX (Sokolov et al., 2017; Schmahmann, 2019; Voogd, 2022). Studies on CCAS have also confirmed these trends. In patients with this condition and with isolated cerebellar pathology, it has repeatedly been confirmed that when the posterior lobe is damaged, they have significant cognitive deficits and perform worse on cognitive tasks such as verbal fluency, visuospatial functions, and executive functions (Ahmadian et al., 2019; Schmahmann, 2019). It is believed the

cognitive functions of the cerebellum work primarily through the olivocerebellar system in which input from the brain is received by the inferior olive and sent to Purkinje cells via climbing fibers (Schmahmann, 2019; Voogd, 2022). Being that Purkinje cells serve as the only source of output from the cerebellar cortex, this leads researchers to believe that these cells specifically in the posterior lobe could be key players in feedback to the rest of the brain with regards to modulating cognitive functioning.

Purkinje cells also appear to have an important role in cognitive performance in aging individuals. Studies examining cognitive performance in older adults compared to young adults have reported a negative correlation with performance with increasing age and significant effects of cerebellar gray and white matter volume on performance (Bernard et al., 2015; Cooper et al., 2023). Although it has not been determined whether it is the quantity and/or physical change that affects their function, it is apparent that there are significant effects on Purkinje cells with increasing age. In their study examining the aging cerebellum, Andersen et al. (2003) determined that the overall mean volume of Purkinje cells decreased about 30%, with the greatest reduction being in the anterior lobe. Another study reported shrinkage of the Purkinje cell soma and reduced height and width of dendritic arborizations of these cells in older individuals (Zhang et al., 2010). Increasing age appears to correlate with a decrease in Purkinje cell volume in the anterior lobe and vermis, however it is still unclear whether this finding is applicable to the posterior cerebellum (Sjöbeck et al., 1999; Andersen et al., 2003; Zhang et al., 2010). As a whole, the cerebellum does appear to change with older age, but the understanding of how these changes affect cognition is varied. It has also been reported that there is

neuronal loss, decreased weight, reduced gray and white matter volume, and decreased synaptic activity of Purkinje cells (Sjöbeck et al., 1999; Andersen et al., 2003; Bernard and Seidler, 2014). It should be noted that the reduction in gray and white matter does not occur at the same rate. MRI studies have reported that there is a greater decrease in white matter volume than in gray matter volume, and the former decreases at a faster rate (Han et al., 2020; Romero et al., 2021). These changes have also been tied with cognitive and motor declines. In a review, it was reported that when older adults had greater cerebellar volume, they performed better in cognitive tests, such as processing speed and executive function, compared to those who had smaller cerebellar volume (Bernard and Seidler, 2014). Other studies have reported different findings, however, with large cerebellar volume in some regions, such as gray matter in the posterior cerebellum, resulting in poorer cognitive performance (Bernard et al., 2015; Cooper et al., 2023). This indicates there could be specific regions within the posterior cerebellum that have a greater effect on cognitive functions than others and that selective activity of Purkinje cells in the cerebellar cortex could be key to identifying those regions.

SPECIFIC AIMS / OBJECTIVES

There are two objectives of this study. The first is to determine whether there is specific cellular activation in the cerebellum as a result of performing a cognitive task and where this activation is present. The second is to determine if there is an aging effect the cerebellum. Prior studies have determined there is activation in the cerebellum while performing cognitive tasks as well as in neurodegenerative conditions, but few have looked into the direct relationship between this structure and cognition (Schmahmann, 2019; Habas, 2021). Most studies that have examined the relationship between the two have done so through the use of various MRI methods including resting state MRI and functional MRI, presenting findings that confirm the activation of the posterior cerebellum during cognitive functioning (Habas, 2021; Liang et al., 2020). Although helpful in determining the cerebellum's role in cognition, there is no standard definition of where exactly the border of white and gray matter volumes lie, making it difficult to definitively identify where activation is present. It has previously been established that the function of the posterior cerebellum has a significant impact on cognitive performance in individuals with conditions such as CCAS and Alzheimer's Disease, but the reason why is not understood as it was previously believed to only serve as a mediator in motor functions (Schmahmann, 2019; Lin et al., 2020). It is, therefore, necessary to first understand what role the cerebellum plays in cognition during normal functioning to understand its effects in pathological conditions. Additionally, cognitive ability varies between older individuals and if the cerebellum does play a significant role in mediating cognition, it would also be imperative to examine how its performance is impacted during

normal aging. Through this study, it is expected to gain a better understanding of the role the cerebellum has in cognition itself as well as during normal aging. This will be achieved by quantifying neural activity in the posterior cerebellum of Rhesus macaque monkeys with different ages using a cellular marker and comparing this with the score from each subject's cognitive impairment index.

METHODS

Subjects

Nine macaque monkeys (*Macaca)*, six males and three females aged between 15.2 to 32.9 years old, were used for this study (Table 1). Prior to sacrifice, the monkeys performed the spatial delayed recognition span task (sDRST) – a cognitive task designed to assess spatial working memory (Salter et al., 2015) – during which their DRST scores were determined. Prior to this study, these monkeys were assessed on a battery of tests that produce a cognitive impairment index. Their cognitive impairment index was measured to determine their baseline cognitive function. Both the DRST and the CII scores were later compared with the findings of this study. All procedures and care of the animals were approved by the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* and the Institutional Animal Care and Use Committee of the Boston University Medical Campus.

Table 1. Subjects

Subject	Sex	Age at Sacrifice	Hemisphere(s) Sectioned	Cognitive Impairment Index (CII)
AM383	F	23	R, L	**
AM303	M	15.2	R, L	1.168
AM369	F	27	R, L	3.193
AM372	M	20.8	R, L	2.853
AM389	F	23.4	*L	2.948
AM391	M	20.4	R, L	1.681
AM390	M	20.1	R, L	1.108
AM402	M	32.9	*R	0.66
AM410	M	31.5	R, L	7.357

*One hemisphere was not available for use in this study.

**CII score was not available

DRST Task, Immediate Early Gene Activation, and Perfusion

Prior to perfusion, the subjects had completed the spatial delayed recognition span test, a delayed non-match sample task, of which the procedures were previously described in another study (Rosene et al., 2005) and are as follows. In this task, the subjects had to identify a stimulus presented in different locations in order to receive a reward. Specifically, a treat (e.g., candy, raisins) was placed in one well of a disk, which was covered by another disk. To prevent influencing the subject during the task, a Wisconsin General Test Apparatus (Harlow and Bromer, 1938) was used to prevent the monkey from being able to clearly see the researcher. The monkeys' task was to remove the cover and identify which well contained the food. While maintaining the correctly identified disks in the same position as originally placed, a treat was then placed in a new location covered by another disk and the monkeys had to identify the where new location of the treat was each time. Each monkey performed ten trials per day for ten days, totaling a span of 100 trials. The task was over once the monkeys either successfully identified nine disks consecutively or if they made an error. Based on performance in these trials, a DRST score was determined. A cognitive impairment index score was also obtained from all subjects, except AM383. The cognitive impairment index (CII) is an aggregation of scores that displays the cognitive ability of an individual. In aged animals,

a score between one and two indicates successful (healthy) aging and a score between two and five indicates unsuccessful (impaired) aging.

The monkeys were perfused 3 hours after the task was completed to allow time for expression of the c-Fos protein. They were heavily anesthetized with sodium pentobarbital and euthanized via exsanguination during transcardial perfusion-fixation of the brain using cold Krebs-Henseleit buffer (4°C), followed by 4% paraformaldehyde (Estrada et al., 2017; Rosene et al., 1986). The brains were blocked in situ in the coronal plane, then removed intact, weighed, photographed, and post-fixed in 4% paraformaldehyde for less than 24 hours (Estrada et al., 2017; Rosene et al., 1986; Orczykowski et al., 2018). To eliminate freezing artifact, the brains were immersed in cryoprotectant, then flash-frozen in -75°C isopentane and stored in -80°C until ready for cutting (Rosene et al. 1986; Estrada et al. 2017; Orczykowski et al. 2018).

Tissue Acquisition

The cerebella of all monkeys were previously cut into hemispheres along the vermis and stored at - 80°C until ready for use. Both hemispheres, when available (discussed later), from each animal were used. Once the cerebella were taken out of the freezer, they were immediately placed on dry ice to prevent any damage to the tissue while being cut. All cerebella were cut on a microtome into series of 40µm thick coronal sections (12 series for two animals, 8 series for one animal, and 24 series for seven animals; discussed later). All series were collected in a 0.1 M phosphate buffer with 30% glycerol, and then stored at -20°C until ready for immunohistochemical staining. One

hemisphere of the cerebellum of three monkeys was not available for use. One hemisphere of two monkeys (AM389 and AM402) had previously been used in another, unrelated, experiment. Due to this, only twelve series were collected from each. The third monkey (AM383) had one hemisphere cut sagittally, so that hemisphere was omitted from this study. This monkey only had eight series collected from the available hemisphere. For the other monkeys with both hemispheres available, twenty-four series were collected (12 series for the left hemisphere, 12 series for the right hemisphere). There was one monkey with both hemispheres still intact at the time of cutting, so its series were not split into right and left hemispheres (24 series collected for the whole cerebellum).

Immunohistochemistry

For the purpose of this study, immunohistochemistry using c-Fos antibody was used to visualize cellular activation in the cerebellum, and the tissue was batch-processed to help ensure consistency. About one-eighth of Series 1, four to six tissue sections, from each hemisphere of each monkey was stained. Due to Series 1 being used in a different, and unrelated, experiment, Series 5 of one monkey (AM383) was used instead. The tissue was removed from storage, then thawed. While the tissue was thawing, 2 L of 0.05 M TBS (pH 7.6, Sigma-Aldrich) was made in preparation for washes. Trays with wells were used to separate the tissue sections throughout the process and, in all steps, the trays were placed on a rocker to ensure full coverage. For the washes, each monkey had its own designated column with three rows of wells that were filled about halfway with the TBS.

The trays were placed on a rocker, set at a low speed, and washed in TBS for 10 minutes. This was repeated two more times for a total of three washes. For each wash, the tissue was moved from one row to the next to ensure complete removal of glycerol. The tissue was then quenched in TBS containing 1% H_2O_2 for 20 minutes. This was followed by another set of three washes. After washing, the tissue was placed in a blocking solution containing 10% Normal Goat Serum (NGS) and 0.4% Triton-X, then incubated for 1 hour. The tissue was then incubated for 1 hour in polyclonal primary antibody (c-Fos polyclonal from rabbit, 1:2000; Calbiochem) in stock solution containing 2% NGS and 0.1% Triton-X. After incubation, the trays were wrapped in parafilm and stored at 4°C on a rocker for 48 hours. Following this, the tissue was removed from the antibody solution and washed in TBS 3 times. It was then incubated in biotinylated secondary antibody (anti-rabbit IgG, H + L, made in goat; 1:600; Vector Labs) diluted in a stock solution containing 0.1% Triton-X and 2% NGS for 2 hours. The tissue was then washed 3 times in TBS, followed by a 1-hour incubation in an avidin biotinylated horse-radish peroxidase complex (Vectastain ABC Elite; Vector Labs), and another set of washes after. To visualize cell activity, the tissue was incubated in a chromagen solution containing Nickel (II) sulfate, 3,3'-Diaminobenzidine (DAB, SigmaFast), and H_2O_2 for about 4 minutes. The tissue was then immediately removed from the solution into TBS for washes. After the final wash, the tissues were placed in new wells containing TBS and stored at -20°C for two overnights before being mounted onto gelatin-subbed slides. Following mounting, the tissue was air-dried for two days. Once dry, the slides were dipped twice in 100% ethanol to dehydrate the tissue and then twice in xylene to clear the

slides. After, the slides were cover-slipped with permount (Thermo Fisher Scientific) and dried in a fume hood for about 4 days before being viewed under a microscope.

Digitizing and Measurements

In preparation for analysis, the posterior lobe (lobule VIIA) and the dentate nucleus were chosen as the regions of interest. All slides were individually digitized using a microscope and the slide scanner function on the program NIS-Elements. To normalize the data, one slide of each hemisphere per animal was chosen for analysis. One hemisphere from four monkeys (AM402, AM389, AM383, and AM391) was not included due to an aforementioned issue. One hemisphere of AM391 was not included due to the absence of lobule VIIA in the tissue selected. The 'Measurement' function of the program was used to measure the area of lobule VIIA and to determine the number of Purkinje cells within that area. The area was determined by drawing a contour around the Purkinje cell layer and the molecular layer in lobule VIIA.

Statistical Analysis

The data was analyzed by comparing the association between age and four groups: Purkinje cell numbers, Purkinje cell density, cognitive impairment index, and DRST score. The relationship between both DRST scores and CII scores and active Purkinje cells and density was also determined. To determine significance, a Pearson correlation analysis was done and a p-value of less than 0.05 was considered to be significant.

RESULTS

Activation Patterns

Compared with the anterior lobe, lobules in the posterior cerebellum showed greater Purkinje cell activation. There were few to no activated cells in the anterior lobe, whereas in the posterior lobe there was clear activation with several active Purkinje cells present. In all samples, specific activation was seen in some regions of lobule VIIA than in others. There was scattered activation of Purkinje cells, but activation was not consistent throughout the lobule. All samples had clusters of highly activated Purkinje cells in each hemisphere except the right hemisphere of AM391 and the left hemisphere of AM410. The left hemisphere of AM372, however, showed Purkinje cell activation in nearly the entire lobule (Fig. 13a). The activation of Purkinje cells also varied within the subfolia. In almost all cases, activation was present in one half of the subfolia, whereas the other half only had either very light activation or no activation at all (red arrow in Fig. 13a). When visible, the dentate nucleus had a large concentration of highly active Purkinje cells in the entire region. Similar to the Purkinje cells, granule cell activation was also present in the form of clusters. Activation of granule cells was present in both regions with active and inactive Purkinje cells. There were two animals that did not perform the DRST behavioral task, AM303 and AM369. The data from these two were compared with that of another pair of animals, AM390 and AM372, who did perform the task. Since bilateral samples were available for both pairs, activation patterns of the left and right hemispheres of these samples were compared. In the monkeys who performed the behavioral task, there appeared to be greater

activation in one hemisphere than the other (Fig. 13a, 13b), whereas those who did not perform the task showed little difference between the hemispheres (Fig. 13c, 13d).

Task-Dependent vs Task-Independent

Cellular activation of two subjects that performed the DRST task (AM390 and AM372) were compared with two subjects who did not perform the task (AM303 and AM369). There was greater cellular activation in the subjects who performed the task compared to the subjects who did not, however, this association was not statistically significant, $r(2) = .88$, $p = .12$ (Fig. 10). Additionally, there was no statistically significant difference in Purkinje cell density between those who completed the task and those who did not, $r(2) = .39$, $p = .61$ (Fig. 11). It was determined that there was no significant difference in activation of the hemispheres in the task group (*Mdn* = 0.164) and the non-task group (*Mdn* = 0.053). A non-parametric Mann-Whitney U test indicated that there was no statistically significant difference between left and right hemisphere activation in the monkeys who performed the task and the monkeys who did not, $U(N_{Task} = 2, N_{Non\text{-}Task} = 2) = 3$, $z = .43$, $p = .67$. There was also no statistically significant difference between Purkinje cell activation in the active hemisphere of the monkeys in the task group (*Mdn* = 123) and either hemisphere of the monkeys of in the non-task group (*Mdn* = 92.5), $U(N_{Task} = 2, N_{Non\text{-}Task} = 2) = 4$, $z = .98$, $p = .33$. A Mann-Whitney U test analysis comparing the Purkinje cell density in the monkeys who performed the cognitive task (*Mdn* = 1.85) to the monkeys who did not (*Mdn* = 1.7) indicated there was no statistically significant difference between the two, $U(N_{Task} = 2, N_{Non\text{-}Task} = 2) = 2$, $z = -.41$, $p = .68$.

Impact of Age on Number of Purkinje Cell Numbers

For each animal, except four (AM402, AM389, AM383, and AM391), the area and the number of activated Purkinje cells present in lobule VIIA was determined for both hemispheres (Table 2). Age appears to have a negative correlation with activated Purkinje cell numbers in lobule VIIA, but in this case there was no statistically significant effect of age, $r(12) = -.46$, $p = .09$. The largest number of Purkinje cells in lobule VIIA was present in the monkeys aged between 15 and 25, whereas monkeys aged around 30 years had fewer cells present (Fig. 2). In the samples that had both hemispheres present, there was a difference in how many cells were active in one hemisphere versus the other (Table 2). The most notable of these were AM410 and AM372, which had a difference of around 50 cells between the left and right hemispheres.

Impact of Age on Purkinje Cell Density

There was no statistically significant effect of age on the number of Purkinje cells per area in lobule VIIA, $r(12) = .29$, $p = 0=.33$. Similar to the relationship between age and Purkinje cell numbers, there appears to be a slight negative correlation between age and Purkinje cell number per area, with the largest concentration of cells present in monkeys aged between 15 and 25 (Fig. 3). There was one outlier in which the right hemisphere of monkey AM410 had a much greater number of cells present than the other monkeys.

Association Between Age, CII Scores, and DRST scores

There was no statistically significant association between either age and the cognitive impairment indices of the subjects, $r(6) = .45$, $p = .27$, or age and DRST score, $r(5) = -.17$, $p = .71$. There was a weak positive correlation between increasing age and higher CII scores. As age increased, so did the cognitive impairment index score. There was one exception to this. AM402 had a CII score of 0.66, which was lowest score of all subjects tested (Fig. 4, Table 3). Age and DRST score showed a weak negative correlation. Increasing age appeared to be associated with a lower DRST score, with worse performance in the behavioral task seen in the older monkeys (Fig. 7, Table 4).

CII Scores vs Active Purkinje Cells and Density

Although not statistically significant, there was a moderate negative association between CII and Purkinje cell density, $r(11) = -.50$, $p = .07$. A higher CII score correlated with a lower Purkinje cell density (Fig. 6). There was also a weak negative association between CII and Purkinje cell numbers in lobule VIIA, $r(11) = -.39$, $p = .17$. A higher CII score correlated with lower Purkinje cell numbers (Fig. 5). AM389 did not follow this trend as its Purkinje cell numbers were much higher than that seen in the other subjects.

DRST Scores vs Active Purkinje Cells and Density

There was no statistically significant association between either DRST score and active Purkinje cells, $r(8) = .13$, $p = .71$, or DRST score and Purkinje cell density, $r(8) = -.36$, $p = 0.30$. There was little to no association between DRST scores and active Purkinje

cells, with a majority of the scores centering between 2 and 2.5 (Fig.8). In contrast, there was a weak negative association between DRST scores and Purkinje cell density, with a greater DRST score being associated with a lower density (Fig. 9). In comparing only the active hemisphere of each monkey, there appeared to be no statistically significant relationship between either DRST and active Purkinje cells, $r(4) = .01$, $p = .98$, or DRST and Purkinje cell density, $r = .50$, $p = .31$. Although, the latter did appear to have a moderate association with each other.

Table 2. Lobule VIIA area measurements and Purkinje cell numbers.

Animal	Area (μm^2)	Purkinje Cell Density	PC/Area (μm^2)
AM410R	714486.86	68	9.5 x 10^-5
AM410L	5259151.52	10	0.19 x 10^-5
AM390L	7689232.41	114	1.5 x 10^-5
AM390R	8036212.62	104	1.3 x 10^-5
AM303L	6357848.7	109	1.7 x 10^-5
AM303R	5509244.42	88	1.6 x 10^-5
AM369R	5611430.85	97	1.7 x 10^-5
AM369L	5751602.99	97	1.7 x 10^-5
AM402R	3453883.28	55	1.6 x 10^-5
AM389L	8328601.71	200	2.4 x 10^-5
AM372R	3376233.15	74	2.2 x 10^-5
AM372L	3930480.75	132	3.3 x 10^-5
AM383R	5316084.12	100	1.9 x 10^-5
AM391R	6434999	92	1.4 x 10^-5

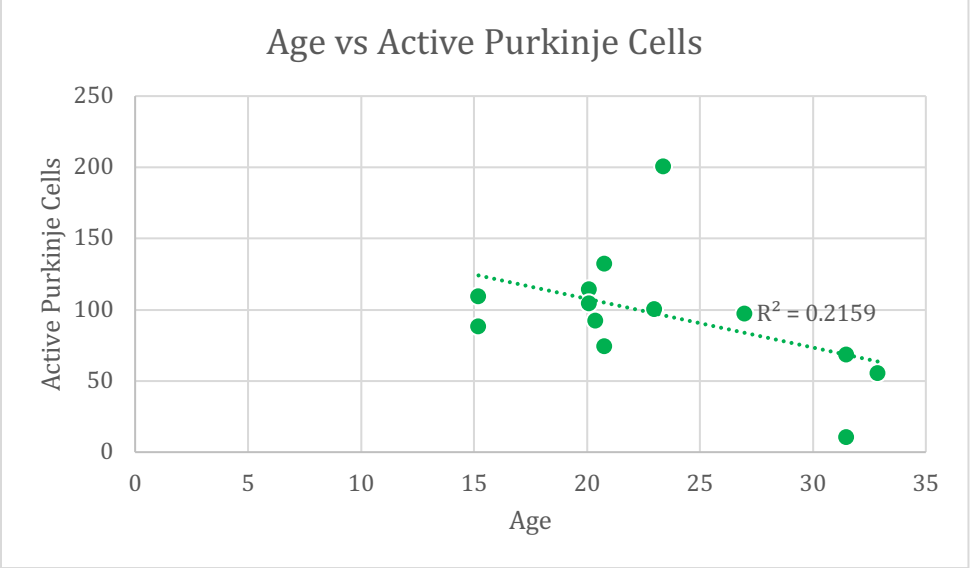


Figure 2. Association between age and active Purkinje cells.

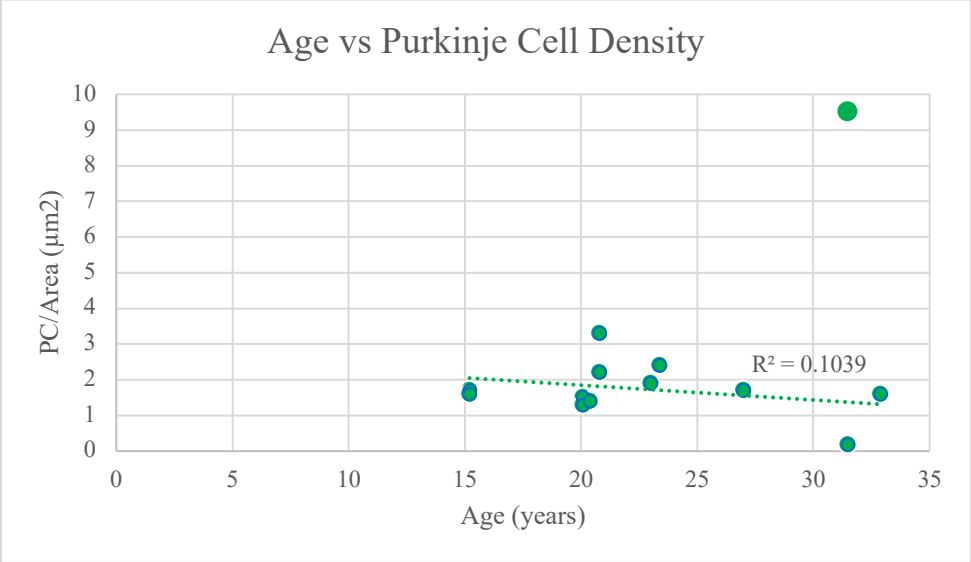


Figure 3. Association between age and Purkinje cell density.

Table 3. Individual Cognitive Impairment Index (CII) Scores.

Animal	Age	CII

AM410	31.5	7.357
AM390	20.1	1.108
AM303	15.2	1.168
AM369	27	3.193
AM402	32.9	0.66
AM389	23.4	2.948
AM372	20.8	2.853
AM383	23	*
AM391	20.4	1.681

*CII score was not available.

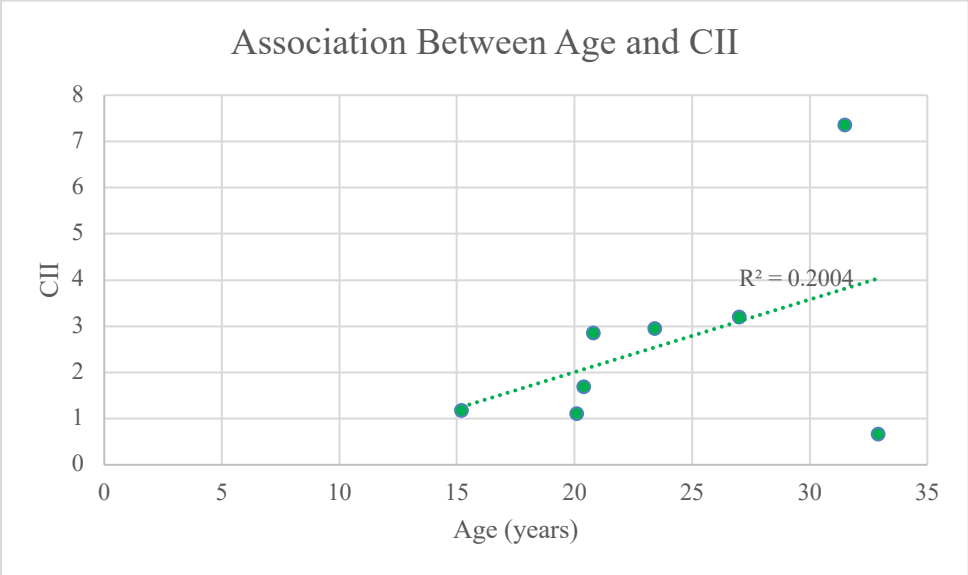


Figure 4. Association between age and CII score.

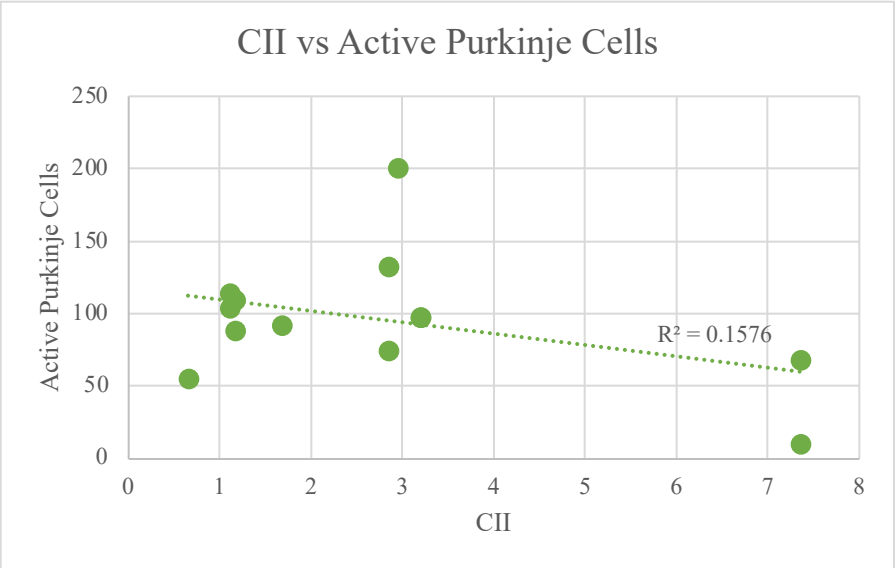


Figure 5. Relationship between CII score and active Purkinje cells.

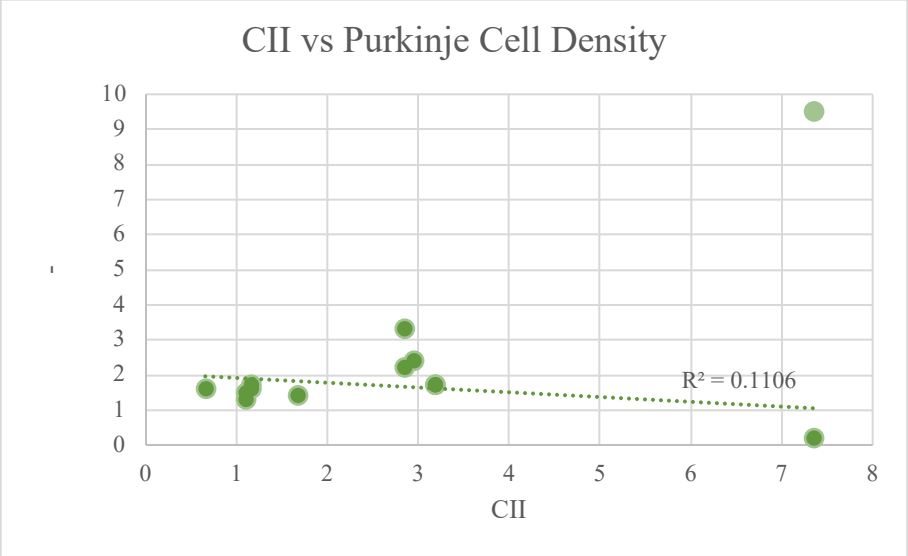


Figure 6. Relationship between CII score and Purkinje cell density.

Table 4. Individual DRST scores.

Animal	Age	DRST

AM410	31.5	1.8
AM390	20.1	3.04
AM303	15.2	*
AM369	27	*
AM402	32.9	2.53
AM389	23.4	1.95
AM372	20.8	2.1
AM383	23	2.26
AM391	20.4	2

*DRST score was not available.

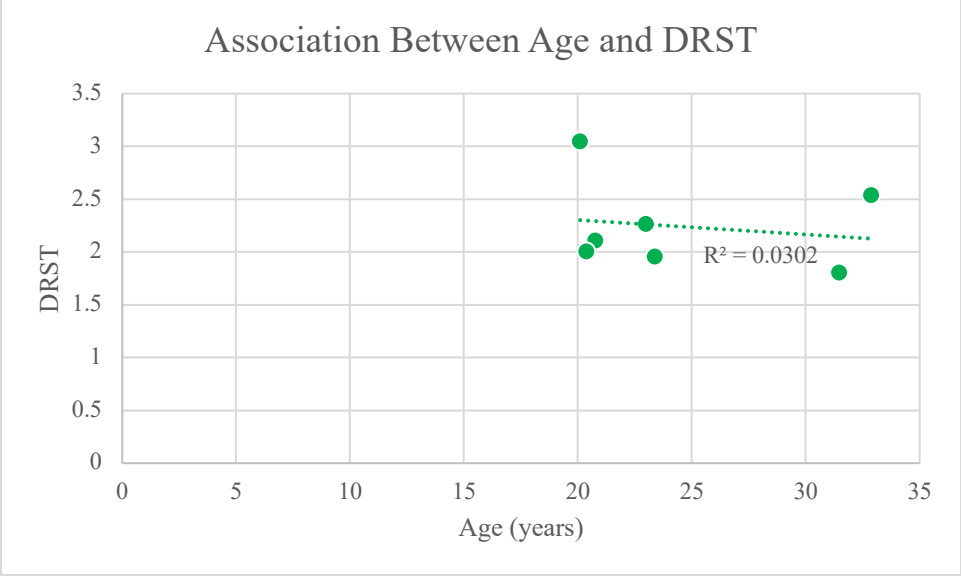


Figure 7. Relationship between age and delayed recognition span test scores.

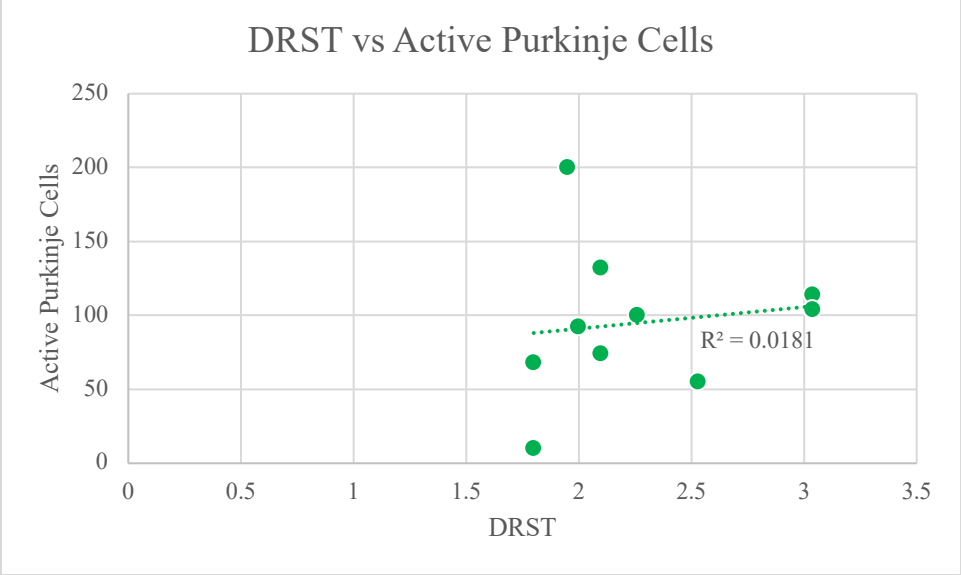


Figure 8. Relationship between DRST scores and active Purkinje cells.

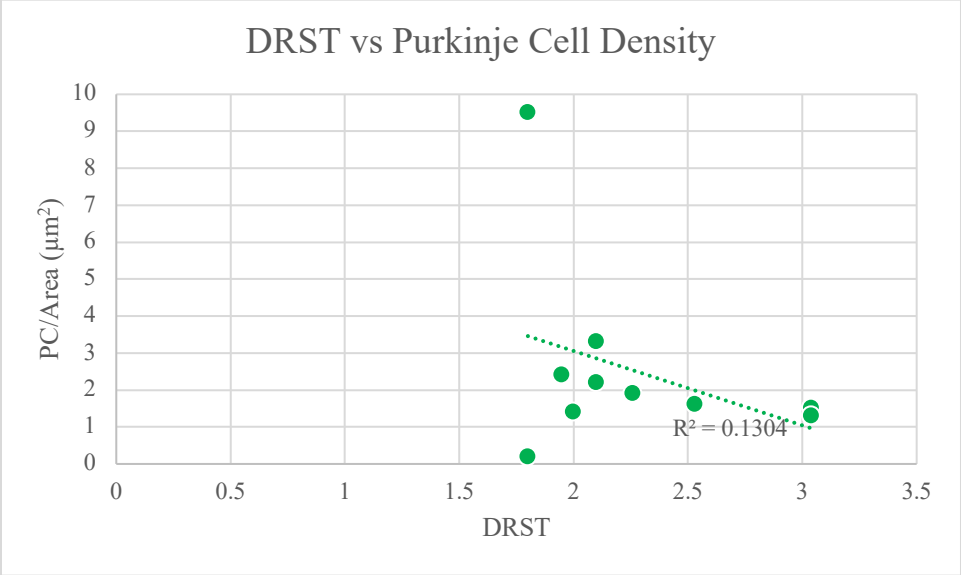


Figure 9. Relationship between DRST scores and Purkinje cell density.

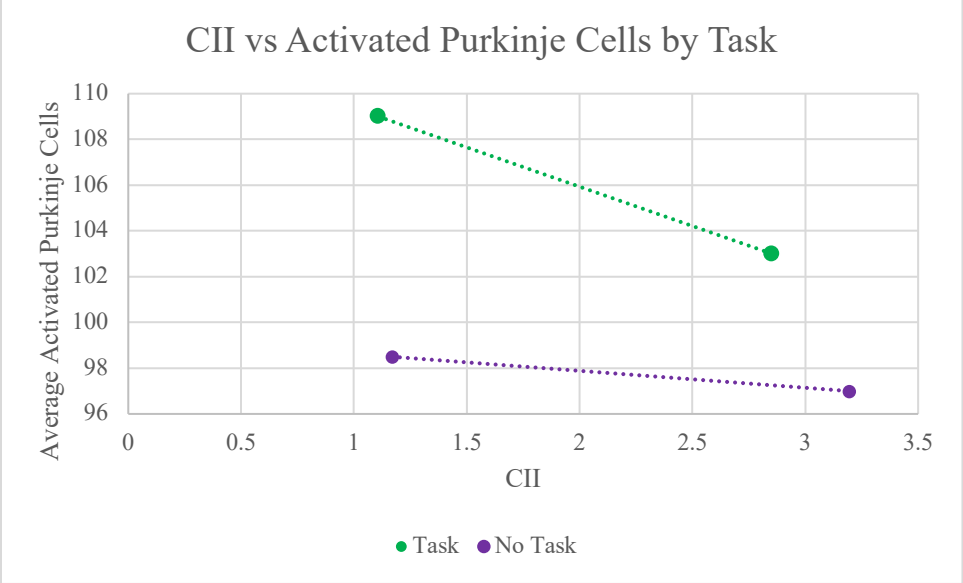


Figure 10. Task-related impact on relationship between CII score and average cellular activation.

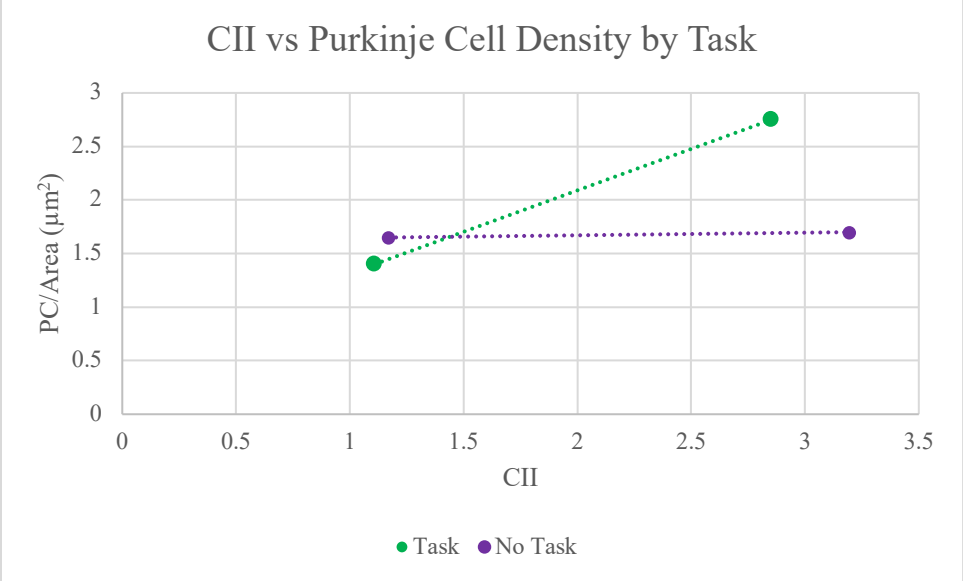


Figure 11. Task-related impact on relationship between CII score and average Purkinje cell density.

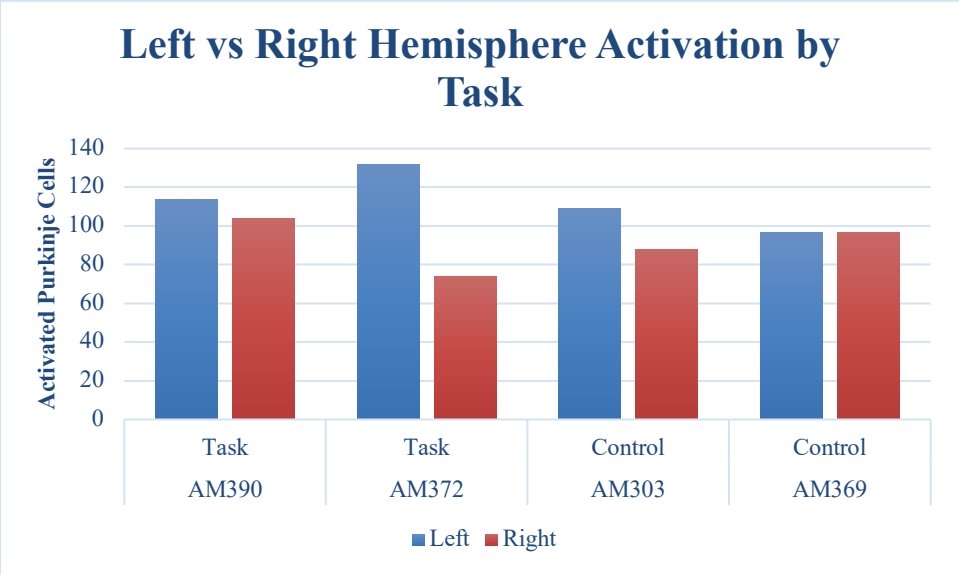
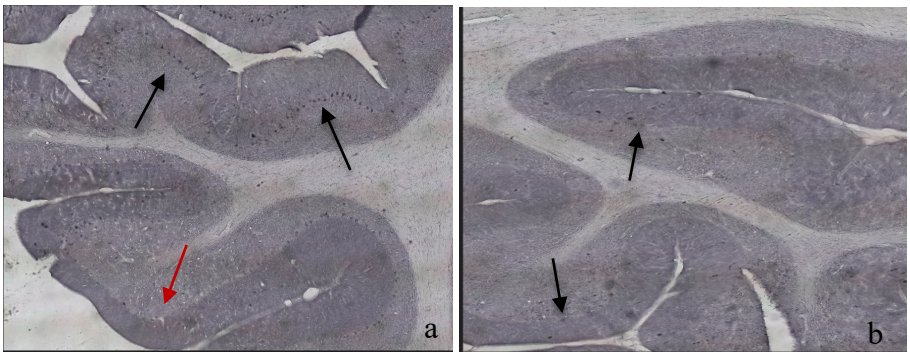


Figure 12. Cellular activation in the left vs right cerebellar hemisphere in task-dependent and task-independent conditions.



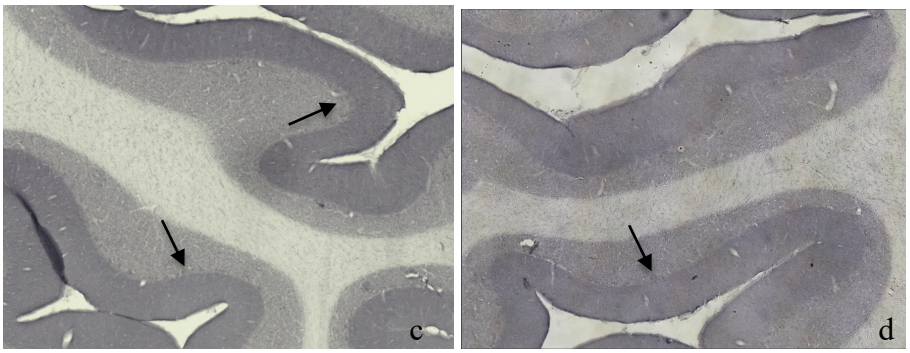


Figure 13. Cellular activation in lobule VIIA in task vs non-task subjects.
Images comparing cellular activation of one task-dependent monkey (AM372) and one task-independent monkey (AM303): (a) AM372 left lobule VIIA, most active hemisphere displaying active Purkinje cells (black arrows), one side of the lobule is more active than the other (red arrow); (b) AM372 right lobule VIIA, least active hemisphere with some Purkinje cell activation (black arrows); (c) AM303 left lobule VIIA, few active Purkinje cells (black arrows) (d) AM303 right lobule VIIA, few active Purkinje cells (black arrows).

DISCUSSION

The two objectives of this study were to determine whether age has a relationship with CII and cellular activation in aged animals and whether there is specific cellular activation in the posterior cerebellum as a result of performing a cognitive task. In this study, we noted differences in activation between the left and right hemispheres – several animals had greater Purkinje cell activity in one hemisphere compared to the other (Table 2). When comparing two groups of subjects who either performed the DRST task or did not perform the task, it appeared that there was a difference in average cellular activation of the left and right hemispheres between the two groups (Fig. 13a-d). However, as this finding was not significant, it is difficult to interpret whether there could be an impact of performing a cognitive task on cellular activation of the left and right hemispheres. It should be noted that there were differences in intensity of activation of Purkinje cells in which some cells were much more darkly stained (Fig. 13a) and others were active but more lightly stained (Fig. 13b,c). Therefore, there could be qualitative differences in cell count, which should be accounted for in future studies to determine a more accurate representation of activity in the lobules. As seen in Figure 12, there is little difference between activation in the left versus right hemispheres in the subjects who performed the task compared to those who did not. Given such a small sample size, it can neither be confirmed nor denied that doing a cognitive task impacts one hemisphere more than the other.

Some evidence has shown that there are disproportionate changes between the regions of cerebellum (e.g., left vs right hemisphere, anterior vs posterior lobe) and that

these changes often are not evident until mid-adulthood (ages between 40 and 59) and old age (ages 60 and up) in humans (Luft et al., 1999; Han et al., 2020; Stalter et al., 2023). Given that there was a slight negative correlation between increasing age and Purkinje cell numbers and density, it is possible that age was starting to affect activation in the posterior cerebellum, but the subjects had not yet reached an age in which significant impairment was evident. Since both hemispheres were not available for all subjects in this study, direct comparisons between the left and right lobule VIIA were not able to be determined. Prior studies have determined that lobules in the left and right hemispheres are affected differentially with increasing age, but most have not examined the cellular differences between them (Escalona et al., 1991; Han et al., 2020). Future studies would need to be conducted to determine the effect of aging on cellular activation between the left and right hemispheres as well as why one hemisphere is more active than the other.

The findings of this study did not show a statistically significant result supporting the hypothesis that age has an association with CII scores in aged animals. However, there was a weak positive correlation. As age increased, there was an increase in CII score, indicating that cognitive impairment became worse over time. This falls in line with what we predicted and provides further evidence that the posterior cerebellum may be correlated with cognitive functioning during normal aging. More studies will need to be conducted with a greater sample size and larger range of ages to determine whether this is a consistent finding in the non-human primate model. In humans, based on MRI studies, it is evident there are changes that occur in the cerebellum that seem to be associated with cognitive dysfunction in aged individuals compared to younger subjects

(Han et al., 2020; Jernigan et al., 2001). As the macaque cerebellum is similar to that of the human cerebellum, this finding could also be translated into future studies examining human cognitive function in aging individuals.

There was a weak negative correlation between CII scores and Purkinje cell numbers and a moderate negative correlation between CII scores and Purkinje cell density. Although these findings did not show statistical significance, they do suggest that there could be a relationship between CII score and neuronal activation in the posterior cerebellum. If there were a correlation between the two, it would have indicated that greater cognitive impairment is related to a decrease in the activation of cells in this region of the cerebellum. The data in this study suggest that a lower cognitive impairment score is associated with greater Purkinje cell activation. Considering the majority of the subjects were aged between 20 and 27 and only two subjects were aged around 30 years, it is uncertain whether this finding will be consistent in other studies. It will be beneficial in future studies to have a wider range of ages in order to determine whether this relationship is accurate. The delayed recognition span test (DRST) score is a direct behavioral metric, so the relationship between the DRST and age, as well as cellular activation, was compared in addition to the CII score. Unlike with CII, as age increased, the DRST score decreased, which is not unusual as this indicates that performance was decreased in the older subjects. Due to such a small sample size, however, more studies are needed in order to determine whether this finding is accurate. There was little association between DRST and active Purkinje cells, however the subjects who performed this task were close in age, so most of them had similar scores. This finding,

therefore, was not unexpected as older subjects would be expected to show the lowest scores and lowest activation of cells. The relationship between DRST and Purkinje cell density showed a weak negative correlation, however, as mentioned above, the scores are similar between the subject so it is difficult to make a conclusion about whether there is a relationship between the two. It would be helpful to include a larger sample of older subjects in future studies to help determine with greater accuracy whether there is a difference in scores between younger and older samples, and how cell activity is in turn related.

Additionally, there was some support for specific cellular activation in the posterior lobe. Lobule VIIA, where Crus I and Crus II are located, displayed high Purkinje cell activation relative to the lobules in the anterior lobe, which displayed little to no activation. This indicates that the cerebellum does recruit cells in the posterior lobe during cognitive functioning, providing further evidence that the posterior cerebellum could be connected with cognitive function. It was observed in most cases that Purkinje cells were active in clusters throughout the lobule and in some of these regions were clusters of granule cells, whereas in others they were not. It could be possible that a single cluster of granule cells could be responsible for activating multiple clusters of Purkinje cells, which may explain why in some regions of the folia few to no granule cells were present. Other studies have noted this finding as well and have attributed these clusters to how the cells migrate during development (Redies et al., 2011; Karam et al., 2001). Purkinje cells are functionally organized in longitudinal zones. As such, it is possible that the clusters of these cells in addition to granule cells are due to this

organization. More studies would need to be conducted to determine why clusters form as well as how activation patterns are related.

The cerebellum has become a region of interest for neurodegenerative conditions in which cognitive function is heavily affected. This is due, in part, to the association between posterior cerebellar damage and cognitive deficits. In order to understand the relationship between the posterior cerebellum and cognition, it is necessary to examine how they are associated during normal functioning and aging. Although the findings of this study were not significant, they give insight into the relationship between these two factors. To our knowledge, this is the first study that shows there could be a correlation between both aging and cerebellar function and, most notably, between cognitive processing and cellular activation in the posterior cerebellum. This study has given evidence that the cerebellum does have a role in cognitive processing, as seen with the heightened activation of Purkinje cells in monkeys who performed the DRST task compared to the little activation in monkeys who did not perform the task (see Fig. 12 and Fig. 13). If the findings of this study are consistent in future studies, it would indicate that the cerebellum is a key player in modulating cognitive processing in addition to the prefrontal cortex. This would be an important factor to consider in studies examining neurodegenerative conditions in which cognition is significantly impaired. There were some limitations to this study that made it difficult to compare cognitive function and cellular activity between each animal, including unavailability of cognitive and behavioral scores for all subjects and both hemispheres not being present. In addition to consistency of variables, it would be beneficial for future studies to have a much greater

sample size for comparisons as well as a larger range of ages to better understand how it relates to functioning in the cerebellum and when significant changes occur.

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