

Role of perineuronal nets in the cerebellar nuclei:
Insights from histology and behavior

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Abstract

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Perineuronal nets are structures in the extracellular matrix that previous work implicates in either inhibiting synaptic plasticity or protecting neurons from oxidative damage. We use histological techniques to explore the distribution of perineuronal nets in different areas of the central nervous system. We find that perineuronal nets do not necessarily surround high activity cells with large oxidative loads. We do find that they are somewhat more prevalent in motor areas, and are most prevalent in the cerebellar nuclei.

The cerebellum is important for motor control and learning. We study the role of perineuronal nets in the cerebellar nuclei using a long term saccade adaptation task. Long term saccade adaptation changes the size of eye movements. It mimics recovery from damage to the motor areas of the nervous system or muscles, that make movements too small.

We find that dissolving perineuronal nets in the cerebellar nuclei, where they are extremely abundant, does not change the speed, magnitude or persistence of long term adaptation.

This indicates that in the saccade-related part of the cerebellar nuclei, perineuronal nets are not important for inhibiting plasticity and thereby do not maintain saccade gain.

Therefore, we cannot summarize role of PNNs as exclusively either neuroprotection or inhibition of plasticity. Perineuronal nets could be important for both or either, depending on the region. This work will enable future investigation of perineuronal net function.

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Chapter 1

BACKGROUND

Despite over a century of study, the role the cerebellum plays in motor control remains poorly understood. We know that impairing cerebellar output makes movements variable, inaccurate, and slow and we know a great deal about the structure of the cerebellum. Still, we do not know how cerebellar neurons process incoming signals to make movements consistent, accurate, and fast.

Work from our lab, described below, shows that cells in the cerebellar nuclei are more densely surrounded by perineuronal nets than are neurons in the cerebellar cortex. Perineuronal nets are large extracellular structures that, in other parts of the brain, are involved in ending developmental critical periods. Developmental critical periods are time windows during which the brain is more plastic, i.e., sensory experience can change synaptic connections. With this series of projects, I use the cerebellum to explore the role of perineuronal nets. The unique properties of the cells in the cerebellum allow me to investigate which types of cells perineuronal nets surround. Also, the prevalence of PNNs in the cerebellar nuclei raises the possibility that they are there to inhibit plasticity after a critical period of motor development. By investigating the role of perineuronal nets in the cerebellum I hope to, in turn, gain insight into the function of cerebellar nuclear neurons and their contribution to making movements accurate.

1.1 *Perineuronal Nets*

1.1.1 Structure

Perineuronal nets (PNNs) are large extracellular structures that surround some neurons. They are composed of a combination of proteins and proteoglycans, which are secreted

by both neurons and glia throughout early postnatal development [78][13]. Different areas of the central nervous system have different complements of perineuronal net proteins[23]. However, they all have four elements in common - hyaluronan, tenascin-R, link proteins and chondroitin sulfate proteoglycans (CSPGs)[43][12][44]. Hyaluronan forms the scaffold, or backbone of the perineuronal net structure to which CSPGs adhere. The CSPG-hyaluronan connection is stabilized by link proteins. Tenascin-R then forms cross-links between several of these structures (see Fig. 1.1A).

There are four different CSPGs found in PNNs in the central nervous system: neurocan, versican, brevican and, most frequently, aggrecan[12]. All of these belong to a family of proteoglycans called lecticans. These share an N-terminal immunoglobulin (G1) domain, but differ in the number of glycos-amino-glycan side chains they have. They also exhibit different isoforms of the core proteins. The G1 domain mediates binding to hyaluronan and the C-terminal domain mediates binding to tenascin-R[4][80][47].

PNNs grow in around neurons between postnatal days 7-14 in rat[13] and days 5 - 90[15] in primates and will often encapsulate the entire soma of a neuron, as well as its proximal dendrites (see Fig. 1.1B). Openings in a neuron's PNN sheath appear only at points of synaptic contact.

1.1.2 *Function*

Many studies support the idea that perineuronal nets are involved in ending the critical period of synaptic plasticity during development[62][12][22][17][5]. Critical periods in neuronal development are windows of time during which experience guides the formation of appropriate synaptic connections. Because the connections that form change depending on what experience the animals receive, a critical period is considered a time of activity-dependent synaptic plasticity. PNNs finish forming at approximately the same time that critical periods end and synaptic connections mature[31][51]. Artificially extending the critical period by preventing animals from acquiring experience results in a delay in perineuronal net formation[32][57]. Dissolving perineuronal nets can result in at least a partial 'return' to early-developmental levels of synaptic plasticity[62][27]. This suggests that PNNs contribute

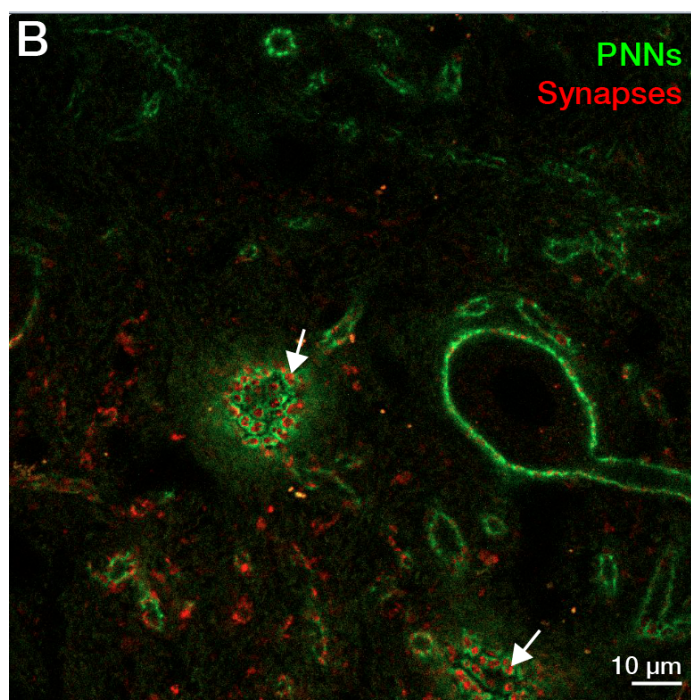
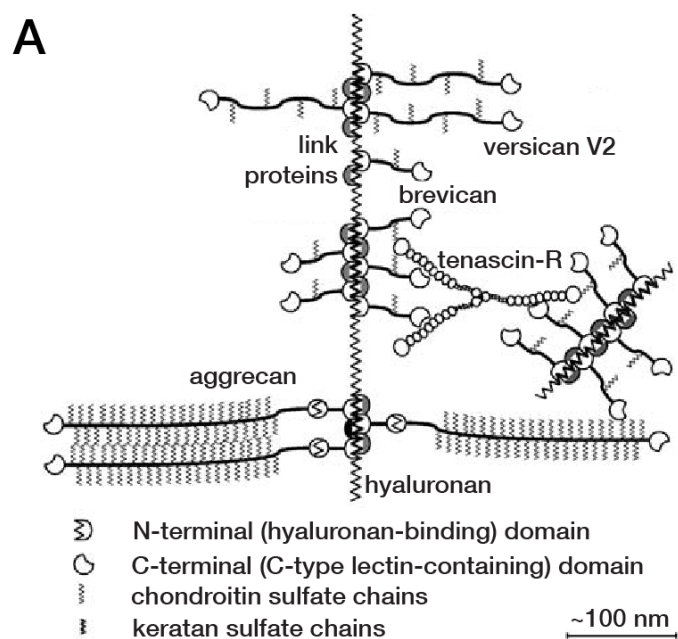


Figure 1.1: Perineuronal Nets A: Model of how perineuronal nets could be structured. Modified from Rauch et al (2004)[64] B: Perineuronal nets surround synapses. Cerebellar nuclear neurons stained for CSPGs (green, *Wisteria floribunda agglutinin*-fluorescein 1:500) and synapses (red, anti-SV2 1:100). Arrows indicate nuclear neurons on which PNNs surround synapses.

to neuronal maturation during development by inhibiting plasticity.

PNNs could be achieving this by either acting as a structural barrier to formation of new processes or synapses, or, by inhibiting the formation of new synaptic contacts through signaling across either the presynaptic or postsynaptic membrane. Several CSPG-ligands have been found that may mediate an inhibitory signal from PNNs: for example contactin-1[52], LAR[18] and PTP- σ [73].

Some studies suggest alternative roles for PNNs. They could serve a neuroprotective function for highly active neurons that are susceptible to oxidative stress[10]. Or, they may act as cation sinks to balance the ion milieu around neurons with high fluctuations of ion exchanges[34]. These functions would not necessarily preclude their involvement in plasticity as well.

1.1.3 Distribution

Several papers describe perineuronal nets as primarily surrounding parvalbumin-positive inhibitory interneurons[5][60][54][45]. We recently showed, however, that, in the cerebellum at least, perineuronal net presence seems related to the amount of inhibitory input a cell receives[15]. This result conflicts with previous literature and therefore we performed a survey of perineuronal nets across different regions of the primate CNS. In chapter two, we profile which cell types are surrounded by PNNs and speculate on the possible implications of those results. We also discuss the comparative distributions of PNNs across different areas of the central nervous system and find that perineuronal nets are far more prevalent in the cerebellar nuclei than elsewhere in the brain.

1.2 The Cerebellum

1.2.1 Anatomy

The cerebellum is a phylogenetically old region of the brain. It is easily identifiable by its specialized cells and nearly stereotyped architecture.

There are two regions of the cerebellum: the cerebellar nuclei and the cerebellar cortex. Both areas receive input from the same excitatory fibers originating from a variety of sources

including the spinal cord and several structures in the brain stem. The largest input to the cerebellum is the pontine nuclei. All sources except the inferior olive send mossy fibers to the cerebellum. The inferior olive sends climbing fibers. Cerebellar nuclear neurons also receive substantial inhibitory input from Purkinje cells in the cerebellar cortex.

The cerebellar cortex itself is comprised of three layers of cells. The deepest layer is the granule cell layer. Granule cells receive mossy fiber input and relay it to other cells in the cerebellar cortex by way of parallel fibers that traverse the superficial molecular layer. Inhibitory neurons in the molecular layer synapse onto local cells, including the large dendritic trees of Purkinje cells. Purkinje cell bodies form the intermediate (Purkinje cell) layer. The entire network of the cerebellar cortex is reduced to one output: the activity of Purkinje cells which project exclusively to cerebellar or vestibular nuclear neurons. See figure 1.2 for more details.

1.2.2 Role in Motor Control

Despite the well-described and elaborate circuitry of the cerebellar cortex, we know little about how the cerebellum contributes to motor control. We do know that it is important for making accurate movements. Not only do many studies show that movements are severely impaired after cerebellar damage[46][33], but there is also a great deal of evidence demonstrating the importance of the cerebellum for motor learning. Motor learning is the ability to alter movements through training or feedback to make them more appropriate. It is important for rehabilitation after injury and in compensating for muscle weakness. The cerebellum has also been shown to be necessary for a number of experimental learning situations such as vestibular-ocular reflex adaptation[3][63], eye-blink conditioning[81][74] and voluntary movement adaptation (see below)[28][42][59]. All of these studies show that lesions of the cerebellar nuclei and cortex prevent animals from changing their movements based on feedback.

1.3 Saccades

Our lab studies the influence of the cerebellum on movements by looking at saccades. Saccades are rapid eye movements that aim our foveas to a point of interest. Saccades are

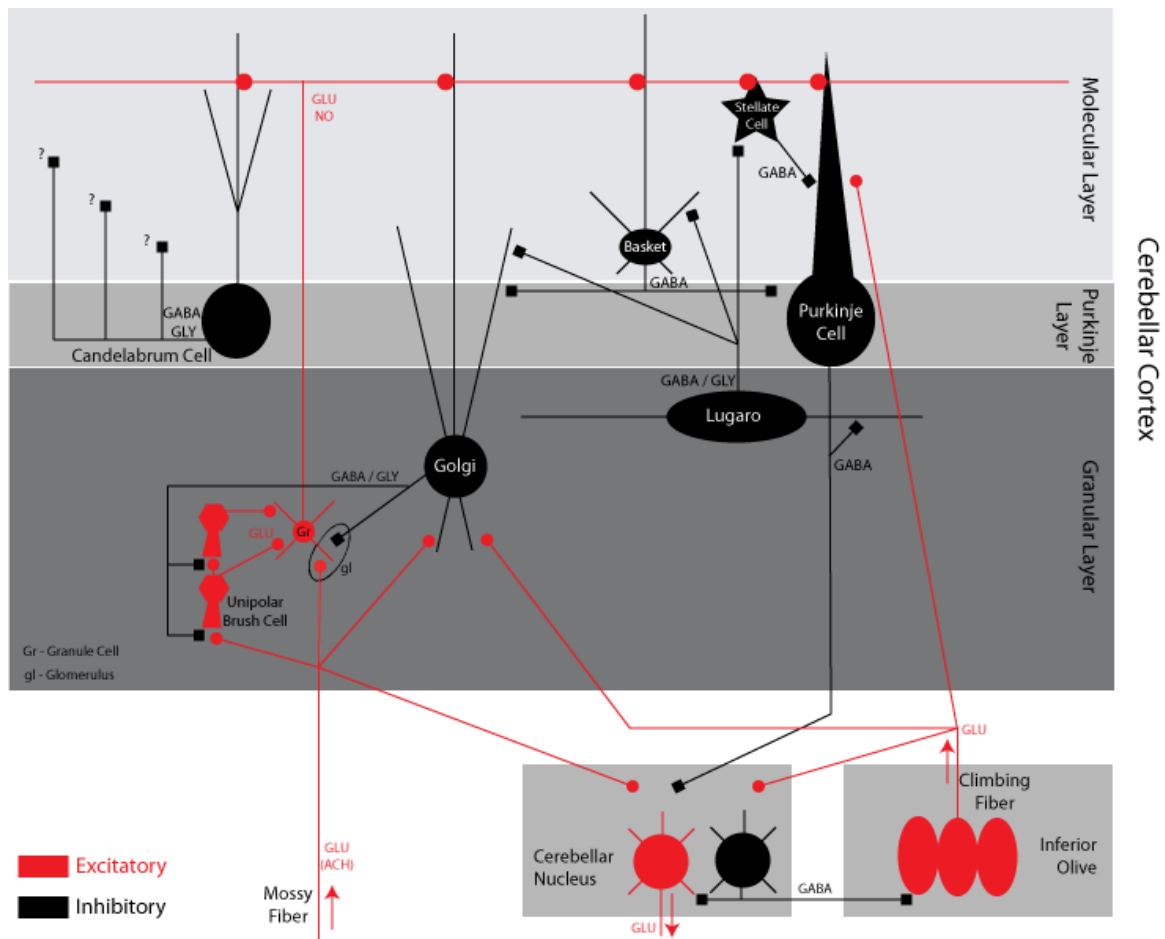


Figure 1.2: Cerebellar Anatomy: Red cells and fibers are excitatory. Black cells and fibers are inhibitory. Synapses are labeled with specific neurotransmitters: GABA (γ -aminobutyric acid), GLY (glycine), GLU (glutamate), ACH (acetylcholine), NO (nitric oxide). Modified from Geurts et al (2003)[24]

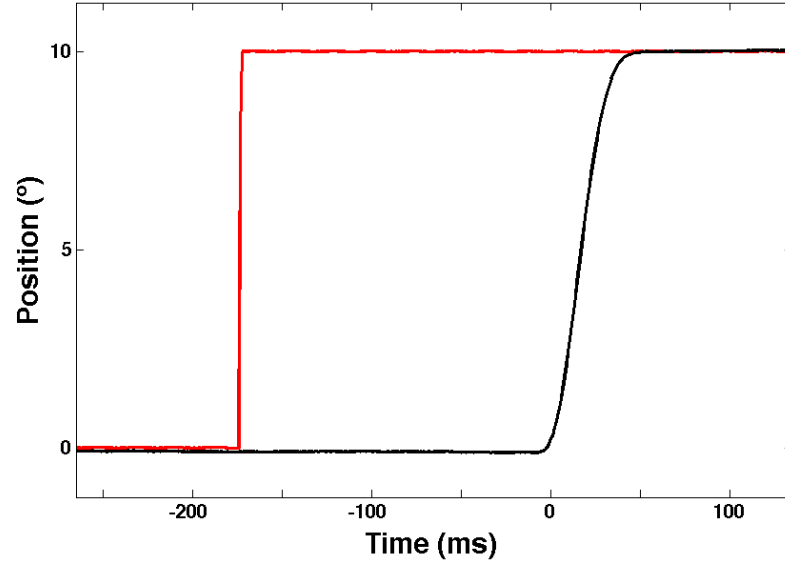


Figure 1.3: 10° rightward saccade. Red: Target position against time. Black: Saccade position against time.

a good model system because 1) their range is essentially limited to two dimensions, 2) they are controlled by only six muscles and 3) their neuronal premotor network is relatively well-characterised compared to other motor systems.

Saccades are target-directed and are measured in degrees of visual angle. We can train animals to make saccades by having them fixate on a dot on a screen. When the dot jumps to a new location the animal's eyes move (after a short reaction time delay) to fixate on the target's new position. Saccades are extremely rapid and extremely stereotyped (see Fig. 1.3).

1.3.1 Adaptation

Many labs have shown the importance of the cerebellum for motor learning tasks such as adaptation and conditioning. Animals adapt their movements after injury to compensate for deficiencies. We can simulate this behavior experimentally by giving animals misinformation about their performance that trains them to change their behavior[50]. Usually

such experiments last only a few hours and elicit effects on saccades that are transient. In chapter three, we describe a procedure called long term saccade adaptation that can change the size of saccades much more than a single session of adaptation can.

1.3.2 Saccade System of the Cerebellum

Two midline cerebellar areas contribute to accurate saccade execution: the caudal end of the midline (fastigial) cerebellar nuclei (CFN), and a region of the cerebellar cortex called the oculomotor vermis (OMV). Several lines of evidence show that these areas are important for saccades. The activity of neurons in both these areas modulates during saccades[21][61]. Permanent or temporary impairment of these areas causes saccades to become inaccurate[67][76][77]. Electrically stimulating these areas results in non-target-directed saccades[25][48].

These areas are also thought to contribute to saccade adaptation. Recent work shows that the activity of neurons in the OMV and CFN changes during adaptation[35] [40][41]. Further, temporary ablation of these regions prohibits acquisition of new learned movements through adaptation, and can extinguish the learning of recently adapted movements[28][42].

Because the changes we see in long term saccade adaptation are not persistent, it is possible that a baseline level of saccade accuracy is set during development by connections to CFN cells. In the fourth chapter we use the LTSA paradigm to explore the role of the extremely abundant PNNs found in the CFN in maintaining baseline saccade accuracy.

Chapter 2

PERINEURONAL NETS IN THE CEREBELLAR NUCLEI

2.1 Summary

Perineuronal nets (PNNs) are extracellular molecules that form around neurons during development. They surround neuronal cell bodies and proximal dendrites and are thought to stabilize synapses and inhibit the formation of new connections. Previous work has characterised the important role of perineuronal nets for plasticity in the visual system and spinal cord of rats. PNNs may surround different proportions of neurons in different brain areas. Still, no previous work describes the prevalence of PNNs in different parts of a monkey's brain.

In this study we use immunohistochemistry to survey the distribution of perineuronal nets in representative areas of the primate brain. We find that perineuronal nets are most dense in the cerebellar nuclei. In contrast, in the cerebral cortex only 10% or fewer of neurons are surrounded by perineuronal nets in all areas that we examine. Prevalence of perineuronal nets around parvalbumin-positive inhibitory interneurons varies considerably between different areas of the brain. These findings contribute to the growing body of knowledge about which neuronal cell types are surrounded by perineuronal nets, and what functions PNNs serve.

2.2 Introduction

Perineuronal nets are large extracellular molecules that surround neuronal cell bodies and proximal dendrites. They consolidate around neurons near the end of developmental critical periods and are thought to restrict plasticity through a variety of mechanisms, including stabilizing synapses and inhibiting neuronal sprouting[37]. Although different perineuronal nets consist of different proteins, chondroitin sulphate proteoglycans, or CSPGs, are common to all.

Previously, our lab showed that perineuronal nets surround a high proportion of neurons in the cerebellar nuclei of the non-human primate *Macaca mulatta* beginning between 5 days and 3 months after birth[15]. Here we describe the prevalence of perineuronal nets around neurons in 25 different areas throughout the brains of adolescent and adult rhesus monkeys, *Macaca mulatta*.

2.3 Materials and Methods

We collected small pieces of brain from nine rhesus macaques (*Macaca mulatta*) perfused with 4% paraformaldehyde in 0.1M phosphate buffer followed by 10%, 20% and 30% sucrose solutions for cryoprotection. All procedures were approved by the University of Washington Animal Care Committee and conformed to the recommendations from the Institute of Laboratory Animal Resources and the American Association for Accreditation of Laboratory Animal Care.

After perfusion, the brain rested in 30% sucrose for several days until it sank. We then took pieces from the 25 locations, indicated in Figure 2.1. From these pieces, we cut 25 μ m floating sections using a cryostat. We exposed each section to both a solution marking perineuronal nets and another marking neurons. To stain for perineuronal nets, we use Wisteria floribunda agglutinin conjugated to fluorescein (WFA-Flscn, 1:500; Vector Labs FL-1351). WFA is a lectican that binds to the long sugar side-chain components of CSPGs[29]. We used either NeuN (mouse monoclonal Neuronal nuclei N, 1:500; Millipore Corp MAB377) or Avidin conjugated with Texas Red (Avidin-TxRd, 1:500; Invitrogen A-820) as a neuronal stain. We used NeuN to label all brain areas except the cerebellar nuclei and avidin to label cerebellar nuclear neurons, which are not antigenic for the NeuN antibody [49][56]. Although Avidin is not a standard neuronal stain, co-staining with NeuN in regions outside the cerebellar cortex suggests that Avidin marks a slightly larger number of neurons than Neu-N does. We also stained for a subset of GABAergic inhibitory interneurons with an anti-parvalbumin antibody (1:500, Sigma: P3088). We exposed the sections to the primary antibodies on consecutive days to maximize signal. In the case of NeuN, sections were additionally exposed to the secondary antibody Alexafluor 568 (1:1000; Invitrogen A-21124). Sections were mounted with fluorimount media and imaged on a Zeiss

Axioskop 2 confocal microscope using LSM 5 Pascal software. The same excitation and acquisition parameters were used for all sections from a single staining session.

We quantified the fraction of neurons with perineuronal nets in a representative area by counting the number of cells with WFA staining and dividing it by the total number of neurons. Four individuals separately counted neurons and PNNs on each image and we averaged the results. Counts from all individuals were similar. We then used these values to obtain an average percentage of neurons surrounded by perineuronal nets across monkeys. We used the same procedure to count parvalbumin-positive and WFA-positive cells, however we did not calculate percentages from the counts.

We collected tissue from eight male rhesus macaques (ages 5, 5, 6, 7, 8, 9, 11 and 21 years) and one female (age 12 years). Because the tissue from these animals was primarily assigned to other projects, we were not able to collect samples from every area in every animal. We did, however, acquire samples from a minimum of four different animals for each area for the PNN/neuron comparisons.

2.4 Results

2.4.1 Proportions of neurons surrounded by PNNs in different areas of the brain

We surveyed areas of the non-human primate cerebral cortex, subcortical structures, brain-stem, and cerebellum for presence of perineuronal nets around neurons. WFA recognizes the glycos-amino-glycan moieties of the proteoglycans that make up perineuronal nets, and is therefore considered to be a general stain for CSPGs. Figure 2.1 shows the areas we examined. Figure 2.2 shows the percentage of neurons surrounded by perineuronal nets in each of these areas.

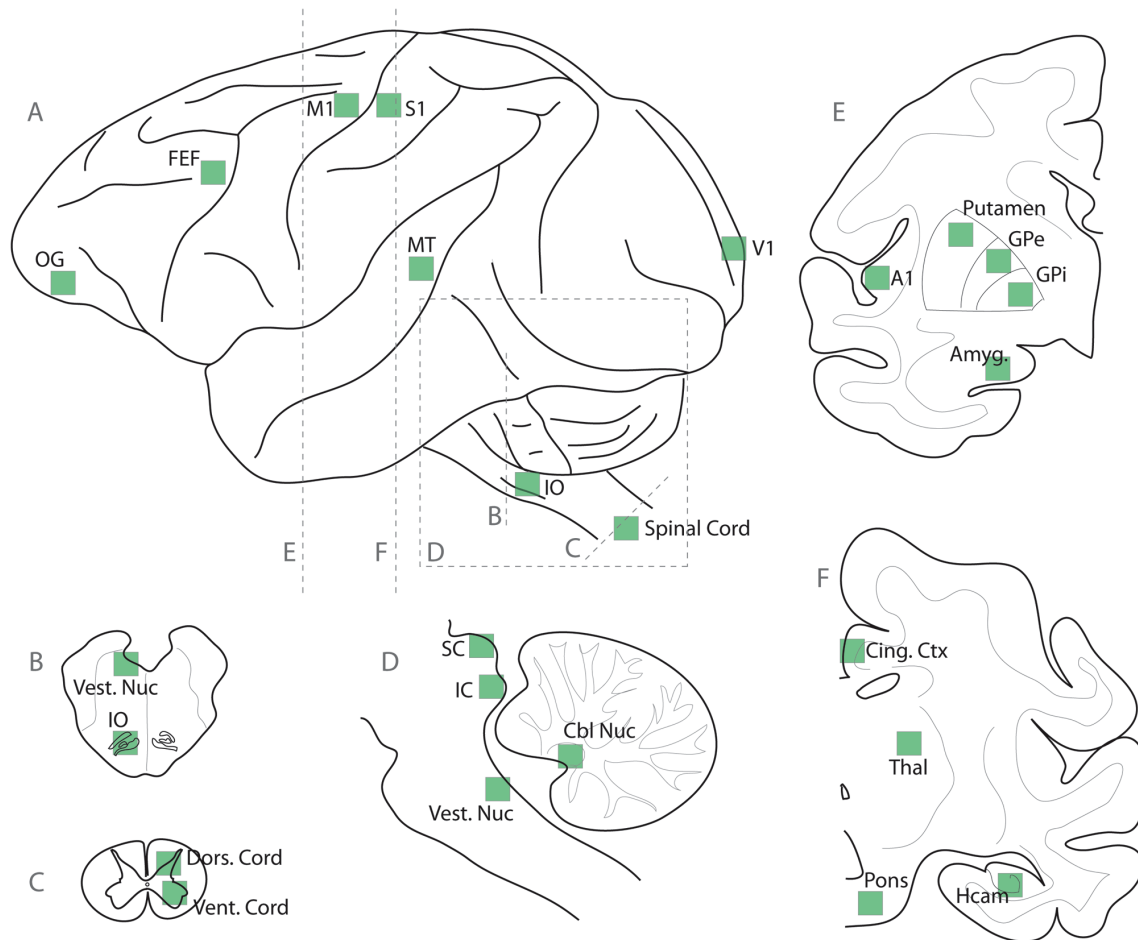


Figure 2.1: Locations where tissue was collected. **A** shows a lateral view of the whole brain. The dashed lines represent planes for the other subfigures. Samples were taken from the orbital gyrus (OG), the frontal eye field (FEF), primary motor cortex (M1), primary somatosensory cortex (S1), primary visual cortex (V1), medio-temporal cortex (MT), the inferior olive (IO) and the spinal cord. **B** shows the brainstem. Samples were taken from the inferior olive (IO) and the vestibular nuclei. **C** shows the spinal cord. Samples were taken of both dorsal and ventral spinal cord. **D** shows a sagittal representation of the brainstem and cerebellum. Samples were taken from the superior colliculus (SC), the inferior colliculus (IC), the cerebellar nuclei (Cbl Nuc) and the vestibular nuclei. **E** shows the left half of the brain in a frontal plane. Samples were taken from primary auditory cortex (A1), the putamen, the external globus pallidus (GPe), the internal globus pallidus (GPi) and the amygdala (Amyg). **F** shows the right half of the brain in a frontal plane. Samples were taken from the pons, the hippocampus (Hcam), the thalamus (Thal) and cingulate cortex (Cing. Ctx).

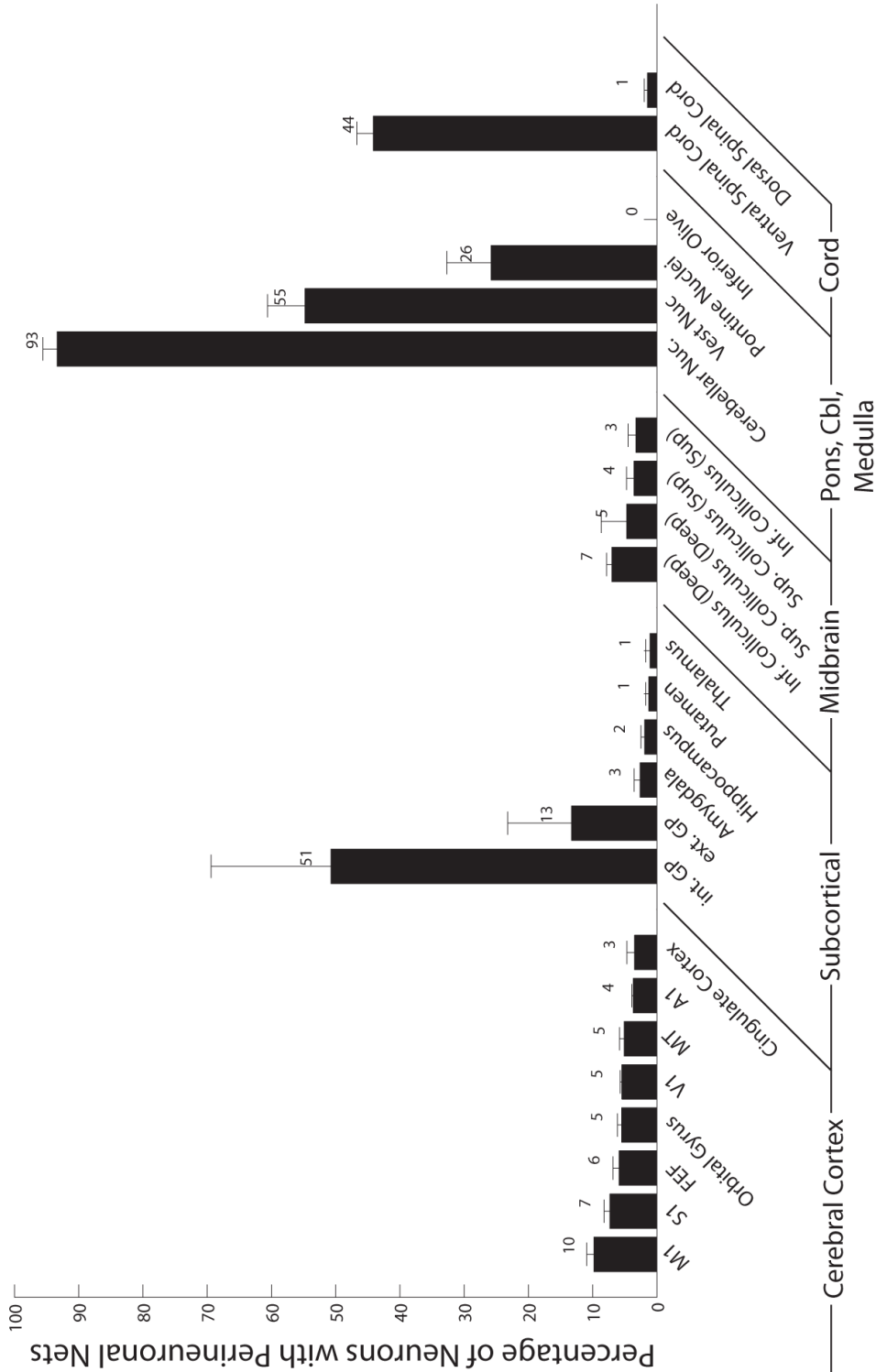


Figure 2.2: Prevalence of perineuronal nets in different parts of the brain. Each bar represents the mean percentage of neurons that were surrounded by perineuronal nets in different parts of the brain. The values, rounded to the nearest percentage, are shown above each bar. Averages and standard error are calculated from representative sections from across monkeys.

Cortex

We stained primary sensory cortices (A1, S1 and V1) as well as primary motor cortex (M1) for perineuronal nets (WFA) and neurons (α Neu-N). Figure 2.3 shows examples of labeling of perineuronal nets and neurons in these four cortical areas. In these and all other areas of cerebral cortex, perineuronal nets surround only a small fraction of neurons, less than 10%. The nets surrounded cells more frequently in layers three and four than in other layers. Figure 2.4 demonstrates this pattern in V1. The pattern held for all areas of cortex. In addition, in M1 the large projection neurons (Betz Cells) in layer five were often surrounded by perineuronal nets.

We also examined other areas of primate cortex i.e., cingulate cortex, orbital gyrus, area MT, and the frontal eye field (Fig 2.5). Approximately 5% of neurons co-stained with the perineuronal net marker in these cortical areas.

Of all the areas of cerebral cortex we examined, perineuronal nets were the most abundant in primary motor cortex.

Subcortical

Perineuronal nets surrounded very few cells in the amygdala, hippocampus, and thalamus. Figure 2.6 shows examples of labeling in these areas. The distribution of perineuronal nets in the basal ganglia varied depending on the subregion. There were virtually no perineuronal nets around cells in the putamen, but the majority of cells in the globus pallidus did have them. The quality of the labeling differed between external and internal globus pallidus. WFA labeling was strong and sharply defined cells in the internal globus pallidus, but in the external globus pallidus (GPe), the staining was more diffuse and weaker. This made it difficult to accurately count the number of neurons with perineuronal nets and is why our counts for GPe are so variable. Figure 2.7 shows examples of labeling in the basal ganglia.

Brainstem and Cerebellum

As figure 2.2 shows, an average of 93% of cells in the cerebellar nuclei were surrounded by perineuronal nets: the highest percentage of any area we examined. Figure 2.8 shows an

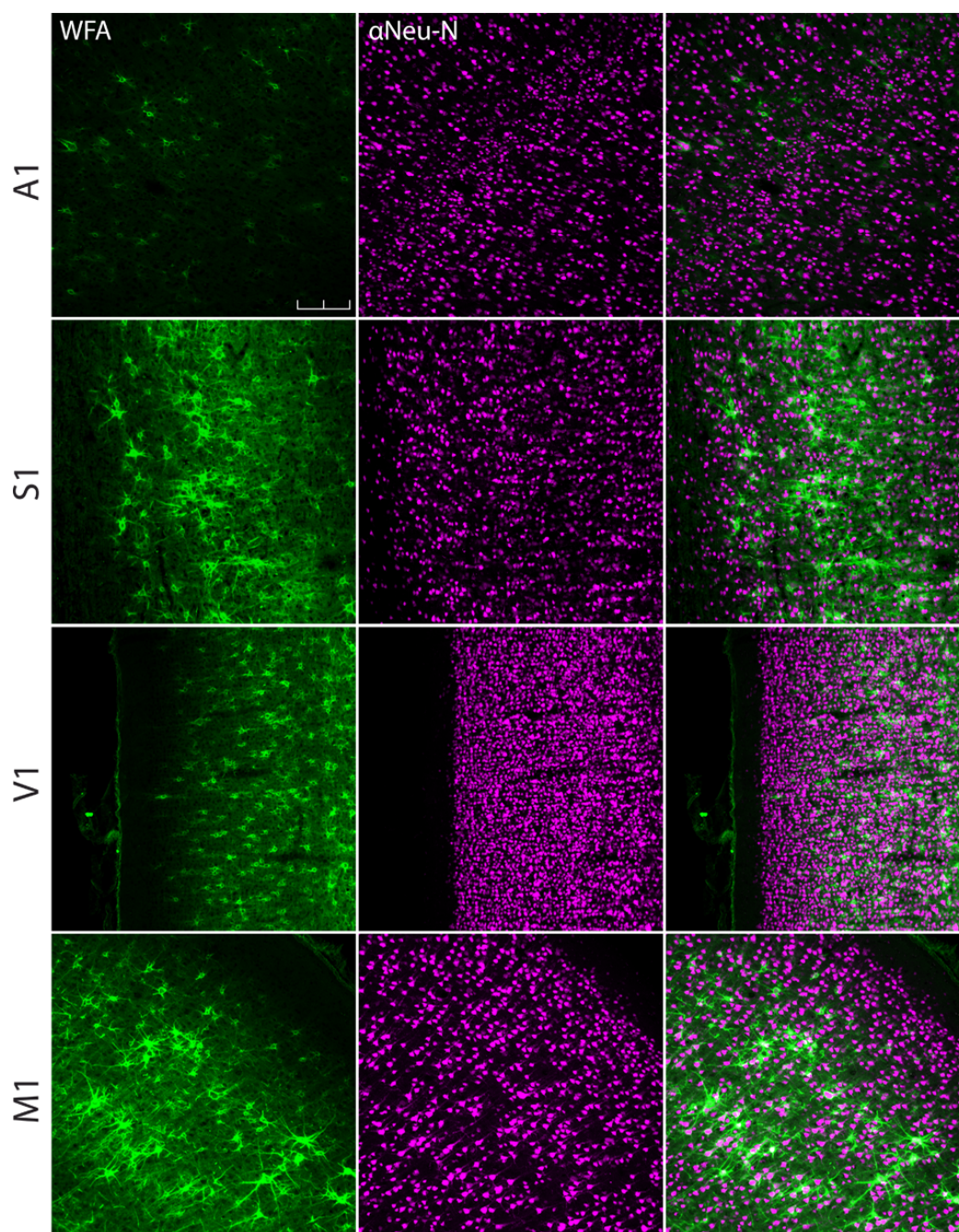


Figure 2.3: Perineuronal nets in primary sensory and motor cortex. The left panels show perineuronal nets stained with WFA. The middle panels shows anti-NeuN-labeled neurons. The right panel shows merged images of the two. Abbreviations: primary auditory cortex (A1), primary somatosensory cortex (S1), primary visual cortex (V1) and primary motor cortex (M1). Scale bar = $200\mu\text{m}$.

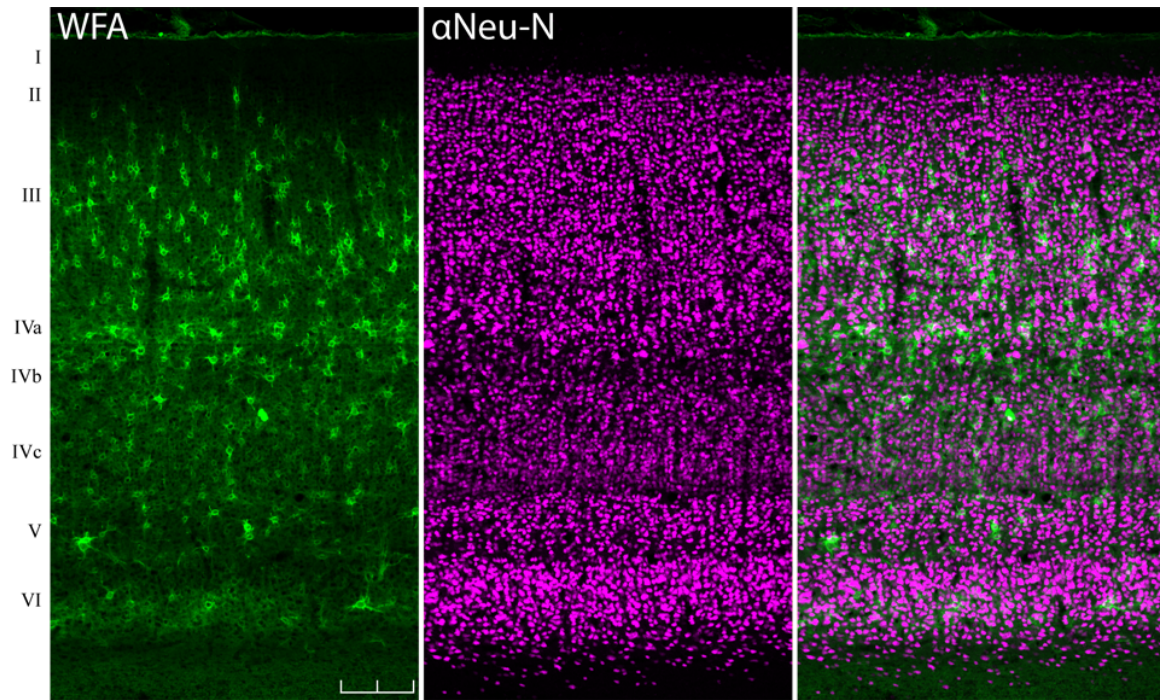


Figure 2.4: Perineuronal nets in primary visual cortex layers. The left panels show perineuronal nets stained with WFA. The middle panels shows anti-NeuN-labeled neurons. The right panel shows merged images of the two. Scale bar = $200\mu\text{m}$.

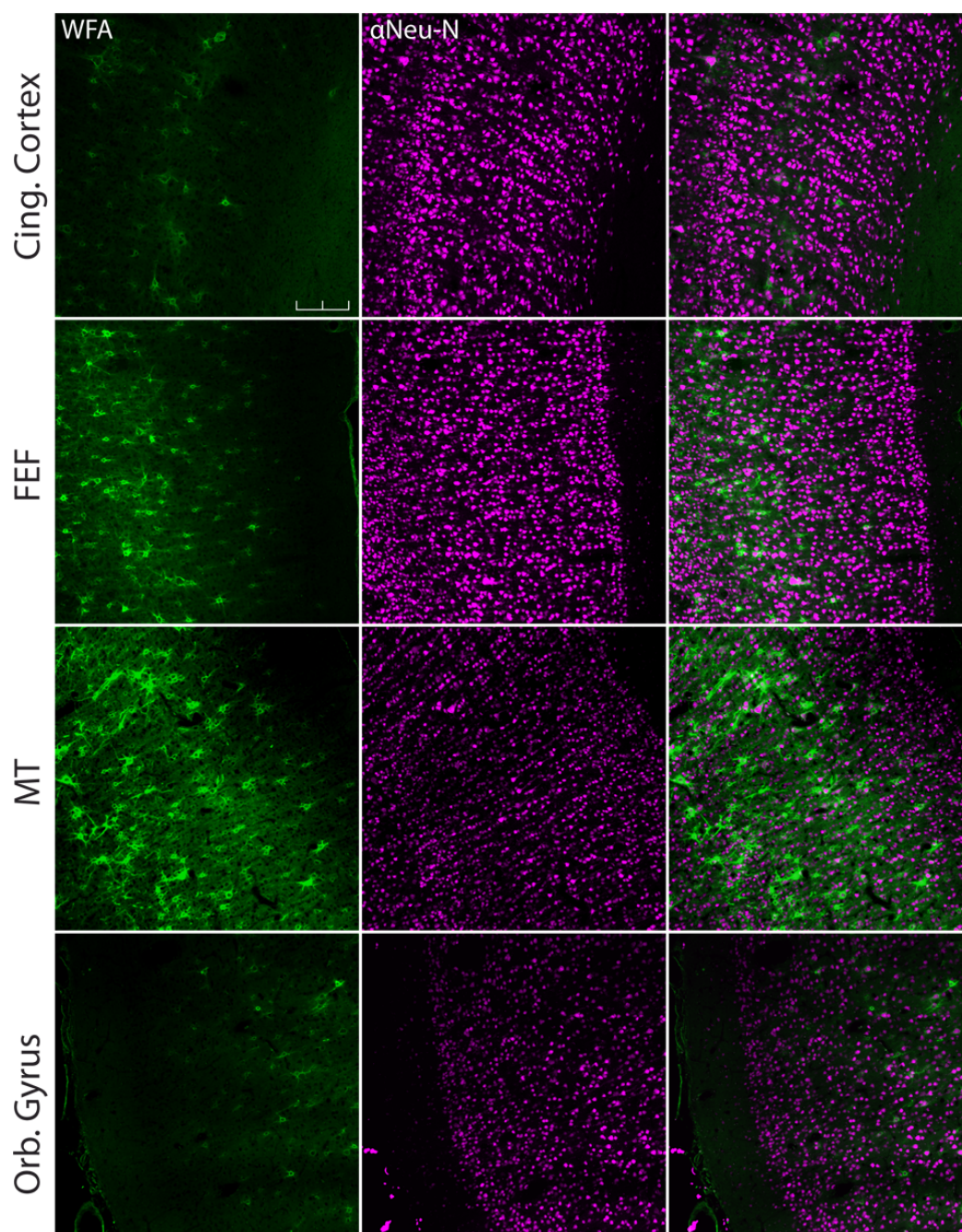


Figure 2.5: Perineuronal nets in cortex. The left panels show perineuronal nets stained with WFA. The middle panels shows anti-NeuN-labeled neurons. The right panel shows merged images of the two. Abbreviations: cingulate cortex (Cing. Ctx), frontal eye field (FEF), medio-temporal cortex (MT) and cortex from the orbital gyrus (Orb. Gyrus). Scale bar = $200\mu\text{m}$.

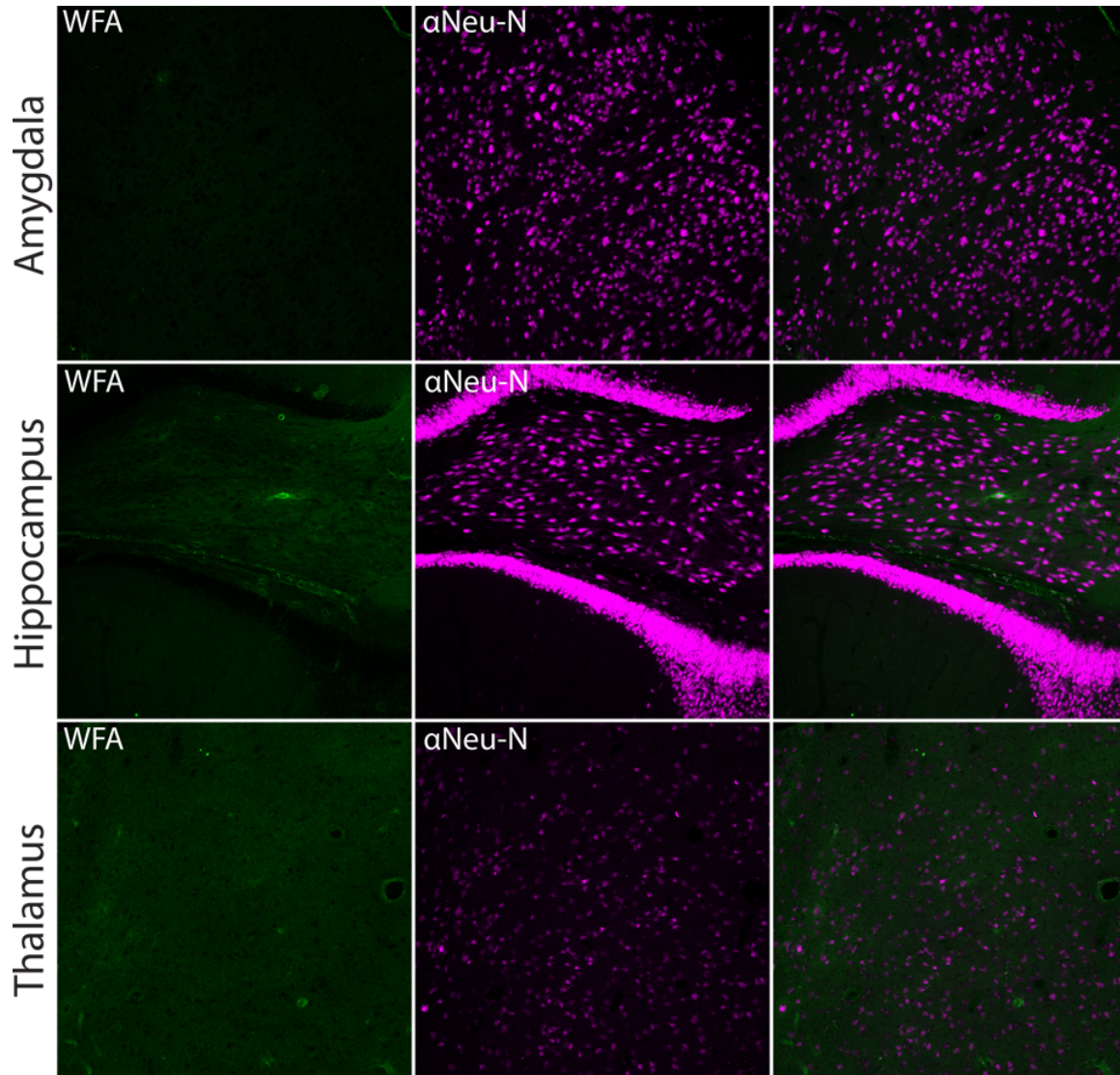


Figure 2.6: Perineuronal nets in subcortical areas. The left panels show perineuronal nets stained with WFA. The middle panels shows anti-NeuN-labeled neurons. The right panel shows merged images of the two. Scale bar = $200\mu\text{m}$.

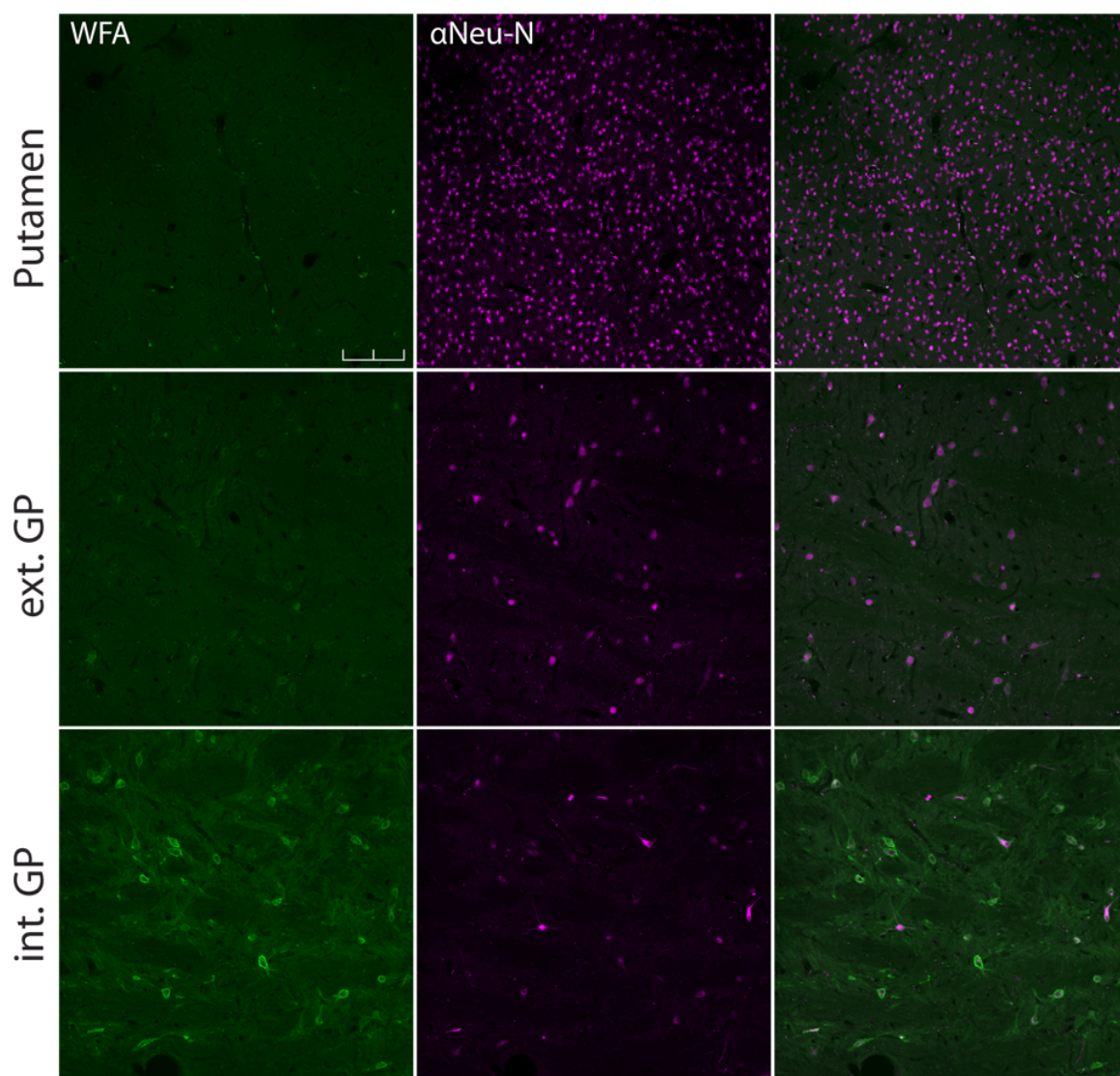


Figure 2.7: Perineuronal nets in the basal ganglia. The left panels show perineuronal nets stained with WFA. The middle panels shows anti-NeuN-labeled neurons. The right panel shows merged images of the two. Abbreviations: external globus pallidus (ext. GP), internal globus pallidus (int. GP). Scale bar = $200\mu\text{m}$.

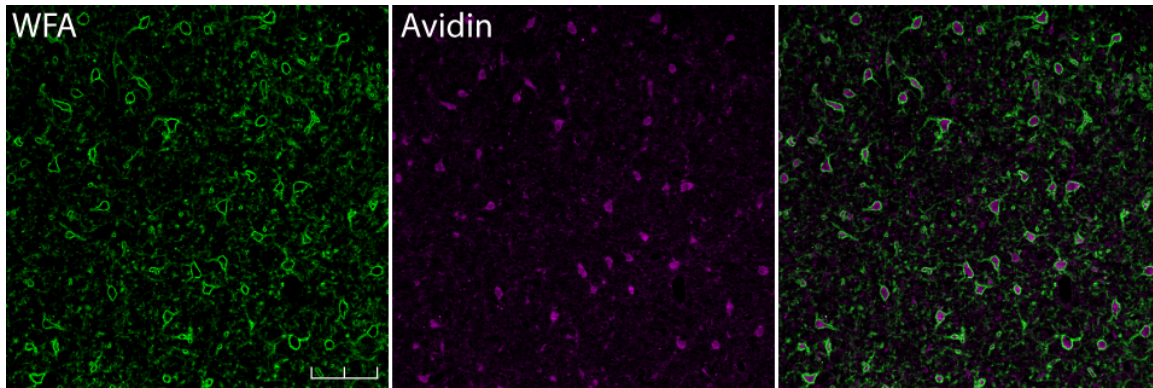


Figure 2.8: Perineuronal nets in the cerebellar nuclei. The left panels show perineuronal nets stained with WFA. The middle panels shows avidin-labeled neurons. The right panel shows merged images of the two. Scale bar = $200\mu\text{m}$.

example of labeling here. The area with the second most dense population of PNN-labeled neurons was the vestibular nuclei (55%). The top row of Figure 2.9 shows an example of labeling here. The vestibular nuclei are similar to the cerebellar nuclei in receiving direct input from Purkinje cells in the cerebellar cortex. Our estimate of perineuronal net density combines both the medial and lateral vestibular nuclei. Nonetheless, we observed more neurons with perineuronal nets in the lateral nuclei.

Approximately 25% of neurons in the pontine nuclei were surrounded by perineuronal nets. In addition to labeling around specific neurons, WFA also diffusely stained the area between cells. This diffuse labeling was also noticeable in other areas of the central nervous system as well, ie in the ventral horn of the spinal cord and to some extent the internal globus pallidus. Neurons in the inferior olive were completely free from perineuronal net labeling; the only area that we examined for which this was true. The bottom two rows of Figure 2.9 show examples of labeling in the pontine nuclei and the inferior olive.

In both the superior and inferior colliculus, perineuronal nets surrounded a higher fraction of deeper neurons than superficial neurons. Figure 2.10 shows examples of labeling in the colliculi.

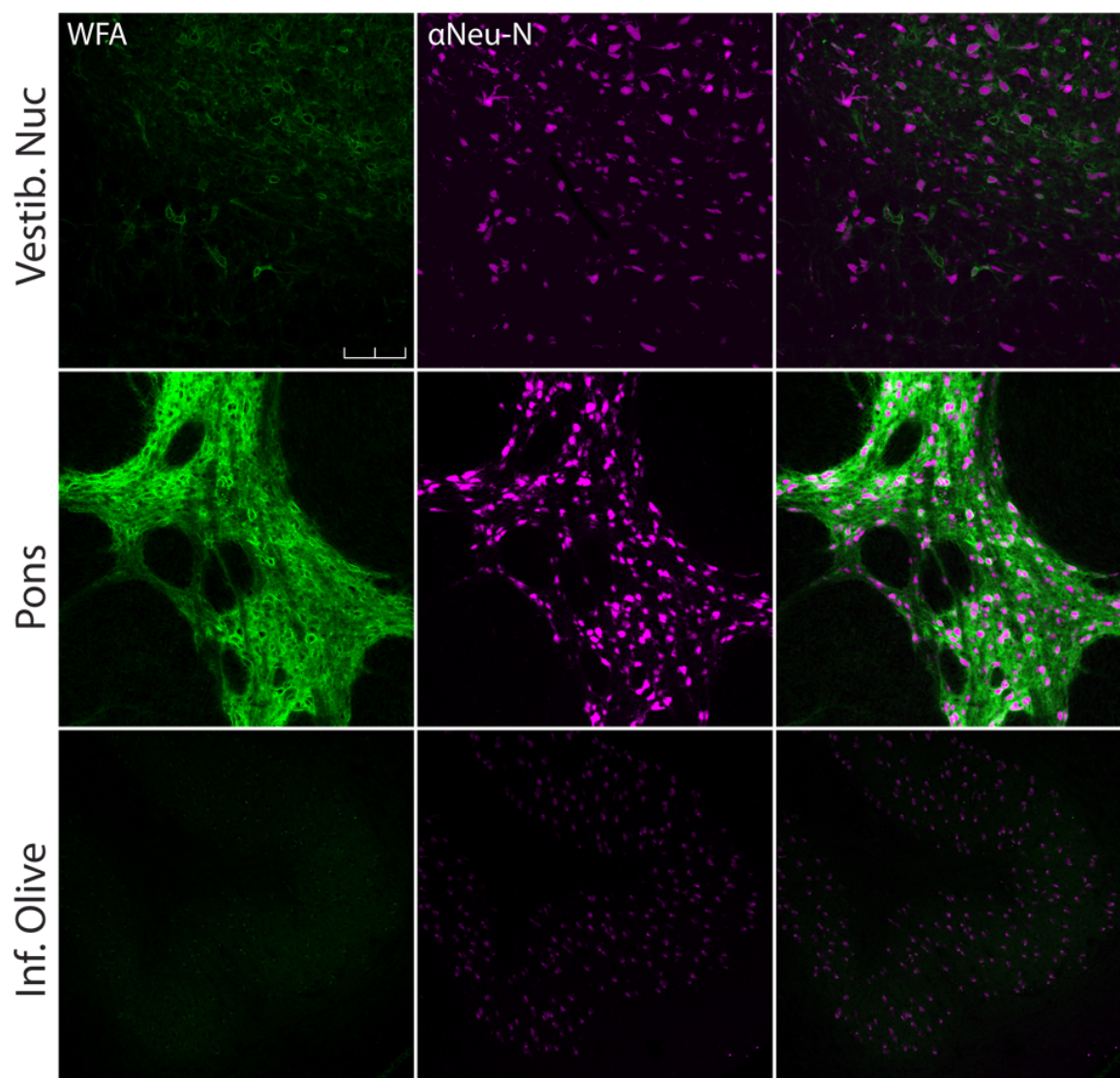


Figure 2.9: Perineuronal nets in the brain stem. The left panels show perineuronal nets stained with WFA. The middle panels shows anti-NeuN-labeled neurons. The right panel shows merged images of the two. Abbreviations: vestibular nuclei (Vestib. Nuc), inferior olive (Inf. Olive). Scale bar = $200\mu\text{m}$.

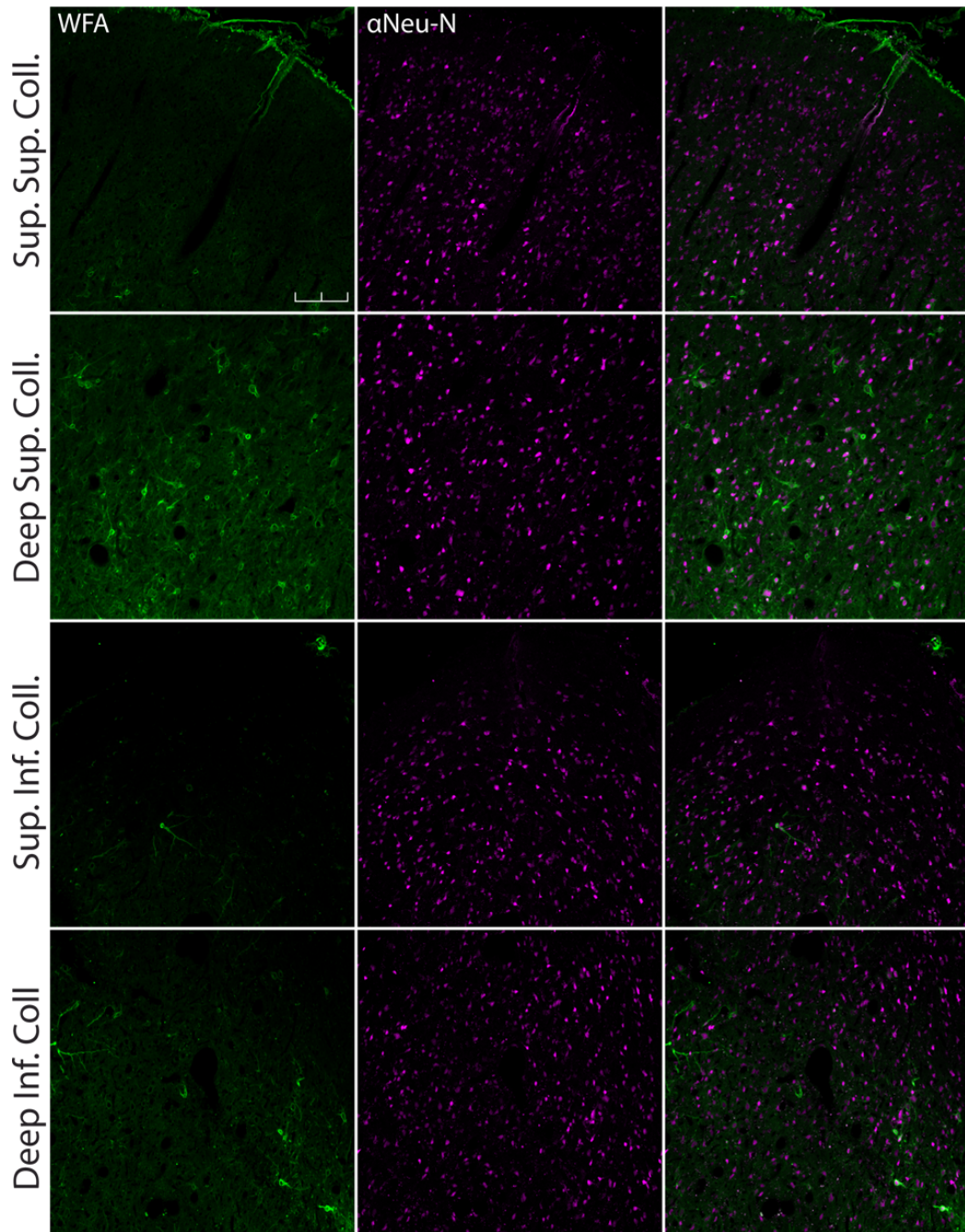


Figure 2.10: Perineuronal nets in the colliculi. The left panels show perineuronal nets stained with WFA. The middle panels shows anti-NeuN-labeled neurons. The right panel shows merged images of the two. The top two rows show superficial and deep layers of the superior colliculi. The bottom two rows show superficial and deep layers of the inferior colliculi. Scale bar = $200\mu\text{m}$.

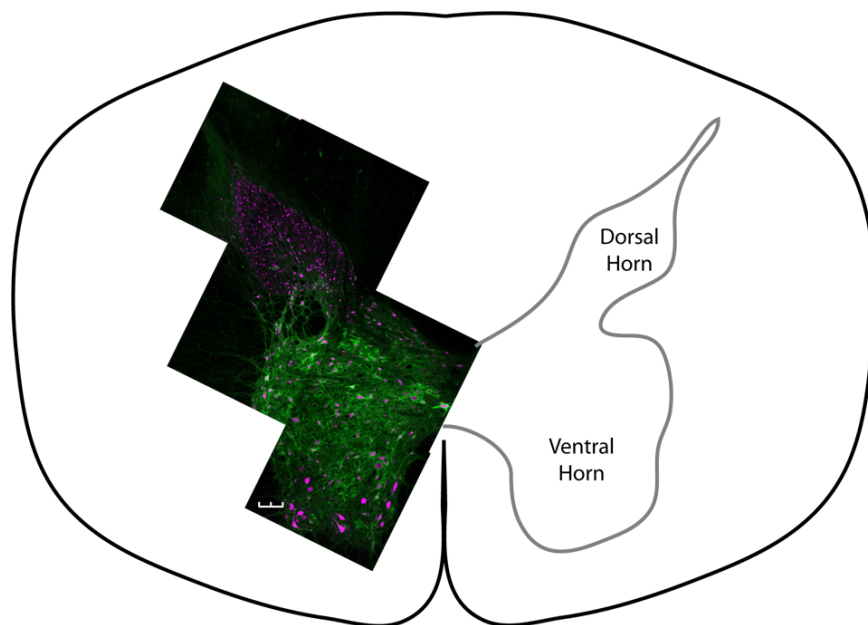


Figure 2.11: Perineuronal nets in a schematic of the spinal cord. A composite image of perineuronal nets (stained with WFA) and neurons (stained with anti-NeuN) is superimposed on the left of a schematic of the cervical spinal cord. Scale bar = $200\mu\text{m}$.

Spinal Cord

Almost 50% of neurons in the ventral horn of the cervical spinal cord (predominantly motoneurons) were surrounded by perineuronal nets while almost none of the neurons in the dorsal horn (sensory neurons) had nets. Figure 2.11 shows a composite picture of labeling in the dorsal and ventral horns.

2.4.2 PNN presence around parvalbumin-positive inhibitory interneurons varies between areas

We also counted the number of parvalbumin-positive cells in a given (0.85mm^2) area for each of the regions shown in figure 2.1 that either did or did not co-stain for PNNs. As is summarized in figure 2.12, we found that 1) Not all parvalbumin-positive neurons are surrounded by PNNs. 2) Not all neurons with PNNs are parvalbumin-positive. 3) These relative frequencies of these populations differed widely between areas.

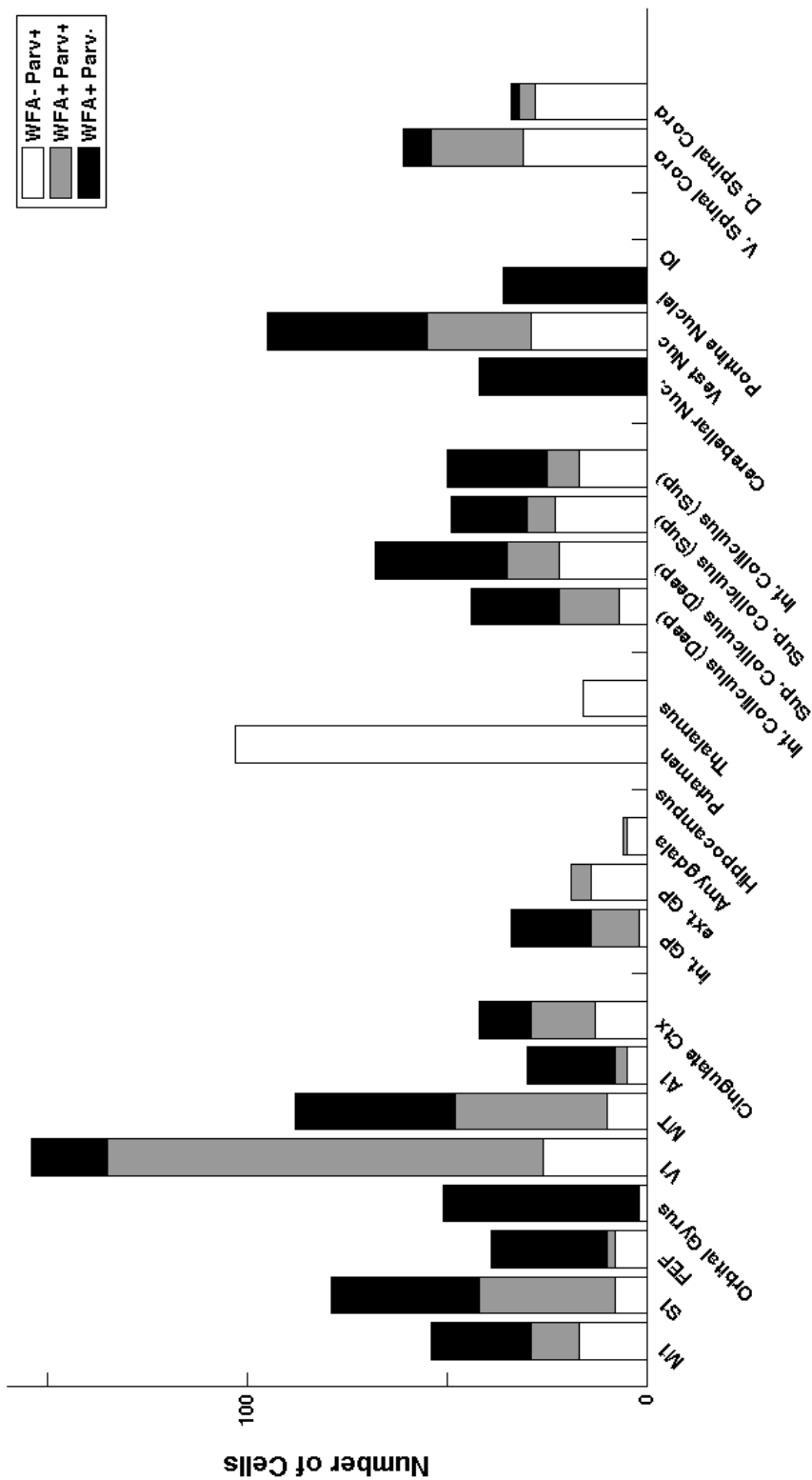


Figure 2.12: Relative frequencies of perineuronal net- and parvalbumin-positive cells in different areas of the brain of one animal. Cells were counted in $0.95\mu\text{m}^2$ areas for each region listed. We tallied cells that stained positive for either PNNs or parvalbumin alone (black and white) as well as cells that co-stained for both markers (grey) separately. Note that we did not collect counts for the hippocampus or the inferior olive, because we had difficulty identifying the structures without a neuronal stain.

For example, in primary visual cortex the populations of PNN-positive and parvalbumin-positive cells overlap extensively. However, in the frontal eye field the two populations were almost completely distinct. (See Fig. 2.13)

In non-cortical regions of the brain, regions in which PNNs are scarce are by no means lacking in parvalbumin-positive cells. For example, Purkinje cells in the cerebellar cortex stained strongly for parvalbumin, but only weakly for PNNs (Fig. 2.14). Similarly, neurons in the dorsal horn of the spinal cord were frequently parvalbumin-positive, but WFA-negative (Fig. 2.15). Also, excitatory projection neurons in the cerebellar nucleus, which are densely surrounded by PNNs, are not parvalbumin-positive (Fig. 2.14).

These results show that although in some areas of the brain PNNs surround mainly parvalbumin-positive inhibitory interneurons, this is not a consistent pattern in most brain areas.

2.5 Discussion

Among the areas that we examined, perineuronal nets generally surrounded a larger proportion of neurons in motor areas than in sensory areas. For example, M1 neurons have more nets than S1, A1, or V1. This trend was especially noticeable in the spinal cord, where we saw that PNNs surround 40-fold more ventral horn cells than dorsal horn cells. Although our results appear to differ from those of Alpár et al. (2006)[2], who found a higher density of perineuronal nets in rat primary visual and somatosensory cortex than elsewhere in the brain, we think this can be explained by our different methodologies. Alpár et al. calculated density by counting cells with nets per 1mm^2 , whereas we counted the fraction of neurons with nets in a given area. Since, there are far more neurons per mm^2 in primary visual cortex than in other areas of the brain, this could easily explain our apparently different estimates. In addition to this trend, we found that the highest proportion of neurons surrounded by perineuronal nets was in the cerebellar nuclei, which is mainly implicated in motor output.

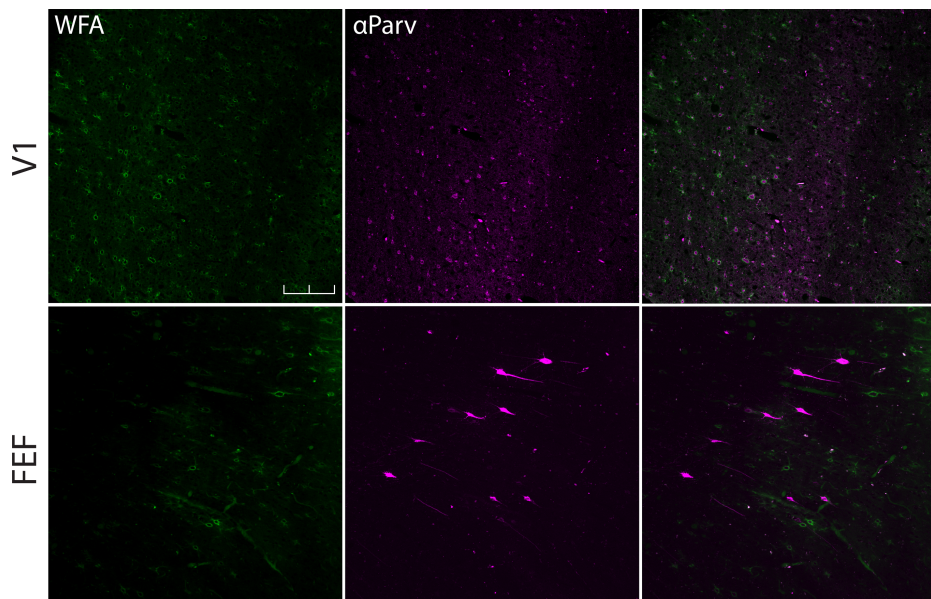


Figure 2.13: Perineuronal nets and parvalbumin-positive cells in cerebral cortex. Upper panels: primary visual cortex (V1). Lower panels: Frontal eye field. The left panels show perineuronal nets stained with WFA. The middle panels shows anti-parvalbumin-labeled neurons. The right panels show merged images of the two. Scale bar = $200\mu\text{m}$.

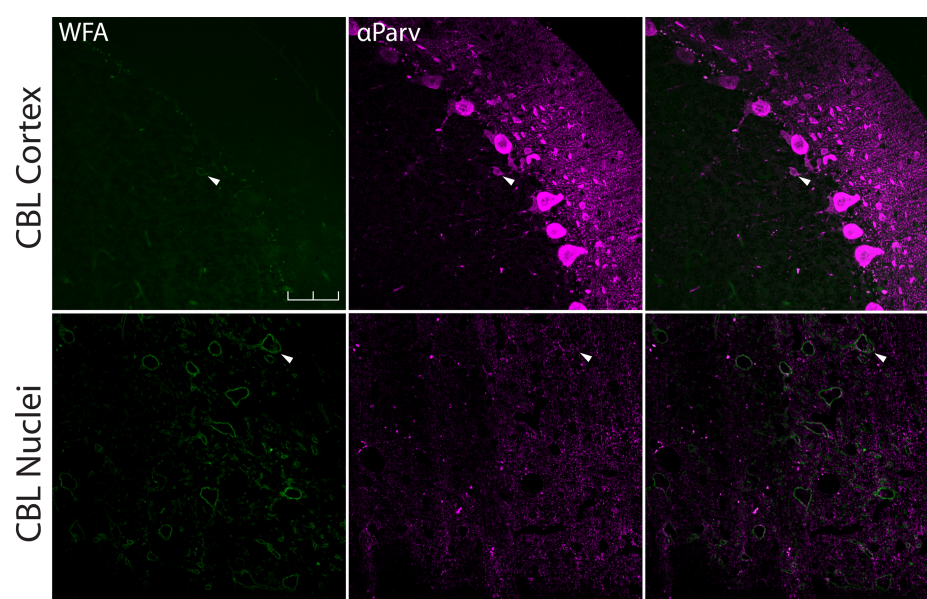


Figure 2.14: Perineuronal nets and parvalbumin-positive cells in the cerebellum. Upper panels: cerebellar cortex. Lower panels: cerebellar nucleus. The left panels show perineuronal nets stained with WFA. The middle panels shows anti-parvalbumin-labeled neurons. The right panels show merged images of the two. Large parvalbumin-positive Purkinje cell bodies are evident in the cerebellar cortex Scale bar = $400\mu\text{m}$.

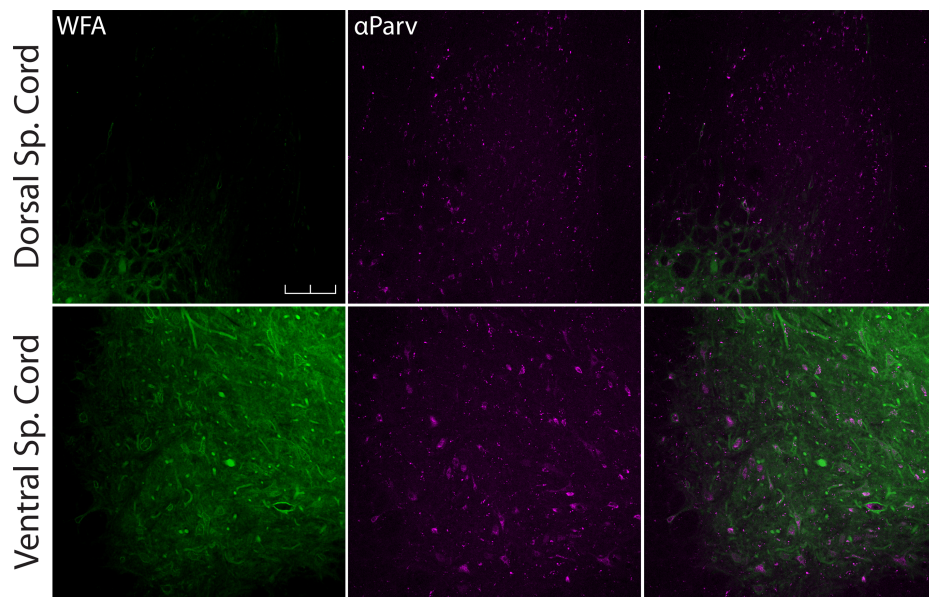


Figure 2.15: Perineuronal nets and parvalbumin-positive cells in the spinal cord. Upper panels: dorsal horn of spinal cord. Lower panels: ventral horn of spinal cord. The left panels show perineuronal nets stained with WFA. The middle panels shows anti-parvalbumin-labeled neurons. The right panels show merged images of the two. Scale bar = $200\mu\text{m}$.

2.5.1 *Implications of differential perineuronal net expression for plasticity*

Perineuronal nets are thought to play a role in restricting plasticity. They mature around neurons during developmental critical periods ([13] [5]). Supporting this view is the observation that dissolving perineuronal nets with the enzyme ChABC causes sprouting and allows restructuring of connections that is similar to what occurs during critical periods in development ([62][22][27]). Given the putative role of PNNs in inhibiting plasticity, why would they surround more neurons in motor areas than elsewhere in the brain? It is not surprising that there would be a paucity of nets around neurons in, for example, the hippocampus, because neurons there are thought to be involved in new memory formation. Restricting sprouting and connectivity for those neurons would be counterproductive. It is possible that because sensory input is, compared to motor output, more changeable, that it is beneficial to have fewer nets present in sensory areas. Those areas might require more flexibility in synapse formation than motor areas.

The cerebellum is traditionally considered to be a motor structure, however it is strongly implicated in motor adaptation. For example, neurons in the saccade-related part of the cerebellar nuclei modulate their activity during saccade adaptation ([35]). The direction of this modulation is consistent with current models of how muscle activations would need to be changed to affect saccade size ([66]). Thus, the cerebellar nuclei represent a paradox. They are the region with the most neurons surrounded by perineuronal nets but may participate in motor adaptation that requires plasticity. Further experiments to explore the importance of these dense nets will be very informative about both PNNs and the cerebellar nuclei.

Also unanswered is why the inferior olive, unique among the 25 areas that we examined, contained no neurons surrounded by PNNs. If PNNs inhibit plasticity, then their absence in the inferior olive may indicate that significant synaptic reorganization is possible there in adult animals.

2.5.2 *Targets of perineuronal nets*

Many studies have shown perineuronal nets surround fast-spiking parvalbumin-positive interneurons ([54] [5] [51] [17] [60] [10]). Although our data supports these results for some

areas, it also shows that the presence of parvalbumin in a cell is not a prerequisite for PNN presence, nor vice versa.

Härtig et al (1999)[30] and Horn et al (2002)[34] suggest that perineuronal nets surround neurons that either sustain or achieve very high firing rates. Härtig et al propose that the perineuronal nets might act as a buffering systems for the rapid cation exchanges that occur in the extracellular space local to highly active neurons. This is consistent with our findings, since neurons in the inferior olive fire at very low rates (approximately 1-2Hz) while neurons in the cerebellar nuclei are tonically active at rates between 60-100Hz. It still remains to be established, however, why some neurons, such as Purkinje cells in the cerebellar cortex, which have similarly high tonic firing rates are not surrounded by perineuronal nets at all.

Past studies show that in the cerebellum, perineuronal nets are frequently around neurons that are the target of inhibitory input ([15] [79]). We found that neurons in the inferior olive were never surrounded by any perineuronal nets. Previous work ([7]), however, shows that inferior olive cells receive substantial GABAergic input from neurons in the contralateral cerebellar nuclei. We can therefore conclude that there is not a simple relationship between the amount of inhibitory input a cell receives and the degree to which it is surrounded by perineuronal nets.

Because no single explanation can currently account PNN presence around neurons throughout the central nervous system, we cannot discount the possibility that perineuronal nets serve different functions in different regions of the brain.

2.5.3 Bearing on previous findings

Finally, two previous studies restore critical period-like plasticity in V1 ([62]) and the amygdala ([27]) of adult rats by dissolving perineuronal nets in these areas. Dissolving PNNs in V1 allowed visual experience to change in the distribution of visual input to V1 and to improve the acuity of a rat's occluded eye ([62]). Dissolving them in the amygdala allowed experience to erase fear conditioning in adult rats; conditioning which was previously thought to be permanent ([27]). These striking changes in behavior and brain structure occur after removal of PNNs from brain areas in which, in macaque, only 5% (V1) and 3%

(amygdala) of neurons are surrounded by PNNs.

It is possible that PNNs surround many more neurons in V1 and amygdala of rat than they do in macaque. To get an indication if rat and macaque V1 and amygdala have very different perineuronal net densities, we assessed the proportion of PNNs in V1 and amygdala of one rat using the same methods that we used for macaques. We found that in this animal PNNs surrounded 3.5% of V1 neurons and <1% of amygdala neurons.

Thus, it is likely that the two previous experiments that strongly increased the plasticity of V1 and amygdala neuron did so by dissolving perineuronal nets around a very small proportion of the cells in these structures. Corollary to this, if formation of PNNs indeed closes critical periods (see Galtrey and Fawcett, 2007[22] for review), then critical periods in V1 and the amygdala end when PNNs form around only a small proportion of neurons in these structures.

2.5.4 Conclusion

In summary, the distribution of perineuronal nets is very different in different areas of the central nervous system. No generalization accounts well for these differences, because we do not yet fully understand the functions of cells in these regions, or of PNNs themselves. It appears that in some areas PNN presence around only a small proportion of neurons is sufficient to block plasticity.

Chapter 3

LONG TERM ADAPTATION OF SACCADDES**3.1 Summary**

Motor learning adjusts movement size and direction to keep movements accurate. A useful model of motor learning, saccade adaptation, uses intra-saccade target movement to make saccades seem inaccurate and elicit adaptive changes in saccades. In the most studied saccade adaptation procedure, which we call short-term saccade adaptation (STSA), monkeys decrease or increase the size of their saccades by tracking 1000 - 2000 adapting target movements in a single saccade session. STSA elicits rapid changes of limited size and duration. Larger, more persistent reduction in saccade size results from adapting saccades daily for 19 days, a procedure that we call long-term saccade adaptation (LTSA). LTSA mimics the demands of rehabilitation more closely than does STSA and, unlike STSA, produces changes that could maintain long-term accuracy. Previous work describes LTSA that reduces saccade size in monkeys. Though convenient to study, size-reducing LTSA is not a good model for rehabilitation because few injuries necessitate making movements smaller. Here we characterize size-increasing LTSA and compare it, in the same monkeys, to size-reducing LTSA. We found that size-increasing LTSA can double saccade gain in 21 days, and is slower than size-decreasing LTSA. In contrast to a single size-decreasing STSA, a single size-increasing STSA does not prevent additional saccade size increase at the normal rate when a monkey continues to track adapting target movements. We conclude that size-increasing LTSA is slower than size-decreasing LTSA but can make larger changes in saccade size. Size-increasing and size-decreasing LTSA use distinct mechanisms with different performance characteristics.

3.2 Introduction

When movements are repeatedly inaccurate because of injury, aging, or growth, the brain changes motor commands to improve performance. In the lab we make voluntary rapid eye movements, saccades, seem to be inaccurate by providing visual feedback that these movements do not end on target. We do this by moving the target to a new location during each saccade. This feedback makes saccades change gradually so that they end closer to their targets. Monkeys usually asymptotically adapt within 1000-2000 saccades. We call this short-term saccade adaptation (STSA). STSA to decrease or increase saccade size is a common model for studying motor plasticity (e.g., [75] [40]).

Despite its utility for studying plasticity, STSA is not a good model of long-term maintenance of movement accuracy. It fades quickly and its size is limited, i.e. one large change precludes subsequent change [69]. Visual feedback that saccades are inaccurate elicits longer lasting changes in saccade size when it persists for 3 weeks [69]. This long-term saccade adaptation (LTSA) does not impair subsequent STSAs, indicating that LTSA and STSA use separate mechanisms. It is not yet clear that LTSA is the mechanism for maintaining movement accuracy in natural settings, but its time course makes it a better model than STSA.

In previous work we characterized size-decreasing LTSA because, in monkeys, size-decreasing STSA is typically faster and larger than size-increasing adaptation [75] [68]. However, despite its convenience, size-decreasing adaptation is less useful than size-increasing adaptation for correcting damaged movements. No injury makes saccades too large except damage to the caudal region of the cerebellar fastigial nucleus. Thus, there seems little use outside the laboratory for size-decreasing adaptation. In contrast, many dysfunctions of the oculomotor system, e.g., muscle weakness or nerve damage, make movements smaller, and are therefore potentially improved by size-increasing adaptation.

In summary, size-increasing LTSA is an example of motor learning that will help remedy hypometria, a common problem of impaired movements, and is likely to last long enough to keep movements accurate. Here we describe size-increasing LTSA in monkeys. We found that size-increasing LTSA changes saccade size at about one third the rate of size-decreasing

LTSA but can double saccade gain in 21 days. In contrast to a single size-decreasing STSA, a single size-increasing STSA does not prevent additional increase in saccade size if the monkey continues to track adapting target movements. Though it is slower, the size-increasing LTSA mechanism has more capacity to change saccade size than does size-decreasing LTSA. The difference in the time course of size-increasing and size-decreasing LTSA leads us to conclude that they use distinct mechanisms.

3.3 Experimental Procedures

3.3.1 Subjects and animal preparation

Three juvenile male rhesus monkeys (*Macaca mulatta*) were implanted with a three-turn coil of Teflon-coated wire in one eye in an aseptic surgery. This wire connected to a plug attached to the top of the monkeys skull with metal screws and dental acrylic. In the same surgery we similarly attached head stabilizing hardware which was embedded in dental acrylic. The animals recovered from surgery in their home cages for at least one week after surgery.

3.3.2 Animal training

We used the search coil technique [20] [65] to measure eye position. We trained animals to use saccades to track a small laser spot projected onto a screen 57 cm away. The spot was positioned by two computer-controlled galvanometers. Animals fixated the target for 0.6-2 s, then the target moved to a new position to the left or right. Monkeys received a small dollop of apple sauce reward for keeping their eyes within 2° of the target position as it moved to new locations. Training took place in a light-tight, sound-attenuating booth. Except for the laser spot, animals were otherwise in complete darkness.

3.3.3 Eliciting short-term saccade adaptation (STSA)

To make saccades seem to have missed their targets, and thereby drive adaptation, we moved targets during saccades [50]. Software detected a saccade when eye velocity exceeded $70^\circ/\text{s}$ and moved the target before the saccade ended. To increase saccade size in these experiments

we presented target movements of 8° to the right or left of current eye position. During saccades to these targets we made the target move an additional 8° in the same direction as the initial target movement. Therefore the target started at either a central fixation point between the animals eyes, or 16° to the right or left of this point on a horizontal line. When a monkey tracked this type of target movements its saccades to targets 8° to the right or left became larger than 8° .

To decrease saccade size we presented the 16° target movements. During the saccade that tracked this movement the target moved back 8° in the opposite direction so that it ended 8° away from initial eye position. The target started either at a central fixation point between the animals eyes, or 8° to the right or left of this point on a horizontal line. When a monkey tracked this type of target movements its saccades to targets 16° to the right or left became smaller than 16° . In a single adaptation session we adapted saccades to both the left and the right. Adapting saccades in one direction does not influence saccades in the opposite direction [1], [16], [19], [53] [75], but see [70]: Simultaneously adapting saccades in opposite directions results in a slower time course for human STSA than adapting in one-direction). We therefore treated adaptation of leftward and rightward saccades in the same session as two independent adaptations.

3.3.4 *Eliciting long-term saccade adaptation (LTSA)*

To elicit long-term saccade adaptation we presented size-increasing intra-saccade target movements as described above for about two hours each day on consecutive days. Typically a monkey made 1000-2000 saccades in each direction during each days adaptation. When they were not inside the experimental booth, animals wore opaque blindfolding goggles. Except for a 5 second period at the beginning and end of each daily adaptation session, a monkeys only visual experience was while it tracked the target with intra-saccade target movements. We ended a long-term adaptation when saccades at the beginning and end of the same session were statistically equivalent in at least one direction according to Schuirmanns (1987)[71] TOST equivalence test with $P \leq$ to 0.05 as the criterion for equivalence.

To evaluate any size-increasing capacity remaining after the end of a long-term adap-

tation we presented intra-saccade target movements of an additional 4° on the first day after we ended an LTSA. On the second day after an LTSA ended we presented the same intra-saccade target movements that we did during the LTSA. This allowed the monkeys saccades to return to the size that they were at the end of the LTSA, i.e., $\sim 16^\circ$. Beginning on the third day after LTSA end, we presented normal 8° target movements, i.e., not followed by intra-saccade target movements. We did this on consecutive days until the size of the monkeys saccades returned to normal.

To compare size-increasing to size-decreasing LTSA in the same animal we also elicited size-decreasing LTSA using 16° to 8° intra-saccade target movement. At the end of size-decreasing LTSA we presented normal 16° target movements for two hours each day on consecutive days until the monkeys gains recovered to normal. The monkeys were exposed to normal target movements for a minimum of two months between size increasing and size decreasing LTSA experiments.

3.3.5 Data collection and analysis

We used a CED Power 1401 laboratory interface (Cambridge Electronic Design, Cambridge UK) to digitized voltages proportional to horizontal and vertical eye and target positions at 1kHz. A custom MATLAB (Mathworks Inc, Natick, MA) program identified target and eye movements and measured their properties. This program initially detected large, rapid changes in target position and then scanned forward within the next 500 ms to the nearest eye movement with a peak velocity greater than $100^\circ/\text{s}$. It then identified the times before and after the time of peak velocity where the velocity dropped below $20^\circ/\text{s}$. These were the start and end times of the saccade. The program measured saccade size and divided it by target movement size to calculate gain. We assessed gain change during the course of an LTSA with two measures:

- 1.) Starting gain: the average gain of the first fifty saccades for each day of LTSA and recovery.
- 2.) Absolute gain change: the absolute value of the difference between the mean of the first and last 50 saccades of each days adaptation and recovery.

We then fitted an exponential to both of these sets of values, minimizing the sum-squared error. Parameter A in the following equation is a multiplier that describes the difference between the first point and the asymptotic value of the fit curve. We use this variable to quantify the degree of adaption. g is the gain measurement calculated for each day using either method one or two from above. b is the bias; τ is the rate constant and n is the number of days the adaptation took.

$$Fit(g) = b \pm A \times e^{\frac{-n}{\tau}} \quad (3.1)$$

To test for similarity of gain at the start and end of a single days session, we used the first and last 50 saccades of the session in the two one-sided tests (TOST) procedure of Schuirmann (1987)[71] and considered $P \leq 0.05$ to be significant.

All surgical and behavioral training procedures were approved by the Animal Care and Use Committee at the University of Washington. The animals were cared for by the veterinary staff of the Regional Primate Research Center. They were housed under conditions that comply with National Institutes of Health standards as stated in the Guide for the Care and Use of Laboratory Animals (DHEW Publication NIH85-23 1985) and with recommendations from the Institute of Laboratory Animal Resources and the American Association for Accreditation of Laboratory Animal Care.

3.4 Results

3.4.1 Gain change during long-term saccade adaptation and long-term recovery

Rightward and leftward saccades adapted very similarly in these experiments. For brevity we show the gains of only leftward saccades in Figure 3.1 and subsequent figures. Figure 3.1 shows the gains of leftward saccades for all three monkeys during one size-increasing LTSA. Table 1 summarizes gain changes during adaptation and recovery for both directions of LTSA. It includes information about the total number of days and trials, the initial and final gain values, the absolute change in gain, and multiplier and rate constant for the exponential fits of starting gains (SG, see Figure 3.3) and the rate constant for the fit of absolute change in gain (ACG, see Figure 3.4). All monkeys have initial gains above 1.

Straube et al (1997) [75] documented a similar small but very rapid change in saccade size during the initial trials of adaptation. This may represent a small strategic component of the monkeys saccade planning.

During size-increasing LTSA all three monkeys achieve gains close to 2. Previous work with STSA [75] [68] indicates that monkeys have less size-increasing capacity than size-decreasing capacity. Our findings here indicate that size-increasing LTSA is capable of nearly doubling saccade size. We arbitrarily limited the LTSA that we elicited here to achieving a gain of 2. We have no indication that this is the limit and it seems likely that size-increasing LTSA could more than double saccade gain.

As apparent in Figure 3.1, during the first several daily sessions of LTSA, saccade size usually increased from the start to the end of the session. Overnight gain usually decreased so that gain at the start of each day was smaller than the final gain for the previous day. All three monkeys exhibited this pattern. Saccade gains for monkeys A, B, and C stabilized by day 11, 16 and 20, respectively.

Figure 3.2 shows, the gains of leftward saccades in all three monkeys during recovery from the size-increasing LTSA in Fig. 1. As apparent in Figure 2 saccade gain decreased quickly on the first day of recovery. At the beginning of the next day, and several subsequent days, gains were larger than at the end of the previous day. This pattern was the same for all three monkeys. By the end of recovery, mean saccade gains at the start and end of the session were equivalent and there was no further decay overnight. In one animal we left the goggles on for 35 days after a forward adaptation and measured the recovery without presentation of normal targets. In this case we blanked the target as soon as the saccade was initiated and placed it at the position that the eye landed within 20ms of saccade completion [68]. This indicates zero error to the animal and minimizes feedback. Although there was some recovery (10.6%, 16.6%, 30.6% and 6.2% for four different experiments), eye movements did not fully recover to a gain of 1. In contrast, after a single short term adaptation (day 2 in figure 1 for all monkeys), there is a substantial shift of the eye movements back toward the baseline gain of 1 overnight.

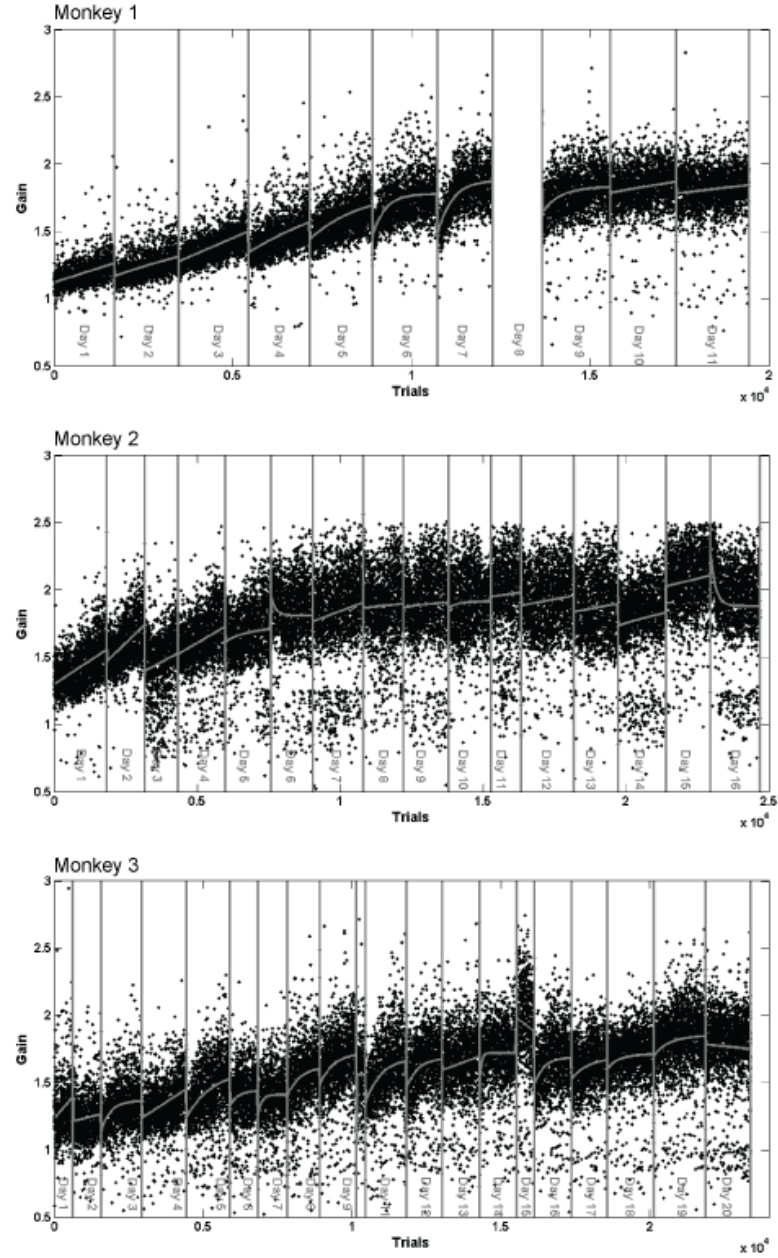


Figure 3.1: Size-increasing adaption. Gains of leftward saccades as monkeys tracked targets that moved from 8° leftward to 16° leftward during each saccade. Thus, complete adaptation would have a saccade gain of 2. Here, as in Figures 2 and 5, saccade gain for each trial is in sequential order. There was an error in data collection during day 8 of monkey As adaption, therefore it is not shown.

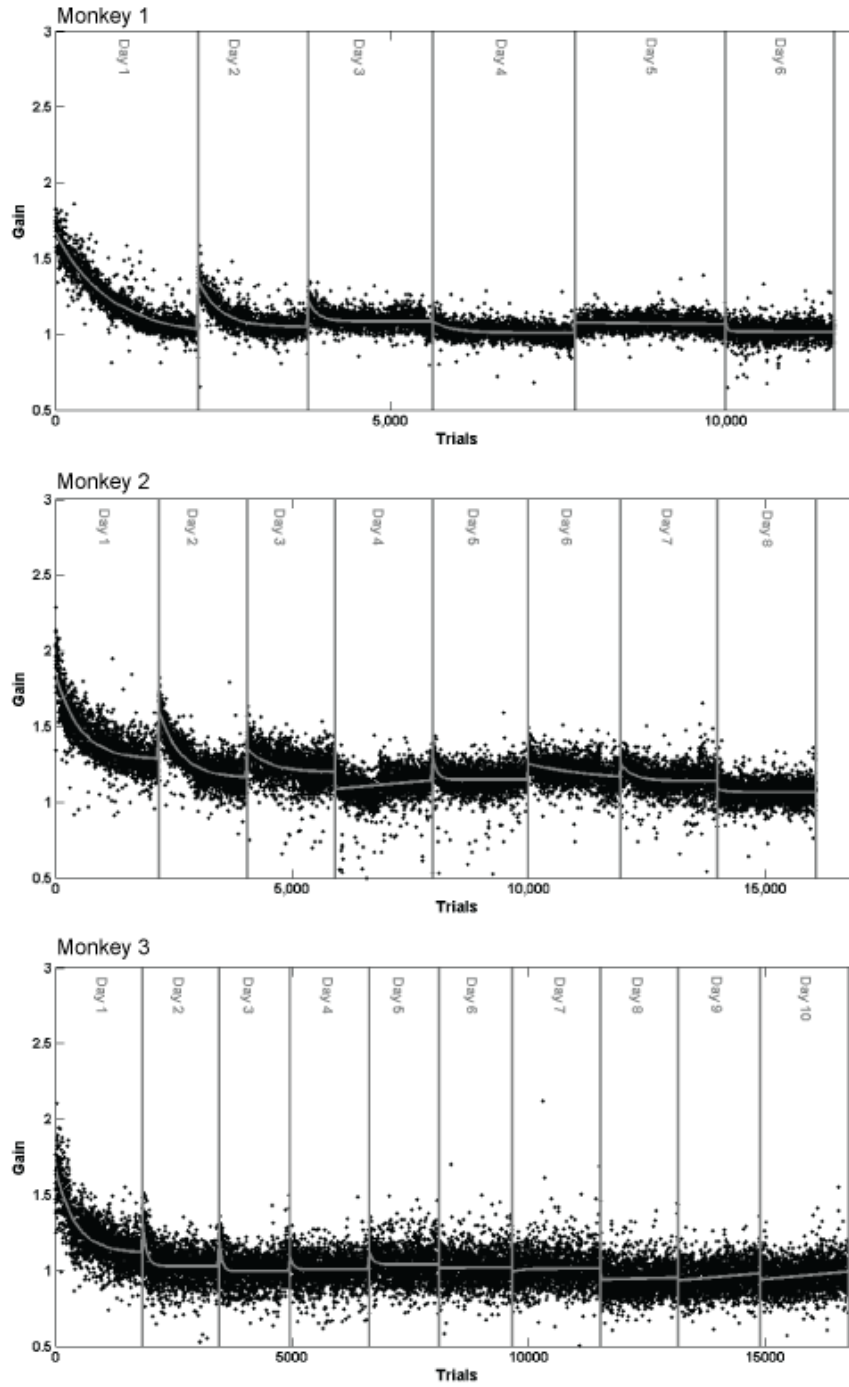


Figure 3.2: Recovery from adaption. Gains of leftward saccades as monkeys recovered from the size-increasing LTSA in Figure 1 by tracking normal 8° leftward target movements.

3.4.2 *Rates of long-term saccade adaptation and recovery*

We characterized the rate of long-term adaptation with two measures of each days adaptation: 1) mean starting gain and 2) absolute gain change. Mean starting gain is the average gains of a days first fifty saccades. We graphed the value of each days average against days of adaptation and fit an exponential curve to this relationship. Figure 3.3 shows these graphs for size-increasing (black) and size-decreasing (grey) LTSAs. Note that size-increasing (8° to 16°) show gains changing from 1 to 2 and size-decreasing (16° to 8°) adaptation shows gains changing from 1 to 0.5. Thus, although the movements are being adapted by the same magnitude, the conversion of the amplitude values into gains causes an apparent compression of size-increasing LTSA compared to size-decreasing LTSA. Changes in mean starting gain during LTSA are orderly in all monkeys for both size-increasing and size-decreasing LTSA. We measured LTSA rate as the rate constant of the exponential curve fit to mean starting gain. Size-increasing LTSA was significantly slower than size-decreasing LTSA in Monkeys A and C. In Monkey B size-increasing and size-decreasing LTSA occurred at approximately the same rate. In all three animals, recovery from size-increasing LTSA was faster than the preceding LTSA.

Our second measure, absolute gain change, is the absolute value of the difference between the average gains of the first and last fifty saccades on the same day. Figure 3.4 shows absolute gain change on successive days of adaptation for both size-increasing (black) and size-decreasing (gray) LTSAs. Absolute gain change did not change as smoothly as mean starting gain during LTSA but the qualitative results are similar. Size-increasing LTSA is much slower than size-decreasing LTSA in monkeys A and C. In Monkey B, size-increasing LTSA is faster than size-decreasing LTSA. Measured with absolute gain change, recovery from size-increasing LTSA was faster than the preceding LTSA in all three animals.

3.4.3 *STSA interactions with previous LTSA and STSA*

Previous work shows that a multi-day size-decreasing LTSA does not impair additional gain decrease during a subsequent one-day STSA, i.e., size-decreasing STSA is normal after size-decreasing LTSA. In contrast, the gain decrease elicited by an STSA is strongly impaired

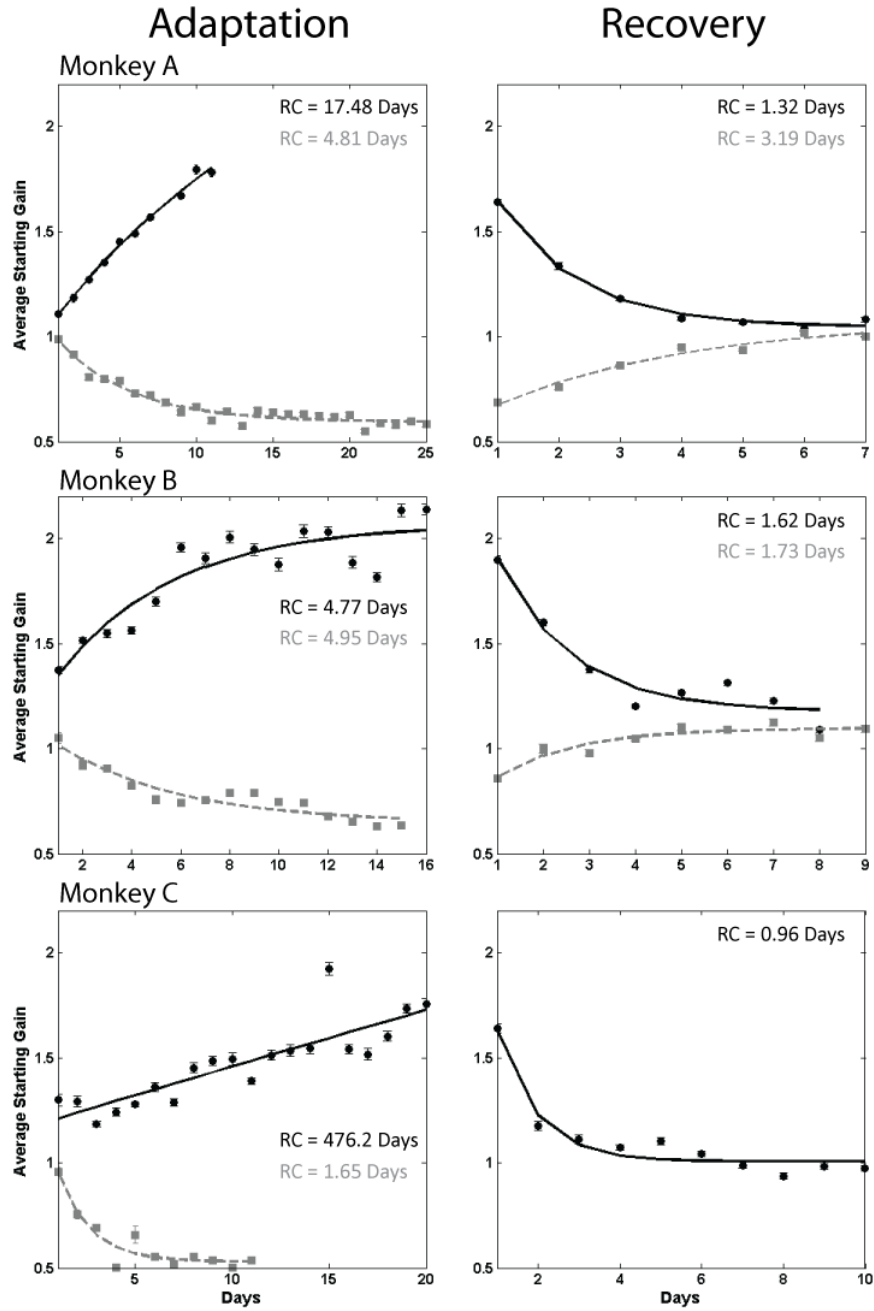


Figure 3.3: Starting gains on each day of size-increasing (black) and size-decreasing (gray) LTSA. Least-squares exponential are fit to each relationship. We did not collect the same recovery data of size-decreasing adaptation for monkey C, therefore it is not shown. RC = rate constant.

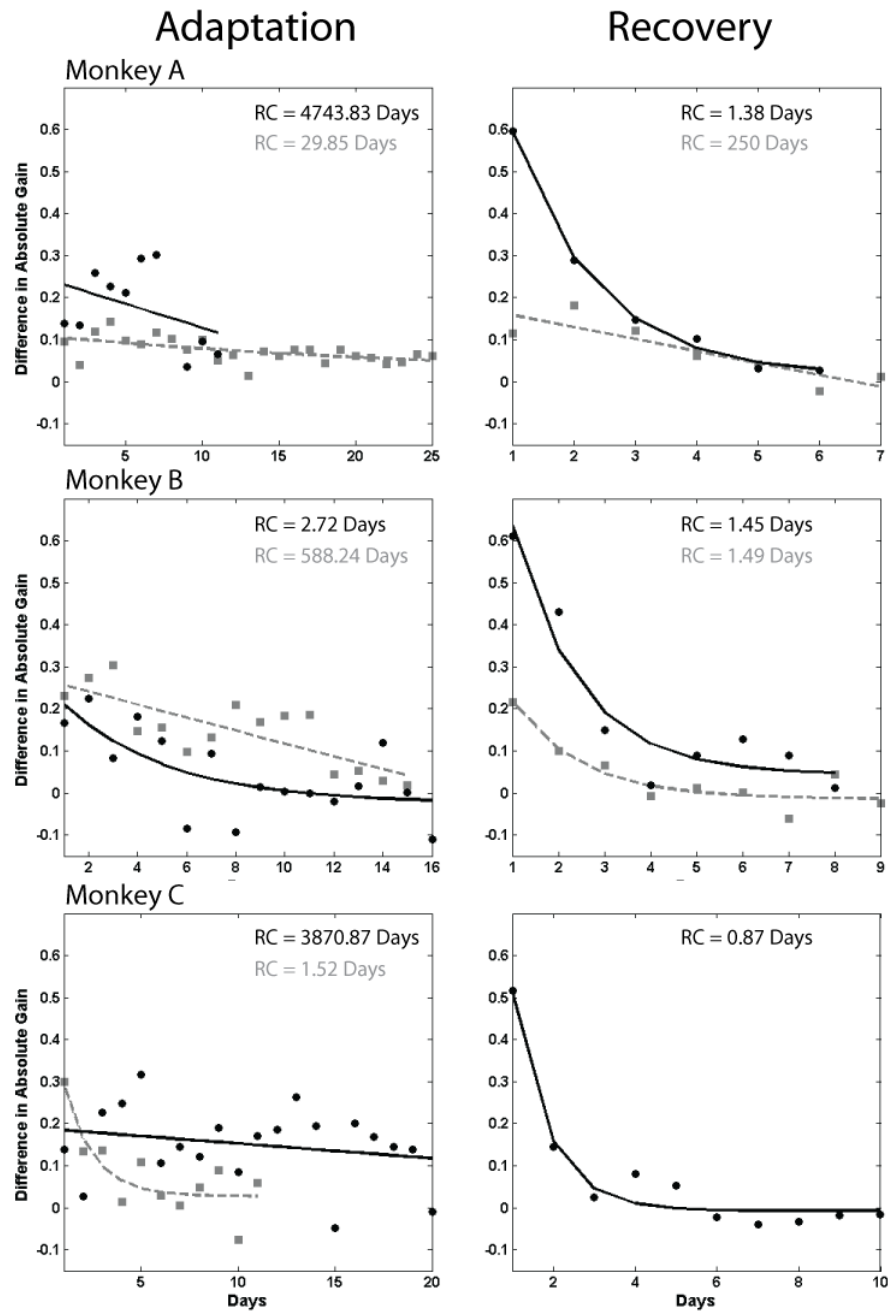


Figure 3.4: Absolute gain change on each day of size-increasing (black) and size-decreasing (gray) LTSA. Least-squares exponential are fit to each relationship. Again, we did not collect the same recovery data of size-decreasing adaptation for monkey C, therefore it is not shown. RC = rate constant.

if that STSA immediately follows a previous size-decreasing STSA. The fact that STSA is normal after LTSA but not after STSA indicates that the LTSA and STSA mechanisms for reducing saccade gain are independent of one another [69].

Here we used a similar strategy to test whether or not size-increasing LTSA and STSA are similarly independent. We elicited size-increasing STSA after a previous size-increasing STSA or LTSA. Figure 3.5 shows data for all three monkeys in the different experiments. The left column shows saccade gains elicited by two consecutive STSAs. During the first, the target moved from 8 to 16 from initial eye position during each saccade. During the second, the target moved an additional 4, from 8 to 20. The center column shows saccade gains elicited on the last day of an 8 to 16 LTSA followed by an STSA during which the target moved from 8 to 20. The right column shows gains elicited during a simple 8 to 16 STSA.

In the left column we see that gain increases substantially during the first adaptation and also during the second. The first gain increase does not preclude the second. In the center column we see that gain increases during an STSA that immediately follows a completed LTSA (i.e., gains are high and stable). Again, previous gain increases do not impair subsequent increases. In the right column, we see gain increases during an STSA in the same monkey. These increases are similar to the increases during the STSAs in the right panels of the left and center columns, indicating that those STSAs were normal.

Table 2 shows the details of the different measurements for these experiments, including the parameters calculated with an exponential fit similar to that described in the method section. In this case the fit is of individual gain values for subsequent trials within a session, not descriptive gain measurements for subsequent sessions. In some cases, the data proved too noisy to be fit with this method and in those cases we have not included the parameters (NA).

3.5 Discussion

The major result of this study is that daily adaptation for 21 days elicits large, persistent increases in saccade gain when we restrict a monkey's visual experience almost exclusively to adapting target movements. With this procedure, both size-decreasing and size-increasing

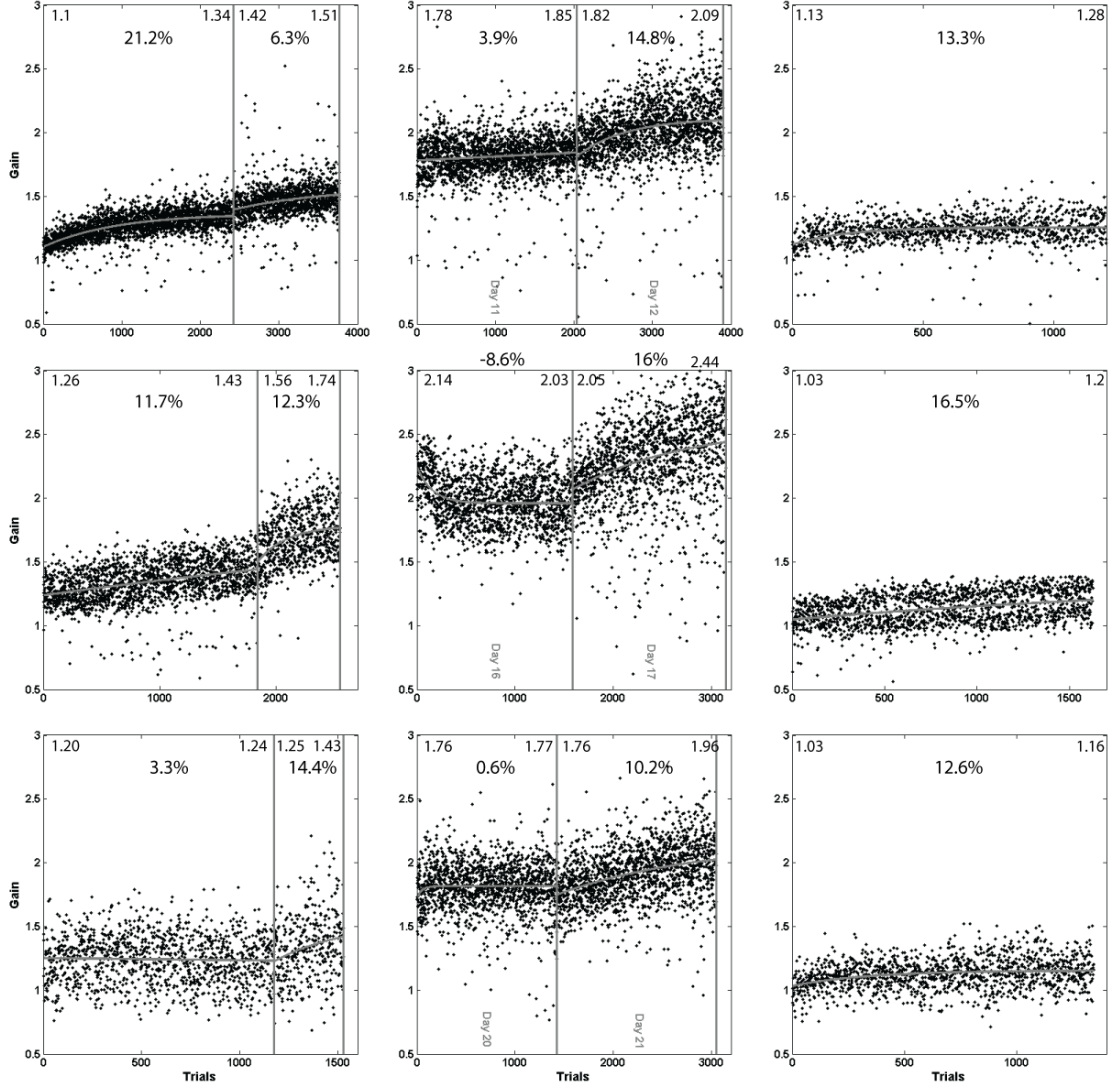


Figure 3.5: Short-term and long-term adaptations. **Left**, Gains of saccades during two consecutive size-increasing STSAs. During the first, the target moved from 8 to 16 during saccades. During the second, the target moved from 8 to 20. **Center**, Gains of saccades during the last day of an 8 to 16 LTSA immediately followed by an 8 to 20 STSA. **Right**, Gains during an 8 to 16 STSA. Gray curves are least square fits of increasing exponentials to saccade gains. Numbers at the top of each panel show the percentage increase in the curve between the beginning and end of the panel. Inserts on the top of each panel illustrate target movement. Numbers at the top of each pane show the average of the 50 trials at the start or end of that epoch.

LTSA cause similarly large, long-lasting gain changes but size-increasing LTSA is slower than size-decreasing LTSA in five out of six experiments and produces larger changes in saccade size. These differences are consistent with, though do not prove, the proposal that size-increasing and size-decreasing LTSA are distinct mechanisms.

If so, then LTSA is similar to STSA and the vestibulo-ocular reflex (VOR) in consisting of at least two different mechanisms. Kojima et al. (2004)[39] argue, based on consecutive size-decreasing and size-increasing STSAs, that size-increasing and size-decreasing STSA rely on distinct mechanisms. Our data support this idea by documenting another difference. A single size-decreasing STSA impairs further gain reductions while a single size-increasing STSA does not impair subsequent size increases. Others have documented a similar dichotomy for gain-up and gain-down VOR adaptation. Boyden et al. (2003)[6] showed different time courses for adapted increases and decreases in VOR gain. They also showed differences in the persistence of the adaptations: recovering from gain-down VOR adaptation with gain-up adaptation was not as successful as the reverse condition. Also, Kimpo et al. (2005)[38] showed that adapted decreases in VOR gain generalized across different frequencies of rotation more than increases did. Together, these findings indicate that the brain uses different mechanisms to increase and decrease movements in several distinct oculomotor behaviors.

We argue[69] that size-decreasing STSA and LTSA are distinct from one another. This is based on our observation that size-decreasing STSA is impaired after previous STSA but not after previous LTSA. We propose that STSA makes a rapid, but temporary, correction to saccade hypermetria, analogous to first aid, while LTSA makes a slower and longer-lasting correction, analogous to a cure. Such an arrangement would provide both rapid and persistent correction for inaccuracy.

Our current observations indicate that we cannot make the same arguments about size-increasing STSA and LTSA. Size-increasing STSA proceeds normally after either STSA or LTSA. Thus, there is no indication that size-increasing STSA and LTSA are distinct. They may rely on the same slow, but very capable mechanism to increase saccade size. If so, then the difference between size-increasing STSA and LTSA is only in how long that common mechanism is engaged by adapting target movements.

Our and previous [69] data show that animals recover from LTSA much more quickly than they adapt. This indicates that LTSA in either direction does not change a pre-configured adaptation state to which the brain defaults more quickly than it adapts. (Note that there remains the logical possibility that by adapting to a single size, we are not engaging the default state.) This putative default state, setting gains at 1, may be plastic given a long enough exposure to adapting stimuli. No current data indicates whether or not it is possible to permanently reset the default state.

Chapter 4

**PERINEURONAL NETS IN THE SACCADRE-RELATED PART OF
THE CEREBELLAR FASTIGIAL NUCLEUS DO NOT MAINTAIN
SACCADRE GAIN**

4.1 Summary

Perineuronal nets (PNNs) accumulate around neurons near the end of developmental critical periods. They may inhibit synaptic plasticity and thereby help end critical periods. PNNs surround a high proportion of neurons in the cerebellar nuclei. These PNNs form during approximately the same time that movements achieve normal accuracy. It is possible that PNNs in the cerebellar nuclei inhibit plasticity to maintain the synaptic organization that produces that accuracy.

We tested whether or not PNNs in a saccade-related part of the cerebellar nuclei maintain accurate saccade size by dissolving them in an adult monkey performing a task that changes saccade size (long term saccade adaptation). We show that dissolving PNNs in this area does not result in faster, larger, or more persistent adaptation. This suggests that perineuronal nets around CFN neurons are not important for maintaining long term saccade gain.

4.2 Introduction

Perineuronal nets (PNNs) form around neurons near the end of developmental critical periods. Critical periods are specific time periods early in life during which the brain is very plastic and experience shapes synaptic organization. Previous reports show that the appearance of PNNs correlates with the end of the critical period. The formation of PNNs may contribute to ending the plasticity of critical periods by inhibiting plasticity. Two recent studies reestablish critical period-like high levels of plasticity by dissolving perineuronal nets in adult rat[62][27].

PNNs surround a higher percentage of neurons in the cerebellar nuclei (93% in rhesus monkey, unpublished data) than in any other part of the brain (e.g. <10% in cerebral

cortex, unpublished data). The high prevalence of PNNs in the cerebellar nuclei raises the possibility that PNNs strongly inhibit plasticity in the cerebellar nuclei. If so, then we expect that if we reinstate critical-period-like plasticity by dissolving PNNs, experience will affect movement because the cerebellar nuclei influence movement. The activity of many of neurons in the cerebellar nuclei correlates with movement, and damage to the nuclei impairs it. Cerebellar nuclear damage can also abolish motor learning that normally compensates for damaged movements, improving their accuracy.

Perineuronal nets form around neurons in the cerebellar nuclei during the first three months of development[15]. A particular part of the cerebellar nuclei, the caudal part of the medial or fastigial nucleus, or CFN, strongly influences saccades[58][61][21][67][26]. During the period prior to the formation of PNNs, rapid eye movements, saccades, in young monkeys become progressively less variable and more accurate (Phillips et al, personal communication). It is plausible that during this same period the visual experience of inaccurate saccades shapes the synaptic organization in the CFN to make saccades less variable and more accurate. In this view, once the brain achieves the organization appropriate to producing accurate saccades, PNNs form around CFN neurons to stabilize the appropriate connections.

In the work described here we sought to implicate PNNs in the CFN in saccade plasticity. To do this we dissolved PNNs in a monkey's CFN by injecting the enzyme chABC into the CFN. chABC dissolves PNNs[62]. After we injected chABC we provided the monkey with visual experience indicating that its saccades were consistently too small. We provided this experience several hours/day for consecutive days. This is a long-term saccade adaptation protocol, described previously[69][55], which causes slow accumulative changes in saccade size over 20-30 days of experience. We compared the size and rate of the change in saccade size when PNNs in the monkey's CFN were intact and after they were dissolved.

In a control experiment, we injected chABC into regions of this monkey's cerebellar nuclei outside the fastigial nucleus and later stained the cerebellum for PNNs. We used this histology to estimate the size of the area within which our injections into the CFN dissolved PNNs. This confirmed that our injections into the CFN would have dissolved PNNs within this entire area. We made these control injections at different times before sacrificing this

animal allowing us to also estimate how quickly PNNs reformed after they were dissolved.

4.3 Methods

4.3.1 Animal Preparation

We implanted one male rhesus macaque (Monkey S, age 9 years) with scleral search coils around both eyes using established methods[20][36] as well as acrylic head-stabilization lugs. In a second surgery we also implanted a recording chamber centered on the midline aimed ventrally 8 mm posterior to ear-bar zero. This put the center of the chamber directly dorsal to the midline between the left and right CFNs. All of our procedures were approved by the University of Washington Animal Care Committee and conformed to the recommendations from the Institute of Laboratory Animal Resources and the American Association for Accreditation of Laboratory Animal Care.

4.3.2 Long-Term Saccade Adaptation

We trained the animal to make saccades for apple-sauce reward. After the monkey became proficient at this task, it underwent two consecutive long-term adaptation and recovery experiments separated by two weeks. During the first experiment the PNNs in the monkey's CFNs were intact. At the beginning of the second experiment, we injected chABC bilaterally into both CFNs to dissolve PNNs.

4.3.3 Eliciting and Measuring Long-Term Adaptation

To elicit long-term saccade adaptation we used a technique modified from McLaughlin' [50] method for adapting saccade size. We trained the monkey to make saccades to track the horizontal movement of a small (0.3°) target spot. During each saccade, the target moved again farther from its starting point. Thus, at the end of each saccade the eye seemed to have fallen short of its target. When a monkey makes repeated saccades to targets that moved like this, the size of its saccades gradually increases[75]. When a monkey tracks such target movements for only a few hours, the elicited change in saccade size fades quickly when the monkey makes saccades to track normal targets, i.e., ones that do not move during saccades,

or even when the monkey makes no visually guided saccades[72] In contrast, if we repeat this adaptation procedure for multiple days, blindfolding the monkey with goggles between its daily adaptation sessions, then saccade size increases day to day[55]. The goggles prevent visually guided saccades outside the booth. The only saccades that the monkey makes are to targets that move during the saccade, so the monkey retains some of the change from previous training. Changes in saccade size elicited by this long-term procedure persist longer than do changes caused by a single day’s adaptation [69][55].

We used the long-term saccade adaptation procedure to adapt Monkey S to make 16° saccades when presented with 8° targets. We measured saccade accuracy by calculating the gain of each saccade. Gain is saccade amplitude divided by initial target amplitude. Thus, for our experiment the gain of its saccades went from ~ 1 to ~ 2 . We adapted Monkey S’ saccades to both the left and the right but, for simplicity, we show only the results of leftward adaptation experiments in this article. The results for rightward adaptation were very similar.

We measured the progress of adaptation by calculating daily gain change. This is the average gain of the last 30 saccades in a daily adaptation session minus the average gain of the first 30 trials of that session. Figure 4.1 shows typical data from one daily adaptation session and how we calculated the daily gain difference. We can then fit an exponential curve of the formula $Fit(g) = b \pm A \times e^{\frac{-n}{\tau}}$ to the daily difference in gain values. The time constant, τ , of this exponential curve is an estimate of the rate of gain change during adaptation or recovery from adaptation. The other variables represent the differences in gain (g), the bias (b) and the number of days the adaptation or recovery took (n).

Long-term adaptation training continued until the average gains of the first 30 trials and the last 30 trials in a session were significantly the same in at least one direction. We use the TOST equivalence test to determine whether the average start gain and average end gain were significantly the same[71]. We then continued to present the animal with adapting target steps for a further two weeks. This allowed time for the perineuronal nets that we dissolved in the second experiment to re-form.

After these two weeks, we again presented normal 8° target movements (i.e., no movement during saccades) to the animal during its daily adaptation sessions while still blind-

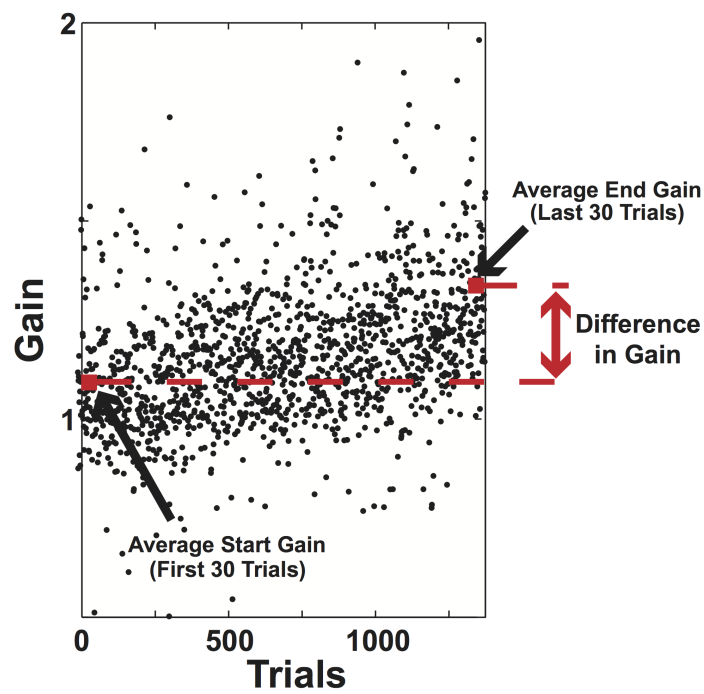


Figure 4.1: Difference in Gain. The difference in gain is calculated by subtracting the average start gain of the first 30 trials from the average end gain of the last 30 trials.

folding it between sessions. We call this procedure Recovery. It elicited a gradual decrease in saccade gain toward the normal value of 1. Recovery sessions continued until the averages of the first and last 30 saccades in a session were significantly the same in at least one direction.

4.3.4 Injection Procedure

We used expoxylite and polyamide-coated extracellular tungsten electrodes with impedances of $\sim 300\text{k}\Omega$ to record from neurons in Monkey S' cerebellar nuclei. We introduced the electrodes into the cerebellum through a guide cannula that ended above the cerebellar nuclei. We identified the CFN by the characteristic burst of action potentials that neurons there exhibit for every saccade. Once we located the CFN on both sides of the cerebellum we drove an injection pipette into this region bilaterally and injected between 1 and $1.6\text{ }\mu\text{l}$ of a solution of (60U/ml) chondroitinase ABC (100330 from Seigaku Corporation) dissolved in 0.9% serological saline. We made bilateral injections on days 1, 4 and 7 of the second long-term adaptation experiment. The pipette consisted of an approximately $200\mu\text{m}$ wide stainless steel hypodermic tube fed through the same guide cannula that we used for the extracellular electrodes.

4.3.5 Saccade Comparisons

In addition to measuring adaptation, we also compared the properties of saccades made both when CFN PNNs were dissolved and intact. To do this, we calculated the saccade velocity and acceleration: the first and second derivatives of the saccade position trace with respect to time. We measured the peak velocity, peak deceleration, and deceleration duration of each saccade in our sample, described below.

To compare the values of these attributes with PNNs intact and dissolved, we performed two-tailed T-tests with Bonferroni correction and considered p-values less than 0.05 indicative of significant differences. We compared samples of saccades that were in the same direction, within 0.1° of a given amplitude and within 1° of given starting position. We also removed the saccades within the lowest 20% of the velocity range of a set that did not

match typical velocity or acceleration profiles.

4.3.6 Histology

To confirm that our chABC injections dissolved perineuronal nets in the primate cerebellar nuclei, we made injections into four sites in the cerebellar nuclei besides the CFNs. At each of these extra-CFN sites we injected the same volume of chABC solution with the same three-injection time schedule that we used for the CFN injections. These additional sites were in the interpositus and dentate nuclei. We injected at each of these additional sites starting at a different times (28, 21, 14, and 7 days) before we sacrificed the monkey. We made two electrolytic lesions at each injection site using a current of 30A for 30 seconds, electrode negative. At each site, we made two marking lesions 1mm below the injection site on the first day of injection and 1mm above the site on the last day of injection.

We sacrificed the animal and perfused it through the heart with 4% para-formaldehyde followed by 10, 20, and 30% solutions of sucrose in phosphate buffer. We used a cryostat to cut $25\mu\text{m}$ frozen sections of the cerebellar nuclei and divided our sections into 4 sets. Thus consecutive sections within one set were $100\mu\text{m}$ apart.

To verify the location of our marking lesions we stained one set of sections for Nissl. We mounted sections on charged slides, dehydrated them with increasing concentrations of ethanol, stained them with toluidine blue pH at 4.1 for ~ 45 seconds, and then rehydrated them. We then dried sections on the slides and cover-slipped them.

We stained another set for perineuronal nets with wisteria floribunda agglutinin (WFA) conjugated with fluorescein (1:500, Vector Labs FL-1351) using standard immunohistochemical techniques[11]. WFA is a lectin that recognizes the sugar-protein moieties of chondroitin sulphate proteoglycans, the main component of perineuronal nets. We also stained this set with avidin conjugated with Texas Red (1:500, Millipore Corp MAB377) to mark cerebellar nuclear neurons. This is not a standard neuronal marker, but others have shown that it labels cerebellar nuclear neurons when other more traditional stains do not[49]. To evaluate PNNs in the vicinity of our chABC injections we made fluorescent images with Zeiss Axioskop 2 confocal microscope using LSM 5 Pascal software.

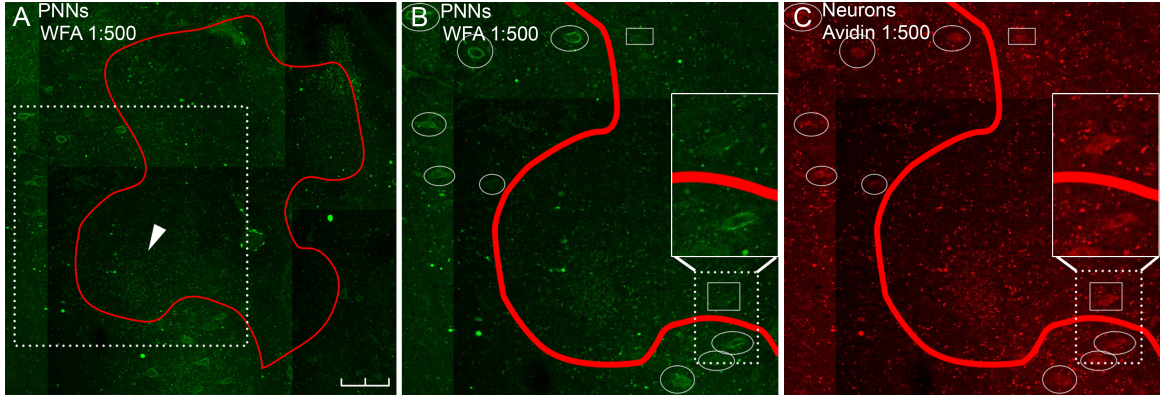


Figure 4.2: Identification of spread of chABC. **A:** The extent of one lesion site is traced in red. PNNs are labeled with WFA (green). The white box indicates the region magnified in panels B and C. Scale bar = $200\mu\text{m}$. We frequently see nonspecific, punctate autofluorescence (arrowhead). This can be extremely bright near the site of an electrolytic lesion. This is not indicative of PNN presence or absence because the stain does not form net-like circles around neurons. **B:** Magnification of square region in A, WFA-stain for PNNs. **C:** Magnification of square region in A, Avidin-stain for cerebellar nuclear neurons[49]. In both B and C, white circles surround neurons with perineuronal nets and small white squares surround neurons without nets. Insets: magnified view of two neurons. The upper neuron does not have a PNN and is therefore included in the injection site. The lower neuron does have a net and is therefore excluded from the injection site.

We estimated the borders of the region within which chABC dissolved PNNs, by identifying the center of the injection sites relative to our marking lesions in the Nissl-stained set and then staining for PNNs and neurons as described above. We traced a border by working outward from the putative injection site until we found neurons surrounded by perineuronal nets (Fig. 4.2). In some areas we could not verify that cells were present but did not have PNNs, because of tissue damage caused by the lesion (Fig. 4.2A, arrowhead). In these situations we extrapolated our estimate based on the information at nearby locations. The evaluation of the enzyme spread was done blind. Independent individuals estimated the border location without knowing which injection they were examining. We placed the border as the consensus of these several estimates. Estimates from different people were similar.

4.4 Results

Figure 4.3 shows the gains of leftward saccades during 35 days of long-term adaptation both when PNNs were intact (4.3A) and when we injected chABC bilaterally into the CFN on days 1, 3 and 7 of the long-term adaptation (4.3B). On day 1 we injected $1.6\mu\text{l}$ into the left and $1.0\mu\text{l}$ into the right CFN. On day 3 we injected $1.4\mu\text{l}$ into the left and $1.6\mu\text{l}$ into the right CFN. On day 7 we injected $1.4\mu\text{l}$ into the left and $1.6\mu\text{l}$ into the right CFN. There is no obvious difference in either the speed or magnitude of adaptation in control and injected conditions.

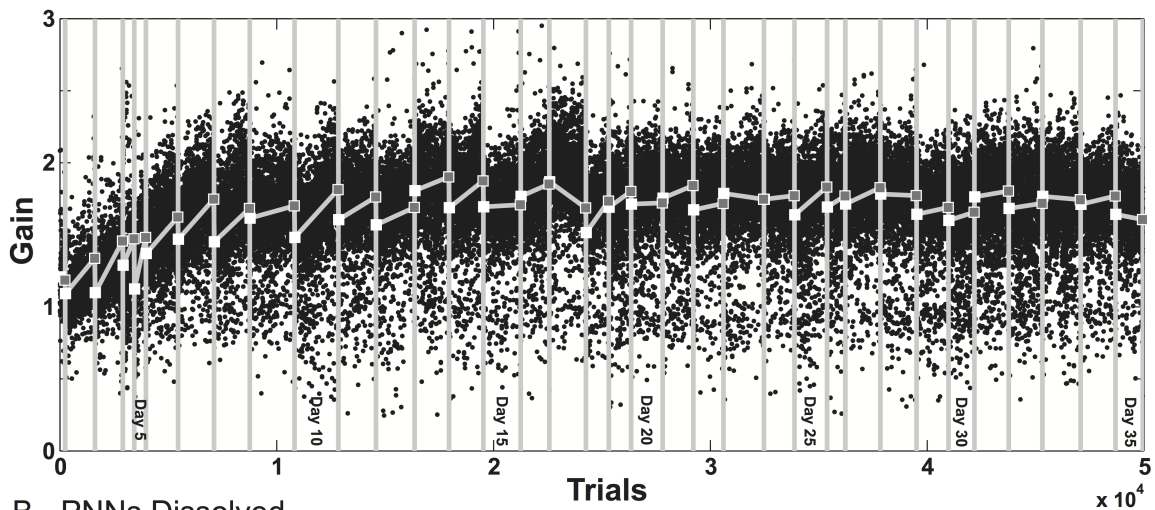
Figure 4.4A shows saccades gains during recovery when CFN PNNs were intact and 4.4B shows gains during recovery after we dissolved PNNs. Like adaptation, recovery progressed at approximately the same rate in both conditions. This shows that saccades with an acquired gain near 2 are not more persistent than normal after we dissolved CFN PNNs and then let them re-form.

Before this experiment we expected that dissolving perineuronal nets in the CFN during long term adaptation would result in either a larger change in gain, a faster rate of adaptation, or a more persistent change in gain. The size of adaptation was very similar whether PNNs were intact or dissolved (average adapted gain of 1.64 ± 0.25 when PNN were intact and 1.62 ± 0.22 when they were dissolved). These values are not significantly different ($p = 0.35$). A larger change in gain would manifest as the animal achieving a gain closer to the target gain of 2.0.

To measure the effect of dissolving perineuronal nets in the CFN on the rate and persistence of long-term saccade adaptation, we compared daily gain change during adaptation with PNNs intact and with PNNs dissolved (Fig. 4.5). We fit an exponential curve to these values for each condition. The time constant of the fit exponential represents the rate of gain change. As Figure 4.5A shows, the rate of change in both conditions varies considerably day to day and was similar in both conditions (time constants of -29.07 and -44.44 days respectively). The animal did not adapt more quickly (have a smaller time constant) when PNNs in the CFN were dissolved.

We anticipated that as PNNs grew back in around CFN neurons during adaptation,

A - PNNs Intact



B - PNNs Dissolved

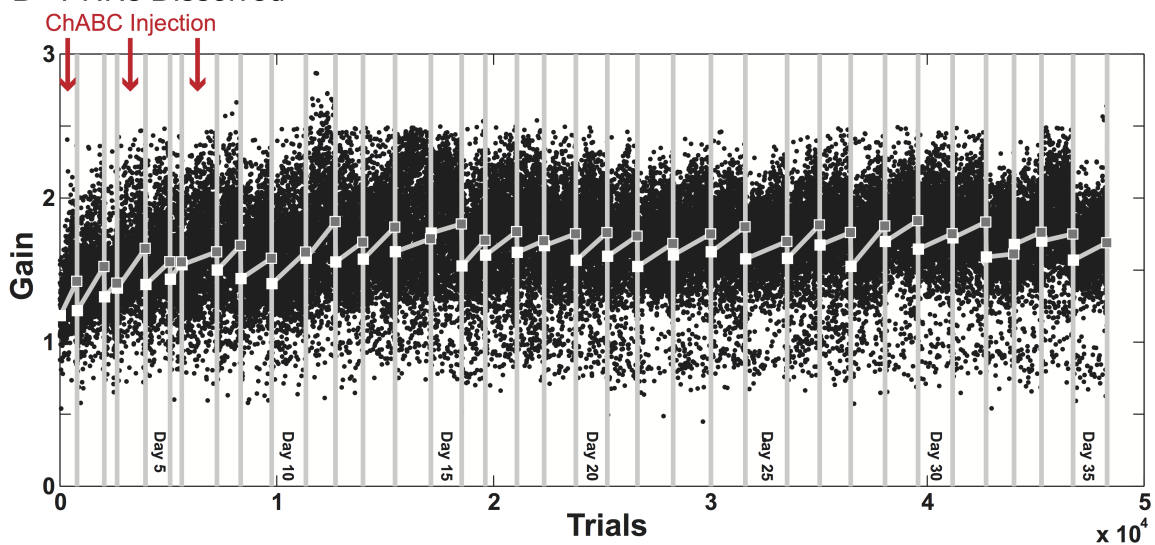


Figure 4.3: Long term saccade adaptation in Monkey S. **A**: Long term adaptation of saccade gain with PNNs intact. **B**: Long term adaptation of saccade gain with CFN PNNs dissolved. ChABC injections occurred on days 1, 4 and 7 of the experiment, red arrows. White squares indicate the average start gain (first 30 trials of a session) of each day's adaptation. Gray squares indicate the average end gain (last 30 trials of a session).

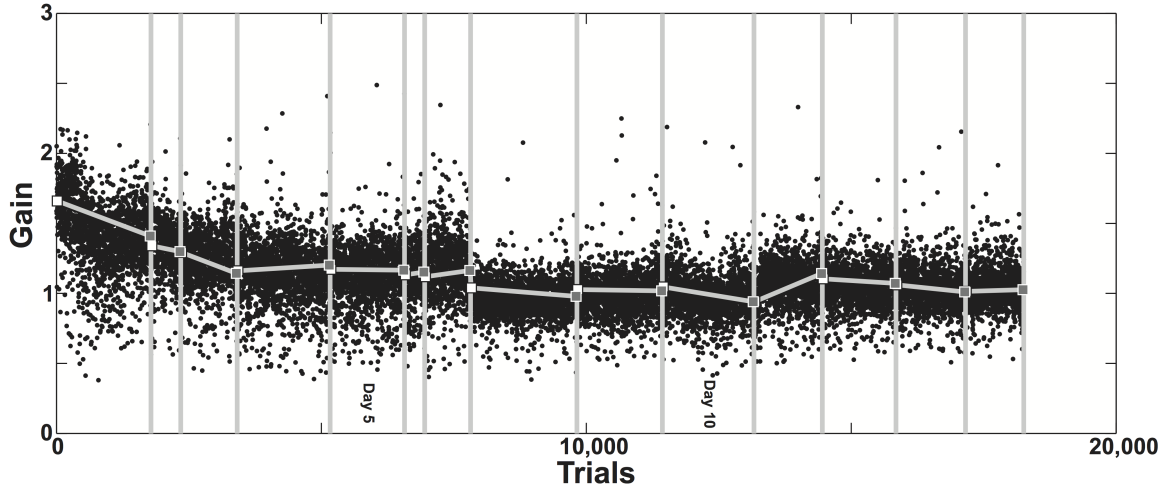
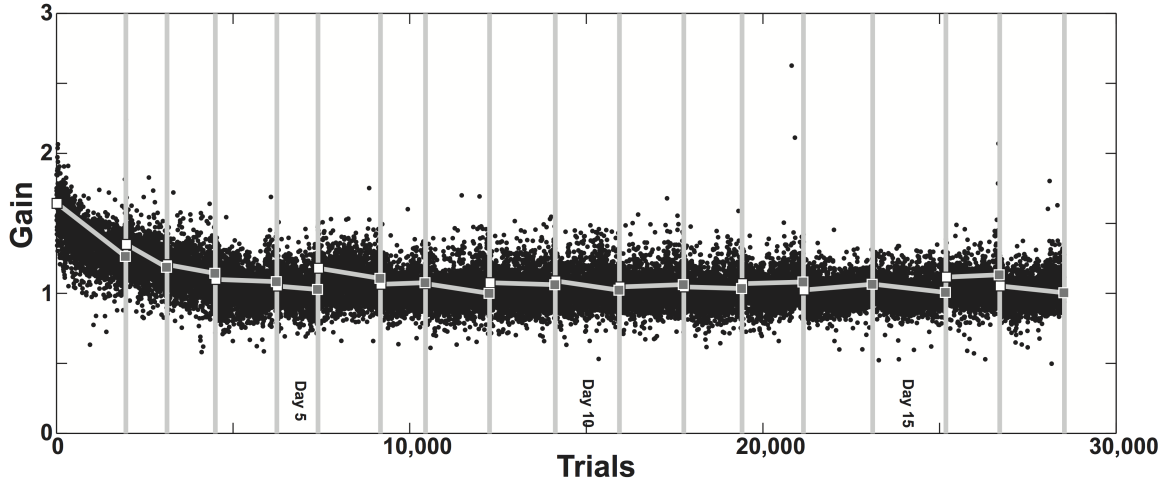
A - PNNs Intact**B - PNNs Dissolved**

Figure 4.4: Recovery of long term adaptation. **A**: Monkey S? gain throughout recovery from long term adaptation with PNNs intact. **B**: Monkey S?s gain throughout recovery from long term adaptation with PNNs dissolved. White squares indicate the average start gain (first 30 trials of a session), gray squares indicate the average end gain (last 30 trials of a session).

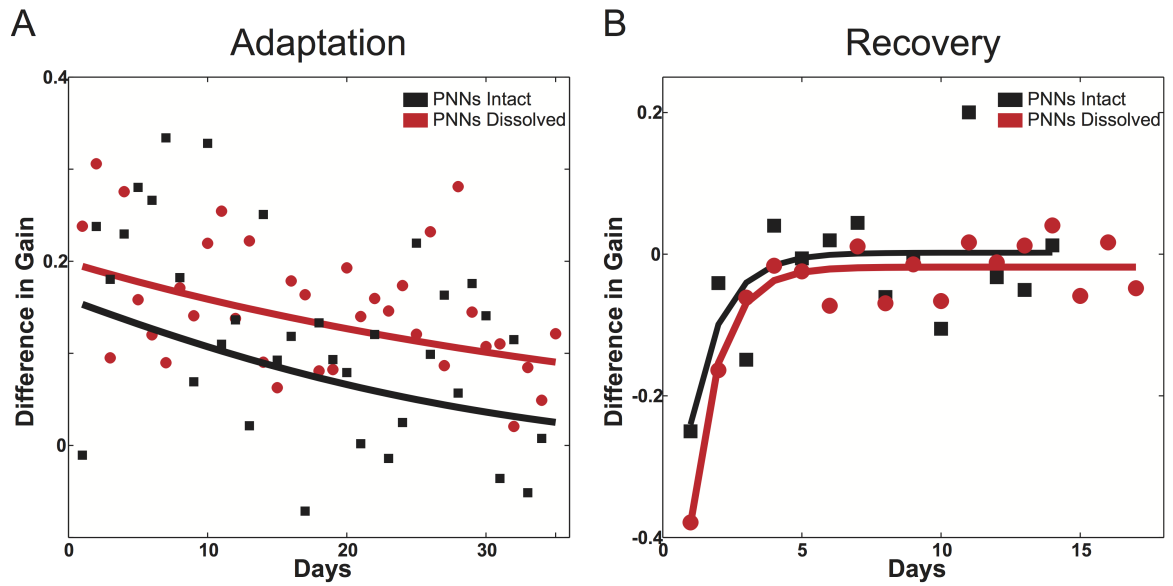


Figure 4.5: Rate of adaptation with PNNs intact and dissolved. **A**: Difference in gain for each day of long term adaptation and consolidation in both control (PNNs intact, black) and chABC (PNNs dissolved, red) conditions. An exponential was fit to both sets of data. **B**: Difference in gain for each day of recovery in both control (PNNs intact, black) and chABC (PNNs dissolved, red) conditions.

synapses appropriate to the new gain level are being maintained. If this were the case, then we should see a slower rate of recovery (return toward gain of 1.0) after we injected chABC to dissolve PNNs. Figure 4.5B shows that adapted gain recovered at very similar rates whether PNNs in the CFN were intact or dissolved and re-formed (time constants of 1.15 and 1.02 days respectively).

4.4.1 Dissolving PNNs did not impair saccades

PNNs surround a large proportion of neurons in the cerebellar nuclei. Without more information about PNN function, it is possible that dissolving PNNs in the CFN could impair CFN function. To assess how well the CFN functions with its PNNs dissolved we examined saccades at three times during each experiment, 1) before adaptation, 2) during days 10-14 of long-term adaptation, and 3) during days 10-12 of recovery. We limited our examination to saccades within a very narrow size range as described in Methods.

We found that, as Figure 4.6A and Tables 4.1 and 4.2 show, both leftward and rightward 8° saccades exhibited significantly lower peak velocities before the long-term adaptation in which we injected chABC than before the control adaptation. Saccades before the chABC adaptation also had significantly lower peak decelerations and longer deceleration durations. These changes are characteristic of impaired CFN function ([26], [9]). Given that we drove electrode tracts through the vicinity of the CFN before the chABC long-term adaptation, we believe that the saccade abnormalities we observed before the chABC adaptation were a result of mild damage to the CFN caused by our electrode penetrations.

To evaluate saccades during adaptation we examined saccades that the monkey made during days 10 to 14 of both the control and chABC adaptations and days 10 to 12 of both recoveries.

We found that saccades made after we injected chABC did not exhibit significantly lower peak velocities, lower deceleration rates, or longer decelerations than compared to control saccades, (Fig. 4.6B, Table 4.1, 4.2). Likewise, saccades during recovery after we injected chABC were not significantly different from control saccades during control recovery. (Fig 4.6C, Table 4.1, 4.2). Thus the only saccades that showed signs of CFN dysfunction were

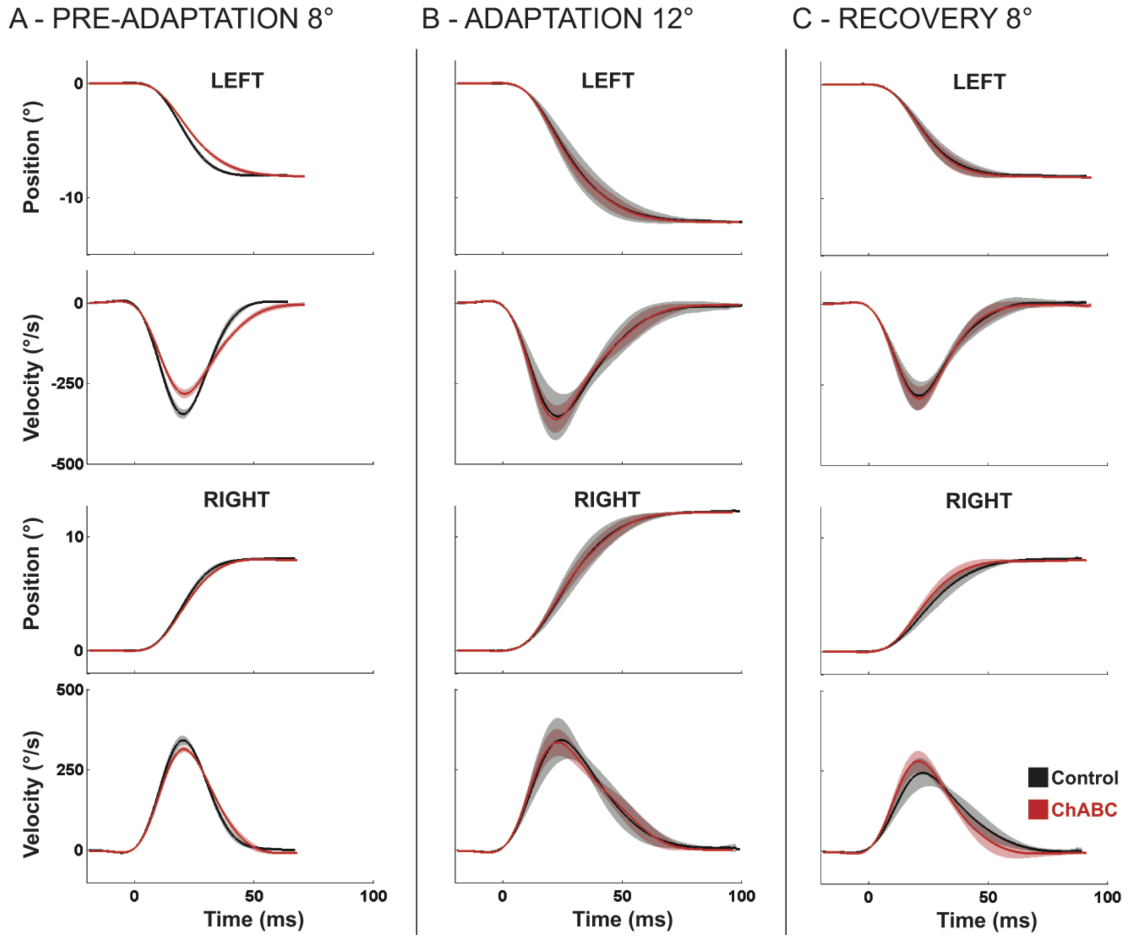


Figure 4.6: Comparison of control and ChABC saccades. **A:** Pre-Adaptation comparison of position and velocity profiles of leftward (top) and rightward (bottom) 8° saccades for control (black) and chABC injection (red) experiments. Traces are averages (line) of several saccades with shading for standard deviation. **B:** Same as A, for 12° saccades pooled across days 10-14 of long term adaptation. **C:** Same as A, for 8° pooled across days 10-12 of recovery.

Pre-adaptation		Peak Vel. ($^{\circ}/s$)	Peak Dec. ($^{\circ}/s^2$)	Dec. Dur. (ms)
Control	Mean	-345.13	18830.77	24.71
	SD	15.10	1878.56	1.25
	N	7	7	7
ChABC	Mean	-282.93	11873.75	36.17
	SD	14.50	1411.85	6.74
	N	6	6	6
T-Test *B		$*7.30 \times 10^{-5}$	$*6.81 \times 10^{-5}$	*0.05
Adaptation				
Control	Mean	-361.17	12841.84	50.00
	SD	60.23	5286.12	71.31
	N	106	106	106
ChABC	Mean	-361.27	12165.39	45.73
	SD	41.22	3042.96	6.59
	N	67	67	67
T-Test *B		5.94	1.72	3.25
Recovery				
Control	Mean	-287.76	12789.19	33.27
	SD	42.15	3913.89	7.53
	N	114	114	114
ChABC	Mean	-294.26	13292.35	31.99
	SD	35.08	3537.12	7.56
	N	195	195	195
T-Test *B		1.00	1.21	1.26

Table 4.1: Comparison of control and ChABC saccade values for leftward eye movements. Peak velocity (Peak Vel.), peak deceleration (Peak Dec.) and deceleration duration (Dec. Dur.). Bonferroni corrected T-Test p-values (T-Test *B) can be greater than 1, but < 0.05 are considered significant, and demarcated with *.

Pre-adaptation		Peak Vel. ($^{\circ}/s$)	Peak Dec. ($^{\circ}/s^2$)	Dec. Dur. (ms)
Control	Mean	342.05	-18829.22	25.92
	SD	14.69	1740.71	1.68
	N	12	12	12
ChABC	Mean	291.97	-13958.45	25.64
	SD	84.50	4206.47	7.72
	N	14	14	14
T-Test *B		$*1.55 \times 10^{-4}$	$*2.99 \times 10^{-5}$	0.29
Adaptation				
Control	Mean	353.95	-13310.55	44.31
	SD	55.15	4014.68	5.03
	N	64	64	64
ChABC	Mean	336.65	-10414.35	44.81
	SD	40.99	2863.70	5.83
	N	74	74	74
T-Test *B		0.25	$*2.85 \times 10^{-5}$	3.54
Recovery				
Control	Mean	251.61	-9717.01	38.15
	SD	35.44	3069.82	4.07
	N	54	54	54
ChABC	Mean	280.84	-11783.73	33.26
	SD	32.36	2929.04	7.29
	N	172	172	172
T-Test *B		$*3.83 \times 10^{-6}$	$*2.14 \times 10^{-4}$	$*2.33 \times 10^{-8}$

Table 4.2: Comparison of control and ChABC saccade values for rightward eye movements. Peak velocity (Peak Vel.), peak deceleration (Peak Dec.) and deceleration duration (Dec. Dur.). Bonferroni corrected T-Test p-values (T-Test *B) can be greater than 1, but < 0.05 are considered significant, and demarcated with *.

Location	Days before Sacrifice	Volume of 60U/ml ChABC (μ l)
Left Dentate	28	1.2
	25	1.2
	22	1.2
Right Dentate	21	1.32
	18	1
	15	1.2
Left Interpositus	14	1.2
	11	1.2
	8	1.3
Right Dentate	7	1.2
	4	1.26
	1	1.2

Table 4.3: ChABC injection schedule, volumes and locations.

before we injected chABC into the CFN. We conclude that dissolving PNNs did not impair PNN function as reflected in saccades.

4.4.2 *ChABC degrades perineuronal nets in macaque cerebellar nuclei*

Table 4.3 shows the size of the injections into the four sites outside the CFN. We found that our chABC injections completely dissolved perineuronal nets 7 days after our injection series started. PNNs were still absent at 14 days. By 21 days they had started to reform and by 28 days they were present in areas very close to or within an injection site(Fig 4.7). We conclude that PNNs in the CFN were absent during a significant proportion of long-term adaptation and that they re-formed well before the end of adaptation.

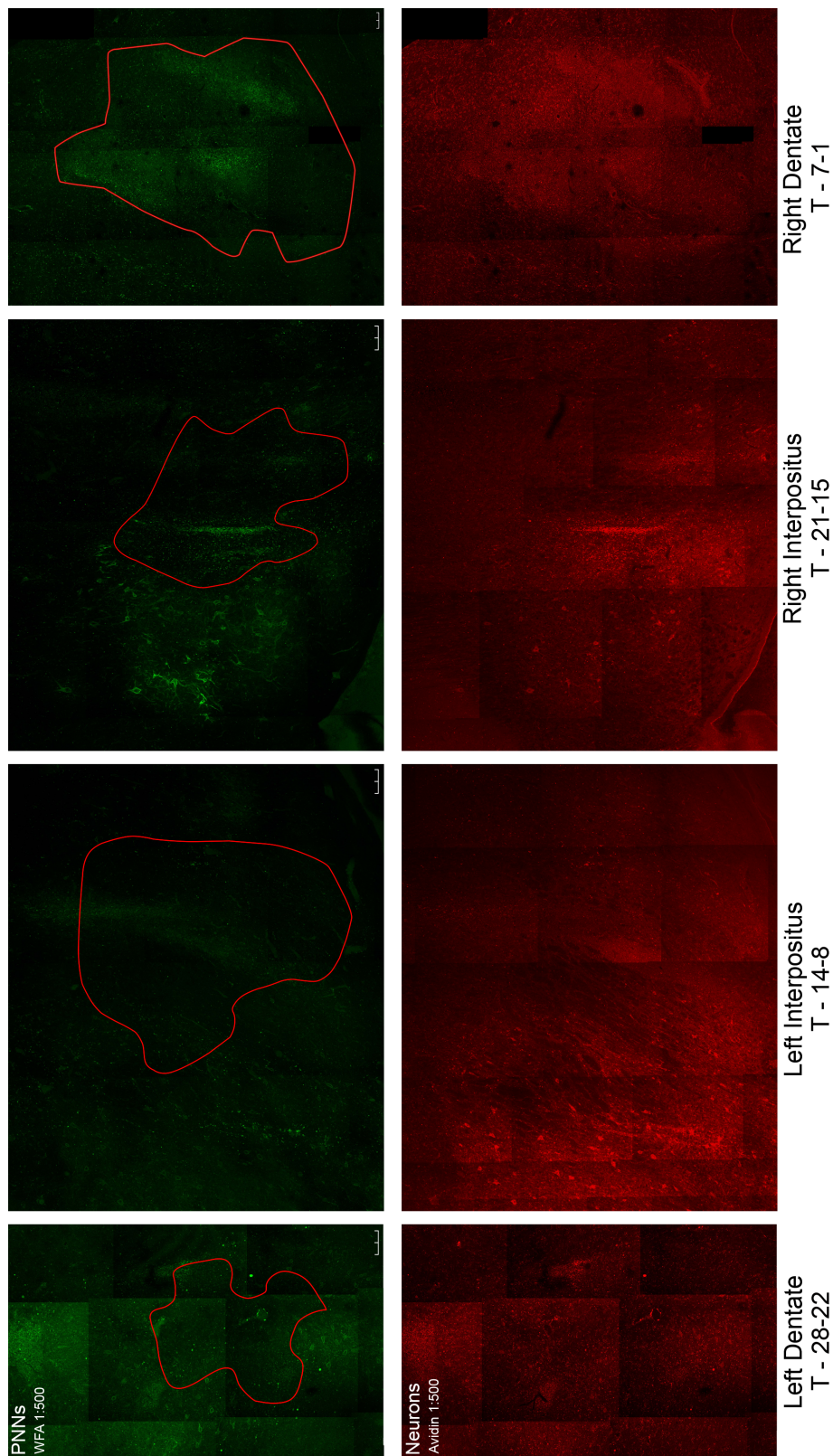


Figure 4.7: Degradation and reformation of PNNs in the cerebellar nuclei. Top panels show staining of four different areas of the cerebellar nuclei for perineuronal nets with WFA-Fluorescein. The proposed maximal extent of the spread of the enzyme is indicated in red. The time of the injections before sacrifice of the animal is noted below each column in days. Bottom panels shows the same areas stained with avidin to indicate locations of cerebellar nuclear neurons. All scale bars = $200\mu\text{m}$. Note that the right dentate is at a different scale to accommodate the entire injection. Small regions for which we lacked a photo are filled in with black.

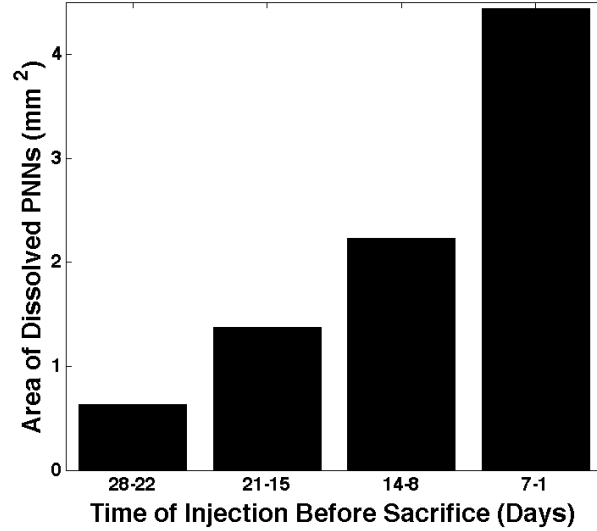


Figure 4.8: Estimate of spread of chABC. Calculated area (in mm²) without PNNs from figure 4.7 for each injection site. Scale bar = 200 μ m.

We measured the distance from the presumed center of an injection site within which chABC dissolved PNNs (Fig 4.8, Table 4.4). There was no evidence that any PNNs were present in the right dentate one day after a series of three injections. The enzyme dissolved PNNs over an area of approximately 4.4mm² and within 1mm horizontal distance of the presumed center of the injection site (i.e. $\sim$ 2mm total media-lateral spread). Each CFN is approximately 1.5mm in diameter, therefore we can assume that we dissolved perineuronal nets throughout the majority of the CFN.

4.5 Discussion

This study shows that dissolving perineuronal nets in the CFN does not affect the rate, size, or persistence of long term saccade adaptation and does not impair CFN function as reflected in saccade attributes. Our control injections outside the CFN show that we dissolved perineuronal nets in the CFN and that the nets had sufficient time to reassemble during the two week consolidation period of the long term adaptation. Therefore, we conclude that perineuronal nets around CFN neurons are not important for setting saccade gain.

Location	Area of Spread (mm^2)	Max. Horiz. Spread (mm)	Max. Vert. Spread (mm)
Left Dentate (T 28-22)	0.63	0.78	0.97
Right Interpositus (T 21-15)	1.37	1.12	1.32
Left Interpositus (T 14-8)	2.23	1.53	1.79
Right Dentate (T 7-1)	4.44	2.14	3.02

Table 4.4: Extent of ChABC spread.

Perineuronal nets surround more neurons in the cerebellar nuclei than anywhere else in the brain. If PNNs in the CFN are not inhibiting plasticity of saccade size, then what could they be doing? Others have suggested that the role of perineuronal nets is not purely to inhibit plasticity. It is possible that they surround highly active cells (such as cerebellar nuclear neurons, which fire at a baseline rate of 50-100Hz) to provide a cation sink[30][34]. In this view the presence of PNNs is not tied directly to modifying the movement-related output of nuclear neurons, but is instead a result of their cellular physiology.

Still, our result stands in clear contrast to other studies in which dissolving perineuronal nets strongly increased plasticity and changed performance. Pizzorusso et al [62] showed that degradation of perineuronal nets in V1 of an adult rat restores critical period levels of plasticity. This effect was present even though only a small fraction of neurons in the visual cortex was surrounded by perineuronal nets (unpublished data) and thus likely to be affected. Gogolla et al's study [27] showed that dissolving PNNs in the amygdala allows extinction of the conditioned fear response in adult rats. This extinction is normally only possible in young rats. Like V1, the amygdala contains only a small proportion ($\sim 2\%$) of neurons surrounded by PNNs. One way to reconcile our data with these previous studies is to propose that in the CFN perineuronal nets are necessary, but not sufficient to end plasticity. Perhaps an additional factor contributes to inhibition of synaptic plasticity. Thus, unlike visual cortex, another component needs to be reversed in the CFN to restore plasticity. The removal of the PNNs alone is not enough.

Another possibility is that PNNs around CFN neurons mediate plasticity of some features of saccades but do not directly contribute to the change in gain during long term adaptation. We think this possibility is less likely because previous work shows that 1) CFN cell activity modulates with single session saccade adaptation [35] and 2) CFN inactivation with the GABA-agonist muscimol abolishes single session saccade adaptation[28].

There is some indication in our data that dissolving PNNs in the CFN changes saccade speed and deceleration. Saccades were slower before the chABC experiment than they were before the control experiment. This is likely due to mild CFN damage caused by our electrode penetrations. After chABC injections, saccade velocity profiles were no longer abnormal. They were very like control saccades. The improvement of saccades from mildly impaired before adaptation to normal during and after adaptation raises the possibility that dissolving PNNs in the CFN allowed synaptic reorganization that repaired the effects of the damage that we did with our electrode penetrations.

If electrode damage had been extensive enough to significantly impair CFN function, then our chABC injections would not have had a substrate on which to act. We do not think that this happened. If it had, then saccades would have been more impaired than they were and Monkey S would not have been able to adapt at all.

There are at least two alternative explanations for improvement in saccades during and after chABC injection. First, the improvements in saccade velocity and deceleration after injection might result from other compensatory mechanisms that operate with or without dissolving CFN PNNs. Second, it may be that, because Monkey S' saccades are so variable these results are an artifact of the small sample size of pre-adaptation saccades. Currently we can only say that our results do not eliminate the possibility that dissolving CFN PNNs allowed improvement of slightly impaired saccades.

It is interesting to note that during development PNNs accrue within a period of about 90 days, while in an adult monkey they grow back within three weeks. This means that production of PNN components is not restricted to a particular time period during development. This is consistent with previous studies that describe the reassembly of perineuronal nets in other areas of the brain in other animals[8][14][17]. There is no vertebrate homologue to the bacterial enzyme chondroitinase, however matrix metalloproteinases may be able to

break down CSPGs and allow remodelling of the PNNS in adult animals[82].

4.6 Conclusion

In summary, we show that dissolving perineuronal nets in the saccade-related part of the cerebellar nuclei, the CFN, does not significantly change the rate, size, or persistence of long-term saccade adaptation. Dissolving PNNs in the CFN or in other parts of the cerebellar nuclei had no noticeable effect on behavior. Apparently, these neurons function at least approximately normally for up to 21 days without PNNs. Perineuronal nets reform within 21 days of being degraded in the adult primate brain. We have yet to understand the role of the dense PNNs in the cerebellar nuclei.

Chapter 5

CONCLUSION**5.1 Summary**

These three studies describe our efforts to explore the role that perineuronal nets play in the cerebellar nuclei by studying CFN cells and long term adaptation of saccades.

In chapter one we described the distribution of perineuronal nets at 25 locations throughout the central nervous system and established that prevalence varied considerably. If there was any pattern, it was that PNNs are more prevalent in motor areas than elsewhere in the brain, and especially abundant in the cerebellum. Looking at co-labeling of cells with PNNs and parvalbumin showed that, although in the cerebral cortex PNNs often surround highly active GABAergic interneurons, the situation was not so clear in subcortical structures. We frequently found parvalbumin positive neurons that stained weakly for PNNs or not at all. These included Purkinje cells in the cerebellum that fire at rates between 50-100Hz. Therefore, being a highly active cell is also not a sufficient condition for having a perineuronal net. Our results suggest that PNNs may serve different purposes in different parts of the central nervous system.

In chapter two we studied long term adaptation to make saccades larger. Long term size-increasing adaptation resembles recovery from muscle weakness or injury more closely than other forms of adaptation. We found that monkeys increase the size of their eye movements much more with long term adaptation than with short term size-increasing adaptation. Also, we found that for size-increasing adaptation, the brain may employ the same (cerebellar) mechanism for both short term and long term learning. We also demonstrated, that animals need less time to recover to their original gain values than they need to adapt away. This suggests that there is a default gain level for eye movements, which could be set during development.

In chapter three, we combined insights from chapters one and two. We performed

an experiment to determine if perineuronal nets in the cerebellar nuclei are necessary to maintain baseline saccade gain. We used long term saccade adaptation to change an animal's saccade size and then attempted to 'fix' the saccade gain at the new value by dissolving perineuronal nets during the adaptation and allowing time for them to re-form. We did not observe any changes in the speed, magnitude or persistence of long term adaptation when PNNs in the saccade-related part of the cerebellar nuclei were dissolved compared with long term adaptation when PNNs were intact. We did find that saccades which had become slightly slower (probably due to mild CFN damage) sped up after PNNs were dissolved. They re-acquired velocities that matched control saccades. This suggested that PNNs in the CFN may be important for maintaining other aspects of saccades, such as speed. Since dissolving PNNs in the saccade-related part of the cerebellar nuclei did not produce critical period-like plasticity, our result indicates that absence of PNNs is not, by itself, enough to make this region of the cerebellar nuclei plastic.

5.2 *Future Directions*

From our first project, we can say that there is not a simple relationship between a region's function and PNN presence, or between a cell's level of activity and its presence. From our third study, we can say that it appears that PNNs are not necessarily important for inhibiting plasticity. Our results suggest that PNNs serve different functions in different areas of the brain.

Future investigation of the functions of PNNs in different parts of the nervous system are necessary. One such study would be to follow up on the possibility raised by our results and determine if dissolving PNNs speeds repair of saccades impaired by damage to the CFN or other saccade-related brain areas.

Another possibility is that baseline gain is partially set in the CFN, but plasticity needs to be restored at an additional site in the brain before behavioral changes are evident. The most likely second site is the saccade-related cerebellar cortex, the OMV, that has previously been shown to be important for adaptation[66][40]. Numerous studies have already shown that synapses in the cerebellar cortex, particularly parallel fiber - Purkinje cell synapses, undergo changes that are associated with learning. Although Purkinje cells themselves are

sparsely populated with PNNs, there are cells in the granule cell layer that are PNN-positive. The fact that dissolving PNNs in V1, where they are relatively rare, had a strong affect on behavior is, in this case, encouraging. We could simultaneously dissolve PNNs in the CFN and OMV and perform a similar long term saccade adaptation experiment. If dissolving PNNs in both these areas is sufficient to restore infant-like levels of plasticity to the saccade system, we should see a change in long term adaptation speed, magnitude or persistence.

A third possibility is that PNNs do not act to inhibit plasticity in the cerebellar nucleus at all. However, as chapter one describes, it is also not enough to simply say that PNNs surround highly active cells, like cerebellar nuclear neurons, for neuroprotection. If they are serving a combination of these roles for different areas, it would suggest that Purkinje cells are in a unique situation. In other areas, PNNs may either be important for suppressing plasticity or for neuroprotection. That Purkinje cells do not receive such neuroprotection, despite their high firing rates, suggests that the cost of having their synaptic connections stabilized is too high. It would be interesting to explore whether addition of PNNs to Purkinje cells affected an animal's motor learning ability. This could be accomplished with genetic tools that upregulate expression of PNN proteins in the cerebellar cortex.

Our research suggests that PNN functions could be highly varied and future studies will be very informative about the roles they play in the central nervous system.

Chapter 6

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Chapter 7

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