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Research Report

Cerebellar dentate nuclei lesions alter prefrontal cortex dendritic spine morphology

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ABSTRACT

Anatomical tracing studies in primates have revealed neural tracts from the cerebellar dentate nuclei to prefrontal cortex, implicating a cerebellar role in nonmotor processes. Experiments in rats examining the functional role of this cerebellothalamocortical pathway have demonstrated the development of visuospatial and motivational deficits following lesions of the dentate nuclei, in the absence of motor impairment. These behavioral deficits possibly occur due to structural modifications of the cerebral cortex secondary to loss of cerebellar input. The current study characterized morphological alterations in prefrontal cortex important for visuospatial and motivational processes following bilateral cerebellar dentate nuclei lesions. Rats received either bilateral electrolytic cerebellar dentate nuclei lesions or sham surgery followed by a 30-day recovery. Randomly selected Golgi-impregnated neurons in prefrontal cortex were examined for analysis. Measures of branch length and spine density revealed no differences between lesioned and sham rats in either apical or basilar arbors; however, the proportion of immature to mature spines significantly decreased in lesioned rats as compared to sham controls, with reductions of 33% in the basilar arbor and 28% in the apical arbor. Although expected pruning of branches and spines did not occur, the results are consistent with the hypothesis that cerebellar lesions influence prefrontal morphology and support the possibility that functional deficits following cerebellar dentate nuclei lesions are related to prefrontal morphological alteration.

1. Introduction

Evaluations of cerebellar functioning have considered possible contributions beyond motor and classical conditioning functions, including those related to cognitive and motivational processes (O'Halloran et al., 2012; Rapoport et al., 2000; Schmahmann and Pandya, 1997; Strick et al., 2009). The dentate nuclei represent a particularly interesting and important cerebellar output source with significant connectivity to nonmotor cerebral regions.

The dentate or lateral nuclei of the cerebellum are the largest of the deep nuclei and researchers have traced the

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feedforward and feedbackward loops of the pathway between the dentate nucleus and cerebral cortex using a herpes simplex virus type 1 in Cebus monkeys (Zemanick et al., 1991). In a retrograde tracing study, Middleton and Strick (1997) found that injections in areas 46 and 9 of Cebus monkey prefrontal cortex revealed cerebellar ventral dentate nuclei projections to these areas via mediodorsal thalamic nuclei. The dentate nuclei also project to the red nuclei, where some fibers continue toward the thalamus and others project toward the brainstem. In either case, projections from the thalamus to the prefrontal cortex feed back to the cerebellum via the pons, completing a neural loop hereafter referred to as the cerebellothalamocortical (CTC) pathway. The existence of such a pathway indicates that in addition to receiving prefrontal information, the cerebellum exerts influence over prefrontal regions of the cerebral cortex. Furthermore, primate anatomical studies have demonstrated that the dentate nuclei possess anatomically distinct "output channels" that project to both motor and non-motor areas of the prefrontal cortex (Kelly and Strick, 2003; Middleton and Strick, 2000, 2001).

Although tracing studies have not targeted the rat CTC pathway per se, evidence based on overlapping anatomical projections strongly implicates homologous loops. In rats, most of the CTC pathway projections from the cerebellar dentate nuclei terminate in the thalamic ventroanterior–ventrolateral (VA–VL) nuclei complex, along with the ventromedial (VM) nucleus (Angaut et al., 1985; Aumann et al., 1994, 1996; Haroian et al., 1981). A review of rat prefrontal connectivity indicates that projections exist between the VA–VL complex and VM to dorsolateral prefrontal and dorsal anterior cingulate cortex, as well as projections between VM with infralimbic, prelimbic, medial orbital, and dorsal and ventral agranular insular cortex (Uylings and van Eden, 1990). Herkenham's (1986) autoradiographic studies of these "nonspecific" thalamic nuclei demonstrate significant projections to various areas of the frontal, parietal, and occipital cortices. The VM displays widespread projections mainly to layer I (particularly frontal and parietal) cortex, and VL prefrontal projections synapse mainly to cortical layers I and II/III. Loss of cerebellar input to any of these areas should result in morphological alterations including pruning of dendritic spines and branches, particularly in distal aspects of the arbor, as these areas demonstrate an increased susceptibility to pruning subsequent to loss of input (Fawcett et al., 2001; Kolb and Whishaw, 2000).

Research examining the functional roles of the CTC pathway has shown that disruption in rats via dentate nuclei or other lateral cerebellar lesions leads to visuospatial and motivational deficits in the absence of motor impairment. Noblett and Swain (2003) observed visuospatial learning deficits in rats with focal dentate nuclei lesions as measured by Morris water maze (MWM) performance. These results echoed those of similar studies in which gross lateral cerebellar lesions led to MWM deficits (Joyal et al., 2001; Petrosini et al., 1996). In a second experiment, Noblett and Swain introduced a delayed spatial alternation T-maze pretraining paradigm expected to reduce deficits caused by cerebellar lesions by strengthening frontal spatial abilities based on literature implicating the importance of rat prefrontal processing on delayed (Wikmark et al., 1973) and spontaneous (Lalonde, 2002) spatial alternation. As expected, lesioned rats pretrained on the T-maze demonstrated behavior similar to that of sham rats, whereas lesioned rats not exposed to the T-maze performed similarly to those in the first experiment. A morphological assessment of prefrontal cortex was not completed; however, other studies have demonstrated spatial maze deficits subsequent to prefrontal cortical lesions (de Bruin et al., 2001; Kolb et al., 1983; Winocur and Moscovitch, 1990) and lesions of thalamic VL and VM nuclei (Jeljeli et al., 2003).

Consistent with the lesion literature, several mutant mouse strains with varying specificity of cerebellar damage display spatial deficits (Lalonde, 1997; Lalonde and Strazielle, 2003). Some cerebellar mutants also display abnormal exploratory behavior, attributed to motivational deficits (Lalonde et al., 1988a,b; Caston et al., 1998). These motivational deficits are particularly intriguing given sparse historical connection of the cerebellum and motivational processes. To explore this relationship further, Bauer et al. (2011) designed a study to assess motivation in CTC-disrupted rats. These rats received lesions similar to those of Noblett and Swain (2003), which introduced relatively focal cerebellar damage compared to the widespread anomalies seen in the various mouse mutants. Results of two experiments (Bauer et al., 2011) demonstrated that bilateral electrolytic lesions of the cerebellar dentate nuclei lower breaking points on an operant conditioning progressive ratio schedule and decrease open field exploration as compared to sham controls, in the absence of motor impairment. These results demonstrate hedonic and purposive motivational reduction and support a CTC role in global motivational processes. Similar deficits in open field exploratory behavior, attributed to motivational dysfunction, have been demonstrated by cerebellectomized rats (D'Agata et al., 1993). In addition, a recent study revealed that transient deactivation of the dentate nuclei via bupivacaine infusion disrupts effort-based decision making (Peterson et al., 2012).

Studies examining a role of frontal cortex in motivated behaviors have revealed similar results. Adult rats with neonatal medial frontal lesions of anterior cingulate, infralimbic, and prelimbic cortices demonstrated lower breaking points on a progressive ratio reinforcement schedule as compared to shams (Schneider and Koch, 2005). Similar lesions in adult rats induce decreased exploration of a vertical hole-board (Mogensen and Divac, 1993) and a propensity to select a low-effort/low-reward path over a high-effort/high-reward path in a T-maze paradigm requiring effort-based decision making (Walton et al., 2002). Reviews (Kolb, 1984, 1990) of rat frontal abilities indicate several lesion-induced dysfunctions that suggest a motivational component, including abnormalities in social, affective, and spontaneous behaviors.

Based on a convergence of the anatomical connectivity of the CTC pathway with the behavioral dysfunctions demonstrated subsequent to damage to this circuit, the visuospatial and motivational deficits observed following cerebellar dentate nuclei lesions are proposed to occur secondary to loss of cerebellar input to frontal cerebral areas necessary for appropriate functional processing. This prompts an investigation of cerebral morphological alterations subsequent to cerebellar lesions.

In particular, prefrontal cortex (PFC) is expected to undergo dendritic pruning after cerebellar lesions, leading to a decrease in dendritic spines and branches. To assess this putative anatomical substrate of functional alteration, the current study characterized morphological alterations in PFC following bilateral cerebellar dentate nuclei lesions. It was hypothesized that PFC of lesioned rats, as compared to sham rats, would demonstrate distal (2nd+order) dendritic morphological alterations including decreased average branch length and decreased spine density. These results were expected in both apical and basilar arbors.

2. Results

2.1. *Lesion verification*

All lesioned brains demonstrated damage in the target coordinates and immediate surrounding tissue, whereas sham brains did not demonstrate appreciable damage. All rats were included in analyses based on these results. Examples of lesioned tissue are displayed in Fig. 2.

2.2. *Dendrite branch morphology*

Neurons expressing 10% or higher unnatural ends (see Section 4.4.1) were removed from all branch morphology analyses. This resulted in a loss of 22 neurons, six from sham animals and 16 from lesioned. Data were averaged across individual neurons within an animal to compute a representative value for each metric. All subsequently discussed values are presented as mean±standard error.

2.2.1. *Neuron characteristics*

A summary of the unnatural ends and gross neuron dimension data is located in Table 1. Lesioned and sham values did not differ significantly on any characteristic measures including average neuron height, width, depth, percent of unnatural ends, number of zero-order branches (“stems”) or number of bifurcations.

2.2.2. *Branch length*

To assess branch length averages, values representing the total sum of branch lengths for each tree and order were created for each neuron, and then a representative mean for each rat was computed by averaging across all 10 neurons. Comparisons of distal (2nd+order) branch length sum averages revealed no significant differences between lesioned and sham brains, in either basilar or apical arbors. Few arbors demonstrated 4th and higher order branching so these data are not reported. Exploratory analyses of proximal basilar branches (0th and 1st order) revealed insignificant differences; these data are included in Table 2 and Fig. 3.

2.3. *Spine density and maturity*

Examination of second order terminal ends revealed a pattern of morphology consistent across both basilar and apical arbors. Specifically, while total spine densities remained unchanged between lesioned and sham rats, the density of mature (short) spines tended towards increased levels and the density of immature (long) spines tended towards decreased levels in lesioned rats as compared to shams. A data and analysis summary of the spine density and maturity values is found in Table 3 and graphed in Figs. 4 and 5.

In the basilar arbor, the total spine density did not differ between lesioned and sham rats. The mature density exhibited an insignificant increase of 15.94% whereas the immature density exhibited an insignificant decrease of 14.77% in lesioned rats as compared to shams. When examined as a proportion, these data reveal a significant difference between lesioned and sham values. Specifically, the lesioned proportion of 0.491±0.035 immature spines per mature spine reflects a 32.92% decrease from the sham proportion of 0.732±0.080 immature spines per mature spine: $F_{1,18}=7.596$, $p=0.013$.

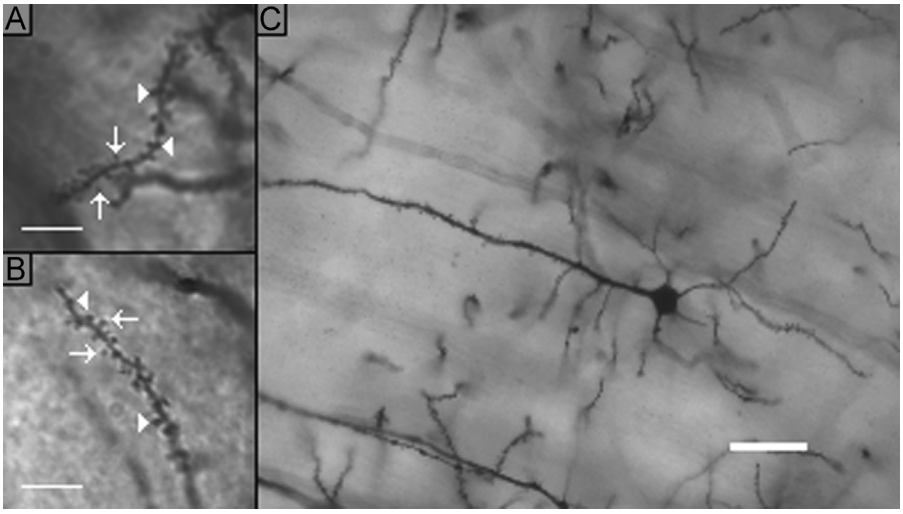

Fig. 1 – (A, B) Examples of 2nd order terminal ends captured at 1000× from sham (A) and lesioned (B) brains. Arrows denote spines categorized as immature whereas arrowheads indicate spines categorized as mature. Differences in proportion were not striking enough across sham and lesioned tissue to reliably identify condition via visual inspection. Scale bar represents 5 μm. (C) Example of Golgi-impregnated PFC tissue from this study magnified at 400×. Scale bar represents 20 μm.

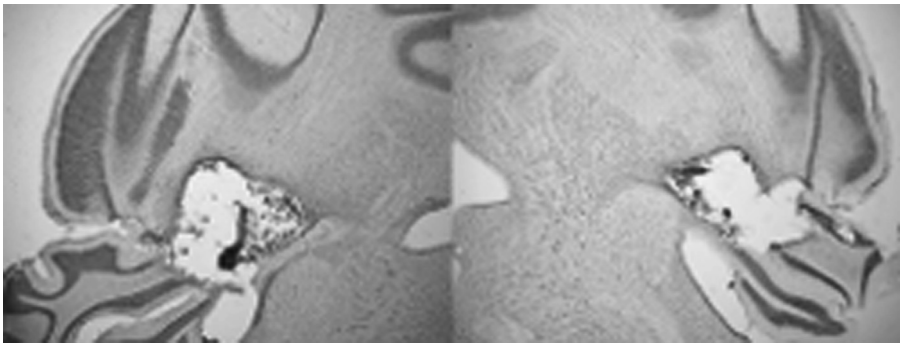

Fig. 2 – Representative cerebellar slices demonstrating damage from electrolytic lesions. All lesioned rats displayed damage in the target coordinates and immediate surrounding tissue whereas sham rats did not display appreciable damage.

Table 1 – Neuron characteristics (mean ± SE).

Measure	Sham	Lesion	$F_{1,18}$	p
Height (μm)	235.14 ± 12.91	258.77 ± 13.44	1.608	0.221
Width (μm)	117.27 ± 5.76	120.72 ± 6.88	0.148	0.705
Depth (μm)	37.92 ± 4.16	34.86 ± 2.49	0.402	0.534
Unnatural Ends (%)	2.68 ± 0.48	3.29 ± 0.62	0.615	0.443
Stems (#)	5.52 ± 0.24	5.85 ± 0.17	1.211	0.286
Bifurcations (#)	19.95 ± 1.79	22.04 ± 1.58	0.771	0.391

Table 2 – Branch length sums (mean ± SE, μm).

Arbor	Order	Sham	Lesion	$F_{1,18}$	p
Basilar	0th	46.36 ± 5.33	56.30 ± 4.79	1.924	0.182
	1st	111.14 ± 6.48	138.21 ± 15.76	2.524	0.130
	2nd	93.25 ± 13.54	105.64 ± 13.80	0.410	0.530
	3rd	35.95 ± 6.23	29.09 ± 4.67	0.776	0.390
Apical	0th	29.48 ± 4.69	37.29 ± 4.50	1.445	0.245
	1st	60.23 ± 6.18	75.71 ± 7.10	2.706	0.117
	2nd	72.32 ± 7.27	75.09 ± 11.89	0.040	0.845
	3rd	70.17 ± 7.01	87.21 ± 13.25	1.291	0.271

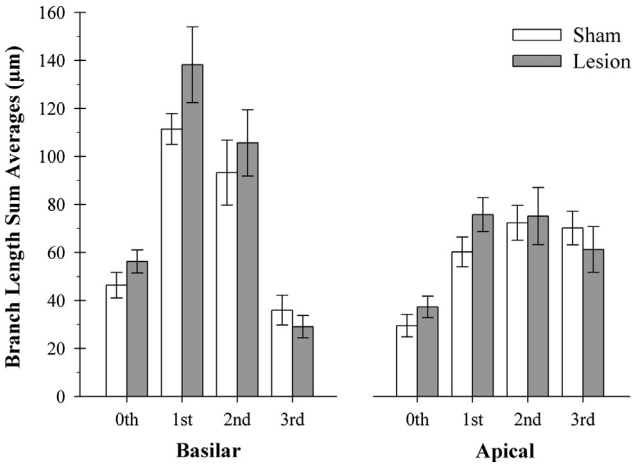

Fig. 3 – Basilar and apical arbor dendrite branch length sum averages by order, expressed in μm. Branches arising from the soma were designated order zero. Comparisons revealed no significant differences between lesioned and sham values in either arbor at any order. Error bars depict SEM.

Results in the apical arbor mirrored those from the basilar arbor, featuring an insignificant difference in total spine density and an insignificant increase in mature spine density of 19.69% along with an insignificant decrease in immature spine density of 13.04% in lesioned rats as compared to shams. Evaluated as a proportion, data reveal significant differences between conditions. Specifically, the lesioned proportion of 0.584 ± 0.042 immature spines per mature spine reflects a 27.27% decrease from the sham proportion of 0.803 ± 0.079 immature spines per mature spine: $F_{1,18}=5.953$, $p=0.025$.

3. Discussion

While the current results do not support the specific hypotheses anticipating branch length and spine density reduction, support was found for the general hypothesis anticipating PFC morphological alterations subsequent to cerebellar lesions. Specifically, lesioned rats demonstrated a greater proportion of mature spines to immature spines as compared to sham rats. This result

Table 3 – Spine density and proportion.

Arbor	Spines	Spine density (mean ± SE spines/μm) Sham	Lesion	$F_{1,18}$	p
Basilar	All	0.609 ± 0.046	0.634 ± 0.049	0.148	0.705
	Mature	0.372 ± 0.032	0.432 ± 0.035	1.615	0.220
	Immature	0.236 ± 0.024	0.202 ± 0.017	1.363	0.258
Apical	All	0.589 ± 0.027	0.625 ± 0.044	0.489	0.493
	Mature	0.342 ± 0.026	0.410 ± 0.033	2.630	0.122
	Immature	0.247 ± 0.013	0.215 ± 0.013	3.149	0.093
Arbor		Spine proportion (mean ± SE immature:mature) Sham	Lesion	$F_{1,18}$	p
Basilar		0.732 ± 0.080	0.491 ± 0.035	7.596	0.013
Apical		0.803 ± 0.079	0.584 ± 0.042	5.953	0.025

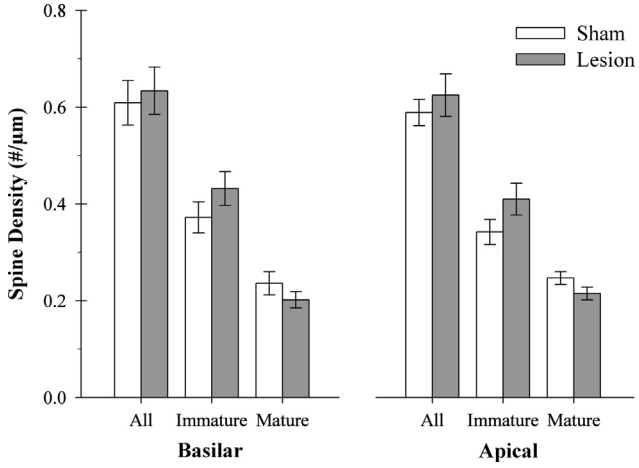

Fig. 4 – Spine densities by arbor and surgical condition. Lesioned and sham values did not differ significantly on density measures in either arbor. Error bars depict SEM.

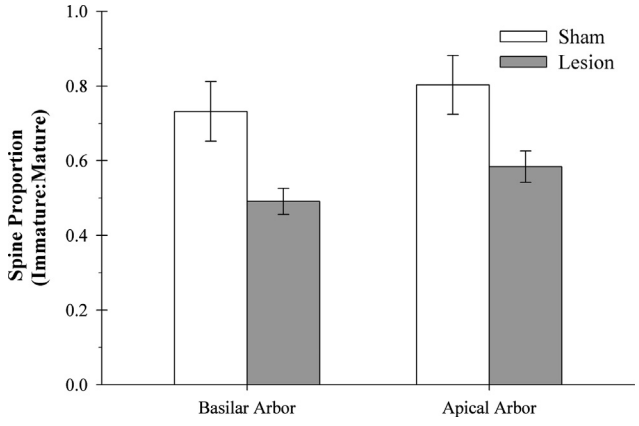

Fig. 5 – Spine proportions by arbor and surgical condition. Significant differences in immature to mature proportion were evident in both basilar and apical arbors. Error bars depict SEM.

was expected, but as a function of decreased total spine density driven by a loss of immature spines. In the absence of the anticipated decreased spine density, the differences appear to arise from a propensity for dendrites in lesioned brains to express a greater proportion of mature spines. These results were detected in both the basilar and apical arbors, suggesting a consistent expression pattern.

The lack of alteration in branch length is likely attributable to the factors of time and/or distance from lesion. Regarding time, dendrite Hbranch retractions following damage are most prominent within 2 weeks after insult and subsequently may exhibit regrowth (Fawcett et al., 2001). Therefore, it is possible that retractions occurred initially and then reversed. In the absence of a time course analysis, this possibility remains an open question. In addition, the distance of the PFC region of interest from the lesion site represents a likely factor contributing to lack of measurable branch length reductions.

The connections from the dentate nuclei to the prefrontal cortex are at best secondary or tertiary in nature, synapsing first in the thalamus or red nuclei. In general, the impact of the loss of excitatory input will most prominently feature on primary contacts, with subsequent connections suffering a reduced impact. It is therefore possible that the loss of input to the PFC caused by dentate nuclei lesions was of such an indirect nature that it lacked sufficient strength to result in large numbers of morphological alterations. Research demonstrating the relative structural integrity of adult pyramidal cell dendritic arbors in barrel cortex following vibrissa removal (Alvarez and Sabatini, 2007; Trachtenberg et al., 2002) supports this possibility; however, a time course analysis would provide additional information necessary to place the current results in a fuller context of alterations over time.

Although a time course analysis of branch morphology would provide interesting information, it may not resolve the issue relating functional behavioral alterations (visuospatial and motivational) observed at 30 days post lesion to morphological alterations if the current result of no differences between sham and lesioned brains is replicated. Although a case may be made for functional behavioral alterations in light of structural retraction followed by extension, one could argue that a return to original dimensions represents an anatomical correction that may precede functional behavioral correction. As such, a time course analysis should involve not only several time points before the currently employed 30-day point, but also several

time points afterwards. Additionally, one cannot discern from the current data to what extent branch morphology in the prefrontal cortex underwent structural reorganization in the absence of length alterations. One must employ a more technologically sophisticated method than a Golgi analysis to address such a question. In lieu of such time and labor-intensive undertakings, perhaps a more prudent analysis for the current and subsequent related studies lies in the more mutable and more readily assessed morphological aspect of dendrites as compared to branch structure: the spines.

Several reviews of spine morphology, density, characteristics, and plasticity (Alvarez and Sabatini, 2007; Bourne and Harris, 2007; Calabrese et al., 2006; Halpain et al., 2005; Wong, 2005) reveal that spines represent the most mutable and responsive morphological attribute of neurons. Indeed, spines can develop, retract, or alter shape on the order of seconds to minutes in response to stimulus addition or subtraction. Longer-term alterations occur on the order of weeks and may persist for months or more. Spines begin as filopodial extensions from the dendrite and upon connection with a stimulus-producing entity (e.g., axon) form a long and spindly "immature" state. As this connection persists over time, the spine morphology tends towards a short and stout "mature" state that demonstrates longer-term stability. Overall spine density and immature to mature proportions are under continual change in response to several criteria including spine structure, spine signaling, axon terminals and glia, spine location and interactions of neighboring spines (local interactions), activity of cells and neural circuits (global activity), and neuron type and brain region (Matsuzaki, 2007). As such, the current results provide a snapshot of spine morphology 30 days post lesion that would benefit from similar data captured at various time points both closer to and farther from the date of lesion induction.

In the current experiment, dendritic spines were expected to demonstrate a density reduction driven by a loss of immature spines in response to a reduction of excitatory input. This would lead to a decreased proportion of immature to mature spines. Although the results reflect a decreased proportion of immature to mature spines, an overall density reduction did not occur. Instead, the current results suggest that PFC dendrites exhibit a tendency to express a greater proportion of mature spines than immature spines in dentate nuclei-lesioned rats as compared to sham rats. This tendency was expressed in both basilar and apical arbors, lending strength to the analyses and implicating a pervasive expression pattern.

As with branch morphology, the likely reasons that a spine density reduction was not observed include time and distance from the lesion (Brown et al., 2007; Kim et al., 2006). To further characterize spine density and morphological alterations subsequent to lesion, a time course experiment should be conducted. However, a lack of findings regarding decreased density in the current data does not explain the significant immature to mature proportional decrease observed in lesioned tissue. Research has shown that spines tend to convert from an immature to mature state after a period of connectivity and activity (Holtmaat et al., 2006), leading some to postulate that immature spines represent "learning" spines whereas mature spines represent "memory" spines (Bourne and Harris, 2007). Indeed, spines tend to express a mature form upon application of long-term potentiation (Matsuzaki, 2007). Such information suggests that the PFC has experienced an increase in excitatory activity leading to a higher proportion of mature spines as measured 30 days post lesion. This interpretation runs counter to our original hypothesis that a loss of cerebellar input would decrease PFC activity, and the current results warrant further study to examine this interesting possibility. Perhaps the tendency towards a mature state represents an increased recruitment of the PFC in an attempt to account for sensorimotor deficits, an interpretation that may fit with Bower's (1997) conceptualization that the cerebellum primarily functions to aid in the acquisition and integration of sensory data. A time-course pattern of electrophysiological activity in conjunction with behavioral observations would provide the best information, as the current results could reflect either a net increase in activity or a compensatory increase following an initial decrease. Notably, although the observed morphological changes did not align exactly with expectations, any change in structure likely reflects a change in neuronal activity that could underlie changes in behavior. Given the behavioral deficits observed in previous studies (Noblett and Swain, 2003; Bauer et al., 2011), one interpretation of the current results suggests that even if increased activity caused spine development towards a mature state, this transition ultimately decreases the potential of both neural and behavioral plasticity.

Although only a single region of interest was assessed for morphological alterations in the current study, an analysis of additional regions of neocortex would assist the goals of relating anatomical and non-motor functional alterations following dentate nuclei lesions. In particular, the posterior parietal cortex demonstrates retrograde tracer overlap in thalamic nuclei with dentate nuclei anterograde tracer, suggesting significant connectivity of these areas (Gianetti and Molinari, 2002). The extent to which this region experiences morphological alterations subsequent to dentate nuclei lesions and contributes to functional alterations remains an open and interesting question. Relatedly, it is important to recognize that incomplete lesions could fail to damage specific outputs of the dentate nuclei and it is possible that minor variations in lesions across animals could lead to null or inconsistent findings, including in the current study. Further research evaluating CTC connectivity and functionality using a rodent model would benefit from a comprehensive map of dentate nuclei output channels based on anatomical tracing data. This map could aid both in the determination of target coordinates and in the verification of appropriate lesion damage.

In summary, the current results augment a growing literature implicating a cerebellar role in non-motor neocortical function by providing anatomical evidence of cerebral morphological alterations subsequent to cerebellar lesions. In particular, these results help implicate a cerebellar role in functions mediated by PFC and help validate the dentate nuclei-lesioned rat model as appropriate for assessing this cerebellar involvement. However, a more robust understanding of these connections warrants significant additional research. In particular, time course studies analyzing morphological alterations in

PFC and other neocortical regions of interest (e.g., posterior parietal cortex) in response to lesions should be performed, perhaps with an emphasis on dendritic spines and in conjunction with behavioral observations. Such studies will help place the current results in a wider context, with the goal of developing a comprehensive understanding of the anatomical substrate of functional abnormalities following cerebellar insult.

4. Experimental procedure

4.1. Region of interest

Anatomical nomenclature and specific coordinates are derived from Paxinos and Watson (1998). The prefrontal region of interest (PFC) was composed of cingulate cortex area 1 (Cg1, the anterior and dorsal cingulate) along with prelimbic cortex (PrL). From anterior to posterior, relative to bregma, these combined areas begin 4.70 mm rostral and end 2.20 mm rostral. Mediolaterally, the areas extend from the medial wall to the ventral orbital cortex, lateral orbital cortex, or forceps minor corpus callosum, depending on rostrocaudal plane. Dorsoventrally, the region begins at or just ventral to the most dorsal aspect of the medial wall to approximately half the distance to the most ventral aspect of the medial wall.

4.2. Subjects

Male Long Evans rats (N=20) underwent either sham surgery (n=10) or bilateral electrolytic lesions (1 mA for 30 s) of the cerebellar dentate nuclei (n=10), 1 day after a three-day administration of an acetaminophen solution used for post-surgical analgesia (Bauer et al., 2003). Rats were anesthetized with isoflurane in oxygen during the procedure. The sham rats experienced the entire procedure with the exception of receiving the lesion-producing current. Surgical coordinates from Paxinos and Watson (1998) were relative to bregma as follows: 11.0 mm posterior, ±3.8 mm lateral, and 6.4 mm ventral (from skull). Food and water (or acetaminophen solution) were available ad libitum. All procedures were overseen and approved by the University of Wisconsin–Milwaukee Institutional Animal Care and Use Committee.

4.3. Tissue preparation

A Golgi procedure based on published protocols (Gibb and Kolb, 1998; Glaser and Van Der Loos, 1981) was employed. The solution was made by mixing 250 ml 5% potassium dichromate in dH_2O with 250 ml 5% mercuric chloride in dH_2O. This mixture was then added to a solution containing 200 ml 5% potassium chromate in dH_2O and 500 ml additional dH_2O. The final solution sat for 5 days and then was filtered to remove precipitate prior to use.

Thirty days post-surgery, rats were sacrificed with carbon dioxide and perfused with 800 ml 0.9% saline. The unfixed brains were removed and the cerebella carefully separated from the cerebra. The cerebella were placed in 10% formalin and the cerebra in 20 ml of Golgi solution.

The cerebella fixed in 10% formalin for at least 5–7 days before vibratome slicing. The cerebra were stored in the Golgi solution in the dark. The solution was replaced after 2 days, and again 3 days after the first replacement. The cerebra remained for 14 days total in the Golgi solution, at which time the solution was replaced with 20 ml 30% sucrose in dH_2O. The brains then remained in the dark for at least 5 days before sectioning.

Coronal slices (100 μm) were taken through the cerebellum via vibratome (Vibratome 1000 Plus). Slices were dried on gelatin-coated slides and stained with a standard cresyl violet/Prussian blue protocol, using a rehydration followed by a dehydration procedure. Slides were examined under a microscope at low magnification (40–200×). Lesion verification was accomplished by drawing damage as seen under the microscope on templates of cerebellar slices adapted from Paxinos and Watson (1998).

For cerebral sectioning, coronal vibratome slices (200 μm) through the frontal lobe were placed in wells of 6% sucrose solution. After sectioning, the slices were rinsed in dH_2O for one min, placed in ammonium hydroxide for 30 min, rinsed in dH_2O for 1 min, placed in Kodak fix for film for 15 min, rinsed in dH_2O for 1 min, dehydrated in 50%, 70%, and 95% ethanol (1 min each), dehydrated twice in 100% ethanol (5 min each), cleared in a solution of 1/3 chloroform, 1/3 HemoDe, and 1/3 100% ethanol and HemoDe for 15 min, and finally placed on unsubbed glass slides and coverslipped using a 50% Canada balsam/50% HemoDe mounting medium. The slides dried for at least 14 days prior to cleaning and viewing under the microscope.

4.4. Dependent measure quantification

4.4.1. Dendrite branch morphology

For each brain, the slices containing PFC were identified. Five of these were randomly selected for use in locating neurons from the left hemisphere, then five randomly selected for use in locating neurons from the right hemisphere. Random sampling was completed without replacement within each hemisphere. All dendrite branch morphology assessment was completed by a researcher blind to experimental conditions.

Within each selected slice, appropriate PFC neurons were identified for analysis using criteria commonly employed in Golgi studies (e.g., Kolb et al., 2003; Robinson and Kolb, 1997; Teskey et al., 1999). These neurons were well impregnated by the stain, easily differentiated from artifact, vasculature, and surrounding cells, and intact within the section. The neurons were also layer II/III pyramidal cells demonstrating both apical and basilar dendritic arborization. The apical arbor projected into layer I and both apical and basilar arbors exhibited 2nd order branching, where branches arising from the soma were designated as zero order with each subsequent order developing after a bifurcation. After all appropriate neurons in a slice were identified at 200–400× magnification, one was randomly selected for further analysis at 1000×. A single neuron was analyzed from each identified slice, for a total of ten neurons per rat (five per hemisphere). Fig. 1 displays an example of a Golgi-impregnated neuron from this study.

Each of the selected neurons was imaged in entirety using an Olympus BX41 microscope with a 100× oil objective. The microscope camera (Diagnostic Instruments, Inc., Model #18.2

Color Mosaic) captured two-megapixel images via commercial software supplied with the camera (SPOT version 4.6).

Methods for computer analysis of neurons were taken from Brown et al. (2005). This procedure utilized a sequence of freeware computer programs in lieu of commercial software.

Multiple image stacks were taken and combined to form a single image stack for the entire neuron. A "tracing" of each neuron was completed by marking coordinates of structural features including the soma, axon, basilar dendrites, apical dendrites, bifurcations, and end points. The completed skeletal tracing was compared to the original image stack to verify accuracy and measures of interest (e.g., branch length by order) were acquired. Brain averages were computed from these quantified values using spreadsheet software and analyzed via Statistical Package for the Social Sciences (SPSS).

Each branch terminal was classified as natural or unnatural based on physical appearance. Natural terminal ends appear fully intact and tapered, and terminate within the slide. Unnatural ends appear broken or cut without tapering and often do not terminate within the slide. Height was calculated as the distance between the top of the apical arbor and the bottom of the basilar arbor. Width was calculated as the distance between the most distal lateral points. Depth was calculated as the distance between the first and last images in the image stack in which the neuron appeared.

4.4.2. *Dendrite spine density and maturity*

Assessments of spine density and morphology were completed on images of 2nd order naturally ending basilar and apical terminal branches. These images were captured independently from those used for branch morphology analyses by a researcher blind to experimental conditions. A total of 20 terminal branches were analyzed per brain, five from each hemisphere in each (apical and basilar) arbor. The method for random slice selection was identical to that employed to select slices for branch morphology assessment. To select individual terminals, the dorsal or ventral 50% of the slice was randomly selected, then an appropriate terminal located that was clear of obstructions and artifact, roughly parallel with the plane of the slide, and at least approximately 15 μm in length. The terminal selection process was identical but independent when selecting terminals for apical and basilar arbors; therefore, apical and basilar terminals most likely came from different neurons. Each terminal was digitally captured at 1000× magnification as a single image.

A researcher blind to experimental conditions independently conducted total spine counts and maturity classification on all images twice, at time points 1 week apart. Intrarater reliability was assessed via Pearson's *r* and comparison of means ± standard error, wherein significant correlations and overlapping mean spreads indicated sufficiently high correspondence. This assessment indicated sound reliability and the values from each rating were averaged for the final analysis values.

Spine density was determined by counting the number of spines and dividing by the total terminal length. Spines were classified as mature or immature using morphological criteria (Irwin et al., 2001, 2002). Briefly, spines characterized as long and spindly were labeled immature, whereas spines characterized as short and stout were labeled mature. The comparison chart used by Irwin et al. (2001, 2002) was employed. Fig. 1 (inset) displays an example of a terminal end from this study with immature and mature spines identified. To differentiate long spines from short branches, a rule was employed such that a protrusion longer than 2.5 times the local average spine length was classified as a branch, whereas shorter protrusions were classified as spines.

4.5. *Analyses*

All analyses consisted of one-way analyses of variance comparing sham to lesion values, conducted in SPSS. An alpha level of 0.05 constrained Type I error. Exploratory analyses of log-transformed ratiometric (spine density and proportion) values yielded the same results and conclusions as non-transformed values. For clarity, the raw (non-transformed) values are presented and discussed. Apical and basilar values were analyzed separately due to differences in information source and function (Spratling, 2002; Teskey et al., 1999).

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