Face Preference and the Effect of Muscimol Inactivation of the Dentate Nucleus on Saccadic Eye Movements

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Abstract

The role of the dentate nucleus of the cerebellum in saccadic eye movements is poorly understood, as is the cerebellum's contribution to social cognition. Based on past data implicating a direct, bi-synaptic projection from the deep cerebellar nuclei to the supplementary eye fields, we have developed an image preference task in which the preference a non-human primate has for looking at images of other primates (both human and non-human) and saccadic eye movement metrics are evaluated. Using this task, the innate image preference and saccade metrics were measured, as were the changes to them after the dentate nucleus was temporarily inactivated via injection of the GABA agonist muscimol. The resultant changes to the preference of face images and the various saccade metrics were differential depending on which nucleus was injected.

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Chapter 1: Introduction

Social cognition is the neural activity and processes that underlie the behavior though which organisms interact with conspecifics. Naturally, the complexity of these neural processes increases dramatically whilst ascending the phylogenetic tree. At the apex of this are primates, who exhibit extremely complex behaviors which can often run counter to what would be seen as evolutionarily prudent. These can include pro-social behaviors such as altruism and cooperation, as well as antisocial behaviors, such as coercion and manipulation. Understanding the neural mechanisms underlying these complex behaviors not only furthers the basic understanding of how neural activity influences behavior, but also can elucidate the mechanisms of various neural and psychological disorders. One particularly strong behavior seen in primates is eye contact and facial preference. This basic behavior is omnipresent in primates and often perturbed in neural and psychological disorders. The precise neural substrate of this facial preference is unknown, but that a newly discovered anatomic pathway connecting structures involved in saccade generation (the deep cerebellar nuclei) to cortical areas known to modulate the more cognitive aspects of saccades (supplementary eye fields) allows an opportunity to explore this issue.

Here we have undertaken a series of experiments in a non-human primate in which we recorded from the deep cerebellar nuclei (DCN) while the subject performs our visual preference task. Our goal was to find task-related activity in the DCN. We also measure the effect of the inactivation of cerebellar nuclei on the task. We used targeted muscimol injections to inactivate the dentate, interpositus, and fastigial nuclei in separate experiments, and we saw the preference for face images decrease relative to saline injections and no injection controls. In the present study, we devised a task to test monkeys' preference strength for looking at images of faces when simultaneously presented with an alternate

The aim of this project is twofold: to pursue the motoric and cognitive contributions of the deep cerebellar nuclei to voluntary (saccadic) eye movements in non-human primates. We have done this through the use of a custom behavioral task that presents the monkeys with a simultaneous choice of targets and monitors the target choice and saccade direction. We subjected the non-human primate subjects to this task while we either recorded the neural activity in the deep cerebellar nuclei or inactivated those nuclei, and we examined the behavior—most importantly target choice—of the monkeys while undertaking the recording and inactivation.

Overview of cerebellar anatomy

The cerebellum is a unique structure in the brain. It has an extremely regular organization. The overall structure of the cerebellum can be divided into the cortex and the deep cerebellum. The cerebellar cortex is highly foliated and can be grossly subdivided into ten lobules, I-X, from anterior to posterior. The cortex has three distinct layers: the molecular layer (the outermost layer), the Purkinje cell layer, and the granular cell layer (innermost layer). Below the granular cell layer is white matter until the deep cerebellar nuclei. The principal cell type in the cortex is the Purkinje cell. The somas of Purkinje cells are located in the Purkinje cell layer (which can also contain candelabrum cells and Bergman glia), and their dendrites are in the moleclular layer. The dentritic arbor of each Purkinje is constrained to a single parasaggital plane, spreads widely within that plane. Purkinje cell axons project through the granular cell layer toward the deep cerebellar nuclei. Cell types that are present primarily in the molecular layer include: stellate and basket interneurons. Cell types that are present primarily in the granular layer include: granular cells, Golgi cells, Lugaro cells, and unipolar brush cells.

Purkinje cells receive their primary input from neurons in the inferior olive via the climbing fibers, which enter the cerebellum via the inferior cerebellar peduncle and terminate on the dendrites of the Purkinje cells in the molecular layer. Each Purkinje cell is innervated by a single climbing fiber, but a single climbing fiber can innervate many Purkinje cells. The cerebellar cortex is also primarily innervated by two other systems: parallel fibers, which originate from granular cells and extend into the molecular layer, and mossy fibers, which originate from several locations in the brain and spinal cord and terminate in the granule cell layer. Purkinje cell activity is modulated primarily by the climbing fiber system and parallel fiber system, and Purkinje cells send their projections to the deep cerebellar nuclei.

Projections from cerebellar nuclei eye movement-related cortical structures

Cell labeling and axon tracing experiments, undertaken primarily by the Strick group, have shown the deep cerebellar nuclei to send projections to multiple cortical areas. Of particular relevance are the projections from the ventral aspect of the dentate nucleus to Areas 46 and 9 in the prefrontal cortex via the thalamus (Middleton & Strick, 1994), which are regions associated with executive functions, such as motor planning and working memory. This is in contrast to projections for more dorsal neurons in the dentate to motor areas, such as primary motor cortex (M1) and ventral premotor area (PMv) (Strick, Dum, & Fiez, 2009). Similar experiments have also shown projections from the ventral dentate nucleus into the frontal eye fields (FEF) of macaques (Lynch et al., 1994), a region associated saccadic eye movement motor commands, but it has been noted that the region containing the dentate neurons that project to the FEF does not overlap with the region of the dentate that sends projections to prefrontal cortex (Strick, Dum, & Fiez, 2009).

Lu, et al. (Lu, Miyachi, & Takada, 2012) have shown through the use of transneuronal retrograde labeling with rabies virus that the deep cerebellar nuclei

send projections via the thalamus to the supplementary eye fields (SEF). In particular, ventral aspects of the medial portion of the dentate nucleus and the posterior interpositus nucleus send a multitude of projections to the SEF, whereas dorsal portions of those same nuclei send projections to M1. These findings were furthered in additional projection studies (unpublished data, shown below, **Fig. 1**) that showed strong labeling in the ventral dentate and posterior interpostitus, as well as a sizeable subset of cells in the caudal-most 1/3 of the dorsal dentate nucleus, after injection of a transneuronal retrograde virus into the SEF. This finding suggests that there may be two or more output channels from these nuclei: a dorsal motor channel and a ventral cognitive channel.

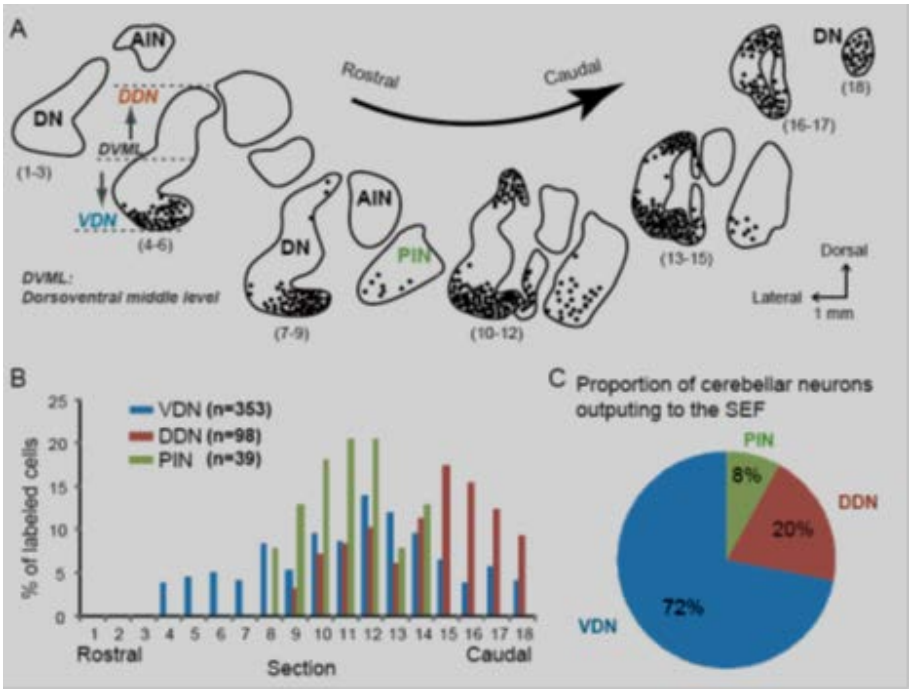

Figure 1: A. Example sections showing labeled neuron locations. **B.** Distribution of labeled cells across rostro-cadal axis of nuclei. **C.** Total percentages of labeled cell by nucleus subdivision. AIN: Anterior interpositus nucleus. DN: Dentate Nucleus. DDN: Dorsal aspect of dentate nucleus. PIN: Posterior interpostitus nucleus. SEF: Supplementary eye field. VDN: Ventral aspect of dentate nucleus.

The SEF is a region of the frontal lobe of the cerebellar cortex that exhibits activity related to the cognitive control of saccades. The SEF has been shown to have activity that responds to context-dependent visual stimuli. Lesions of the SEF do not affect the mechanics of saccade generation or execution, unlike lesions to the FEF. Additionally, functional imaging of autistic individuals during eye movement tasks show reduced activation of the Supplementary Eye Fields (SEF) (Takarae, Minshew, Luna, Krisky, & Sweeney, 2004). Two of the hallmarks of autism are a disinterest in social interaction and a reduction of eye contact. This reduction in SEF activity, coupled with the anatomical abnormalities in the cerebellums of autistic patients (described below), could be indicative of a loss-of-function phenotype for this neural pathway.

Contribution of the deep cerebellar nuclei to saccadic eye movements

There are 3 deep cerebellar nuclei. From medial to lateral, they are the fastigial, posterior and anterior interpositus, and dentate. They are bilateral and exhibit different functions. The majority of information known about the cerebellum's contribution to eye movements is based on studies of the medial posterior cerebellar cortex, the so-called oculomotor vermis (lobules VI and VII), and the caudal aspect of the fastigial nucleus to which it projects. A brief summary of the fastigial nucleus' involvement in saccadic eye movements is below, but this study will focus primarily on the interpostius and dentate nucleus, as there is very little known about those two nuclei and how they contribute to eye movements.

Much of the knowledge related to the deep cerebellar nuclei's contribution to eye movements has been gleaned from the behavioral deficits seen after focal lesion to the nuclei. Lesions of the fastigial nucleus have been shown to decrease saccade speed and accuracy and increase saccade size and speed variability. During unilateral lesion/inactivation, saccades toward the contraversive direction are smaller and ipsiversive saccades are larger (Robinson & Fuchs, 2001). Such results are seen in both induced lesions in monkeys and human stroke patients.

Bilateral lesion/inactiviation of the fastigial nucleus results in increased saccade amplitude in all directions, but with the most pronounced effect on the horizontal component (Robinson & Fuchs, 2001).

Similarly, the ventral aspect of the posterior interpositus nuclei have been shown to contribute to vertical saccades via inactivation studies (Robinson, Straube, & Fuchs, 2000), which demonstrated increased length of upward saccades and decreased length of downward saccades, with little to no change in horizontal saccades or saccade velocities. Similar effects were seen in the fastigial and interpositus nucleus during experiments in which a cooling probe was implanted in the medial cerebellum, between the fastigial and interpositus nuclei (Vilis & Hore, 1981). This same study did not see any effect of on the dentate nucleus.

Less is known about the contribution of the dentate nucleus to eye movements, but dentate neurons have been implicated in responses to visual stimuli by implying their response to increase Purkinje cell activity during visual stimulus presentation. Similarly, during presentation of temporally specific stimuli, dentate neurons were shown to have increased firing rates in response to steady repetitive stimuli, in a manner opposite to sensory adaptation, such that the change in firing rate would be smaller if the expected stimulus was omitted (Ohmae, Uematsu, & Tanaka, 2013). Muscimol injection into the same recording site delayed detection of the missing stimulus and saccade latency during saccades. These findings implicate the dentate nucleus as responding to stimulus prediction and saccade generation.

Muscimol injections into the central and dorsal aspects of the dentate during a sequential button press task disrupted the memory of old (pre-injection) learned sequences, but not new (post-injections) sequences, and only when the hand ipsilateral to the injection site was used (Lu, Hikosaka, & Miyachi, 1998). Anticipatory saccades were also noted to be disrupted, particularly when the

ipsilateral hand was used. These effects were not seen when muscimol was injected into the ventral aspect of the dentate.

The evidence that either the interpositus or dentate nuclei modulate saccades is much less solid. I am aware of only one full publication on the role of the interpositus nucleus in saccades (Robinson et al., 2000) showing that lesions in the posterior interpositus primarily affect vertical saccades causing hypermetria of upward saccades and hypometria of downward saccades. The role of the dentate nucleus in saccades has never been studied in detail though we do know that there are projections from dentate to the frontal eye fields (Lynch, Hoover, & Strick, 1994) that provide a route through which it might influence saccades and that neurons in the lateral dentate respond to visual stimuli (Chapman, Spidalieri, & Lamarre, 1986; Marple-Horvat & Stein, 1990). Taken as a whole, the experimental data from the deep nuclei show that they do play a role in saccades but the details of this process are lacking for all but the fastigial nucleus.

Sociality in primates

Laboratory studies

Research in the laboratory has furthered the notion that non-human primates have strong social cognition. It has been shown that primates seem to have a natural interest in the faces of other primates. Experiments with rhesus macaques found that the monkeys scan faces in patterns similar to humans (Gothard, Erickson, & Amaral, 2004; Nahm, Perret, Amaral, & Albright, 1997; Parr, 2011). Studies with chimpanzees have shown that primates are strongly interested in images of humans (Fujita & Matsuzawa, 1986; Tanaka, 2003). Furthermore, experiments in which monkeys were raised with no exposure to faces (masked experimenters), the subjects still demonstrated an innate interest in faces when exposed to them alongside control images (Sugita, 2008), which suggest that monkeys have an innate interest in faces. Social isolation studies performed during the wild west days of science by Harlow demonstrated that

even infant non-human primates have a strong innate interest in sociality that seems to exist from birth (Harlow, Dodsworth, & Harlow, 1965). These studies have demonstrated that primates have inbuilt mechanisms for face preference and face scanning that are conserved during evolution.

Ethological studies

Social cognition is the study of the neural processes that underlie interpersonal behaviors. Certain species, notably humans and other primates, have a very well-developed sense of social cognition. Ethological studies have shown that monkeys in the wild are more attuned to social considerations than they are to their surrounding environment (Cheney & Seyfarth, 1985). They are keen to understand things like dominance structure, who treated them affinitively, etc., but they are less cognizant of things that should be critical for surviving in the wild, e.g. evidence that a predator is in the area. Their interest in social relationships can override attention to important survival cues, such as the presence of a predator in the vicinity. Strong social bonds between female baboons in the wild has been shown to correlate strongly with increased infant survival, and these bonds transcend group rank, dominance, and environmental factors (Silk, Alberts, & Altmann, 2003).

Potential relevance to human disease syndromes

Due to the novelty of this new cerebello-SEF pathway, this project provides new insight into potential mechanisms underlying several debilitating human diseases, such as schizophrenia and autism. The behavioral phenotypes of these disorders, and others, often feature poor eye contact. Understanding how such a face preference/eye contact system works could lead to new breakthroughs in treatment for such disorders.

Autism is a devastating neurological disorder of unknown cause, unclear pathogenesis and without an effective treatment. Autism currently affects 1 in 68

children, and is the fastest growing developmental disorder in the US, with no known cure (Community Report, 2016). One of the primary defining features of autism is a lack of eye contact, as was noted by Lovaas (Lovaas, 1987) in his foundational paper describing his technique for treating autism through behavior modification. The defining feature of autism is a profound deficit in social behavior including a lack of interest in social bonding and impaired language development. There are several lines of physiological evidence that implicate the cerebellum in autism (Wang, Kloth, & Badura, 2014). Autopsy data from autistic individuals show reduced Purkinje cell count and cerebellar volume (Bauman & Kemper, 2005; Courchesne, Yeung-Courchesne, Press, Hesselink, & Jernigan, 1988; Ornitz & Ritvo, 1968; Palmen, van Engeland, Hof, & Schmitz, 2004). Further, functional imaging of autistic individuals during eye movement tasks show reduced activation of the Supplementary Eye Fields (SEF) (Takarae et al., 2004), a region associated with cognitive control of eye movements.

We are limited with existing rodent models of the disorder because they cannot fully recapture this defining feature of the disorder. Similarly, we are limited with what research can be done ethically with human patients. We believe that establishing a non-human primate model of autism will be an excellent compromise if the innate sociality of non-human primates be harnessed to peel back the layers of mystery obscuring the source and mechanism underlying autism. We believe this study provides a baseline assay for social interest in primates that is robust, quantifiable, and easily implemented in a laboratory setting. Furthermore, our anatomical and neurophysiological findings provide a neural substrate through which the cerebellum can contribute to the cognitive control of eye movements, and deficits of this system could be the physiological mechanism through which autism acts.

Summary

Based on previous work done by members of this lab and others, we predict that there will be movement related activity in all of the deep cerebellar nuclei. In particular, we expect a large proportion of fastigial nucleus neurons to respond to horizontal eye movements, and the majority eye movement-related neurons of the interpositus nuclei to be specific to vertical eye movements. We also predict that movement-related activity in the dentate nucleus will be primarily confined to the dorsal aspect of the dentate. In regards to neural activity related to the cognitive aspects of the task (e.g. during stimulus presentation, activity selective to a specific target stimulus, etc.) will be present primarily in the ventral aspect of the dentate nucleus and the posterior interpositus nucleus.

We expect changes related to saccade dynamics (e.g. saccade amplitude/length, velocity, etc.) after muscimol injections into the fastigial, interpostitus, and dorsal aspect of the dentate nucleus, and we expect to see changes in target preference after inactivation of the ventral aspect of the dentate nucleus and the ventral aspect of the posterior interpostius nucleus.

Chapter 2: Methods

Behavioral training using operant conditioning

Two male rhesus macaques (*Macaca mulatta*) monkeys were used as subjects for this study. They were trained from a young age to respond to positive reinforcement and were first trained to sit in custom-made primate chairs.

Task

We trained two male monkeys to sit in front of a computer screen and gaze at a center fixation point (60 pixel radius). Eye position was measured with an EyeLink II system. After fixating at the center target for 500-700 ms, then they were simultaneously presented with two outer targets (**Fig. 2.A.).** The center of each target was 275 pixels from the center of the fixation point. In early training the targets were colored circles (100 pixel radius), and during testing they were images (see below for categorization). After maintaining center fixation for another 500-700 ms, the center fixation point disappeared (go signal) and the monkey would saccade to either of the two outer targets and hold for 200 ms to receive a reward. The monkeys were rewarded an equal amount regardless of which image they made a saccade to.

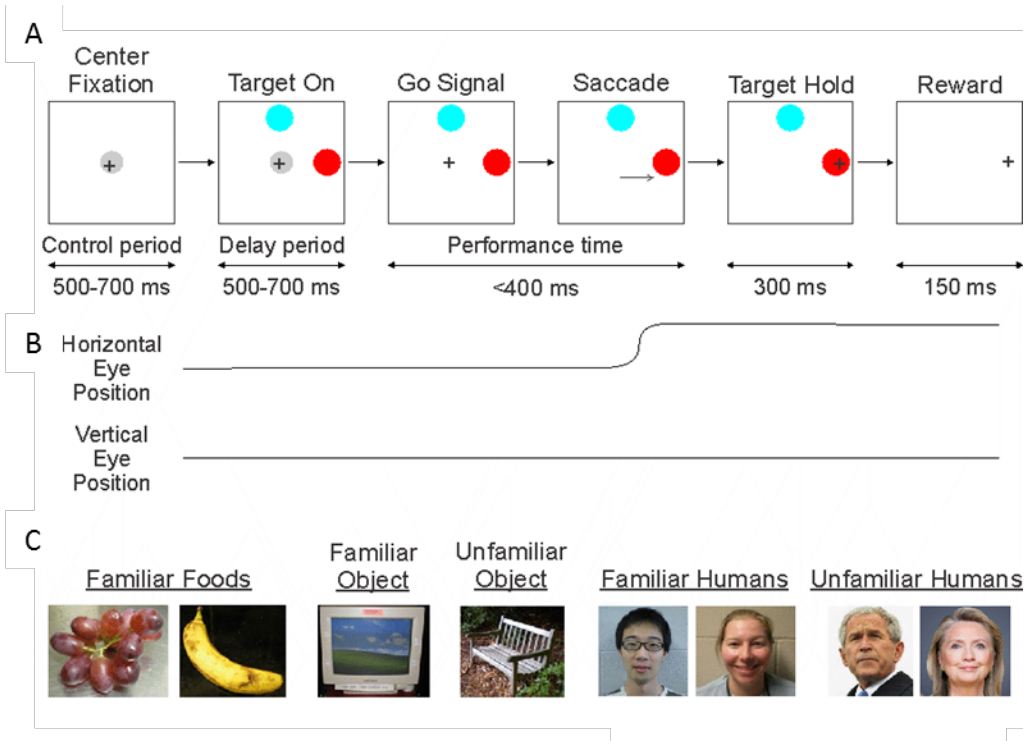

Figure 2: **A.** Task schematic and time course. **B.** Example eye position during task. **C.** Sample images and categories.

The target pairs were either presented in the up (90°) and right (0°) directions, down (270°) and left (180°) directions, up and left directions, down and right directions, left and right directions, or up and down directions simultaneously. Initially, the animals were trained using colored circles as the targets (100 pixel radius), and after familiarization with the task, the circles were masked and images were superimposed over the circles. The circles still functioned as the actual targets, but they were covered by the images and thus hidden from the monkeys. Trials containing single targets were interleaved throughout the two target task, as well.

The two main image categories were faces and non-face objects. These categories were further sub-divided into familiar human face, unfamiliar human face, familiar monkey face, unfamiliar monkey face, food, and non-food object

(**Fig. 2.C.**). The familiar faces were images of humans and monkeys that the subjects knew from personal experience and daily interactions (e.g. experimenters, cagemates). The unfamiliar faces were of humans and monkeys that the subjects have never met or seen before. The images of food were of foods and treats that they monkeys receive regularly and enjoy (e.g. apples, grapes, marshmallows). The non-food object images were of toys and objects that they monkeys encountered daily or have no experience with (e.g. balls, primate chair, bicycles, musical instruments). Three main types of trials were conducted: Pairs of non-face images, a face image paired with a non-face image, and trials with a single face or non-face image.

MRI

The overall premise of this procedure is to obtain an MRI from fully anesthetized subjects mounted into a nonmagnetic stereotactic array. The MRI is of critical importance because the resulting images will guide the placement of the recording chamber and selection of recording sites.

The monkeys were anesthetized with a 7:3 ketamine (4.0–5.0 mg/kg) and xylazine (1.0–2.0 mg/kg) cocktail before being mounted prone into a nonmagnetic stereotactic array. The resultant images were used to identify the locations of the deep cerebellar nuclei, and vitamin E markers on the MRI stereotactic array were used as reference points to plan placement of the recording chambers.

Surgery

The main goal of the surgery was threefold: install a sealable recording chamber, perform a craniotomy within the bounds of the chamber, and install the provisions for a head holding apparatus. A rectangular chamber (internal dimensions of 19 mm x 34 mm, see **Fig. 3**) was placed straddling the monkeys' midline, with its

anterior-posterior center over the medial aspect of the deep cerebellar nuclei (determined from MRI results).

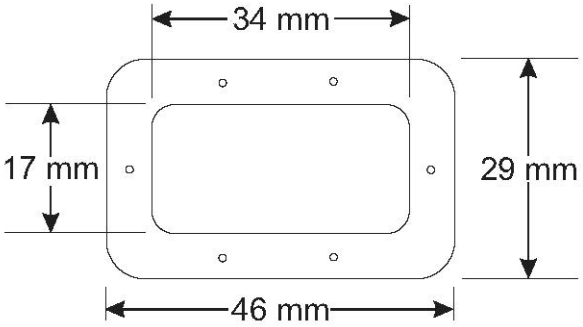

Figure 3: Dimensions of recording chamber.

The monkeys were sedated with ketamine (4.0–5.0 mg/kg) and xylazine (1.0–2.0 mg/kg) intramuscularly, and then general anesthesia was induced by intravenous injection of pentobarbital sodium. Surgical procedures were conducted in aseptic conditions. The monkeys were mounted in a stereotactic array, and, after exposing the skull, 15–20 holes were drilled and tapped approximately three-fourths of the way through it. Titanium posts were installed using titanium screws that were inserted into the aforementioned holes and fixed in place with dental acrylic resin. The screws acted as anchors for the dental acrylic, to which the delrin recording chamber was affixed to the skull. The posts would be used to affix a head holding apparatus (halo).

A craniotomy was performed on the skull inside of the recording chamber. A Foredom rotary tool with spherical fluted cutting burr was used to cut away the acrylic resin and skull inside the recording chamber, exposing the dura.

The monkey received Meloxicam (0.2 mg/kg, subcutaneous), Enrofloxin (5 mg/kg), Buprenex (0.005-0.02 mg/kg), and Buprenex SR (0.25 mg/kg) the day of

the surgery, and Meloxicam (0.1 mg/kg, subcutaneous) for two days after the operation.

Recording

Single-cell tungsten microelectrodes (~4 MΩ resistance) from FHC were mounted in a Narishige micromanipulator. A custom delrin cylindrical adaptor allowed the round micromanipulator to be attached to the rectangular recording chamber. Anterior-posterior and medial-lateral coordinates, determined from the MRI, were set on the micromanipulator, and the electrodes were driven through the dura with the use of a protective guide tube. The electrodes were driven from dorsal to ventral through the cerebrum into the ventral cerebellar cortex. The characteristics of the neural activity were noted during the descent to facilitate physiologically mapping the region and to corroborate the structural MRI. Upon arrival in the deep cerebellar nuclei, individual cells were isolated during the task with an Alpha Omega spike sorter. A separate computer ran the task, recorded the behavioral metrics (including eye position), and the neural signal.

Electrolytic lesions were induced after the completion of neural recording. Locations were chosen based on their nucleus/anatomical region and the presence of task dependent activity. Tungsten microelectrodes were driven to the sites in the same manner as when recording neural activity. Upon arrival at the chosen locations, a 30-60 second square wave pulse of 100-400 mA current was injected into the site. The monkeys' physical response to the stimulus was noted. Sites were identified after histological preparation using a microscope.

Injections

The same Narishige electrode micromanipulator was used to place the tip of an injection tube at the same sites at which task-related activity was found. We used muscimol, a GABA agonist, to inactivate local brain activity.

Dry muscimol powder was dissolved in saline with and brought to a concentration of 5 µg/µl. All injections were carried out with an injection tube that was inserted into the cerebellum using the Narishige micromanipulator. Our device for pressure injections was a stainless steel tube connected to a Hamilton syringe (10 µl) by a polyethylene tubing (ID: 0.3 µm). We used a stainless steel tube (ID: 0.10 µm, OD: 0.3 µm, length: 80 mm) with a sharp angle at the tip. The injection tube was set up the same way as the electrodes were, except the tube tip was placed ~500 µm lower than the electrode tip because—due to the injection tube tip being cut at an angle—the opening was not actually located at the very tip of the tube.

Before being mounted in the Narishige micromanipulator, the injection tube and the Hamilton syringe first were filled with silicon oil. Before an injection experiment, a small amount of muscimol solution was aspirated into the injection tube. The depth of the injection was determined by the data obtained in the preceding recording sessions. During injection, the muscimol solution was pressure injected in ten steps (0.2 µl for each step) with 25 s between steps. The total amount injected was 2.0 µl (5 µg/µl) for each site (Lu et al., 1998). We collected the behavioral data for up to 3 hr after each injection. As a control for muscimol, we injected the same volume of saline under the same conditions.

Data analysis

Animal behavior (eye position, reaction/movement times, target choice, etc.) and saccade dynamics were imported into Matlab. Median performance times were calculated based on the amount of time taken to saccade to a given target after the go signal. Cumulative preference was determined by counting the number of trials in which the subject made a saccade to a given image/image category. ANOVAs were used to determine if there were differences between saccade metrics before and after injection. The neural data was analyzed several ways. ANOVAs were used to compare neural firing rates between behavioral epochs

(center hold, target on, reaction, movement, target hold, late phase) across all trials of a given set. Unbalanced ANOVAs were performed comparing firing rates between trials for each direction in each behavioral epoch. This determined if the cell had differential firing rates for different saccade directions during a given behavioral epoch. Similar analyses were performed comparing firing rates between trials towards each target during each behavioral epoch. Also, since target choice is intrinsically linked to saccade direction, 2-way ANOVAs were performed comparing the interaction between target and direction.

Sacrifice and perfusion

The aim of the perfusion is to preserve the neural tissue in as pristine a shape as possible for further histological analysis. Both monkeys underwent the following procedure of transcardiac perfusion.

The monkeys were anesthetized via an injection of a ketamine/xylazine (7:3) cocktail. Following anesthesia, the monkeys were placed supine and their chest cavities were opened via a rostro-caudal incision along their anterior rib cage. After exposing the heart, a ~1 cm incision was made through the wall of the posterior aspect of the left ventricle. A perfusion needle (connected to a pump via a rubber hose) was inserted into the incision. Simultaneously, an incision was made into right atrium and the aforementioned pump immediately began pumping in 4°C saline through the monkeys' circulatory system. Saline was pumped through the subjects until fluid leaving the circulatory system through the right atrium was clear, at which point the pump was switched to 4°C 10% formalin. Approximately 1000 ml of 4°C 10% formalin was pumped through the subjects. After perfusion with the formalin, the subjects were decapitated and their heads were placed in a 10% formalin solution overnight.

Histology

After removal of the brain, the tissue will need to be processed in order to visualize lesions, nuclei, and electrode tracks. The histology is separated into two distinct phases: sectioning and staining. These two phases will be described separately.

Sectioning:

The brains were sectioned with a Lecia VT100S vibrating microtome. First, a razor blade/scalpel was used to grossly trim down the entire cerebellum to a smaller sample containing the deep cerebellar nuclei (approximately a 2.5 cm x 2.5 cm coronal section). Sample thickness must not exceed 1 cm, otherwise it will not fit beneath the vibrating microtome blade. The tissue sample was submerged in sucrose overnight (or until sample sinks to the bottom, indicating that sucrose has fully impregnated sample). The tissue sample tray of the microtome was filled with 4°C phosphate-buffered saline (PBS), and the tray was surrounded with ice to maintain the PBS bath at 4°C. And the microtome was initially set to 70 Hz vibration speed and 0.25 mm/s transverse speed.

The sample was mounted onto vibrating microtome specimen disc with super glue, allowing the glue to fully dry before fully submerging the sample in the buffer tray. A section thickness of 60 µm was used for the first monkey, but that thickness seemed to lack sufficient structural integrity, so a section thickness of 100 µm was used for the second monkey. One slice was cut at a time to ensure adequate time to remove the slice from the PBS bath and mount on the gelatin-subbed slide. The microtome settings were modified (e.g. slow blade progression speed, hasten vibration speed, etc.) throughout the sectioning if the tissue slices were not of optimum quality. Allow the slides to dry overnight before staining.

Staining:

A 0.1% thionine (Nissl) stain was used, which will mark cell bodies with a bluish/violet dye. The sectioned and mounted tissue was allowed to dry overnight before being subjected to the staining regimen. The slides were moved from one solution bath to another. First, the slides were rehydrated, then dipped in the 0.1% thionine solution, then washed, then dehydrated via an increasing progression of alcohol baths. The specific progression can be seen below. After a final bath of xylene, the stained tissue was sealed with cover slips and a cover slip mounting media.

Procedure:

1. Acetate buffer – 1 minute
2. 0.1% Thionine – 2.5-3 minutes
3. Acetate buffer – Brief rinse

   Dehydrate (10-20 seconds each):
4. 10% Ethanol
5. 30% Ethanol
6. 50% Ethanol
7. 70% Ethanol
8. 95% Ethanol
9. 95% Ethanol
10. 100% Ethanol
11. Xylene
12. Apply cover slip immediately (when slides are still wet)

Chapter 3: Face Preference in Non-Human Primates

Introduction

Studies in nonhuman primates, both in the controlled setting of the laboratory and in their natural environment, have shown that they exhibit a strong preference for looking at objects with high social relevance, particularly faces. Rhesus macaques found that the monkeys scan faces in patterns similar to humans (Nahm et al., 1997; Gothard et al., 2004; Parr, 2011). Similarly, studies with chimpanzees have shown that primates are also strongly interested in images of humans (Fujita and Matsuzawa, 1986; Tanaka, 2003). Furthermore, in experiments in which monkeys were raised with no exposure to faces from birth (masked experimenters), the subjects still demonstrated a preference for faces compared with control images (Sugita, 2008), which suggests that monkeys have an innate, hard-wired interest in faces that has been linked to the existence of a face area in primate inferotemporal cortex (Perrett et al., 1982). However, the preference for looking at faces is present before the appearance of the face area (Livingstone et al., 2017), and in non-human primates raised without seeing faces, the face area fails to develop (Arcaro et al., 2017) making it likely that the behavior is driven by reinforcement in infancy.

Visual preference for viewing faces is one of the core behaviors that contribute to social cognition, which is the set of behaviors and processes that have developed to enable animals to thrive within social groups. The development of social cognition, which reaches its apex in primates, is closely correlated with, and may have been the major stimulus for, increasing intelligence and forebrain size during primate evolution according to the social intelligence hypothesis (Humphrey, 1976). Among the other social behaviors of primates are: recognition of individual characteristics, rank and kinship of others; social manipulation including deception and the formation of strategic alliances;

predicting and understanding the actions of others (Byrne and Bates, 2010). In fact, behaviors related to social cognition appear much more ingrained than other important practices and behaviors that are important for survival; for example, vervet monkeys while very cognizant of the troop's social dynamics frequently ignore or do not appreciate the significance of other environmental cues such a python tracks or a carcass in a tree as an indicator of a leopard nearby (Cheney and Seyfarth, 1985). Similarly, monkeys readily form alliances to achieve social goals but rarely cooperate to hunt or find new sources of food.

The apparent bias in non-human primates toward socially-relevant behaviors documented in the wild has never been tested in controlled laboratory conditions. To do so, we developed a visual preference task in which nonhuman primate subjects are simultaneously presented with two images, and then choose an image by making a saccade toward it to receive a liquid reward. The visual images are drawn from two categories, face and non-face (including ecologically relevant familiar foods and objects) and the subject were rewarded equally for all choices. We show here that non-human primates show an overwhelming preference for images of other primates (human and non-human) even when paired with familiar favorite foods and objects.

Methods

Behavioral task

We trained two male monkeys (*Macaca mulatta*) to sit in front of a computer screen and make eye movements to select one image from pair of successive images. The screen was positioned 45 cm in front of the subjects. Eye position was measured with an EyeLink II system and sampled at 250 Hz. At the beginning of each trial the subject fixated on a visual target at the center of a computer screen. After fixating for 500-700 ms (control period), two targets were presented simultaneously in two of the four potential target locations (Fig. 1a). After maintaining center fixation for another 500-700 ms (delay period), the

center fixation point disappeared (GO signal) and the subjects make a saccade to one of the targets and maintain that eye position for 300 ms to receive a liquid reward; each of the targets were rewarded equally. The pairs of visual images were pseudo-randomly presented among the four possible spatial locations such that in a block of trials any specific image will appear equally at each location. (**Fig. 4.a**).

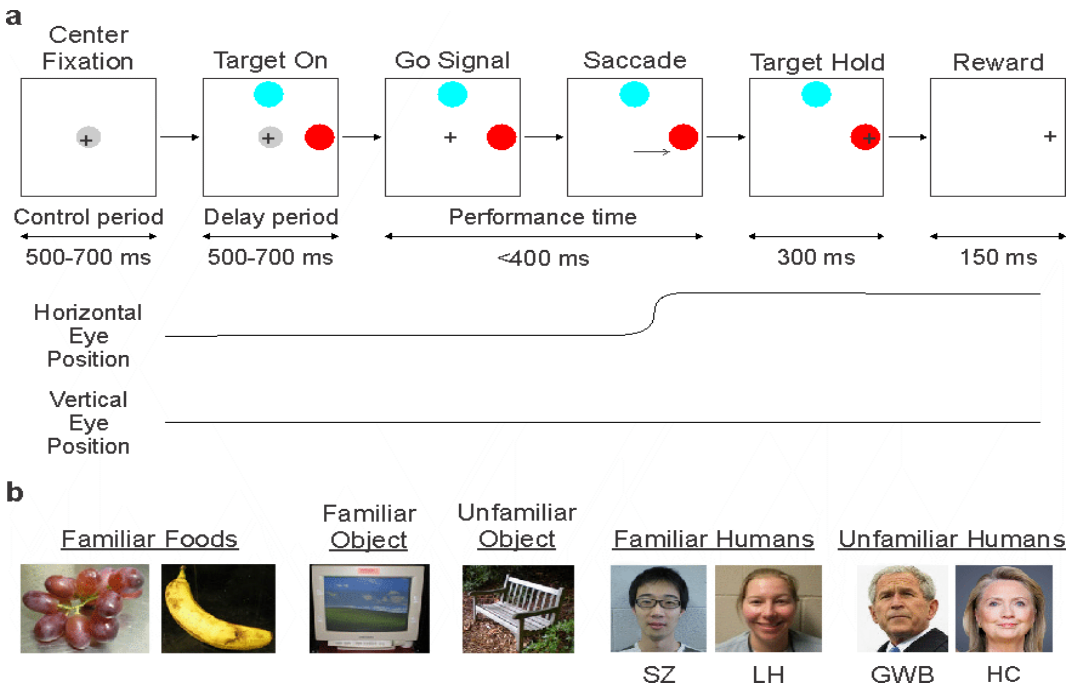

Figure 4: **a.** Time course of a single trial including changes in eye position. **b.** Examples of some of the visual images used in the different images categories.

The main image categories were faces and non-face objects (**Fig. 4.b**). These images were further sub-categorized as familiar human face, unfamiliar human face, familiar monkey face, unfamiliar monkey face, food, and non-food object. The familiar faces were images of humans and monkeys that the subjects knew from personal experience and daily interactions (e.g. experimenters, cage-mates). The unfamiliar faces were of humans and monkeys that the subjects have never met or seen before (e.g. US politicians, non-human primate images

from internet). The images of food were of foods and treats that the subjects receive regularly and enjoy (e.g. apples, grapes, marshmallows). The non-food object images were of toys and objects that the subjects encountered daily (toys and balls in environmental enrichment) or objects with which they had no experience with (e.g., bicycles, musical instruments).

The pairs of visual images were pseudo-randomly presented among the four possible spatial locations such that in a block of trials any specific image will appear equally at each location. Trials were presented in blocks of 120 correct trials and will be two general types: face/non-face pairing, non-face/non-face pairing. The face category can be further divided into subcategories: familiar (human), familiar (monkey), unfamiliar (human), unfamiliar (monkey). The non-face category can be sub-categorized into: foods (familiar and unfamiliar), objects (familiar and unfamiliar). In daily experiments, we typically run 10-15 blocks with similar number of face/non-face and non-face/non-face blocks

Data analysis

Standard statistical approaches (Snedecor and Cochrane, 1989) were used to analyze the data and to determine the significance of the findings.

Results

The two non-human primate subjects performed in a total of 493 experimental sessions comprising a total of 59160 trials. The principal finding across the two animal subjects was that they showed an overwhelming preference for images of human and non-human primate faces compared to all of the other visual images categories with which they were paired.

We found that, when images of a face were paired with images from any of the other categories the subjects preferred the faces in 92.8% of pairing with human faces, and in 89.9% of pairings with non-human primate faces ($p<0.0001$,

binomial distribution fit) (**Fig. 5**). The preference can also be detected at the level of the individual data sets for two given image pairings. The median number of trials in which the human face was preferred in blocks of 120 was 117, and the median number of trials in which non-human primate face was preferred was 114.

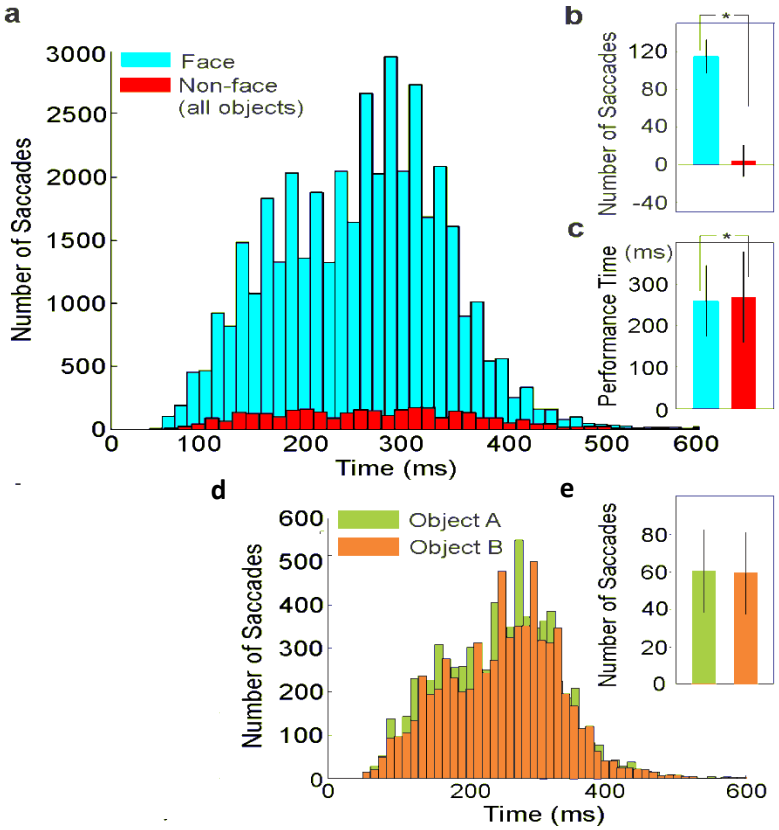

Figure 5: Preference for face compared to non-face visual images. **a.** Histogram of the number of saccades binned by response time for all the experimental sessions with paired face (blue) and non-face (red) images. **b**. Average number of saccades to face and non-face targets in each 120-trial experimental block. **c**. Mean performance time (reaction time and movement time) for saccades to face and non-face, * denotes significant difference at the 0.05 level. **d.** Histogram of the number of saccades binned by response time for all experimental blocks in which both visual images were non-face objects. **e**: The average number of saccades to each of the paired non-face objects.

These results are statistically significant for both the human face image trials ($p<0.0001$, Wilcoxon rank sum), as well as the monkey face image trials ($p<0.0001$, Wilcoxon rank sum). There was no detectable difference between the preferences of the subjects in trials in which two non-face images were paired (**Fig. 5.d**) nor in the median number of trials for non-face image pairings (**Fig. 5.e**). Because the focus of this study was on comparing the strength of the preference for faces compared to other visual image categories including those that were familiar to the subjects and those that might be of high ethological and biological significance, we did not directly compare preferences for different face categories (human and non-human primate, familiar and unfamiliar). Our data, however, may be used to address indirectly the relative preference for human and non-human primate faces by examining the subjects preference when each of these face categories was paired with the same non-face group (**Fig. 6**). We believe that it is not appropriate to do any quantitative comparison among these data groups but, nevertheless, the data suggest that the subject preference for the different face categories was quite similar.

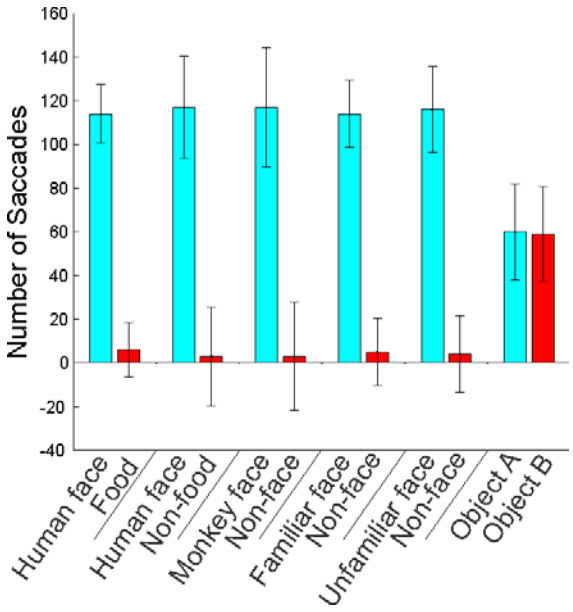

Figure 6: Median number of saccades per experimental block for different visual image categories.

Finally, we found that the preference for primate faces persisted even when these visual images were paired with images of foods that were regularly eaten an enjoyed by the subjects (**Fig. 7**).

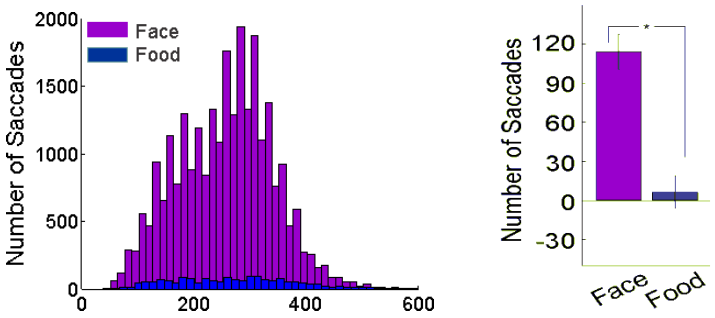

Figure 7: Pairings of face and familiar food objects.

Discussion

Our results in this behavioral component of the project suggest that non-human primates when given a choice between visual images of the faces of other primates and images of a wide variety of other images categories show an overwhelming preference for the facial images to all other categories of including images of familiar objects with which they play and foods that they regularly eat and enjoy. These data suggest that visual preferences in non-human primates are more strongly determined by images that have social significance than by those that are of objects that are essential to survival such as food and is consistent with the observation of Cheney and Seyfarth based on ethological studies in which vervet monkeys seemed more aware of kinship and hierarchy than of environment cues that might be essential for survival.

Face Preference in Primates

Visual preferences in humans and in non-human primates have been studied to open a window into their general cognitive processes but more specifically to probe social cognition. Understanding the perceptual processes of NHP

particularly their ability to correctly categorize different animal species and to recognize individuals within a species was the motivation for this line of scientific inquiry. Sackett (Sackett, 1966)) showed that macaques have a preference for looking at visual images of their own species and this was confirmed by Humphrey (Humphrey, 1974) who suggested by that this property was related to the life history of exposure to other monkeys rather than an innate predisposition. Rhesus macaque monkeys can categorize different species of monkey (Yoshikubo, 1985) and recognize that humans are part of a separate category (Fujita and Matsuzawa, 1986) . Additional work by Fujita and colleagues (Fujita and Matsuzawa, 1986)(Fujita, 1987; Fujita and Watanabe, 1995; Fujita et al., 1997) found that macaque monkeys showed greater interest in the visual images of their own species (conspecifics) than in other monkey species or in humans. However, it is likely that several factors including previous exposure, perceived dominance, and novelty play a role in determining visual preference because chimpanzees bred in captivity by humans prefer images of human faces over those of their own species (Tanaka, 2003) and rhesus macaques show a preference for faces of Barbary macaques and chimpanzees compared to their own species (Méary et al., 2014).

The issue of whether face processing (preference) is innate was addressed comprehensively in an experiment in which infant monkeys were raised in captivity for a period of 6-24 months without ever being exposed to the faces of humans or other monkeys or face-like stimuli (Sugita, 2008). During the 'face deprivation' period the monkeys showed a preference for images of human and monkey faces and were able discriminate between members within each category This finding strongly supports the hypothesis that face processing is an innate characteristic of primates as it is independent of exposure to faces though we acknowledge that the findings of this study have been interpreted differently by others (Arcaro et al., 2017). After a variable period of deprivation, the monkeys were then exposed exclusively either to monkey or to human faces and

they slowly lost the ability to discriminate among members of the non-exposed group showing that though face processing and preference is innate it can be subsequently modified by experience. The neural substrate of face processing in human and non-human primates is thought to be the inferotemporal face area (Perrett et al., 1982). However, signs of face preference and processing are detectable in infant primates well in advance of the complete development of the temporal face area (Arcaro et al., 2017; Livingstone et al., 2017) which suggests that other brain areas can process faces in infancy though with age the inferotemporal cortex appears to be the main substrate for these perceptions.

With some rare exceptions (Sugita, 2008), studies on face preference in non-human primates have not focused on the strength of this preference relative to other images from the natural environment whether ther be neutral, unfamiliar, or images of desired objects. In perceptual experiments in which natural objects have been used the focus has often been on the ability of the non-human primates to place the objects in appropriate categories (Tanaka, 2001). Sugita, in addition to using images monkey and human faces, showed images of unfamiliar non-face objects (houses, household objects, vehicles) that formed a single image category; as might be expected the subjects showed a preference for the face images that increased with the duration of exposure to faces. The development of face processing areas has been assessed in monkeys by presenting images of different image categories including familiar rectilinear objects but relative preference was not tested (Livingstone et al., 2017).

Importance of Social Cognition in Primates

Non-human primates most of whom live in semi-permanent troupes have developed complex social structures to enable them to prosper in such an environment. They recognize individuals, and appreciate the subtlies of rank, kinship, and third-party relationships within the troupe (Cheney et al., 1986). In addition, they are adept manipulative social tactics such as making alliances with

with those who are most likely to be useful in the future (Cheney, 1978), or the use of tactical deception when it confers an advantage (Byrne and Whiten, 1985). Other socially important properties such as understanding and predicting the actions of others have been shown in NHP species. Whether some non-human primates show evidence that they possess a theory of mind, or the ability to infer intentions and desires from actions, is somewhat more controversial (Call and Tomasello, 1998; Byrne and Bates, 2010). Jolly (Jolly, 1966) was to first propose a link between the social development and skills of primates and the development of superior intelligence in primates compared to other species. This concept was later expanded and further developed by Humphrey (Humphrey, 1976) in what has become known as the social intelligence hypothesis that has been fundamental to our recent understanding of primate development.

Methodological Considerations

The subjects in our experiment exhibited an overwhelming preference for faces, and it is conceivable that this preference was driven by the inherent properties of the visual images such as contrast, luminance, and the color palette (Palmer et al., 2013) rather than by the faces themselves. Although we did not control for the level of contrast or luminance in the images, think it highly unlikely that systematic differences in any of these factors among the categories of visual images could have accounted for the very strong preferences we observed. The novelty and familiarity of the visual image can also affect preference (Fantz, 1963) and may even have differential effects of preference dependent on the specific category of image (e.g. faces or geometric figures) being displayed (Park et al., 2010). We used the same set of images throughout the experiment in both animal subjects and did not detect any significant change in our measures of preference over time as the experiment progressed. Finally, the symmetry of the images can also influence preference (Enquist and Arak, 1994); we did not specifically control for this feature but think it unlikely that it was a major contributor to the preference.

Chapter 4: Inactivation of Deep Cerebellar Nuclei

In this experiment, the deep cerebellar nuclei, in particular the dentate nucleus, were the target of these muscimol injections. Past studies have already investigated the effect of muscimol inactivation and destructive lesions of the interposed and fastigial nuclei, showing deficits and alterations in saccadic eye movement and demonstrating the soundness of the approach. There have not been any comprehensive studies examining the effect of dentate inactivation on saccade metrics.

The approximate locations of the deep cerebellar nuclei were identified with an MRI in each subject, and subsequent electrophysiological recording while the subjects were performing the behavioral task provided a more definitive map of the deep cerebellar nuclei and their boundaries. From these recording sessions, the location of neural activity related to the task was identified and which guided us in determining the optimal location for the subsequent of muscimol inactivation injections. Injection, recording, and lesion sites can be seen in the **Fig. 8.** below.

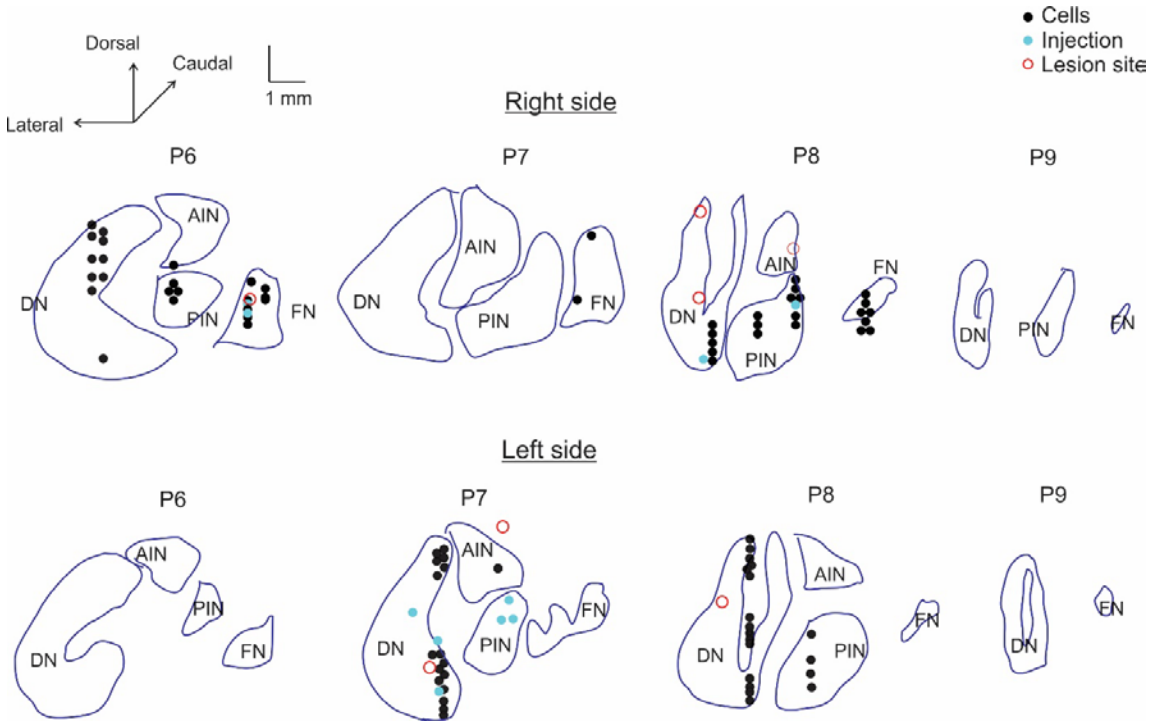

Figure 8: Map of recording, injection, and lesion sites. Shown is a schematic representation of a series of coronal sections of the deep cerebellar nuclei. The outlines indicated the approximate area the nuclei occupy. The P# indicates the distance—in millimeters—that the section was posterior to the zero of the stereotactic array. DN = dentate nucleus, AIN = anterior interpositus nucleus, PIN = posterior interpositus nucleus, FN = fastigial nucleus.

In the presentation of the data, we will focus primarily on the results of dentate inactivation, as this has received the least attention in the literature, and will first explore the effect of inactivation on face preference during the behavioral task, and then outline the effects on the following saccade metrics: reaction time, movement time, saccade length, magnitude and latency of peak velocity, saccade deflection, and saccade spread.

Deep nucleus inactivation reduces face preference

Muscimol was injected at the location of task-related neural activity in PIN and VDN (**Fig. 9.a**) of monkey M007 (One of the animal subjects used for inactivation

and neural recording component of the experiment failed to show any face preference). Immediately after injection, we observed the expected decrease in overall neural activity and concomitantly found a significant decrease in face preference as the subject continued to perform the task; there was no change in the preference after the injections into DDN and AIN as we would have predicted based on our anatomical studies. Control injections in the same locations using saline had no effect on target preference.

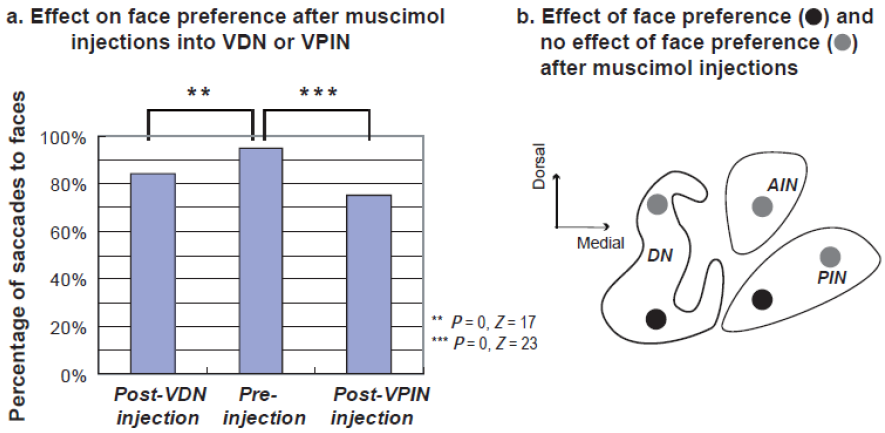

Figure 9: Muscimol inactivation. a. Effect on task performance. **b.** Injection locations and behavioral effects.

Muscimol Inactivation of the Dentate Nucleus

There are several metrics related to saccadic eye movement that will be tracked. The first is reaction time, which is defined as the time after the presentation of the go signal until the time the saccade velocity reaches 20% of its maximum. Next is the movement time, which is defined as the time after the saccade velocity reaches 20% of its maximum until it enters the outer target. Both the reaction and movement time are recorded in milliseconds. Next is the saccade length, which is defined as the eye-centered angle that the eye subtends while moving from one target to another (illustrated as **1** in **Fig. 10.a**). Next are two velocity metrics, the peak velocity, defined as the maximum velocity the saccade

reaches, and the time to peak, how long it takes the saccade to reach this maximum velocity. The final two metrics are representative of how well-regulated is the control of the eye movements. These metrics are the *deflection* of the saccade trajectory relative to an ideal path and the *lateral spread* of the saccades toward a given direction, defined as the visual angle that captures to spread of the trajectories on reaching the target, (illustrated as **2** and **3** in **Fig. 10.a.** and **10.b**, respectively).

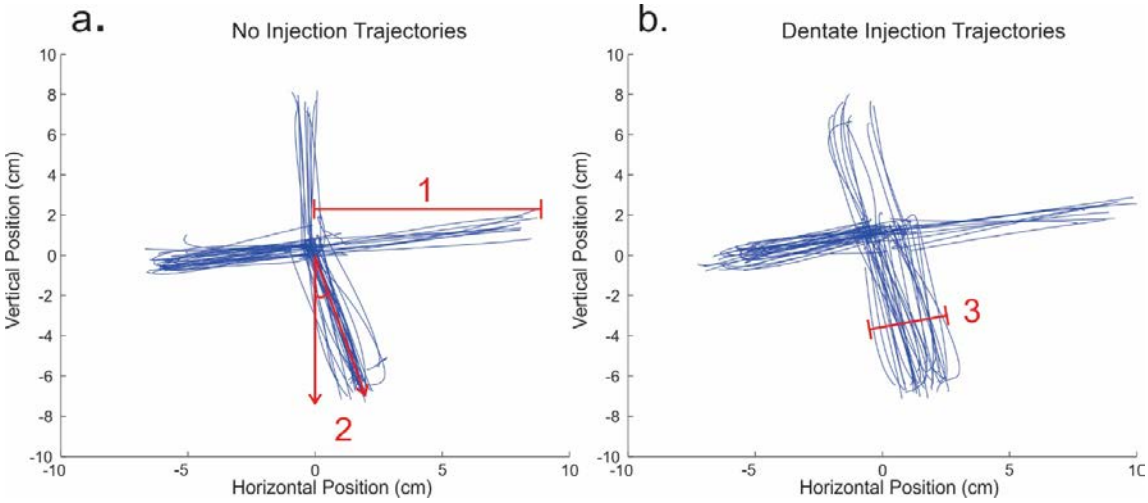

Figure 10: Trajectories of eye position during different experimental conditions. Several saccade metrics are illustrated in red. **a.** Trajectories during a session where no injection occurred. 1. Saccade length, 2. Deflection. **b.** Trajectories during a muscimol injection into the dentate nucleus. 3. Spread.

Reaction time

Because of the relatively liberal spatial windows governing the performance of the behavioral task, we chose to define reaction time at that time (after the GO signal) when the saccade velocity reached 20% of its peak.

Reaction times were increased for saccades in all four directions.

In general, RTs were increased for saccades in all directions though the effects were not entirely consistent across injection sites. The changes in RT for the rightward saccades are shown in **Fig. 11**. A summative figure for all reaction time changes can be seen in **Fig. 12**.

Rightward Saccades: There were significant increases in RT for rightward saccades increased in 6/8 of the sessions and exhibited no change in 2/8 sessions. The injection sites for two sessions in which no change was seen in rightward reaction time were both in the left dentate and, for the injection sites for the six sessions that exhibited an increase in rightward reaction time, three were the right dentate and three were in the left dentate. There was no change in reaction time during rightward saccades exhibited in the single saline injection session (right side injection).

Upward Saccades: An increase in reaction times was seen in 6/8 injection sessions, three of which were during injection sessions in the right dentate, and three of which were during injection sessions in the left dentate. The muscimol injections were in the left dentate during the two sessions in which no change was seen.

Leftward Saccades: Reaction times for leftward saccades were increased in 5/8 sessions. Three of those sessions were left side injections and two were right side injections. 3/8 sessions exhibited no change in reaction time. Two of those sessions were left side injections and one was a right side injection.

Downward Saccades: Reaction times for downward saccades were increased in 6/8 sessions. Much like the other directions, three of those sessions were left side injections and three were right side injections. Downward reaction times were decreased in one session and exhibited no change in one session. Both of these were during left side injections. Similarly to the leftward reaction time, an

increase in downward saccade reaction times was seen during the saline control session (right side injection).

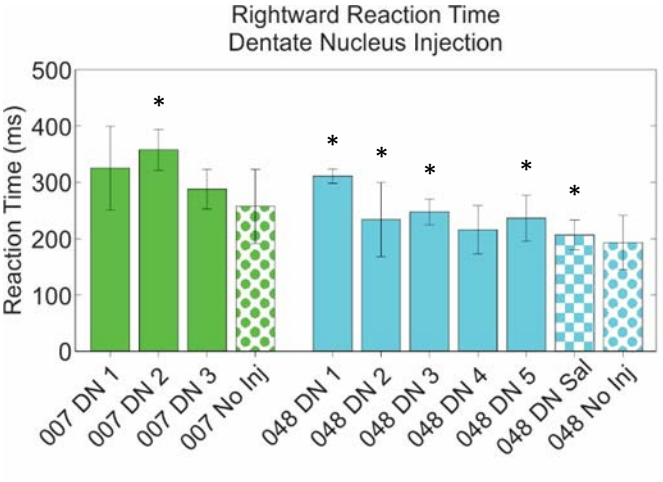

Figure 11: Mean reaction times of rightward saccades during dentate injection of muscimol. 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

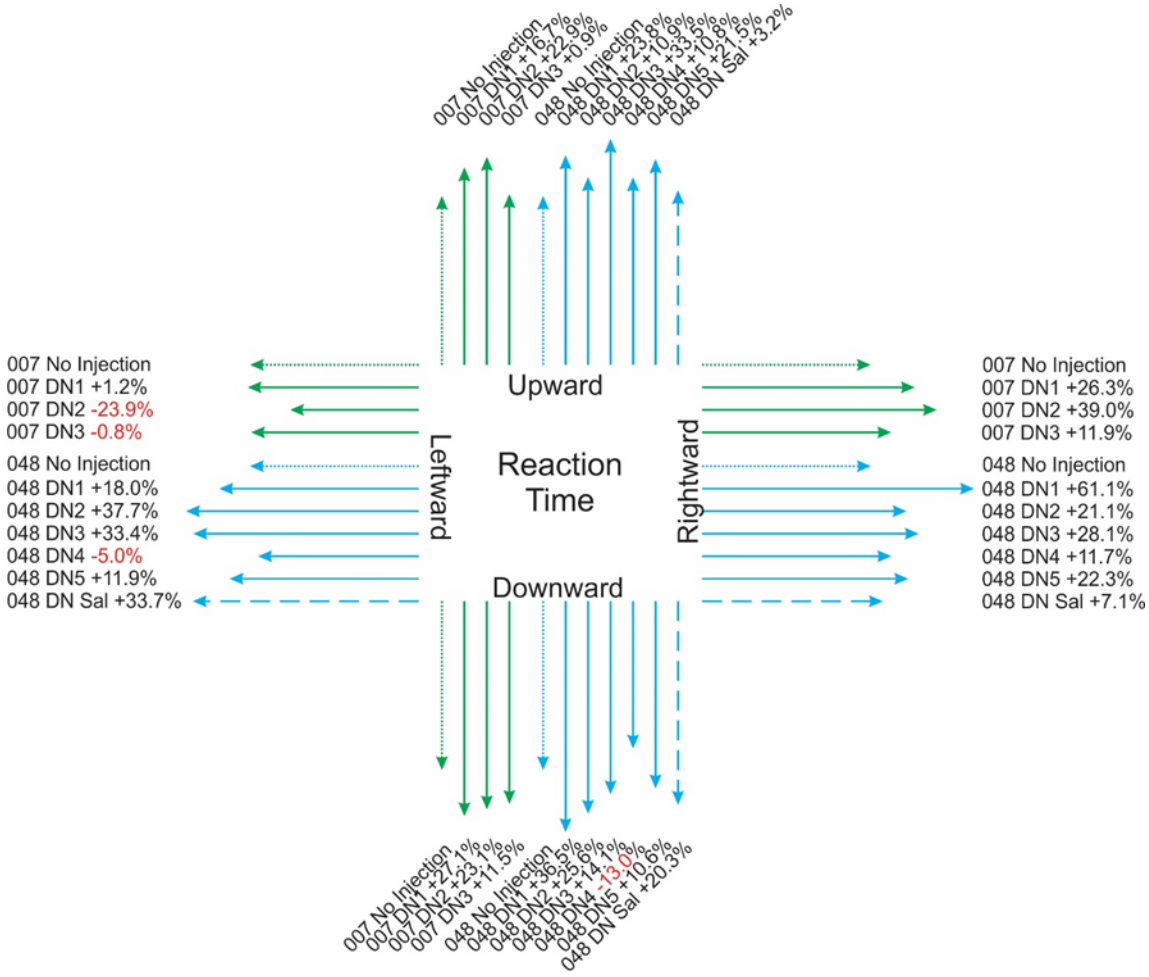

Figure 12: Summary of proportional reaction time changes during dentate injection of muscimol. Reaction time changes are presented as a percentage increase or decrease relative to the no injection times (broken dotted line). 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

Effect on saccade dynamics: Movement time

The movement time is the time after the eye position leaves the center target until the eye position enters the outer target (in milliseconds). Movement times during rightward saccades exhibited no change during the majority of the injection sessions, and there were a few prominent cases of increased movement times during the upward and leftward saccades. The changes during downward saccades were more variable, exhibiting both increases and decreases. A summary of these changes can be seen in **Fig 14**.

Rightward: Movement times were increased in one session, which was during a left side injection, otherwise no change was seen in rightward movement times (during three right side injection sessions and four left side injection sessions). There was no change in rightward movement times during the saline injection session (right side injection).

Upward: Movement times were increased in 4/8 sessions, two of which were during right side injections and two were during left side injections. No change was seen during the other four sessions (one right side injection, three left side). There was in increase in upward movement times during the saline injection session (right side injection).

Leftward: Movement times were increased in 3/8 sessions. Two of these sessions were during left side injection and one was during a right side injection. Movement times decreased in only one session, which was during a right side injection. No change was seen during the remaining 4/8 sessions (three were left side injections and one was a right side injection), but there was an increase in leftward movement times during the saline injection (right side injection). These changes can be seen in **Fig. 13.**

Downward: Movement times: were increased during 2/8 sessions (one right side injection and one left side injection) and decreased during 2/8 sessions (both left side injections). No change was seen in the remaining 4/8 muscimol injection sessions (two right side, two left side), but there was an increase in downward movement times during the saline injection (right side injection).

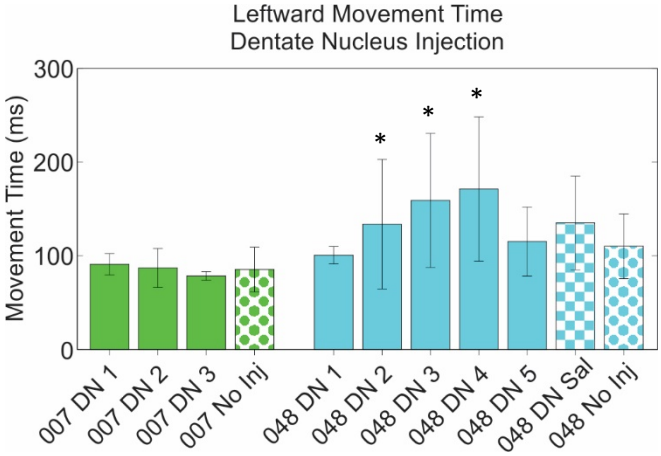

Figure 13: Significant increase in mean movement times during dentate injection of muscimol. 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

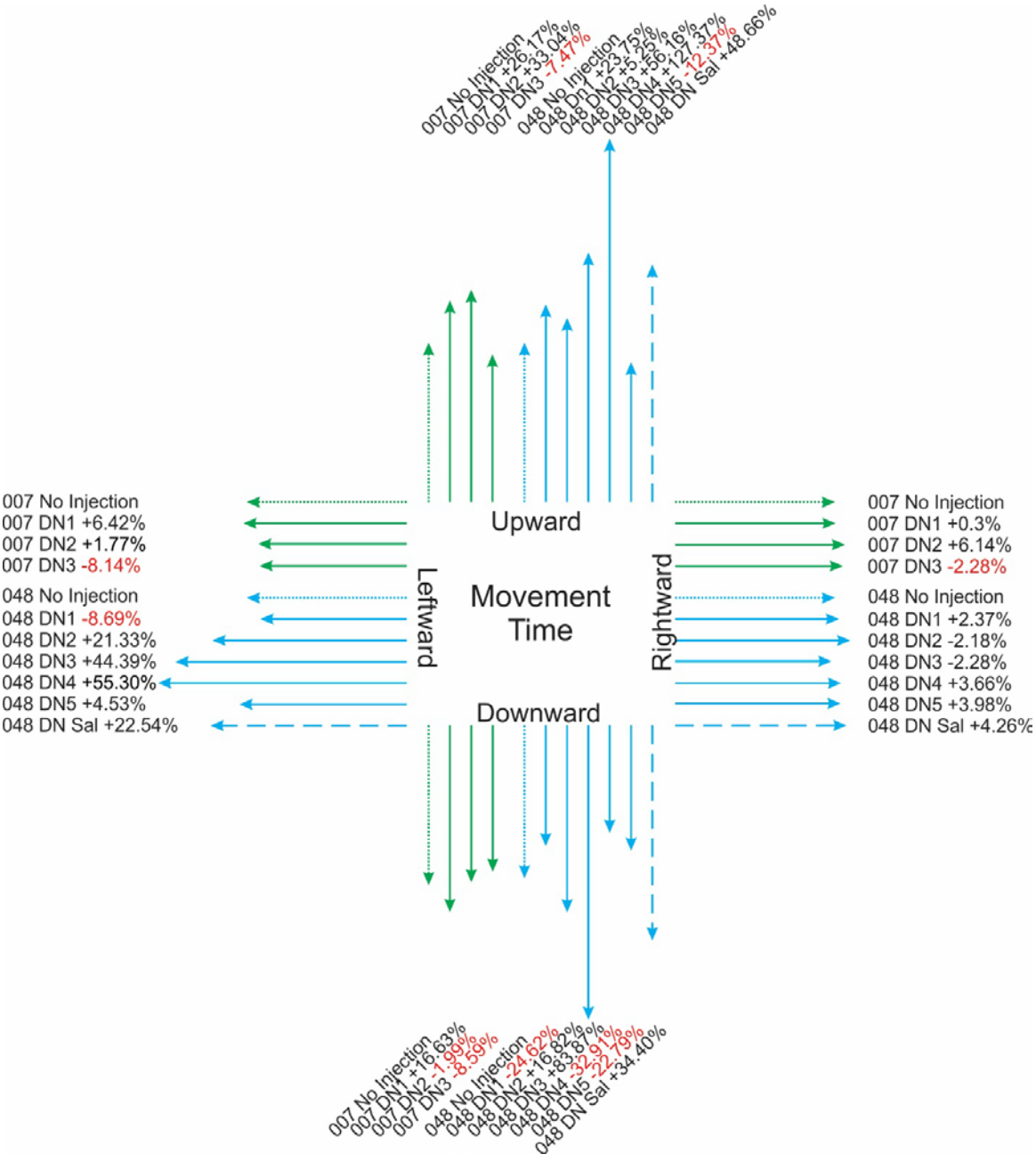

Figure 14: Summary of changes in movement time during dentate injection of muscimol. 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

Effect on saccade dynamics: Saccade length

Next is the saccade length, which is defined as the visual angle that the eye subtends while moving from one target to another. Saccade length in all directions was unaffected in almost half of the sessions. In the cases in which saccade length changed, the change was an increase in all directions except downward, during which the change was a decrease in length (during a right side injection). The mean saccade lengths for each direction per session can be seen **Fig. 16**.

Rightward: Saccade length was increased in 4/8 sessions (three left side injections and one right side injection), but exhibited no change during 4/8 sessions (two left side injections, and two right side injections). There was an increase in saccade length during the saline injection (right side injection). The changes in rightward saccade length can be seen in **Fig. 15**.

Upward: Saccade length was increased in 5/8 sessions (three left side injections and two right side injection), but exhibited no change during 3/8 sessions (two left side injections, and one right side injection). There was no change in saccade length during the saline injection (right side injection).

Leftward: Saccade length was increased in 5/8 sessions (four left side injections and one right side injection), but exhibited no change during 3/8 sessions (one left side injections, and two right side injection). Leftward saccade length increased during the saline injection (right side injection).

Downward: Saccade length was decreased in 3/8 sessions (two left side injections and one right side injection), increased during 1/8 sessions (left side injections), and exhibited no change in 4/8 sessions (two right and two left). Downward saccade length increased during the saline injection (right side injection).

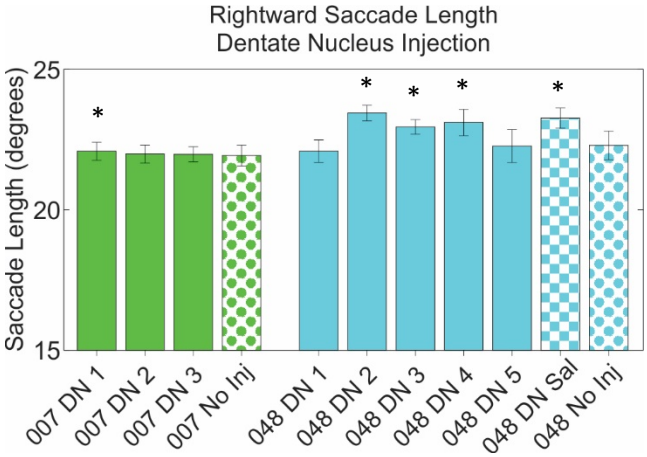

Figure 15: Marginal increases in mean rightward saccade length during dentate injection of muscimol. 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

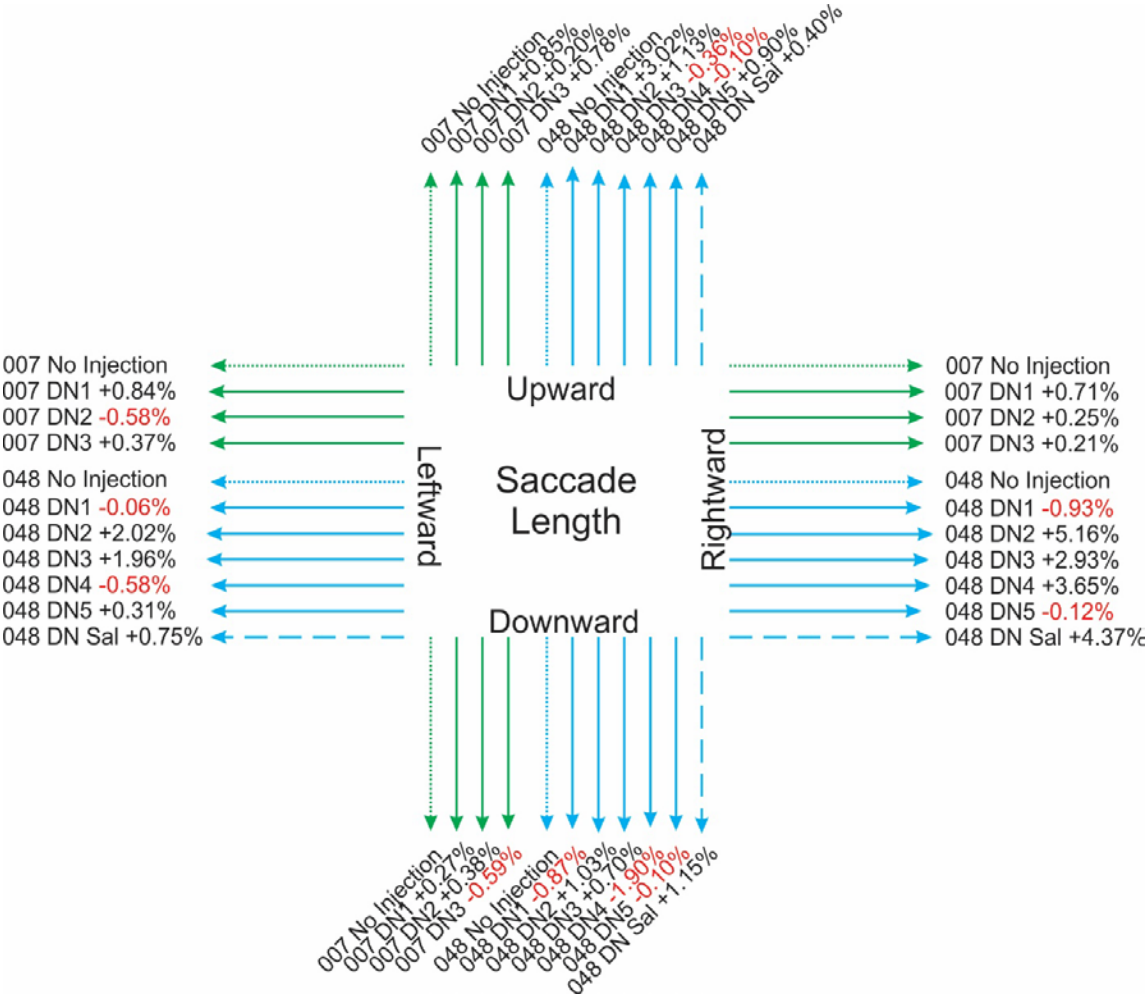

Figure 16: Summary of changes in saccade length during dentate injection of muscimol. 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

Effect on saccade dynamics: Peak velocity

Rightward: The peak velocity in the rightward was increased in 4/8 sessions (three left side injections and one right side injections) and exhibited no change in 4/8 sessions (two left side injections, tow right side injections). The rightward peak velocity increased during the saline session (right injection).

Upward: The upward peak velocity increased in 4/8 sessions (two of which were right side and two of which left side), decreased in 1/8 session (right side), and exhibited no change during 3/8 sessions (all left side). The upward peak velocity increased during the saline session (right side injection). These changes can be seen in **Fig 17**.

Leftward: The leftward peak velocity increased in 3/8 sessions (one left side, two right side), decreased in 3/8 sessions (two left side, one right side), and had no effect in 2/8 sessions (both left side injections). There was a decrease in leftward peak velocity in the saline session (right side injection).

Downward: The downward peak velocity increased in 3/8 sessions (two left side injections, one right side) and exhibited no change in 5/8 sessions (three right side injections, two right side). There was no change in the saline session (right side injection).

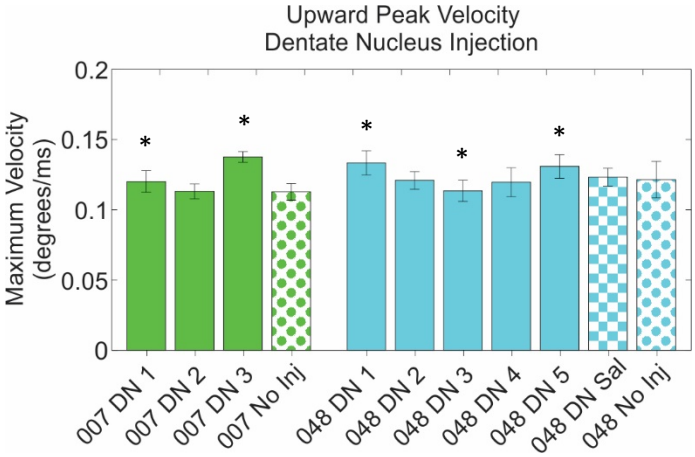

Figure 17: Occasional increases in upward peak velocities during dentate injection of muscimol. 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

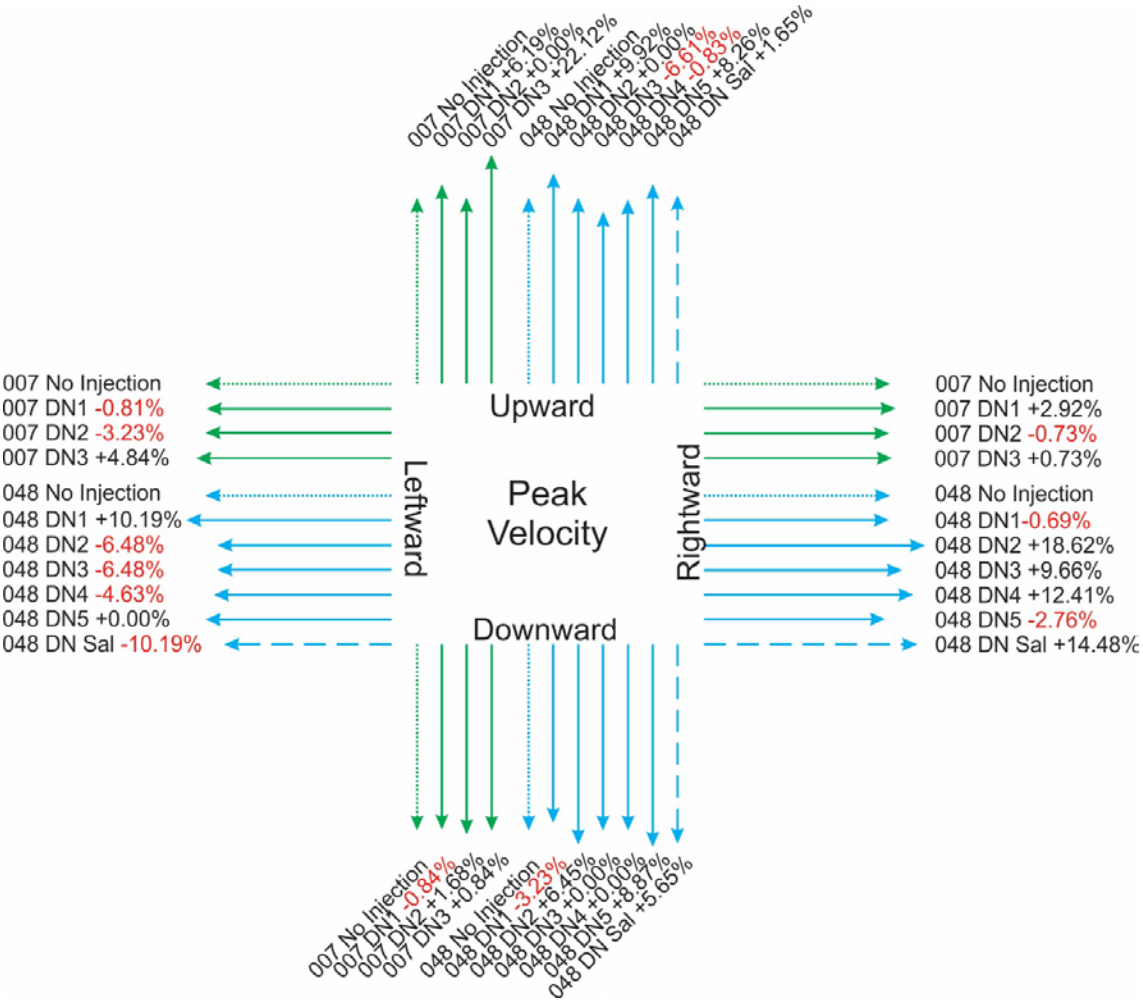

Figure 18: Summary of changes in peak velocity during dentate injection of muscimol. 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

Effect on saccade dynamics: Time to peak

Rightward: The time to peak velocity in the rightward direction was increased in 5/8 sessions (two right side injections, three left side) and exhibited no change in 3/8 sessions (one right side, two left side). There was no change in the rightward time to peak during the saline session (right side injection).

Upward: Time to peak increased in 2/8 sessions (one right injection, one left), decreased in 1/8 sessions (left side), and exhibited no change in 5/8 sessions (two right, three left). The saline session showed no change (right side injection).

Leftward: Time to peak increased in 4/8 sessions (three were left side, one was right) and exhibited no change in 4/8 sessions (two left and two right). There was an increase in the time to peak during the saline session (right side injection).

Downward: Time to peak in the downward direction increased in 5/8 sessions (two right side injections and three left side injections), and had no change in 3/8 sessions (one right side injection and two left side). The time to peak in the downward direction increased during the saline session (right side injection).

Effect on saccade dynamics: Deflection

Rightward: Deflection during saccades in the rightward direction increased in 4/8 sessions (all four were during left side injections), decreased in 1/8 sessions (right side injection), and show now change in 3/8 sessions (two left side injections, one right side). The deflection increased during the saline session (right side injection). Table 5 and Figure 7 show the changes of saccade deflection during muscimol injections into the dentate.

Upward: Deflection during saccades in the upward direction increased during 6/8 sessions (two right side, four left side) and exhibited no change in 2/8 sessions (one right and one left). There was also an increase during the saline injection session (right side injection). Changes in upward deflection can be seen in **Fig. 19**.

Leftward: Deflection during leftward saccades increased in 4/8 sessions (three left side injections, one right side) and exhibited no change in 4/8 sessions (two

left side injections, two right). Deflection increased during the saline injection session (right side injection).

Downward: Deflection during saccades in the downward direction decreased in 5/8 sessions (three right side injections, two left), increased in 1/8 sessions (left side injection), exhibited no change in 2/8 sessions (both left side injections), and increased during the saline injection session (right side injection).

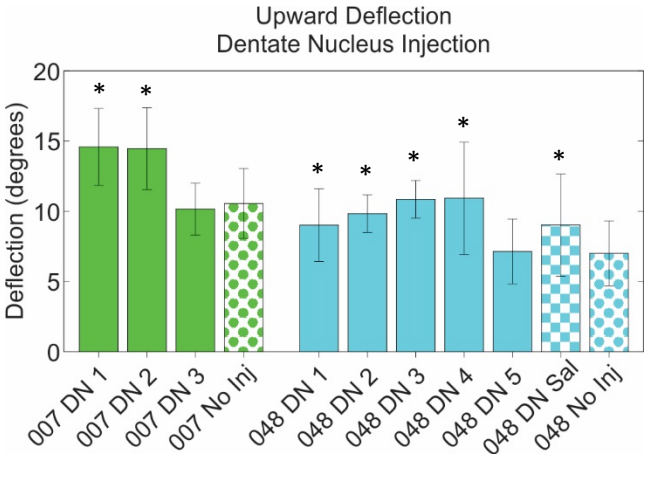

Figure 19: Regular increases in upward saccade deflection during dentate injection of muscimol. 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

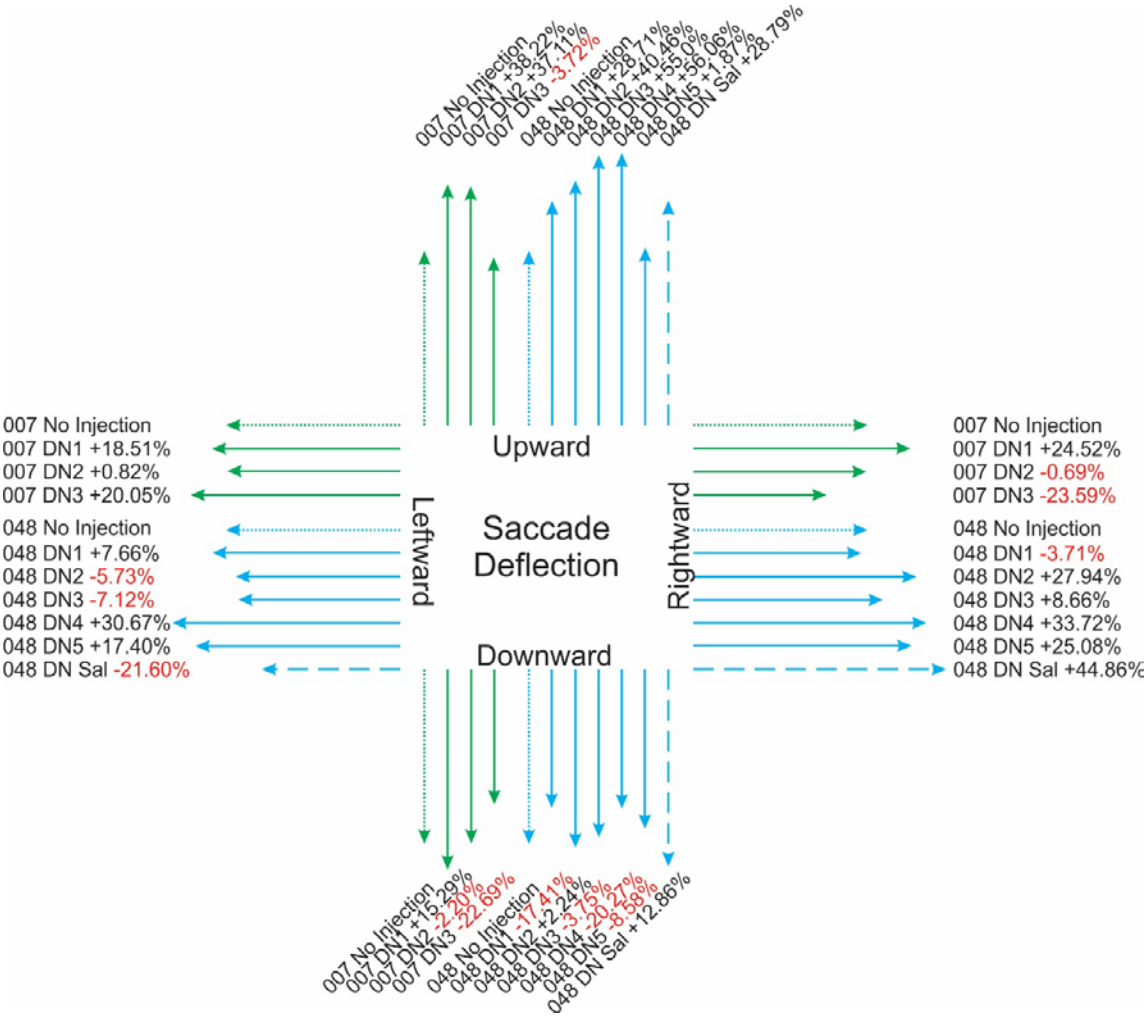

Figure 20: Increase in mean saccade deflection during dentate injection of muscimol. 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

Effect on saccade dynamics: Spread

Rightward: The lateral spread of saccades in the rightward direction increased in 3/8 sessions (all left side injection sessions), exhibited no change in 5/8 sessions (three right side injections, two left side), and increased during the saline session (right side injection).

Upward: The lateral spread of saccades in the upward direction increased in 5/8 sessions (two right side injections, three left side), exhibited no change in 3/8 sessions (one ride side injection, two left side), and increased during the saline injection session (right side injection).

Leftward: The lateral spread of saccades in the leftward direction increased in 3/8 sessions (all left side injections), exhibited no change in 5/8 sessions (two left side injections, three right side), and exhibited no change in the saline session (right side injection). These changes can be seen below in **Fig. 21**.

Downward: Downward saccades increased in only 1/8 session (left side injection) and also only decreased in 1/8 session (right side injection). There was no change in 6/8 muscimol sessions (two right side injections and four left side injections) nor the saline session (right side injection).

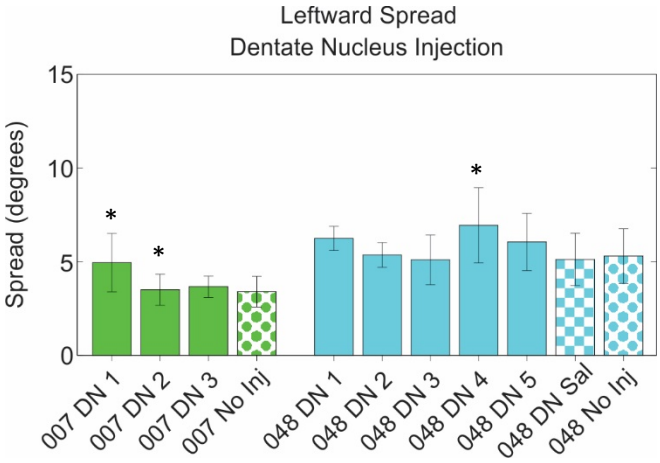

Figure 21: Limited increases in leftward saccade spread during dentate injection of muscimol. 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

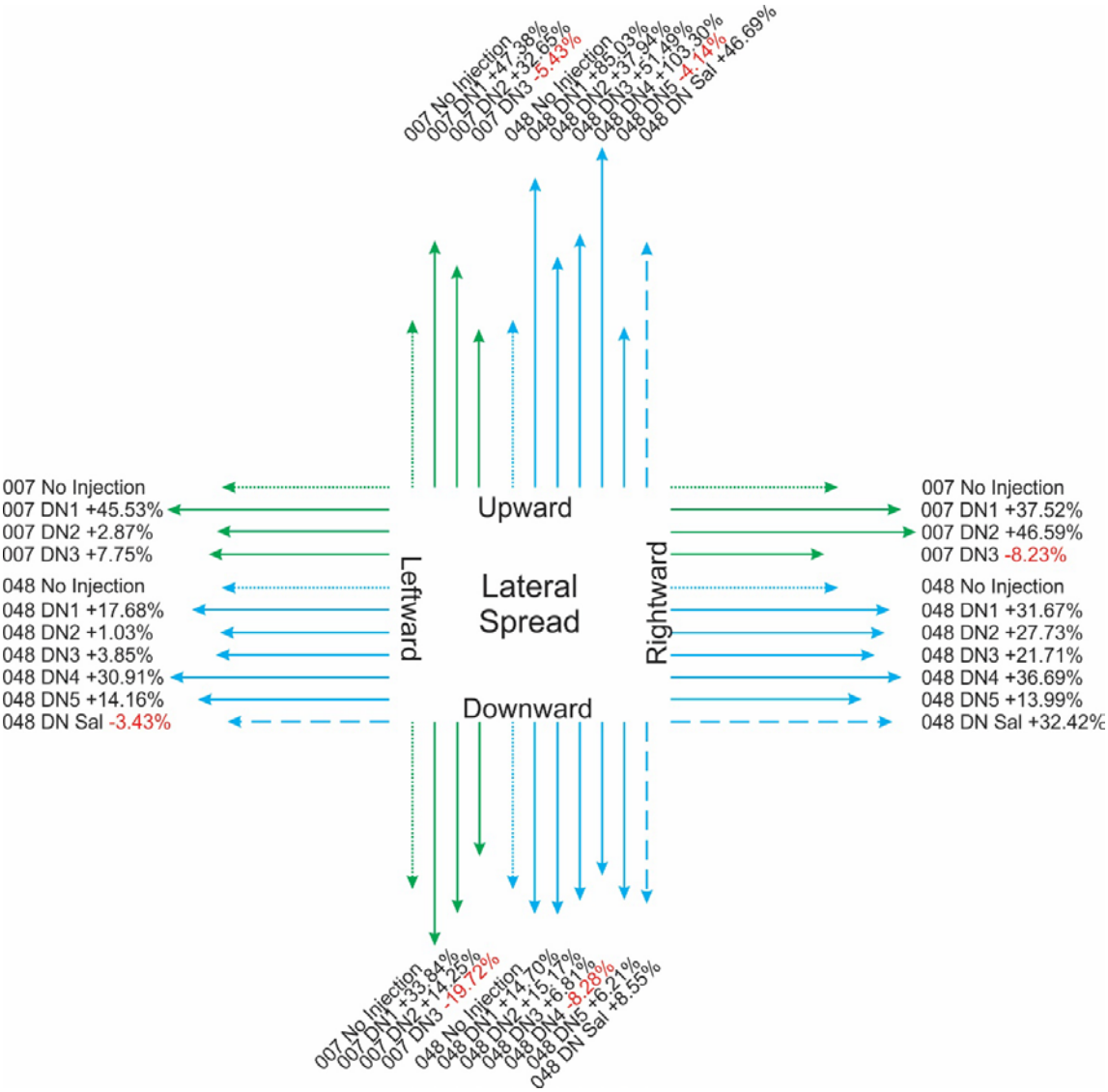

Figure 22: Increase in mean lateral spread during dentate injection of muscimol. 007 = monkey 007, 048 = monkey 048, DN # = muscimol injection session into the dentate nucleus #, DN Sal = saline injection session into the dentate nucleus, No Inj = data from non-injection sessions.

Discussion

Lesions and Neural Inactivation

One of the oldest techniques of brain research is to simply lesion or otherwise damage an organism's brain in a specific way and observe the resultant change in behavior. This approach dates back centuries and continues to be relevant in modern medicine, as human neurological disorders occur in patients who have experienced an accident or underwent a stroke. Two prominent cases in modern neuroscience are that of the frontal lobe obliteration of Phineas Gage and the double hippocampectomy of the epileptic HM. These types of studies comprise some of the most definitive loss-of-function experiments available.

One principal drawback of lesioning the brain is its irreversibility. The central nervous system, in general, does not regenerate from damage, so lesioning a neural structure one a "one time only" ordeal. This can be acceptable when simpler model systems are used because they are, for financial and logistical reasons, more easily used in large numbers, but this is generally not the case when working with non-human primates. Typical non-human primate studies only utilize 2-3 subjects, so the undertaking of reversible procedures is of paramount importance. Previous researchers have demonstrated the usefulness of muscimol injections to achieve a temporary lesion effect in non-human primates. Muscimol is a GABA agonist that binds to GABAA receptors, activating the signaling cascade of the most common inhibitory neurotransmitter, GABA. The use of sophisticated injection devices allows precise placement of small quantities of muscimol in the brain of non-human primate subjects, providing the ability to selectively inactivate restricted regions neural tissue. The effect of muscimol is temporary as it is eventually metabolized and flushed from the system, but while it is present it mimics the effect of a permanent loss-of-function lesion. This technique provides the tools necessary to undertake repeat loss-of-function lesion studies in the same animal across multiple sessions.

Dentate Nucleus and Saccades

The role of the dentate nucleus in saccades control is unknown, has never been studied in detail, and there are no published studies on the effects of lesioning or reversible inactivation on saccadic behavior The information we do have about the dentate and saccades has been largely gleaned from incidental observations in studies that were exploring other issues. We do know that there are projections from dentate to the frontal eye fields (Lynch, Hoover, & Strick, 1994) that provide a route through which it might influence saccades and that neurons in the lateral dentate respond to visual stimuli (Chapman, Spidalieri, & Lamarre, 1986; Marple-Horvat & Stein, 1990) which suggests that the structure might also be important for saccade control. Several past studies regarding the role of the cerebellum in saccadic eye movement have clearly implicated the dentate nucleus as a source of influence. Ron and Robinson (1973) were able to elicit saccades through electric stimulation of the dentate. The extremely short latency of their findings (~5 ms) suggests a monosynaptic projection to motor fibers. More recent studies, such as Uematsu, et al. (2017), have shown shortened latencies after microstimulation of the dentate nucleus. Other work from the same group (Omhae et al., 2013) has shown the dentate may be involved in the timing of saccades toward the predicted location of targets of interest. These findings corroborate some of the results we report here.

A recently published study (Rosini, et al., 2017) on saccadic abnormalities in patients with cerebrotendinous xanthomatosis , a metabolic disorder characterized by damage to the dentate nucleus, is the only work we are aware of that has focused primarily on the relationship between the dentate and saccades. The authors showed that the patients has less precise saccades with increased directional errors and longer latencies than normal controls, results that generally consistent with what we report.

One of the primary conclusions that can be drawn from these data is that few of the changes in the saccade dynamics were strongly consistent. Oftentimes an effect would be present in only ~50% of sessions, with the remaining ~50% of sessions showing no effect or, occasionally, the opposite effect as seen in the majority/plurality of sessions. This could be explained with a weak argument pertaining to the relatively large size of the nuclei when compared to the volume of neural tissue affected by the infusion of muscimol. Although the injection sites were chosen based on the activity patterns seen there, i.e. the presence of movement-related activity, it is possible that the likelihood of hitting the exact same location two sessions in a row is low. In a similar vein, the injection tube is much larger than the tungsten electrodes used for the neural recording. The tube, while albeit small profiled to have a sharp point, is still 3-5 times the diameter of the tungsten electrodes. That large diameter object barreling through the neural tissue could have killed off some of the targeted neural population. Furthermore, the profile of the injection tube tip was cut at an angle, much like a syringe, to facilitate traveling through the tissue. This profile raised the hole in the injection tube ~1mm dorsal of the very tip and added a directionality to the muscimol infusion relative to a flat, square cut tube end (see **Fig. 23** below), resulting in the muscimol missing the targeted neuron(s).

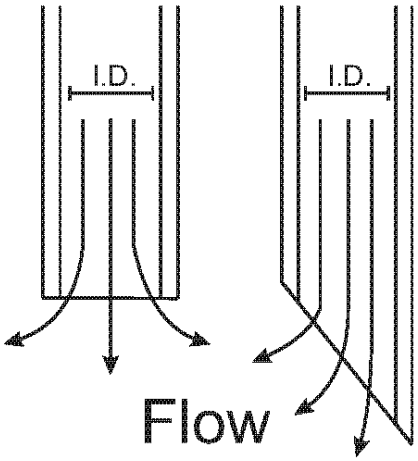

Figure 23: Injection tip flow pattern. Hypothetical square cut injection tip and resultant flow pattern (left) and actual angled cut injection tip and flow pattern (right). Not to scale.

When consistency in results does occur, most of the changes are increases in saccade dynamics, e.g. increase in saccade length, saccade velocity, time to peak, etc. The muscimol injections into the deep cerebellar nuclei should be inhibiting neural activity, so it might be counterintuitive to see increases in the saccade metrics. This counterintuitive result can be explained by recognizing that the majority of the cerebellum's output is inhibitory, and, thus, the inhibition of such activity via application of muscimol yields a net increase of activity: i.e., inhibition of inhibition.

When the laterality of injections of injection sites is considered, then a marginal pattern emerges from the effect of muscimol on the dentate nucleus. A higher proportion of effects are seen in the contra-versive/contralateral direction. Consider rightward saccade length. There were increases in 4/8 sessions. Of those four sessions, three of them were during injections into the left dentate and only one of which was in the right side. There was no change in the remaining four sessions, two of which were right side injections and two of which were left side injections. This suggests that the output of these cells in the dentate innervate areas that either suppress ipsi-versive saccadic eye movement or

promote contra-versive saccadic eye movement, and the inhibition of this inhibition ultimately promotes contra-versive eye movements.

In a general sense of time, movement, and velocity, it should be expected that these parameters should be linked. When movement time is the same, but saccade length increases, then we should expect an earlier time to peak and/or an increase in peak velocity.

Methodological Considerations

It was discovered after the experiments that apparent flushing of the injection apparatus was not a guarantee that the muscimol would be removed from the system. This was discovered when injecting colored dye into the second (M048) monkey's brain. Specifically, what was noticed after the dye was injected and the injection system was "flushed" in the same manner was in between muscimol injections (by running alcohol through the system followed by oil), was that a substantial amount of dye remained in the system. This implied that the "flushing" of the muscimol from the injection system might have been incomplete, and that extra muscimol could have contaminated later injections, in particular the saline injections.

A refresher on how the injection system apparatus: The injection system is comprised of a metal tube (functioning as the syringe that enters the monkeys' parenchyma) connected to a section of clear plastic tubing, which is in turn connected to a Hamilton syringe. The baseline condition of the system is to be fully loaded with silicon oil. Before conducting an injection, the metal tubing is dipped into the injection solution (either a muscimol solution or just saline) and the Hamilton syringe plunger is slowly drawn out at a rate of 0.2 µl per 30 sec, which will pull the injection solution first into the metal tubing and, subsequently, into the plastic tubing. The total volume drawn into the system is 10 µl or less.

Although only 2 µl are injected into the subjects per session, having extra solution in the system is preferable because the ease and consistency of injection needs to be tested (typically by undertaking at least one “dry run” of the injection procedure) before the sticking the needle into the brains. Having extra solution in the system is also nice in case the Hamilton syringe plunger is accidentally pressed while setting up or transporting the apparatus.

A remedy for insufficient flushing of the injection solution during future sessions would be to add a non-reactive dye or some other colorant to the injection solution (saline and/or muscimol) in order to monitor how much of it is left in the tubing. Additional sessions of injecting dyed muscimol/injection solution could be performed alongside the original injections of non-dyed muscimol to determine if the inclusion of the dye imparts any additional effect(s) on the monkeys’ behavior. If no effect is observed, then the dye could be confidently used to label injection solutions without fear of compromised results. If dying the injection solution is still too contentious, then dying the oil and/or alcohol could be a satisfactory alternative, otherwise all three of the possible solutions in the system are clear, and their borders are poorly visualized, if at all. Anecdotally, though, a dyed injection solution used in conjunction with clear oil provides the best contrast to gauge the presence of remaining injection solution in the system. This is primarily because of the confined space in which the injection solution-oil interface is present. The volume of silicon oil in the injection system outweighs the volume of injection solution by a factor of 10-50:1, and such large quantities of dyed solution could overwhelm the presentation of the otherwise clear injection solution.

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