

ACTIVATION OF THE SUBTHALAMIC NUCLEUS
DURING OBSTINATE PROGRESSION IN RAT PUPS

By

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To Katie, and to my family

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While the underlying anatomy of the connections between the basal ganglia and the midbrain are known, the function of their interaction is poorly understood. The present research interested itself with the contribution of direct projections from the subthalamic nucleus (STN) to the tegmentum. Excitatory projections of the STN share, as a target, the substantia nigra *pars compacta* (SNc) and the motor command center of the mesopontine tegmentum with the traditional output nuclei of the basal ganglia: the globus pallidus interna (GPi) and the substantia nigra *pars reticulata* (SNr). Projections from the STN to the midbrain are not abundant, but could be salient in neonates, in whom the inhibitory outflow of the basal ganglia may not be fully developed. Neonatal models of locomotor behavior, combined with assays of cell activity, may shed new light on the development of these neural mechanisms.

In the present experiment an immunohistochemical analysis of the immediate early protein, c-Fos, was performed as a marker for cellular activity in the brains of postnatal day 5 (PD5), PD15 and PD25 rat pups that engaged in decerebrate obstinate progression. Immunoreactivity was greater than age-matched controls in the STN, SNr, and lateral dorsal tegmental nucleus (LDT) at PD15 and PD25. Obstinate progression was exhibited by decerebrate PD5 rat pups, but c-Fos immunoreactivity in the basal ganglia and midbrain was not observed.

Three other age-matched treatment groups were assayed for c-Fos activity: rat pups that received bilateral electrolytic lesions of the GPe, rat pups that received bilateral microinjections of bicuculline (a GABA-blocker) into the subthalamus, and rat pups that received both lesions and microinjections. These manipulations were all intended to disinhibit the STN and increase locomotion. Only rat pups that received microinjections of bicuculline showed an increase in forward progression and c-Fos immunoreactivity in the subthalamus and tegmentum. From these results, it was concluded that elimination of inhibitory projections to the STN is not sufficient to produce obstinate progression. Rather, disinhibition of the SN and tegmentum contributes to the liberation of a network of feed forward circuitry in the brainstem, which results in the production of persistent locomotion.

CHAPTER 1 INTRODUCTION

Current understanding of the role of the basal ganglia during motor output is based largely upon what has been revealed from the etiology of disorders of the basal ganglia and models of these disorders in adult animals (Albin et al., 1989). It is apparent that, in adult animals, the basal ganglia and the cortex are intimately involved with one another in the production of motor behavior. However, it has also become increasingly clear that the basal ganglia directly reciprocate activation from several subcortical motor centers that are capable of activating behavior, independent of cortical involvement (McHaffie et al., 2005). This is demonstrated by animals that totally lack a forebrain, yet display a surprisingly rich repertoire of behavior. Bignall and Schramm (1974) showed that, following mesencephalic transections of kittens, many behaviors emerged at or earlier than the same behaviors in normal kittens. However, these behaviors were characterized by a lack of appropriate stimulus control; the kittens reacted to auditory stimuli by pouncing at non-existent objects, and running full speed off table-tops.

Subcortical forebrain structures appear to be important for the establishment of stimulus control of caudally organized behaviors. In rats, unilateral lesions of the nigrostriatal pathway, which interrupt normal striatal contributions to caudal motor centers, result in both motor and sensory asymmetries, indicating that the basal ganglia are involved in sensorimotor integration and not strictly motor output (Schallert et al., 1982). Similarly, patients with Parkinson's disease, whose motor symptoms are thought to result from reduced nigro-striatal projections, also show sensory-modulated deficits in advanced stages of the disease, such as "freezing" when turning or approaching narrow spaces (Okuma, 2006). Thus, the general picture that emerges is that much of species-typical behavior is organized in the midbrain, but that subcortical forebrain structures, particularly the striatum, contribute importantly to sensorimotor integration.

In neonates, cortical projections are immature. Models of locomotion during early development provide an accessible means of investigating the organization of behavior sub-cortically. One such model utilizes systemic injections of L-3, 4-dihydroxyphenylalanine (L-DOPA). In rat pups, this elicits persistent, very regular locomotor behavior at birth (PD0) through postnatal day twenty-two (PD22) to PD25 (Iwahara et al., 1991; Stehouwer et al., 1994; Grigoriadis et al., 1996). Rat pups that engage in L-DOPA-induced locomotor behavior express dramatic c-Fos immunoreactivity in all areas of the basal ganglia except the substantia nigra *pars compacta* (SNc) and the striatum. The area of densest cell-labeling is the subthalamic nucleus (STN; Staup and Stehouwer, 2006). Interestingly, rat pups with complete transections rostral to the STN and caudal to the striatopallidal complex exhibit automaton-like forward locomotion similar to L-DOPA-induced locomotor behavior. This behavior has been termed ‘obstinate progression’ because, like the behaviors of kittens studied by Bignall and Schramm (1974), it is marked by a stereotypical appearance and persistence in the face of obstacles (Campbell and Stehouwer, 1979; cf. Mettler, 1940; Bailey and Davis, 1942). The continual, patterned locomotor behavior that is observed following decerebration, and after injection of L-DOPA, is in sharp contrast to the paucity and variability of locomotion that is typical of intact, untreated neonates (Altman and Sudarshan, 1975; Cazalets et al., 1990). These manipulations reveal an interaction between the STN, the rest of the basal ganglia, and more caudal brain regions that may represent a common mechanism underlying their similar behavioral outcomes.

The STN occupies a central position in basal ganglia circuitry, intercalated along the rostral-caudal axis between the globus pallidus interna (GPi) and substantia nigra *pars reticulata* (SNr), two nuclei that are traditionally abbreviated together as the primary output nuclei of the basal ganglia. The STN sends excitatory, glutamatergic projections to these nuclei. The GPi/

SNr then make inhibitory, GABA-ergic connections in the forebrain, thalamus, tectum, and brainstem (Parent et al., 2000; Smith et al., 1998).

The striatum is the main receiving center of the striatum, integrating information from not only the cortex, but from the thalamus, brainstem, and other nuclei of the basal ganglia as well (McHaffie et al., 2005). Traditionally, the striatofugal system has been separated along two routes: the direct pathway and the indirect pathway. The direct pathway sends GABA-ergic projections immediately to the GPi/SNr, whereas projections of the indirect pathway synapse first in the globus pallidus externa (GPe). The postsynaptic projections from the GPe then modulate GPi/SNr-activity either straight away, or via inhibitory control over STN neurons, which reciprocate input from the GPe, and whose collaterals find as their targets the GPi/SNr. The direct pathway is thought to originate primarily from GABA-ergic medium spiny cells that co-express the peptide neurotransmitters, dynorphin and substance P, and where excitatory, D1-dopamine receptors are localized. In contrast, the medium spiny neurons of the indirect pathway co-express enkephalin, and are dense with inhibitory, D2-dopamine receptors (Smith et al., 1998; Grillner et al., 2005). However, it has become clear that the pathways of the basal ganglia are not so austere. There is evidence that D1 and D2 receptors are expressed by medium spiny cells of both the direct and indirect pathways, and that many of these neurons project to both the GPe and the GPi/SNr, while other striatal projections make their way to areas outside the basal ganglia, as far caudal as the tegmentum (Parent and Hazrati, 1995a; Winn et al., 1997; Parent, 2000).

In the mesopontine tegmentum of the rat, there is a less discrete area of the brain known as the 'mesencephalic locomotor region' (MLR). The boundaries of this area of the midbrain are functionally defined by the ability of chemical or direct electrical stimulation to elicit forward

progression (Garcia-Rill et al., 1986b). The exact coordinates of the MLR vary between species. In the rat, the MLR consists of a collection of nuclei, most notably the pedunclopontine nucleus, but also including portions of the lateral parabrachium and cuneiform nucleus (Skinner and Garcia-Rill, 1984, Garcia-Rill, 1991). Among the species studied, the area most commonly associated with the MLR has been the caudal *pars compacta* portion of the pedunclopontine (PPNc). The presence of an MLR in species as diverse as lampreys (Sirota et al., 2000), primates (Castiglioni et al., 1978), and cats (Shik et al, 1966), with similar connections to the basal ganglia and medulla suggests that this brain region has been conserved throughout evolution. There is growing evidence of a similar locomotor command center in humans (Hathout and Bhidyahasiri, 2004; Lee et al., 2000).

Many studies of the rat brain have traced connections between the basal ganglia and tegmental areas in and around the parabrachium, where the PPNc/MLR is located (Garcia-Rill et al., 1986a; Semba and Fibiger, 1992). The presence of direct input from extrapyramidal nuclei varies within this small region, where heterogeneous cell types of several nuclei are interdigitated with one another (Mesulam, 1989; Inglis and Winn, 1995). The ill-defined boundaries of these nuclei make it difficult to correlate structures and functions. In rats, the area of the tegmentum that is reported to share the greatest abundance of reciprocal connections with the basal ganglia is a collection of primarily non-cholinergic nuclei just medial to the lateral boundary of the PPNc. Some authors have included this region as part of the PPN, while others have distinguished it as the ‘midbrain extrapyramidal area’ (MEA; Hallanger and Wainer, 1988; Lee et al., 1988; Steininger et al., 1992).

Stimulation of areas medial to the PPNc and the MEA, around the locus coeruleus (LC) and the lateral dorsal tegmental nucleus (LDT), have also been shown to elicit locomotion

(Garcia-Rill, 1988; Jordan, 1998; Laviolette, 2000). Although some consider the LDT and the PPNc two limbs of the same midbrain cholinergic activating complex, the LDT is not included as part of the traditional, 'lateral' MLR (Woolf and Butcher, 1986). The classic, lateral PPNc/MLR projects first to the medial reticular formation and then bilaterally along the ventrolateral funiculus of the spinal cord. In cats, a 'medial' MLR has been characterized based upon its projections to the medulla and spinal cord (Shefschik et al., 1984; Steeves and Jordan, 1984). The projections of the medial MLR are thought to correspond to the pontomedullary locomotor strip (PLS), which synapses diffusely in both medial and lateral areas of the reticular formation. The postsynaptic neurons of the medial PLS/MLR then send their projections from the medulla ipsilaterally along the dorsolateral funiculus of the spinal cord (Noga et al., 1991; Whelan, 1996). However, it is not entirely clear whether the medial PLS/MLR corresponds to a region similar to the MEA in rats, to the LDT, or both.

The predominant contribution from the basal ganglia to the midbrain is GABA-ergic (Semba and Fibiger, 1992; Steininger et al., 1992). The GPi/SNr exercise tonic inhibitory control over several motor command centers (Grillner et al., 2005). Microinjections of GABA-blockers into the MLR disrupt this tonic inhibition, inducing locomotion in adult rats (Garcia-Rill, 1985; Milner and Mogenson, 1988). Though the extent of direct, glutamatergic innervation of the tegmentum by the STN in adult rats is unclear, at least a meager projection of fibers from the STN reaches the PPNc, capable of depolarizing it (Takada et al., 1988; Granata and Kitai, 1989). However, the primary targets of the STN remain the GPe and the GPi/SNr (Kita and Kitai, 1987; Robledo and Fèger, 1990).

There are several cortical and subcortical circuits that loop through the striatopallidal complex and the STN, balancing inhibition and excitation to modulate inhibitory output from the

GPi/SNr to behavioral centers (Parent and Hazrati, 1995a; McHaffie et al., 2005). Although there is scarce evidence of a direct striatosubthalamic pathway, the GPe serves as an intermediary between the striatum and the STN (Canteras et al., 1990). Elimination or inactivation of the GPe increases the firing rate of cells in the STN, demonstrating the inhibitory control that the GPe exercises over the STN (Hassani, 1996; Ryan and Clark, 1992). This cascade of inhibitory projections (fig. 1-1) agrees with the Jacksonian view that subcortical forebrain areas inhibit caudal centers involved in behavioral activation (e.g. Campbell, 1969; Moorcroft, 1971). The STN is an important exception to this rule, providing a depolarizing influence to the caudal substantia nigra and PPNc/MLR (Canteras et al., 1990; Inglis and Winn, 1995; Woolf and Butcher, 1986). Disinhibition of the STN in adult rats by means of pallidal lesions or direct application of GABA-antagonists increases neuronal activity in the STN and its target structures, but an increase in locomotion, such as with decerebrate obstinate progression, has not been reported among the many behavioral effects (Robledo and Feger, 1990; Ryan and Clark, 1992; Jeljeli, 1999; Periér et al., 2002). It is unclear, however, whether the same procedures would fail to elicit locomotion in young rat pups.

Although L-DOPA-injected PD15 rat pups exhibit an increase in expression of c-Fos immunoreaction product in the STN and GPi/SNr, it is without apparent inhibition of the PPNc/MLR, where an increase in c-Fos is also evident. Administration of L-DOPA does not elicit locomotion or an increase in c-Fos expression in the midbrain of PD25 rat pups, suggesting the MLR may be more susceptible to excitation at PD15 than at PD25. Local delays in the maturation of inhibitory strength may produce just such a developmental effect, allowing direct projections from the STN to the PPNc/MLR to have a greater influence on the behavior of neonates than adults (Staup and Stehouwer, 2006). This may result from the absence of fibers or

synapses that have yet to develop, or the influence of exuberant connections on local circuits before synapse withdrawal. It is also possible that cells in the midbrain of rat pups have a less negative equilibrium potential for Cl⁻, compromising the inhibitory action of ionotropic GABA receptors, or even producing excitation (Clayton et al., 1998; Rivera et al., 1999). Whatever the mechanism may be, immature GABA-activity in the midbrain of rat pups would facilitate the effect of excitatory projections to that area of the brain. This is consistent with a body of work that has demonstrated the inhibitory influence of cholinergic and serotonergic neurotransmitter systems in the brainstem of rats does not reach maturity until late in the third postnatal week (Fibiger, 1970; Mabry and Campbell, 1974). The action of the reticular activating complex operating unchecked by forebrain inhibitory centers is thought to be reflected in the high level of spontaneous locomotor activity exhibited by rat pups around PD15 (Campbell, 1969; Moorcroft, 1971). Weak inhibitory projections from rostral brain areas to caudal motor centers may also provide a necessary background against which L-DOPA is able to elicit persistent locomotor behavior in rat pups (Staup and Stehouwer, 2006). Due to the similarity of behaviors expressed by decerebrate and L-DOPA-injected neonates, it has been suggested that L-DOPA treated rat pups may be functionally decerebrate (Stehouwer and Van Hartesveldt, 2000).

In this study, complete pre-subthalamic /post-pallidal transections were performed to replicate the scarce behavioral data that is available on decerebrate obstinate progression in rat pups, and to provide a more quantitative analysis (Campbell and Stehouwer, 1979). This study also sought to extend the available knowledge of obstinate progression by performing an assay of cellular activity on the intact, caudal brain regions of decerebrate rat pups. It was hypothesized that the transections would disinhibit the STN, and that this disinhibition would be represented by a similar high level of c-Fos immunoreaction product as has been exhibited in the STN of L-

DOPA-injected rat pups (Staup and Stehouwer, 2006). Furthermore, any disinhibition of the STN that produces enough summation of excitation in the PPNc/MLR to overcome opposing inhibition should result in locomotion. Toward this end, bilateral electrolytic lesions of the GPe were performed on rat pups to disrupt inhibition of the STN, while leaving the inhibitory influence of the GPi/SNr over the midbrain locomotor nuclei intact. Another group of rat pups received bilateral microinjections of bicuculline (a competitive GABA-antagonist) to the STN, disrupting inhibition of the STN without eliminating other influences of rostral brain structures. A final experimental group received both microinjections of bicuculline to the STN and lesions of the GPe. It was anticipated these manipulations would produce locomotion in rat pups where they have not in adults because of the greater ease of activation of the PPNc/MLR in neonates, which has been demonstrated with the L-DOPA preparation (Staup and Stehouwer, 2006).

The manipulations were performed at three ages: PD5, PD15 and PD25, in three separate groups of rat pups. The behavior of these rat pups immediately after recovering from surgery was compared to that of sham-treated rat pups from the same age groups. The first two age groups were chosen based on a previous report that decerebrate obstinate progression was not observed in PD5 rat pups, but was prevalent at PD15 and PD20 (Campbell and Stehouwer, 1979). Instead of PD20 rat pups, though, PD25 rat pups were chosen in order to test whether decerebrate obstinate progression disappeared or attenuated at that age, as seen with L-DOPA-induced locomotor behavior (Staup and Stehouwer, 2006). It was hypothesized that, at PD15, the direct excitatory impact of increased STN activity on the midbrain locomotor nuclei would be greater than the indirect inhibitory influence provided by the GPi/SNr, and that a greater increase in locomotion would be observed as a result of the experimental manipulations at PD15 than at PD5 or PD25.

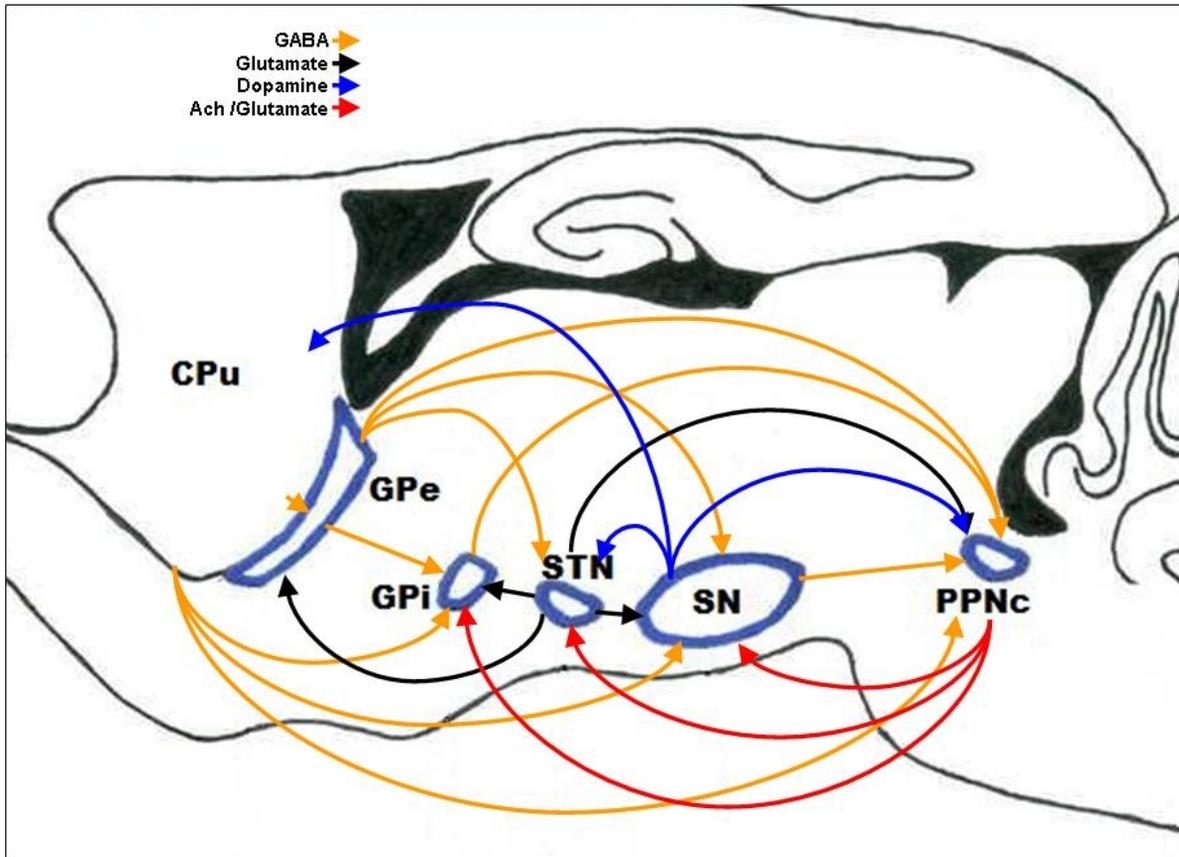


Figure 1-1. Subcortical circuitry of the basal ganglia and tegmentum. This circuit diagram illustrates the powerful inhibitory influence (yellow arrows) the striatopallidal complex exercises over more caudal, excitatory circuits in the intact rat: caudate-putamen /striatum (CPu); globus pallidus externa (GPe); globus pallidus interna (GPi); subthalamic nucleus (STN); substantia nigra (SN); pedunculopontine *pars compacta* (PPNc).

CHAPTER 2 MATERIALS AND METHODS

Subjects

A total of 95 Sprague-Dawley rats born at the University of Florida were used for this experiment. Dams and sires were paired for a week and then separated. Throughout the third week being apart from the sires, dams were checked twice daily for new pups. Pups were considered PD0 on the day they were born. On PD5, litters were culled to nine pups with at least four males and four females in each litter, when possible. The pups were tested on PD5, PD15 or PD25, at which time they were assigned to one of five treatment groups: transection, microinjection, lesion, microinjection and lesion, and sham. The sample size for each age/treatment group is given in Table 2-1.

With the exception of pre-subthalamic transections, which were performed prior to the other treatments, each treatment group contained at least one subject from a given litter, and a minimum of two litters were represented in each treatment group. Data for the transected animals were obtained before testing the other surgery groups in order to localize the area in the subthalamus of greatest c-Fos reactivity at each age. These data were then used to determine where to inject bicuculline in subsequent treatment groups.

Surgeries

General Surgical Procedures

For stereotaxic surgeries, the top of the tooth bar and the center of the ear bars were in the same horizontal plane. Because there were no published data with regard to the stereotactic coordinates of either the STN or the globus pallidus in neonatal rat pups at the ages we performed our surgeries, the initial coordinates were interpolated from Sherwood and Timiras' *A Stereotaxic Atlas of the Developing Rat Brain* (1970) and the fourth edition of Paxinos and

Watson's *The Rat Brain in Stereotaxic Coordinates* (1998). Final coordinates were empirically derived during preliminary research.

On the day of surgery, rat pups that received transections were anesthetized in a bell jar with a 15% halothane/ 85% mineral oil, volume-to-volume mixture. During surgery, anesthesia was maintained with a nose cone that contained cotton balls soaked in the same halothane/oil mixture. For the lesion and microinjection treatment groups, animals were anesthetized in a Plexiglas container ventilated by a Surgivet Classic T³ isoflurane vaporizer with 3% - 4% isoflurane in oxygen at a flow rate of 1L/min. Depth of anesthesia was verified by testing the pedal withdrawal reflex. During surgery, isoflurane was delivered through a nose cone attached to the tooth bar of the stereotaxis at a concentration of 1% - 2% in oxygen at a flow rate of 1L/min.

For P15 and P25 rat pups, an incision approximately 6-8mm in length was made along the midline of the scalp, after they were secured in the stereotaxis. For PD5 rat pups, a midline incision, extending from their eyes to the base of their skull, was made before placing them in the stereotaxic instrument. The scalp of the PD5 rat pups was then separated from the underlying connective tissue and deflected laterally and ventrally until the ear canals were exposed. The PD5 rat pups were then secured in a Stoelting Mouse and Neonatal Rat Stereotaxic Adaptor, with ear bars that were placed directly in the cartilaginous portion of the external meatus, being careful not to rupture the ear canal (Cunningham and McKay, 1993). Cold anesthesia is frequently used with new born rat pups because they recover well from drops in body temperature. In the present study, cold anesthesia was not used, but surgery on PD5 rat pups was performed without a heating source. For PD15 and PD25 rat pups, a heating pad was placed underneath of the surgery area to keep them warm.

Transections

Transections were performed freehand rather than stereotaxically. A burr-hole approximately 2mm in diameter was made in the skull of rat pups over the anterior margin of the superior colliculus. In PD5 rat pups the hole was located 2mm caudal to the bregma suture. In PD15 and PD25 rat pups the hole was located 3mm caudal to bregma. Burr-holes were set 1 to 2 mm lateral to the midline, over the left hemisphere of all subjects, to avoid rupture of the superior sagittal sinus. A sterilized, stainless steel, curved knife was inserted along the midline to the ventrum of the brain case, keeping the curve of the blade in the coronal plane. The knife was withdrawn, rotated 180°, and the procedure was repeated on the contralateral side.

Electrolytic Lesions

The skulls of animals that received lesions of the globus pallidus were trephined on both sides of the midline to allow bilateral access to the targeted nuclei. The location of the burr-hole was determined according to the following coordinates for each age: PD5 (A +1.3mm to +3.1mm, L \pm 2.2mm to \pm 2.5mm), PD15 (A +3.8mm, L \pm 3.4mm), PD25 (A +4.1mm to +5.3mm, L \pm 2.3mm to \pm 3.3mm). Stainless steel electrodes .38 mm in diameter insulated but for 0.3mm at their tip, were connected to a power source and lowered through the burr hole into the brains of the rat pups. Because the globus pallidus is oblong, and the main body of the nucleus extends further along the dorsal-ventral axis than along the mediolateral axis, lesions were performed at three levels in PD15 and PD25 rat pups, and at two levels in PD5 rat pups. The ventral coordinates for each level, as well as the intensity and duration of the current used, were as follows: PD5 (V+3.3mm to +3.8mm at 1.5mA for 8s, V+2.3mm to +2.8mm at 2mA for 10s), PD15 (V+4.2mm at 1.5mA for 10s, V+3.7mm at 1.5mA for 15s, V+3.3mm at 2mA for 15s), PD25 (V+4.7mm at 1.5mA for 10s, V+4.1mm at 1.5mA for 15s, V+3.6mm at 2mA for 15s).

Intracranial Injections

The burr holes of rat pups that received microinjections of bicuculline were located according to the following coordinates for each age: PD5 (A +0mm to +1.5mm, L \pm 1.5mm to \pm 1.7mm), PD15 (A +1.8mm, L \pm 2.2mm), PD25 (A +2.9mm, L \pm 2.3mm). Trypan blue was dissolved in saline, which was then used to create the bicuculline solution. Injections of 0.37 μ g bicuculline / μ L saline were delivered through a 30g injection cannula attached to a 10 μ L #701 Hamilton syringe, which was itself attached to an arm of the stereotaxic instrument. Injections were given over a period of 2-3 minutes, using the following ventral coordinates and volume for each age: PD5 (V +1.0mm to +2.0mm / 1.2 μ L to 1.5 μ L), PD15 (V +2.4mm to +2.6mm / 2 μ L), PD25 (V +3.1mm / 2 μ L). The needle was left in place for one minute following completion of the injection before being removed slowly.

Microinjection and Lesion

For rat pups that received both intracranial injections of bicuculline to the STN and electrolytic lesion of the globus pallidus, surgeries were performed exactly as described above. Microinjections were always performed immediately after creating the lesions, so that drug diffusion would be approximately the same in all subjects during behavioral testing.

Sham Surgeries

Control animals were anesthetized and placed in a stereotaxic instrument. Their scalps were incised and their skulls were trephined in the same manner as the rest of the subjects in their age group, but they did not receive either injections or lesions.

At the end of each surgery, scalp incisions were sutured and subjects placed in an incubated (30°C) recovery chamber while they emerged from anesthesia. Bleeding during surgeries was minimal, and normally stopped by the time the sutures were complete. After demonstrating the

ability to support their own body weight, or once their limbs began alternating (normally a period of 10 to 20 minutes), rat pups were transferred to an arena for behavioral testing.

Behavioral Testing and Analysis

Fifteen and twenty-five day old rat pups were placed in a Plexiglas arena (25 cm high, 55 cm diameter) on a Formica tabletop. To roughly scale the test apparatus to the size of the subjects, five day-old rat pups were tested in a 29½ cm high Nalgene cylinder with a diameter of 30 cm, in an incubator that maintained an ambient temperature between 30°C and 32°C. The test arenas were wiped with a 3% bleach solution following the test of each animal.

The rat pups were videotaped in the arena for 45 minutes. This tape was later used to determine the duration of the behavioral testing session that each subject spent engaged in forward progression and limb alternation. Each test session was divided into three fifteen minute bins to control for abbreviated or otherwise time dependent effects of our manipulations. Using software developed in our lab for the Apple IIe computer, key strokes indicated the onset and the offset of each bout of locomotion and limb alternation.

To assess possible differences in speed between subjects, the image of the arena on the video screen was separated into five equal areas: four sectors around the perimeter and a circular one in the center. In a separate analysis from the measurements of duration, a blind observer recorded the number of times each subject crossed between sectors during their 45 minute testing session. A sector cross was counted when the nose of the rat pup passed the boundary of a sector. This provided us with a relative measure (within age groups) of distance covered during forward progression. Because of growth of the rats and the difference in the sizes of testing chambers used, comparisons of sector crosses across age groups were not made. As with duration, sector results were also divided into fifteen minute bins.

Histology

Preparation of Tissue

After behavioral testing, rat pups were deeply anesthetized with sodium pentobarbital and perfused intracardially with heparinized 0.15 M NaCl, followed by sodium phosphate buffered 10% paraformaldehyde. Brains were removed and post-fixed in a 20% sucrose/10% paraformaldehyde solution. Whole brains were then cut alternately into 80 μ m and 40 μ m sagittal sections with a vibratome.

Transection, Lesion and Microinjection Verification

Sections 80 μ m thick were immediately mounted on slides, dehydrated and cover-slipped. Gross examination was used to exclude subjects with incomplete transections. Subjects whose transections ended caudal to the STN were also excluded from the study.

Images of these sections were projected onto a digitizing tablet that provided a resolution of 29.85 μ m, and the lesions were traced to determine their area. Lesion area was recorded from the sagittal section where the lesion was largest, representing the central part of the lesion. If this section was outside the medial or lateral boundaries of the globus pallidus, the subject was excluded. Subjects whose lesions extended further beyond the globus pallidus than the area of the globus pallidus which was destroyed were also excluded from the study. If there was extensive damage to the internal capsule (i.e. if the nerve tract was completely bisected), those subjects were eliminated in order to minimize any confounding effects of disrupting projections from cortical or more rostral subcortical brain structures.

For microinjections, subjects in whom the injection was placed above the medial lemniscus or outside the internal capsule, which provide the boundaries of the subthalamus, were excluded. Subjects were also excluded if the serial section where the ventral tip of the cannula tract was

found was more than .64 mm medial or lateral to the STN, beyond which trypan blue indicated the drug diffused predominately into areas outside the subthalamus.

Immunohistochemistry

Sections cut at 40µm were rinsed in potassium phosphate buffered saline (KPBS) and incubated with c-Fos antibody (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA) in 0.4% Triton X-100 in KPBS for 48 hr at 4°C. The sections were then rinsed, again with KPBS, and placed in biotinylated goat anti-rabbit IgG (Zymed, San Francisco, CA) for four hours at room temperature. After this, the sections were rinsed a third time with KPBS and placed at 4°C in avidin-biotinylated peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA) overnight. Finally, sections were rinsed in KPBS and placed in sodium phosphate buffer containing 0.03% diaminobenzidine, 0.008% nickel ammonium sulfate, 0.008% cobalt chloride, and 0.0075% hydrogen peroxide. Stained sections were mounted on slides, dehydrated and cover-slipped.

C-Fos Analysis

In the tegmentum, labeled cells were counted in the PPNc and the lateral dorsal tegmental nucleus (LDT). Labeled cells were also counted from areas of the basal ganglia (STN and SNr), as well as from areas of the subthalamic locomotor region: the zona incerta (ZI) and lateral hypothalamus (LH). These nuclei were located with reference to the rat brain atlases of Sherwood and Timaris (1970), and Paxinos and Watson (1998). Nuclei were counted from the sections that stained most densely. Slides were coded randomly and scored by a blind observer.

Sections were viewed under a light microscope at 40X. The images were captured with a Sony video camera (model DXC-151) and displayed on a 275mm x 200mm Sony Trinitron monitor. Every cell on the screen was included in the analysis of each brain area. Labeled cells were gray and smoothly rounded, elliptical in appearance. Clusters of cells had regular,

scalloped edges, allowing the individual cells to be counted. Material that was much darker or much lighter than labeled cells, and had irregular, ragged edges were considered to be an artifact and not counted.

Data Treatment

To minimize the relationship between the means and the standard deviations of the behavioral data, a $\log(X + 1)$ transformation was used. Because body lengths of the subjects and the dimensions of the arena differed between ages, which would confound comparisons of distance data between ages, our analysis of sector crosses was limited to the effects of our experimental manipulations within age groups. A two-way (treatment X age) ANOVA, with an α -criterion of 0.05, was performed for sector crosses to investigate the possibility of an interaction between the independent variables. One-way ANOVAs, with an α -criterion of 0.05, were performed for treatment effects on number of sector crosses at each age for each 15-minute bin, and for the entire forty-five minute testing session. Dunnett's method of posttest comparisons was used for the one-way ANOVAs, with sham-treated rat pups as the controls.

Two-way (treatment X age) ANOVAs, with an α -criterion of 0.05, were performed for measures of timed locomotor behavior for the entire forty-five minute testing session, and for each 15-minute bin. Two analyses of these data were performed. One analysis was only of the time spent engaged in forward progression, while another analysis included time spent lying on their side with their limbs alternating. Pairwise comparisons were made using Tukey's HSD method.

For measures of the duration of limb alternation, a three-way (treatment X age X bin) mixed analysis of variance with bins as the within subjects comparison and age and treatment as the between subjects comparisons was performed. These were followed up by one-way repeated measures ANOVAs for each age and for each treatment group.

Due to differences in cell size and glial proliferation between ages, our immunohistochemical results were also limited to a one-way analysis of treatment effects. We used Bartlett's test to determine if the samples from each data set showed unequal variances. If Bartlett's was significant, the non-parametric Kruskal-Wallis test was used with Dunn's posttest comparisons. For data whose sample variances were equal, one-way ANOVAs were performed with Dunnett's method, using sham-treated subjects as controls. These analyses were performed at each age for treatment effects on the expression of c-Fos in the STN, LH, ZI, SN, PPNc, and LDT averaged between both hemispheres.

Table 2-1. Experimental design and sample sizes

Age	Pre-STN transection	STN microinjection	GPe lesion	STN Injection + GPe lesion	Sham operations
5 days	n=10	n=5	n=2	n=4	n=9
15 days	n=10	n=7	n=4	n=3	n=9
25 days	n=9	n=4	n=5	n=7	n=7

This table demonstrates the experimental design and sample size for each group.

CHAPTER 3 RESULTS

Histological Verification

Transection

Every transection was judged to be complete. Figure 3-1 shows the level of the most rostral and the most caudal transection at each age. The ventral level of each transection was rostral to the subthalamic nucleus and caudal to the GPe. The dorsal levels of the transections were consistently pre-collicular, rarely eliminating the entire thalamus, regularly leaving the posterior thalamic nuclei intact.

Microinjection

Injection sites for rat pups that received microinjections alone are shown in Figure 3-2. Injection sites for rat pups that received microinjections with lesions are shown in Figure 3-4. Most injections did not penetrate the STN. However, our volumes were large (1.5-2 μ L). Upon sectioning the tissue, trypan blue was observed to have diffused 1-2 mm from the injection site, encompassing the STN. Our injections also encroached upon areas neighboring the STN, including the zona incerta (ZI) and the lateral hypothalamus (LH), as well as the SNr. Despite the presence of bicuculline outside the STN, these animals were included in the study because a behavioral effect similar to obstinate progression was evident.

Lesion

For rat pups that received lesions alone, the largest and smallest lesions for each age are shown in Figure 3-3. Refer to Figure 3-4 for rat pups that received lesions with microinjections. Most lesions encroached only minimally upon adjacent brain areas. However, some of the lesions did include portions of the striatum and GPi. In a few cases the lesions appear to have completely destroyed the GPi, while leaving most of the thalamic nuclei intact.

Behavior

As expected, rat pups that received complete pre-subthalamic transections demonstrated the same persistent, automaton-like forward progression that has been reported in other studies. This rapid locomotor behavior was very regular, with few disturbances throughout the duration of the testing session. Subjects usually ran along the wall, but often ran in circles with a diameter less than that of the arena, sometimes as small as twice the snout to rump length of the subject. Further decreases in the diameter of the circling behavior resulted in pivoting about the axis of one of the hind legs. Occasionally PD15 and PD25 rat pups ran in a straight line, altering their course only after running into the wall. PD5 rat pups were more likely to cross the arena than run in circles when not in contact with the arena wall. However, circling and pivoting were also present at PD5.

With the exception of minor bouts of retrogression on only a very few occasions, the behavior of lesioned rat pups was indiscernible from that of controls. However, rat pups that received microinjections of bicuculline and rat pups that received microinjections with lesions demonstrated similar increases in activity. The behavior of these rat pups was not as consistent as that of transected rat pups, with frequent pauses during locomotion as well as extended bouts of grooming, rearing, scratching and hopping. At the beginning of the testing session, these rat pups exhibited twisting of the body axis that often led to barrel rolling, and extreme dorsiflexion that served as the precursor to infrequent series of back flips. Although their behavioral profile was more varied than that of the transected rat pups, microinjected rat pups exhibited the same rapid circling and pivoting that characterized transected neonates. This behavior of microinjected rat pups normally subsided by the final fifteen-minute bin of the behavioral testing session.

Sector Crosses

Using the number of sector crosses as a measure of locomotor behavior, two-way analysis of variance of the number did not reveal an interaction of treatment and age for the 45-minute test session, or for any of the three fifteen minute test session bins. However, one-way analysis of variance and Dunnett's posttest comparisons revealed that, at PD15, across the entire 45-minute testing period, decerebrate rat pups were the only experimental group to demonstrate greater forward progression than control animals ($F=3.95$, $d.f.=4,33$, $p<0.02$). At PD5 and PD25, not only did decerebrate rat pups demonstrate more sector crosses than age-matched controls, but microinjected rat pups and rat pups that received microinjections with lesions did, as well (PD5: $F=19.18$, $d.f.=4,30$, $p<0.0001$, PD25: $F=13.23$, $d.f.=4,32$, $p<0.0001$; fig. 3-5).

As seen in figure 3-5, the same treatment effects were not reflected for each 15-minute bin. Transected PD15 rat pups only exhibited more sector crosses than PD15 sham-treated rat pups during the first bin (Kruskal-Wallis and Dunn's multiple comparisons, $X^2=16.63$, $d.f.=4,33$, $p<0.005$). During the final two bins, none of the experimental treatments, including decerebration, resulted in more sector crosses among PD15 rat pups than was exhibited by age-matched controls. At PD5 and PD25, transected rat pups, microinjected rat pups, and rat pups that received microinjections with lesions all demonstrated more sector crosses than age-matched controls during the first bin (PD5: Kruskal-Wallis and Dunn's multiple comparisons, $X^2=19.37$, $d.f.=4,30$, $p<0.001$; PD25: one-way ANOVA and Dunnett's posttest comparisons, $F=12.16$, $d.f.=4,32$, $p<0.0001$). During the second bin, transected and microinjected PD25 rat pups showed significantly greater sector crosses than sham-treated PD25 rat pups ($F=8.68$, $d.f.=4,32$, $p<0.0001$). During the third bin of behavioral testing, transected PD25 rat pups, and PD25 rat pups that received microinjections with lesions demonstrated more sector crosses than age-matched controls ($F=7.96$, $d.f.=4,32$, $p<0.0005$). At PD5, only transected rat pups demonstrated

more sector crosses during the second and third bin than age-matched controls (one-way ANOVA and Dunnett's posttest comparisons, 2nd bin: $F=4.13$, d.f.=4,30, $p=0.01$, 3rd bin: $F=7.47$, d.f.=4,30, $p<0.0005$).

Duration of Forward Progression

Summing the duration of circling and pivoting across, a two-way analysis of variance revealed an interaction of treatment and age in the expression of timed forward progression across the entire 45-minute testing session ($F=4.99$, d.f.=8,94, $p<0.0001$; fig. 3-6). Tukey's HSD posttest comparisons showed that although PD15 transected rat pups did not spend more time in locomotion than age-matched treatment groups, they did, along with microinjected PD15 rat pups, exhibit greater forward progression than sham-treated rat pups from the PD5 and PD25 age groups, as well as every experimental PD5 group. Sham-treated PD15 rat pups also demonstrated greater forward progression than PD5 and PD25 control animals. There were no PD5 or PD25 experimental groups that exhibited greater forward progression than PD15 control animals. Decerebrate rat pups from the PD5 and PD25 age groups, along with PD5 and PD25 rat pups that received microinjections with lesions demonstrated greater locomotion than their age-matched controls. The only age group to receive microinjections without accompanying lesions and demonstrate greater timed forward progression than age-matched controls was the PD25 rat pups.

An interaction of treatment and age on timed forward progression was found for the first 15-minute bin ($F=3.3$, d.f.=8,94, $p<0.005$; fig. 3-6). Pairwise comparisons revealed the same differences in behavior among the different groups during the first bin that were found for the entire behavioral session, with the exception that transected PD15 rat pups did not exhibit significantly more locomotion than PD5 rat pups that had received both microinjections and lesions. Although an interaction was not found for the second or third bin, a main effect was

evident for both treatment (2nd bin: $F=9.92$, d.f.=4,94, $p<0.0001$; 3rd bin: $F=9.94$, d.f.=4,94, $p<0.0001$) and age (2nd bin: $F=7.51$, d.f.=2,94, $p=0.001$; 3rd bin: $F=8.56$, d.f.=2,94, $p<0.0005$). Pairwise comparisons showed that, during the second bin, transected rat pups exhibited more locomotion than every treatment group except microinjected rat pups, and PD15 rat pups exhibited more locomotion than PD5 rat pups. During the third bin, transected rat pups outperformed every treatment group, including rat pups that received microinjections, and PD15 rat pups outperformed both PD5 and PD25 rat pups.

Duration of Limb Alternation

Occasionally, subjects engaging in decerebrate or bicuculline-induced locomotion collapsed onto their sides, no longer moving forward, but limbs still exhibiting patterned alternation. It should be noted that PD5 rat pups barely supported their own body weight, and fell onto their side early during the behavioral testing session more often than PD15 or PD25 rat pups. However, they persisted in struggling to right themselves and continue moving forward throughout the session. The form of locomotion also varied between ages. There was greater relative activity of the forelimbs than of the hind limbs at PD5 than there was at older ages. This was particularly evident while the neonates were lying on their side. Also, synchronous movement of the hind limbs (i.e. galloping) frequently occurred at PD25 but not at younger ages.

When limb alternation that did not contribute to forward progression was included in the analysis of variance of timed locomotor behavior across the entire 45-minute test session, we again found an interaction of age and treatment ($F=5.2$, d.f.=8,94, $p<0.0001$; fig. 3-7). Posttest comparisons revealed similar results to the analysis of timed forward progression alone, with a few additional differences. In this analysis, decerebrate PD15 rat pups demonstrated greater limb alternation than their age-matched controls. Decerebrates were the only experimental PD15 group to exhibit a significant difference from PD15 sham-treated rat pups. However, every

PD15 treatment group engaged in significantly greater locomotor behavior than sham-treated PD5 and PD25 rat pups. Also, save for lesioned rat pups, every experimental PD5 and PD25 group demonstrated greater locomotor behavior than both PD5 and PD25 controls. As with the duration of forward progression, PD15 sham-treated rat pups demonstrated greater limb alternation than PD5 and PD25 control animals, and there were no PD5 or PD25 experimental groups that engaged in significantly greater locomotor behavior than PD15 controls.

An interaction of age and treatment on duration of limb alternation was also evident during the first 15-minute bin of the behavioral testing session ($F=3.92$, $d.f.=8,95$, $p<0.001$; fig. 3-7). Posttest analysis revealed almost the exact same differences as the 45-minute analysis, with the exception that decerebrate PD15 rat pups did not show greater locomotor behavior than microinjected rat pups or microinjected rat pups with lesions from any age group. An interaction during the second and third bins was not found, although main effects were evident for both treatment (2nd bin: $F=15.75$, $d.f.=4,95$, $p<0.0001$; 3rd bin: $F=19.93$, $d.f.=4,95$, $p<0.0001$) and age (2nd bin: $F=14.25$, $d.f.=2,95$, $p<0.0001$; 3rd bin: $F=10.14$, $d.f.=2,95$, $p=0.0001$). During the second bin, decerebrate rat pups, microinjected rat pups, and microinjected rat pups with lesions all exhibited greater limb alternation than those given lesions or sham-treated rat pups. During the third bin, microinjected rat pups and microinjected rat pups with lesions no longer outperformed sham-treated rat pups, and decerebrate rat pups exhibited more locomotor behavior than every other treatment group. During both bins, PD15 rat pups engaged in more limb alternation than PD5 and PD25 rat pups.

A three-way (age X treatment X bin) mixed analysis of variance revealed a significant interaction of treatment and bin on the duration of limb alternation ($F=5.6$, $d.f.=8,95$, $p<0.0001$). One-way repeated measures ANOVAs of the duration of limb alternation for each treatment

group revealed that microinjected rat pups ($F=13.05$, $d.f.=2,16$, $p<0.0001$) and rat pups that received both microinjections and lesions ($F=10.76$, $d.f.=2,14$, $p<0.0001$) declined sharply in the duration of limb alternation across the test session, demonstrating significantly less limb alternation in the second bin than the first, and significantly less limb alternation in the third bin than in the second (fig. 3-8).

Immunohistochemistry

Transected PD5 rat pups exhibited no c-Fos immunoreactivity in the basal ganglia or tegmentum. Kruskal-Wallis and Dunn's multiple comparisons tests revealed the only brain region of PD5 rat pups to express significantly greater c-Fos than age-matched controls was the LDT of microinjected PD5 rat pups, and PD5 rat pups that received both microinjections and lesions ($X^2=18.76$, $d.f.=4,30$, $p<0.001$; fig. 3-11). Although labeling was evident in the subthalamus and SNr of microinjected PD5 rat pups and PD5 rat pups that received both microinjections and lesions, it did not differ significantly from sham-treated PD5 rat pups.

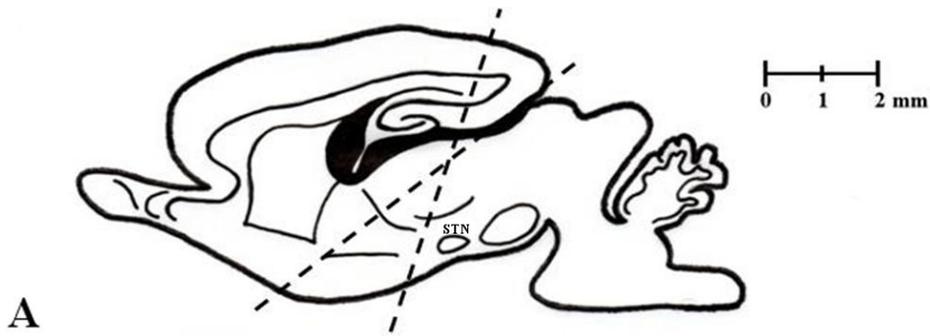
Transected PD15 and PD25 rat pups both exhibited much greater c-Fos immunoreactivity in the STN than age-matched controls (Kruskal-Wallis and Dunn's multiple comparisons tests, PD15: $X^2=21.17$, $d.f.=4,33$, $p<0.0005$, PD25: $X^2=20.21$, $d.f.=4,32$, $p=0.0005$). Dunn's posttest also revealed microinjected PD15 rat pups and PD25 rat pups that received both microinjections and lesions expressed greater labeling in the STN than age-matched controls. However, microinjected PD15 rat pups still expressed significantly less c-Fos in the STN than transected PD15 rat pups, whereas PD25 rat pups that received both microinjections and lesions did not differ significantly with transected PD25 rat pups in STN expression of c-Fos (fig. 3-8).

In other areas of the subthalamus, transected PD15 rat pups expressed greater c-Fos immunoreactivity in the LH than age-matched controls (Kruskal-Wallis and Dunn's multiple comparisons test, $X^2=18.32$, $d.f.=4,32$, $p<0.002$; fig. 3.9). Microinjected PD15 rat pups and

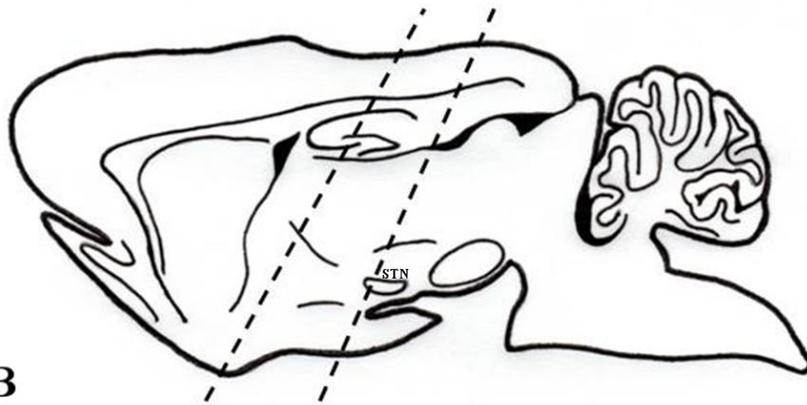
microinjected PD25 rat pups, as well as PD25 rat pups that received both microinjections and lesions, demonstrated greater c-Fos immunoreactivity in the ZI than age-matched controls, which was not apparent in transected animals of either age group (Kruskal-Wallis and Dunn's multiple comparisons test, PD15: $X^2=9.96$, d.f.=4,32, $p<0.05$, PD25: $X^2=18.47$, d.f.=4,31, $p=0.001$; fig. 3.9). Microinjected PD25 rat pups also demonstrated greater c-Fos immunoreactivity in the LH than age-matched controls (PD25: $X^2=17.73$, d.f.=4,32, $p<0.002$).

In the SNr, brain slices of transected rat pups at PD15 and PD25 revealed greater c-Fos immunoreactivity than age-matched controls (Kruskal-Wallis and Dunn's multiple comparisons test, PD15: $X^2=15.67$, d.f.=4,32, $p<0.005$, PD25: $X^2=19.24$, d.f.=4,31, $p<0.001$). No other treatment group at PD15 demonstrated an increase in SNr c-Fos immunoreactivity (fig. 3-10). However, Dunn's posttest comparisons revealed that, at PD25, the SNr of sham-treated rat pups exhibited significantly less c-Fos immunoreactivity than all other treatment groups, including lesioned rat pups.

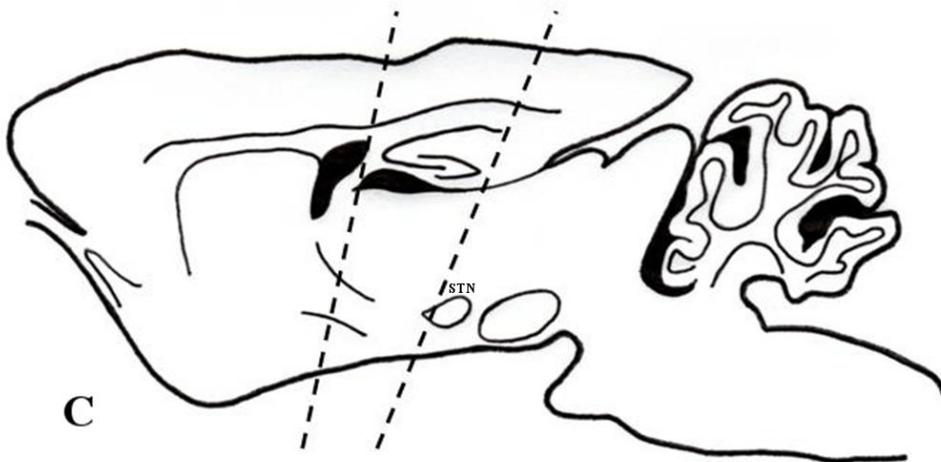
Finally, in the tegmentum, there was no treatment effect at PD15 or PD25 on c-Fos immunoreactivity in the PPNc. However, in the LDT of PD15 rat pups, a treatment effect revealed that transected PD15 rat pups expressed greater c-Fos immunoreactivity than lesioned PD15 rat pups, although not compared to age-matched controls (Kruskal-Wallis and Dunn's multiple comparisons test, $X^2=14.17$, d.f.=4,32, $p<0.01$; fig 3-11). A treatment effect on the expression of c-Fos in the LDT of PD25 rat pups was also evident (one-way ANOVA, $F=4.48$, d.f.=4,32, $p<0.01$). Dunnett's posttest comparisons revealed that, at PD25, transected rat pups, microinjected rat pups, and rat pups that received microinjections with lesion all expressed greater levels of c-Fos immunoreactivity in the LDT than age-matched controls (fig. 3-11).



A



B



C

Figure 3-1. Transections. The most rostral and caudal transection, represented by perforated lines, at A) PD5, B) PD15, and C) PD25.

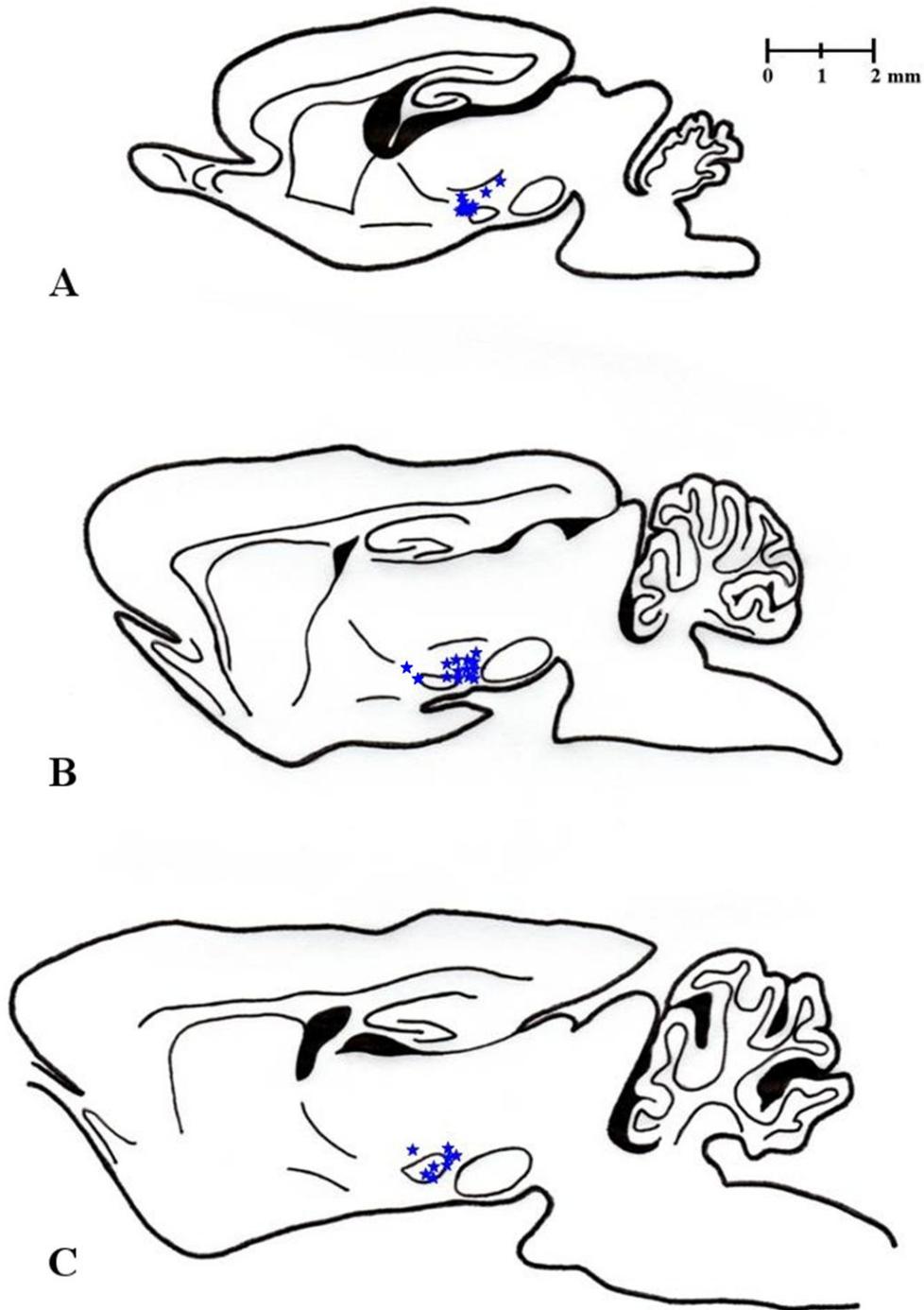


Figure 3-2. Microinjections. Injection sites, represented by the blue stars, for A) PD5, B) PD15, and C) PD25 rat pups that received microinjections alone.

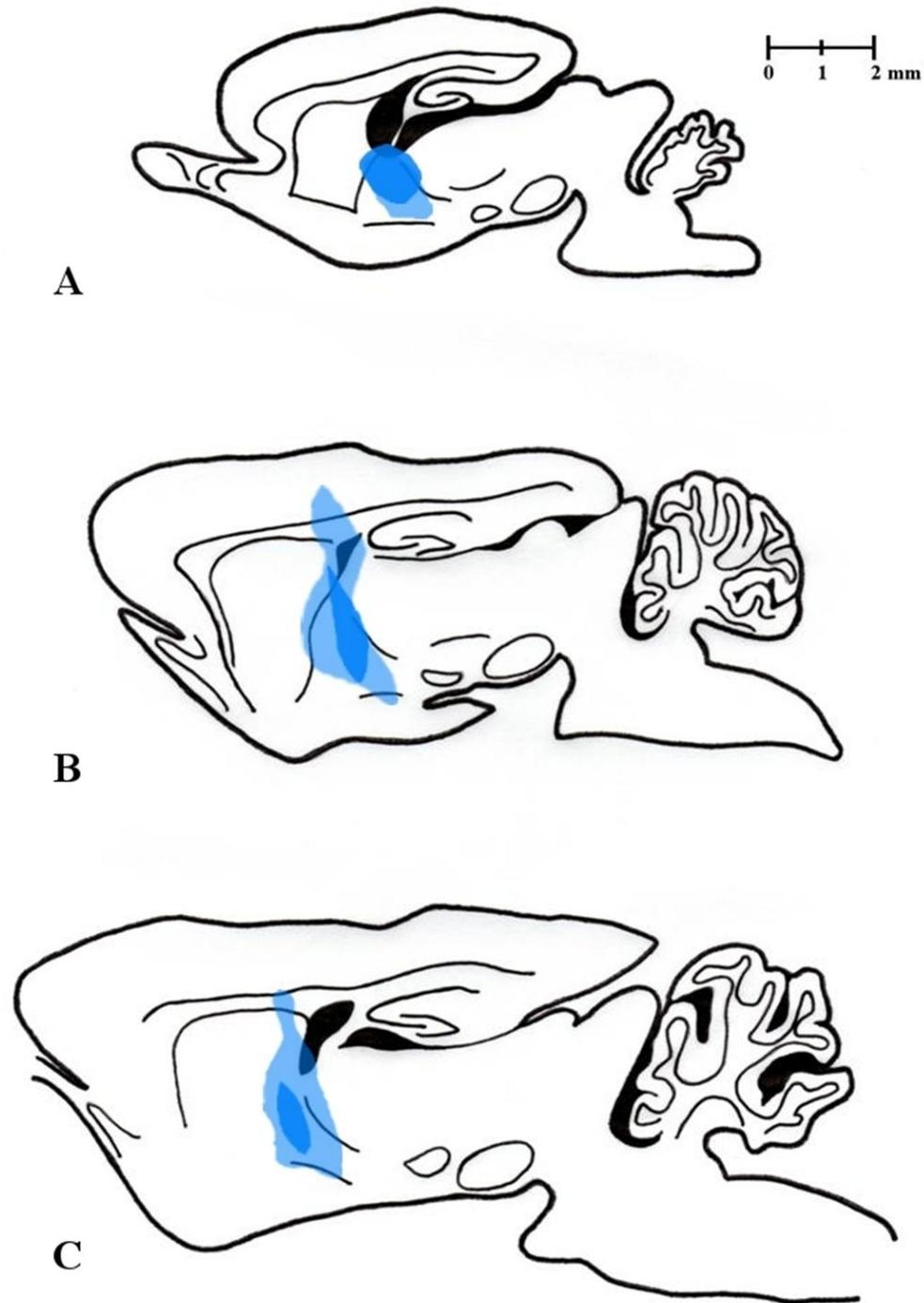


Figure 3-3. Lesions. Largest (light blue) and smallest (dark blue) lesions for A) PD5, B) PD15, and C) PD25 rat pups that received lesions alone.

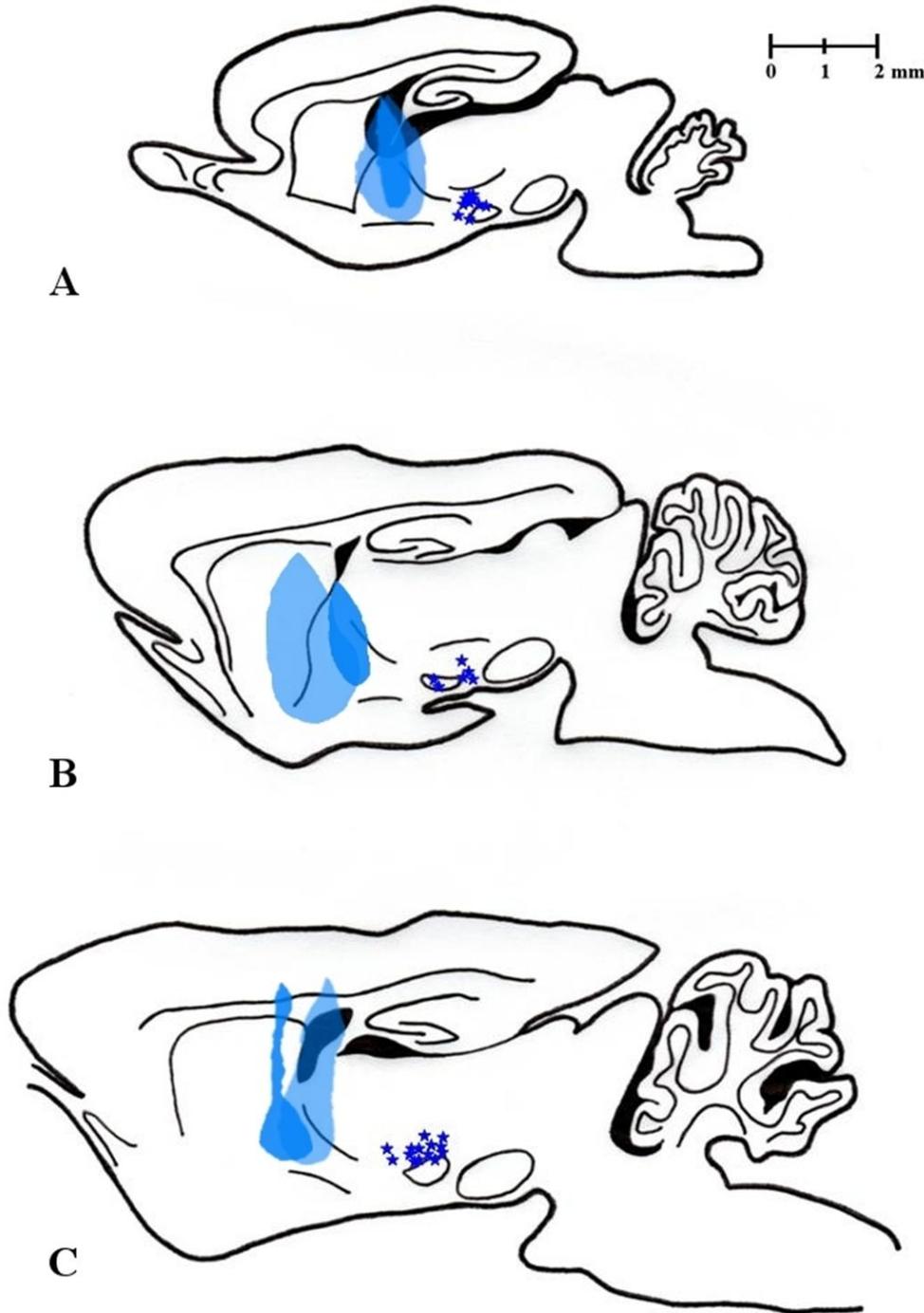


Figure 3-4. Microinjections with lesions. Injection sites (stars), largest (light blue) and smallest (dark blue) lesions for A) PD5, B) PD15, and C) PD25 rat pups that received both microinjections and lesions.

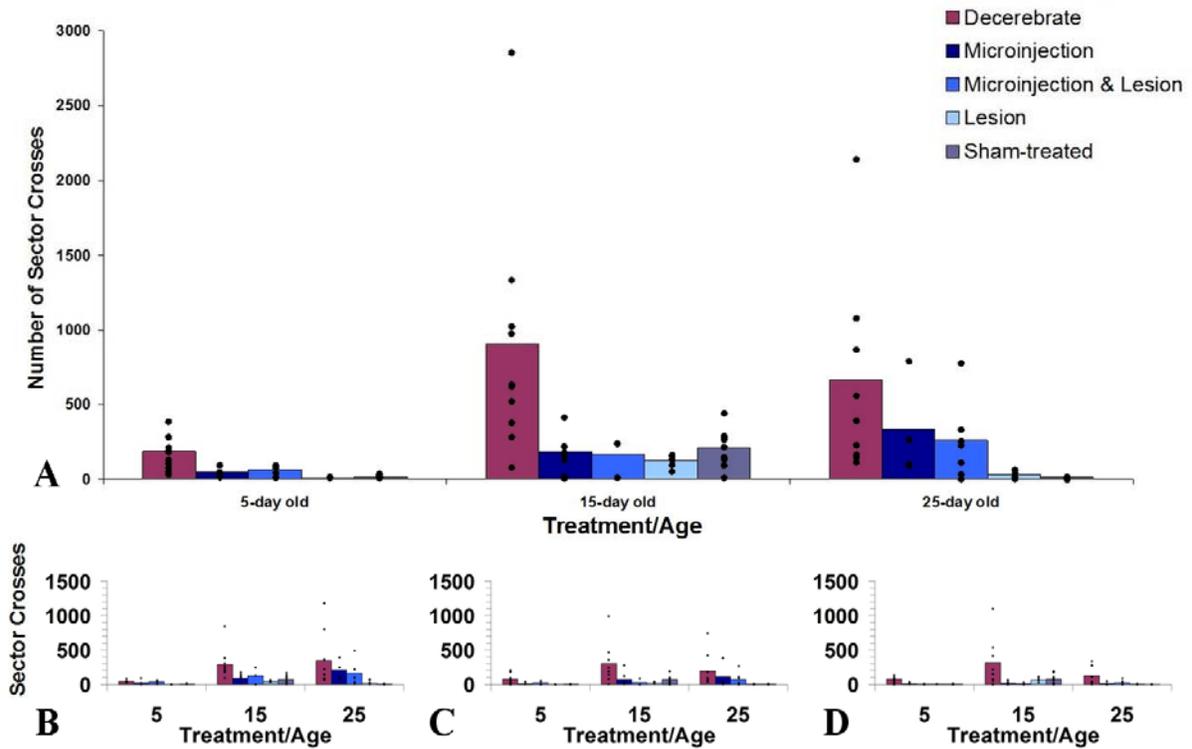


Figure 3-5. Sector crosses. Individual data points and the raw mean number of sector crosses are shown for each treatment/age group across A) the entire forty-five minute testing session, the B) first fifteen minute bin, C) second fifteen minute bin, and D) third fifteen minute bin. At every age, decerebrate rat pups crossed more sectors than age-matched controls. Microinjected rat pups and rat pups that received microinjections with lesions also crossed more sectors than age-matched controls, but only at PD5 and PD25.

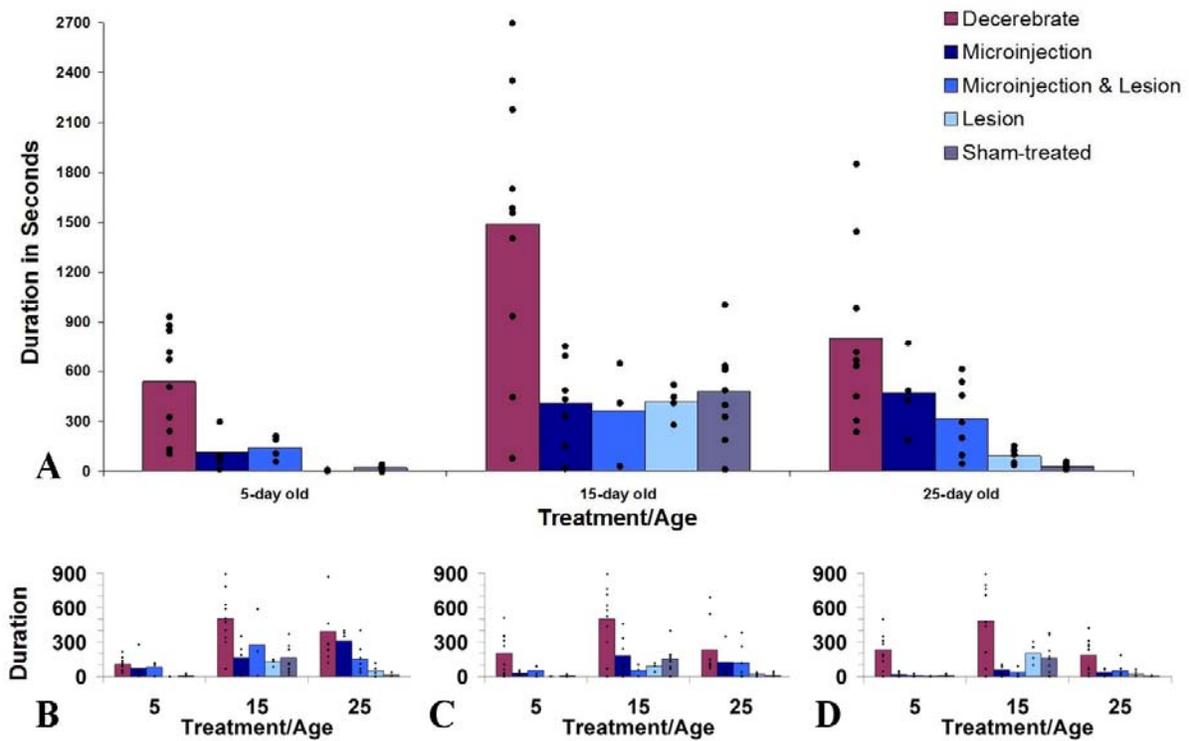


Figure 3-6. Duration of forward progression. Individual data points and the raw mean duration of forward progression are shown for each treatment/age group across A) the entire forty-five minute testing session, the B) first fifteen minute bin, C) second fifteen minute bin, and D) third fifteen minute bin. Decerebrate rat pups and rat pups that received microinjections with lesions spent more time engaged in forward progression than age-matched controls at PD5 and PD25, but not at PD15. However, sham-treated PD15 rat pups engaged in more spontaneous locomotion than sham-treated PD5 and PD25 rat pups.

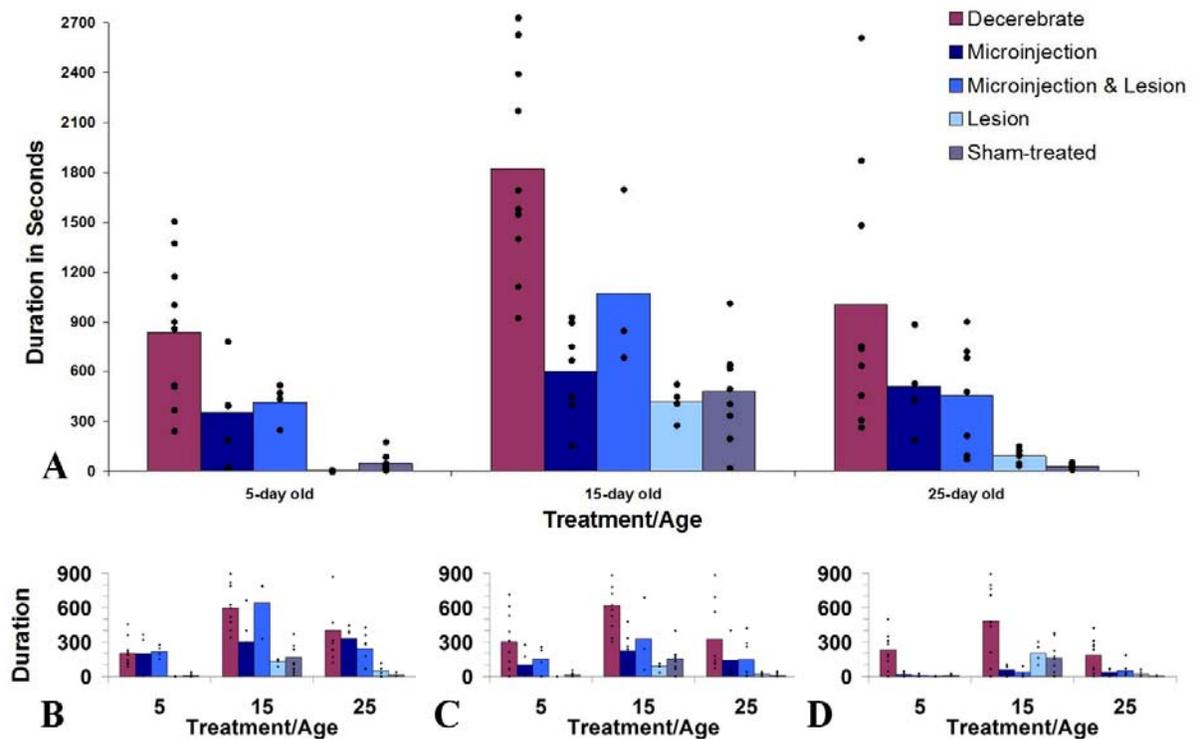


Figure 3-7. Duration of limb alternation. Individual data points and the raw mean duration of limb alternation are shown for each treatment/age group across A) the entire forty-five minute testing session, the B) first fifteen minute bin, C) second fifteen minute bin, and D) third fifteen minute bin. Decerebrate rat pups from every age group demonstrated greater overall limb alternation than age-matched controls. Microinjected rat pups and rat pups that received microinjections with lesions also engaged in more limb alternation than age-matched controls, but only at PD5 and PD25. Sham-treated PD15 rat pups again exhibited more limb alternation than sham-treated PD5 and PD25 rat pups.

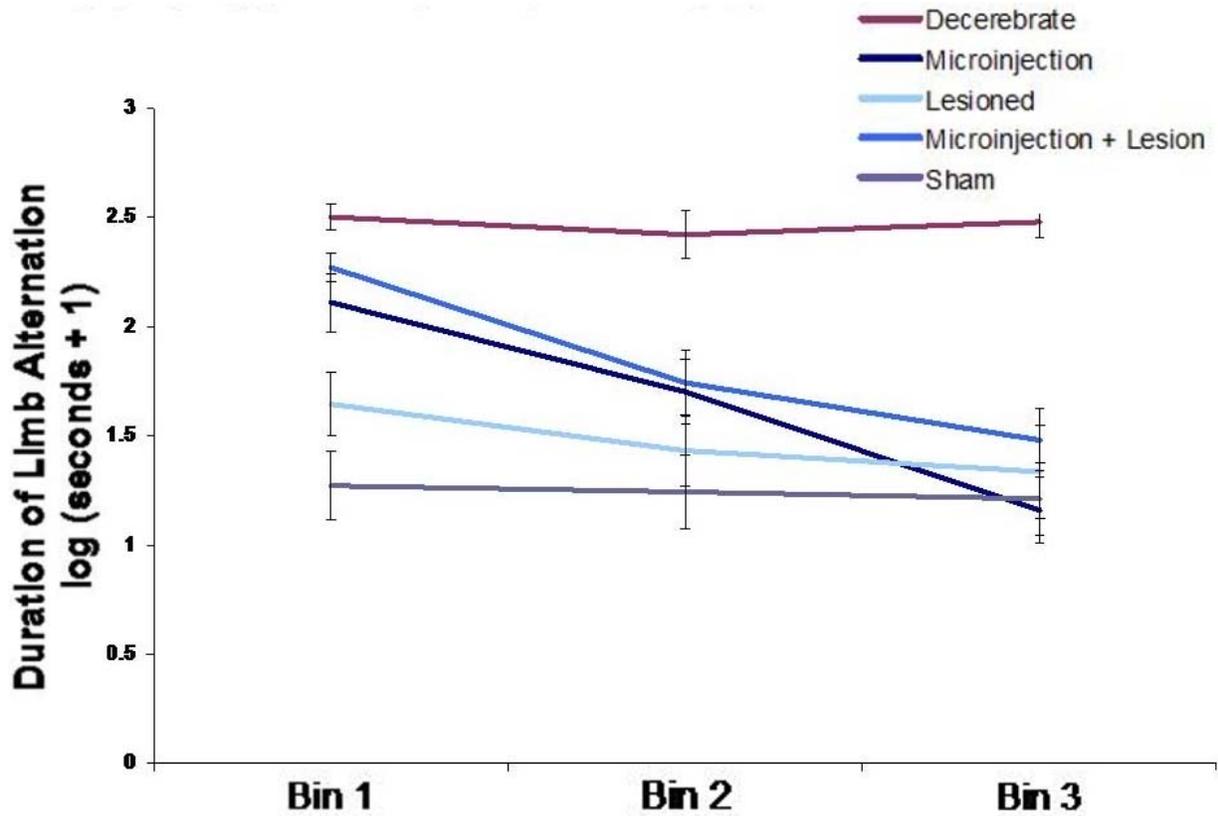


Figure 3-8. Changes in the duration of limb alternation across test session. Microinjected rat pups and rat pups that received microinjections with lesions exhibited a significant decrease in activity during the 45-minute session. Lesioned and sham-treated rat pups were inactive throughout, while transected rat pups showed no sign of a decrease in limb alternation.

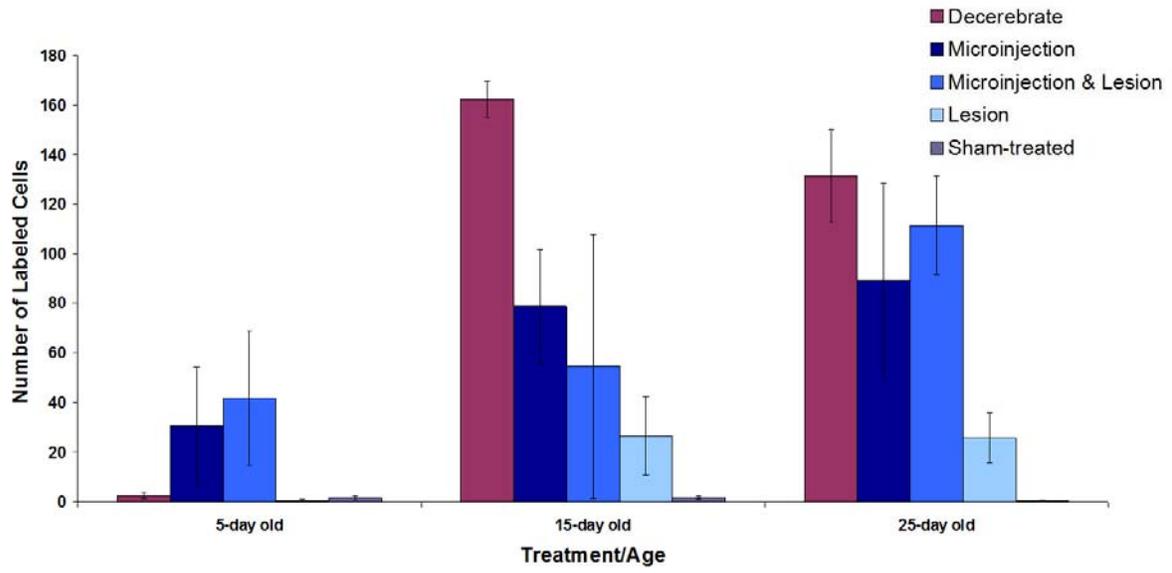


Figure 3-9. C-Fos immunoreactivity in the subthalamic nucleus. At PD25, decerebrate rat pups and rat pups that received microinjections with lesions demonstrated greater c-Fos-immunoreactivity in the STN than age-matched controls. At PD15, microinjected rat pups demonstrated greater c-Fos activity in the STN than age-matched controls, and decerebrate rat pups demonstrated even greater c-Fos activity than microinjected rat pups. No differences were found between any treatment groups at PD5.

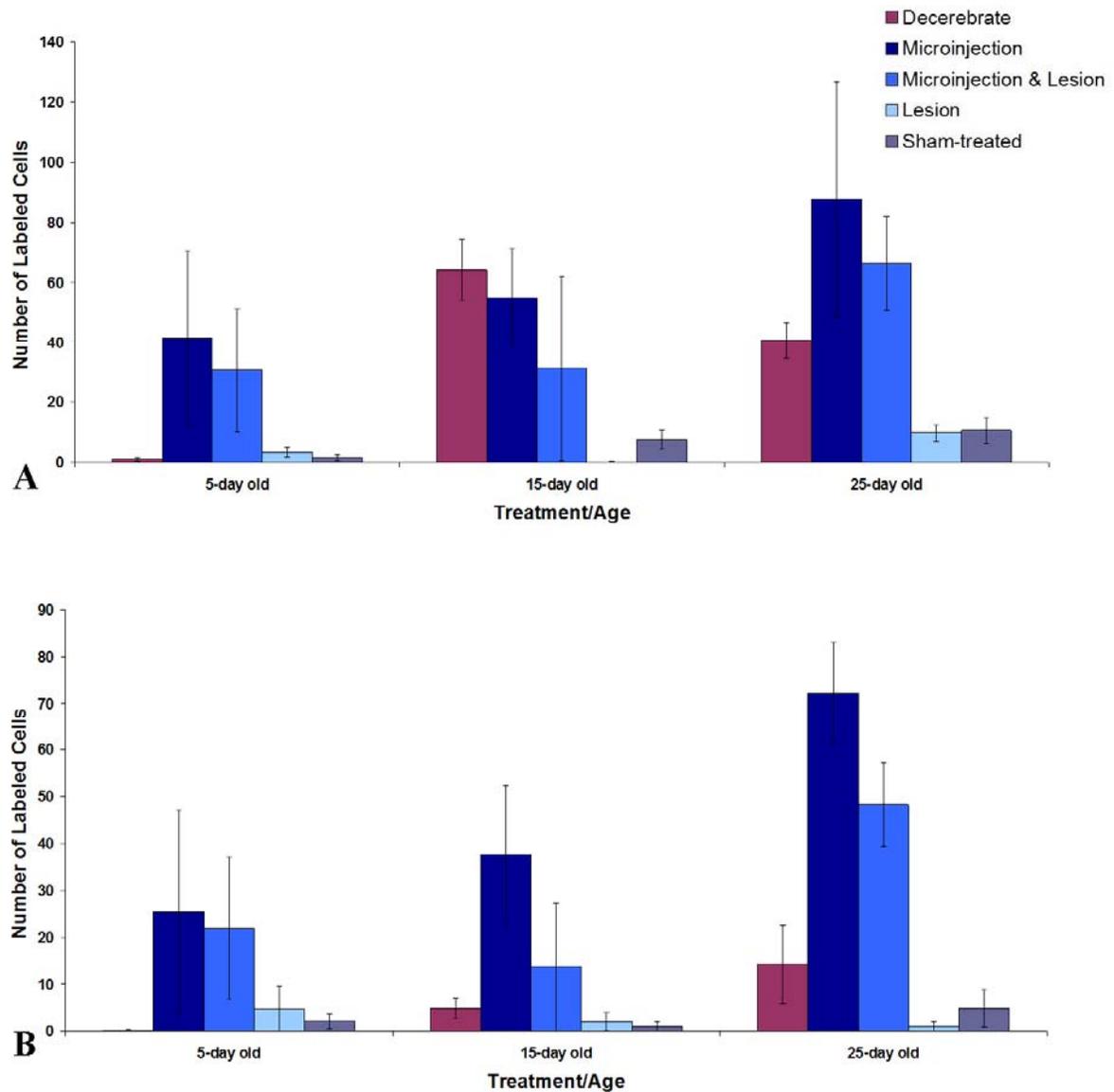


Figure 3-10. C-Fos immunoreactivity in the zona incerta and lateral hypothalamus. A) PD15 and PD25 rat pups that received microinjections, and PD25 rat pups that received microinjections with lesions were the only groups to demonstrate greater c-Fos reactivity in the ZI than age-matched controls. B) Microinjected PD25 rat pups also showed greater c-Fos reactivity in the LH. The only decerebrate group to exhibit significantly more labeling than age-matched controls in an area adjacent to the STN was PD15 rat pups, who displayed higher levels in the LH.

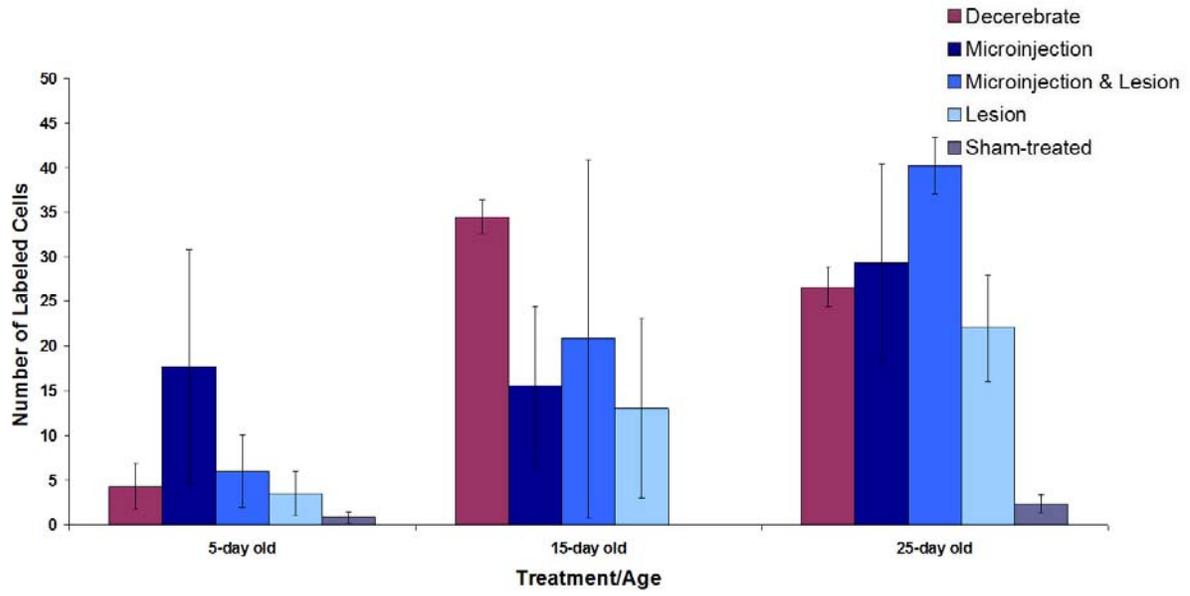


Figure 3-11. C-Fos immunoreactivity in the substantia nigra *pars reticulata*. Decerebrate PD15 and PD25 rat pups exhibited a clear increase in c-Fos immunoreactivity of the SNr. However, only at PD25 did the other experimental manipulations produce significantly greater labeling in the SNr than in age-matched controls.

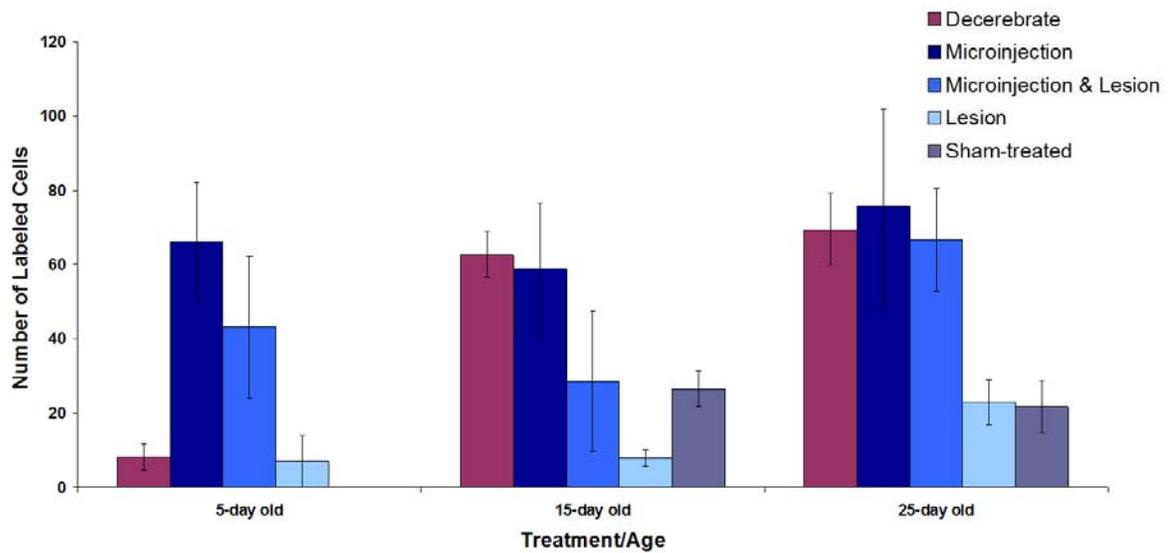


Figure 3-12. C-Fos immunoreactivity in the lateral dorsal tegmental nucleus. At PD5 and PD25, microinjected rat pups and rat pups that received microinjections with lesions demonstrated more c-Fos immunoreaction product in the LDT than age-matched controls. Decerebrate PD25 rat pups did, as well. Similar high levels of c-Fos reactivity in the LDT of decerebrate and microinjected PD15 rat pups fell just short of significance when compared to age-matched controls. This was the only brain area where increases in c-Fos immunoreactivity of microinjected PD5 rat pups achieved significance.

CHAPTER 4 DISCUSSION

Decerebrate obstinate progression and L-DOPA-induced locomotor behavior in rat pups are similar in many regards. Both behaviors are characterized by persistent, coordinated limb alternation, and both result in an increase in c-Fos immunoreactivity in the STN, SNc and mesopontine tegmentum. However, systemic injection of L-DOPA no longer elicits locomotion from rat pups at PD25, while pre-subthalamic transections continue to do so. This difference may be due to brain areas rostral to the STN, which are eliminated in decerebrate rat pups, but whose inhibitory influence depress locomotion in intact L-DOPA-injected PD25 rat pups (Staup and Stehouwer, 2006).

In the adult rat, several areas of the basal ganglia rostral to the STN are known to exert direct inhibitory control throughout the midbrain (fig. 1-1; McHaffie et al., 2005; Parent et al., 2000; Parent and Hazrati, 1995a; Smith et al., 1998). In the present study, complete pre-subthalamic transections, caudal to the GPe, removed striatopallidal influence in the brainstem and remaining diencephalon. In PD15 and PD25 rat pups this resulted in a profound increase in c-Fos reactivity in the STN (fig. 3-8), which was accompanied by an increase in locomotor behavior that mirrored the magnitude of change in cellular activity. Although decerebrate PD5 rat pups exhibited a similar increase in forward progression, c-Fos immunoreactivity in the STN was not detected (fig. 4-2). Within each age group, the behavioral and immunohistochemical results of decerebrate rat pups were remarkably consistent given the variability in the level of the transections, which, in the most caudal examples, bisected the STN (fig. 3-1). The lack of effect from this kind of error, and from nonspecific damage due to the transections, supports the conclusion that the results were a product of the elimination of forebrain structures, and not other, unintended consequences of the experimental manipulation.

It was anticipated that locomotion would be more readily elicited by disinhibition of the STN at PD15 than at PD25 due to immature inhibitory input to the midbrain motor nuclei, and thus greater susceptibility of these nuclei to the excitatory input of the STN. However, transected and bicuculline-injected rat pups only demonstrated greater duration of forward progression than age-matched controls at PD5 and PD25 (fig 3-6). Only when limb alternation, which did not contribute to forward progression, was included in the analysis did transected PD15 rat pups exhibit significantly greater duration of locomotor behavior than age-matched controls (fig. 3-7). Transected PD15 rat pups also covered more distance during behavioral testing, as measured by sector crosses, than age-matched controls (fig. 3-5), indicating that while transected PD15 rat pups may not have spent more time in forward progression than sham-treated PD15 rat pups, their rate of locomotion was increased.

The lack of a treatment effect on the duration of forward progression at PD15 likely resulted from the greater level of spontaneous locomotion demonstrated by untreated rat pups at this age than at PD5 or PD25 (fig. 3-8). This age effect replicates the results of several other researchers (Campbell, 1969; Fibiger, 1970; Moorcroft, 1971; Mabry and Campbell, 1974). In the present study, the upper limit for the duration of locomotion was 45 minutes. As more decerebrate PD15 rat pups approached that limit, measures of duration became less sensitive to actual differences between that group and others. Differences may have been masked altogether as subjects from the other PD15 groups approached the time limit, producing a ceiling effect on measures of duration at that age.

It can only be speculated that the high level of spontaneous locomotion at PD15 may, itself, be a reflection of immature inhibitory input to the tegmentum, and thus greater basal levels of activity in the motor command centers found there. Data from the present study partially support

this possibility, in that the difference between expression of c-Fos in the LDT of transected PD15 rat pups and age-matched controls fell short of significance. As the production of c-Fos tends to accompany *increases* in cellular activity, this may indicate that these cells were already highly active in intact PD15 pups and that transections did not produce an elevation in the production of c-Fos (Kovács, 1998).

Lesions of the Globus Pallidus

Studies of adult rats with bilateral lesions of the GPe demonstrate decreases in motor coordination and spontaneous locomotion (Hauber, 1998; Jeljeli, 1999). This is the logical consequence of disinhibition of the STN in adult rats, which results in increased inhibitory output from the GPi/SNr to their target structures. Contrary to this, the present study predicted that, due to weak inhibitory projections to the midbrain motor command center of neonates, the few excitatory fibers that project directly from the STN to the PPNc/MLR would have a more potent effect in pre-weanling rat pups than in adults, resulting in the production of locomotion. However, although pallidal lesions did not produce a decrease in locomotion at any age in this study, they were no more effective at producing an increase in locomotion than has been reported in studies of adult rats. Destruction of the GPe is capable of producing changes in electrophysiological activity of the STN in adult rats (Ryan and Clark, 1992; Hassani et al., 1996). If such a change was present at the ages investigated in this report, it was not reflected by an increase in c-Fos reactivity. However, in the present study, behavior was measured immediately after recovery from anesthesia, and then recorded for 45-minutes before the rat pups were sacrificed. Elimination of the GPe at different stages of early postnatal development may have more profound long-term effects worth investigating.

It is also possible that this study failed to produce an increase in locomotion with pallidal lesions because neurons in the STN that receive inhibitory projections from the GPe are not the

same neurons that send their axons to the midbrain. Double retrograde labeling of the rat brain has distinguished projections of the thin lateral strip of the STN, which find their target in the PPNc, from the majority of STN projections that send collaterals throughout the rest of the basal ganglia (Takada et al., 1988). Anatomical studies utilizing anterograde labels injected into the pallidum make no mention of projections from the GPe to the lateral strip of the STN (Canteras et al., 1990). While the STN and the GPe share a reciprocal relationship, it may be that STN neurons that do not send fibers to the GPe do not receive input from the GPe, either.

On the other hand, if the lateral strip does receive input from the GPe, there remains the possibility that lesions did not destroy cells of the GPe that send axons to that region of the STN. The GPe is a large, oblong nucleus, the most lateral and medial borders of which do not lie in the same coronal plane. In the adult rat, the pattern of projections from the GPe to the STN approximates a rostrocaudal, mediolateral topography (Parent and Hazrati, 1995b). This would place fibers projecting to the lateral strip of the STN in the most caudolateral aspect of the GPe. Lesions in the present study destroyed the main body of the GPe. However, the far lateral edge of this nucleus extends caudally, well beyond its largest part, and could have remained intact and capable of inhibiting the STN. Other studies report that destruction of more than 50-80% of the GPe results in significant changes in STN cellular activity, and they have used this as their acceptance criterion (Ryan and Clark, 1992; Jeljeli, 1999). Yet, the lateral strip comprises <2% of the STN (Takada et al., 1988). If the portion of the GPe that projects to the lateral strip is similarly small relative to the main body of the nucleus, then it may well have fallen within their margin of error.

However, in the present study, dense c-Fos immunoreactivity was homogenously displayed throughout the entire STN, not just the lateral strip, of transected rat pups (fig. 4-2). Coupled

with the fact that GPe-lesioned rat pups did not exhibit an increase in locomotion or limb alternation and did not display greater c-Fos reactivity in any region of the STN than control animals, it seems unlikely that elimination of pallidal inputs to the STN is wholly, or even mostly, responsible for the production of decerebrate obstinate progression. Furthermore, a significant difference was not found in any measure of locomotor behavior or c-Fos reactivity between rat pups that received both microinjections and lesions, and rat pups that received microinjections alone. What little variability there was between these two experimental groups relative to transected rat pups is most likely attributable to low sample sizes (table 2-1). Based upon these results, it does not appear as though lesions of the GPe provided a major contribution to the production of bicuculline-induced locomotor behavior, either.

The role of the GPe as an inhibitory input to the STN in neonates is further challenged by the high level of c-Fos activity in the GPe of rat pups that engage in L-DOPA-induced locomotor behavior, while demonstrating even higher levels of c-Fos labeling in their STN (Staup and Stehouwer, 2006). This same pattern of c-Fos reactivity is found in rats that engage in intrastriatal 1S, 3R-ACPD (an mGluR agonist) -induced circling behavior (Kaatz and Albin, 1995). As speculated earlier in this report, the failure of an active GPe to inhibit its primary target nucleus in young rat pups may reflect immature GABA-ergic activity from those GPe projections. However, it is also possible that the GPe of intact rat pups may actually produce heightened activity in the STN via GABA_A receptors. In adult rats, multiple inhibitory postsynaptic potentials are capable of creating burst activity in STN neurons via low-threshold Ca⁺ channels and hyperpolarization-induced cation channels. When cells of the STN and the GPe are isolated from the cortex and the caudate-putamen (CPu) *in vitro*, patterned bursts are recorded between their neurons, demonstrating the possibility of an oscillatory relationship

between these nuclei in the absence of descending input *in vivo* (Bevan et al., 2002). However, this potential source of STN-activation in L-DOPA-injected rat pups does not explain the activity demonstrated in the STN of decerebrate rat pups, in whom the reciprocal connection between the STN and the GPe has been eliminated.

The Striatum as an Inhibitory Influence to the Midbrain

Another major source of inhibition rostral to the level of the transection is the striatum (Fig. 1-1). The striatum is organized into sensorimotor, associational and limbic information receiving centers. This information is then processed along parallel pathways that run through the rest of the basal ganglia (Parent and Hazrati, 1995b). Interestingly, rat pups that engage in L-DOPA-induced locomotor behavior, and rats that engage in intra-striatal 1S, 3R-ACPD-induced circling demonstrate a complete lack of c-Fos immunoreactivity in the striatum (Staup and Stehouwer, 2006; Kaatz and Albin, 1995). The dramatic increase in c-Fos immunoreactivity in the GPe, GPi, STN and SNr resulting from both these manipulations implicates the absence of the normal, inhibitory influence of the striatum over the rest of the basal ganglia in the production of persistent locomotor behavior. As further evidence of this, acaudate cats demonstrate an importunate attraction to moving objects, following a target until forced to stop (Villablanca and Marcus, 1975). This behavior of acaudate cats, termed ‘compulsory approach syndrome’ is distinguished from obstinate progression because it is stimulus-tied. As such, it is not considered to be ‘spontaneous’ (Garcia-Rill, 1986). However, increases in spontaneous locomotion after removal of the entire striatum have been reported since the nineteenth century (Mettler, 1940).

Figure 4-1 (from Staup et al., 2005) shows what appears to be diminished c-Fos reactivity in the CPu and nucleus accumbens of L-DOPA-injected rat pups. While this effect was not significant in all areas of the striatum that were investigated, the trend was consistent. This is noteworthy because, while c-Fos immunoreactivity is an accepted measure of cellular activation,

the method is far less reliable for investigating cellular inactivation (Kovács. 1998). Thus, diminished c-Fos immunoreactivity in the anterior dorsomedial CPu was unexpected.

In the same experiment, cell labeling was shown to be significantly lower throughout the striatum of PD15 rat pups than PD25 rat pups (fig. 4-1; Staup et al., 2005). Lower levels of striatal activity in PD15 rat pups may be the product of a greater density of inhibitory D2 receptors relative to excitatory D1 receptors in the rat striatum during the first three postnatal weeks. There are many more D1 receptors in the striatum of adult rats than pre-weanling rat pups, and D1 receptors are generally not outnumbered by D2 receptors in adults as they are in neonates (Tarazi and Baldessarini, 2000). Evidence that L-DOPA neutralizes the striatum of PD15 rat pups, considered with the similarity between L-DOPA-induced locomotion and decerebrate obstinate progression, supports the possibility that elimination of the striatum is responsible for decerebrate obstinate progression, and the claim that systemic L-DOPA in preweanling rat pups produces chemically decerebrate animals (Stehouwer and Van Hartesveldt, 2000).

Deterioration of the GABA-ergic nuclei of the CPu characterizes the early stages of Huntington's disease (HD), which is accompanied by involuntary, choreiform movements (Davis, 1976; Pinel, 1976). While decerebrate obstinate progression is certainly ballistic in nature, it does not share the uncoordinated quality of chorea. However, the irregular behaviors that accompany bicuculline-induced forward progression, and the torsion exhibited by both bicuculline-injected and decerebrate rat pups upon recovering from anesthesia is evocative of the writhing maneuvers displayed by HD patients (Bhidayasiri and Truong, 2008). However, increased activity in the STN, as in decerebrate rat pups, has not been connected to HD. Instead, the disease is normally associated with deterioration and a reduction in STN-activity. These

changes in the STN of HD patients do not occur, though, until later in the progression of the disease, when gliosis has spread to the secondary structures of the basal ganglia (Emerich and Sandberg, 1992).

The primary outflows of the striatum find as their targets the GPe, along the indirect pathway, and the GPi/SNr, along the direct pathway, of the basal ganglia. There are, in addition, several systems of collaterals originating from the striatum that provide direct, dense innervation of other areas of the brain, including the thalamus and the PPN (Smith et al., 1998; Winn, 1997; Parent et al., 2000). The existence of a striatosubthalamic tract has been speculated to explain the increase in c-Fos labeling of the STN after inactivation of the striatum (Kaatz and Albin, 1995). However, there is only scant evidence of a meager projection from the nucleus accumbens to the STN in rats, backed up by only provisional reports in cats and primates (Ohye et al., 1976; Beckstead, 1983; Gronewegen and Berendse, 1990). If inactivation of the striatum does contribute to the genesis of decerebrate obstinate progression by activating the STN, the scarcity of direct striatal projections to the STN would suggest that an intermediary brain area or areas must be involved.

The Role of the Substantia Nigra

Of the striatal targets left intact after decerebration, the SNr extends GABA-ergic fibers to the tegmentum and to the SNc (Parent and Hazrati, 1995a). The tegmentum, which represents the most caudal extent of direct striatal influence, sends glutamatergic and acetylcholinergic fibers to the STN and SNc (Woolf and Butcher, 1986; Winn et al., 1997; Hallanger and Wainer, 1988; Lee et al, 1988). The SNc, which receives only sparse connections from the striatum in the intact rat, reciprocates input from the tegmental nuclei and the SNr, and provides a substantial contribution of dopaminergic fibers to the STN (Smith et al., 1998; Parent et al., 2000). Both D1 and D2 receptors have been localized in the STN, where dopamine has been

shown to have a predominately excitatory effect by reducing GABA-mediated IPSPs. However, dopamine is also capable of reducing glutamate-mediated EPSPs. These effects are carried out in the STN via D2-mediated, presynaptic mechanisms (Shen and Johnson, 2000).

Although a high level of c-Fos activity is expressed in the SNr of rat pups that engage in L-DOPA-induced locomotion, and in adult rats that engage in intrastriatal 1*S*, 3*R*-ACPD -induced circling, these preparations do not demonstrate c-Fos immunoreactivity in the SNc (Kaatz and Albin, 1995; Staup and Stehouwer, 2006). The same conspicuous absence of c-Fos labeling has been observed in the SNc of rat pups from the present study that engaged in decerebrate obstinate progression. Elimination or inactivation of the striatonigral pathway could sufficiently disinhibit the SNr, resulting in inhibition of the SNc (Hajós and Greenfield, 1994). In the absence of GABA-ergic fibers from the GPe, the primary consequence of inhibiting the SNc could be to release the STN from D2-mediated presynaptic inhibition of glutamatergic input from the tegmentum. Between the absence of GABA-inhibition and the increased excitatory input from the tegmentum, the STN could produce enough positive feedback to the SNr to increase inhibition of the SNc, effectively neutralizing dopaminergic output.

Deterioration of the dopaminergic fibers of the SNc and hyperactivity of the STN are primary factors in the etiology of Parkinson's disease (PD). Bradykinesia associated with PD is thought to result from a dysregulation of the indirect pathway, whereby D2-mediated inhibition of the striatum is reduced, increasing GABA-ergic output to the GPe, which disinhibits the STN, producing an increase in the inhibitory output of the GPi/SNr (Jellinger, 2002). Rats that receive 6-hydroxydopamine (6-OHDA) lesions of nigral dopaminergic fibers are used as models for PD (Jolicoer and Rivest, 1992). Loss of these fibers results in an increase in the firing rate and a change in the firing pattern of neurons in the STN (Breit et al., 2006). However, Hassani et al.

(1996) has demonstrated that 6-OHDA lesions produce a much greater increase in the rate of STN firing than pallidal lesions alone, and that, in fact, there is little to no decrease in the firing rate of pallidal neurons in rats that receive 6-OHDA lesions. This suggests that an increase in STN activity after dopaminergic fibers of the SNc have been compromised occurs via an alternate route than the indirect pathway of the basal ganglia. The data from the present report support this conclusion, as lesions of the GPe did not produce an increase in locomotion or an increase in the expression of c-Fos in the STN, but compromise of striatal projections to the midbrain did. The tegmentum may provide one such 'alternate pathway', as lesions of the PPN have been shown to decrease the STN activity of 6-OHDA-lesioned rats (Breit, 2006). However, this influence of the PPN may be unique to the pathological state, as similar lesions of the PPN produce an increase in STN-activity in intact rats (Breit, 2005). It is not entirely clear whether decerebrate rat pups match closer with a model of decreased or increased SNc-activity.

It is apparent that the profound up-regulation of c-Fos in the STN of decerebrate PD15 and PD25 rat pups does not occur by direct disinhibition of the STN through removal of the GPe alone. However, as has been discussed, elimination of striatal projections more closely resembles the anatomy of HD than PD. An increase in dopamine relative to other neurotransmitter systems of the basal ganglia is a distinguishing character of HD (Emerich and Sandberg, 1992), and may have played a more active role in decerebrate obstinate progression than the immunohistochemical results of the present report suggest. In other studies of rats, increases in SNc-activity were not reflected by an increase in the production of c-Fos or c-Fos mRNA (Kaatz and Albin, 1995; Labiner et al., 1993). This is particularly relevant because the pattern of c-Fos reactivity in the STN and SNr of 1S, 3R-ACPD-injected rats reported by Kaatz and Albin (1995) followed inactivation of the striatum, and was similarly robust to what was

found in the present study. No mention was made in that study of the presence or absence of c-Fos reactivity in the tegmentum.

An increase in cellular activity of the SNc in decerebrate rat pups may have occurred after removal of inhibitory projections from the striatum or excitation by direct projections from the disinhibited STN. However, in the intact rat, the SNc does not appear to be heavily innervated by either the striatum or the STN (Kita and Kitai, 1987; Parent et al., 2000). It may be, though, that the confluence of disinhibition that resulted from removal of the striatopallidal complex, along with whatever excitation the SNc received from the STN and mesopontine tegmentum, sufficiently depolarized cells of the SNc to reciprocate their input. The projections from the SNc to the STN and mesopontine tegmentum are dense, and may provide sufficient substrate to establish a system of positive feedback with both the STN and the tegmentum in the absence of inhibition from the striatopallidal complex (Semba and Fibiger, 1992; Hassani et al., 1997).

C-Fos Activity in the Tegmental Nuclei

The PPNc/MEA provides substantial cholinergic/glutamatergic innervation of the SNc (Parent and Hazrati, 1995; Takakusaki, 1996), and reciprocates the input it receives from the STN with a substantial, excitatory projection, as well (Woolf and Butcher, 1986; Canteras, 1990; Inglis and Winn, 1995). This would seem to leave the PPNc/MEA in an ideal position to support cellular activity in the STN of decerebrate rat pups, and to coordinate the behavioral response. However, while some diffuse c-Fos immunoreactivity was observed in the PPNc of most subjects in this study, very few showed dense bands of activity, and a treatment effect was not found. Instead, increased c-Fos expression was observed in the LDT of PD25 rat pups that engaged in decerebrate obstinate progression, and in the LDT of PD5 and PD25 rat pups that engaged in bicuculline-induced forward progression. In contrast to the PPNc, the LDT does not receive projections from the STN (Semba and Fibiger). This pattern of afferentation is not

consistent with the hypothesis that direct projections from the STN are providing activation of tegmental motor command centers during decerebrate obstinate progression, and is a departure from the pattern of c-Fos activity demonstrated by rat pups that engaged in L-DOPA-induced locomotor behavior, where an increase in c-Fos activity was found in the more lateral PPNC (Staup and Stehouwer, 2006).

The PPN is a much more diffuse collection of nuclei than the LDT and it may be that the 40X target region from which c-Fos-labeled PPN cells were counted in the present study was too small, generating artificially low numbers. The LDT and PPN are highly interconnected, and are often conceived of as a single functional unit (e.g., Garcia-Rill and Skinner, 1988), suggesting that activity in one may represent activity in the other. However, muscarine agonists inhibit activity in the PPN and LDT. Given the cholinergic nature of the fiber paths between them, these nuclei may be mutually inhibitory (Garcia-Rill, 1991; Laviolette, 2000). The different pattern of activity in the mesopontine tegmentum produced by L-DOPA-induced locomotor behavior and decerebrate obstinate progression may reflect greater sensitivity to adrenergic arousal by the PPNC of PD15 rat pups, whereas the LDT may be more sensitive to inhibition from the forebrain.

Like the PPN, the LDT shares a reciprocal connection with the SNc (Woolf and Butcher, 1986; Semba and Fibiger, 1992). If the SNc of decerebrate rat pups is active, mutual excitation between the SNc and the STN, and between the SNc and the tegmental motor nuclei may generate enough activation to produce a behavioral response. Once locomotion has been initiated, the activity of tegmental neurons entrained by the efferent copy of motor output from the medulla and spinal cord, along with whatever re-entry information is made directly available to the STN and SN, may provide further positive feedback (Garcia-Rill and Skinner, 1988).

Disinhibition of feed-forward circuitry in the midbrain by removal of striatal influence over the SN and tegmentum explains the dense c-Fos immunoreactivity in the STN of decerebrate rat pups without presuming the existence of a powerful striatosubthalamic projection, for which there is little evidence (Canteras, 1990). A similar pattern of interaction between the STN, SN and tegmentum has been speculated to be responsible for secondary neurodegeneration in the PPN of PD patients, and to exacerbate deterioration of the SNc in later stages of the disease (Rodriguez et al., 1998; Pahapill and Lazano, 2000, Wright and Arbuthnott, 2007). However, these effects are thought to be mediated by direct projections from the STN to the PPN, which does not appear to be active in the decerebrate preparation. Given the possibility of mutual inhibition between the LDT and the PPN, which area of the tegmentum is recruited by excitotoxic feed-forward circuits could be an important distinction between models of basal ganglia disorders, characterizing advanced stages of dysfunction.

Bicuculline-Induced Forward Progression

Contrasting the behavior and level of c-Fos activity in the STN of decerebrate rat pups to those of bicuculline-injected rat pups may offer some insight into the effect an intact and functioning striatum may have on disinhibition of the STN. When considering the impact of GABA_A antagonists on the STN, it is important to recognize that GABA_B receptors, which are present on many axon terminals in the STN, continue to provide presynaptic inhibition of excitatory inputs to the STN (Shen and Johnson, 2001). Also, STN activity elicited in neonates by systemic L-DOPA and by decerebration may reflect changes in the input of several neurotransmitters, not just GABA, to the STN; including dopamine, 5-HT, glutamate and acetylcholine (Cantares et al., 1990; Shen and Johnson, 2000; Shen and Johnson, 2008).

Despite the very high dose of bicuculline used in this study, c-Fos activity in the STN was limited, never showing heterogeneous activity throughout the nucleus, as was characteristic of

decerebrate rat pups. In areas of the STN where cellular activity was scored, the density of labeled cells of microinjected PD15 rat pups, although greater than age-matched controls, was lower than that of decerebrate PD15 rat pups (fig 3-8). Although microinjected rat pups did exhibit more forward progression than control animals, the behavior did not persist throughout the test session as it did with the decerebrate rat pups, and was often interrupted by periods of inactivity, grooming or other behaviors, as noted in our results.

The sporadic locomotor behavior and less-dense labeling of the STN was not unexpected, and may have reflected the continued influence of the striatopallidal complex on the SN and tegmentum, as well as the presence of negative feedback to the STN from the GPe. Such activity may have been the product of limbic and sensory arousal of the still intact forebrain of microinjected rat pups. Furthermore, because cortical projections are immature in neonates, the information that was integrated by the striatopallidal complex of microinjected rat pups likely arrived via subcortical circuits, which are also present in adults and compete regularly with cortical projections for behavioral resources (McHaffie et al., 2005). However, differences between decerebrate and bicuculline-injected rat pups may also be attributed to the inadvertent diffusion of bicuculline, and subsequent activation of GABA-sensitive behavioral centers adjacent to the STN.

The Subthalamic Locomotor Region

The subthalamus refers to a region larger than the STN. This area of the brain is bounded dorsally by the medial lemniscus and rostral-ventral by the internal capsule. Other researchers have reported an increase in locomotion only after application of GABA-blockers to areas within the subthalamus, neighboring the STN, but not the STN itself (Waldrop et al., 1988; Périer et al., 2002). These areas, including the zona incerta (ZI) and the lateral hypothalamus (LH), are of note because they represent a region, similar to the MLR, which is functionally defined by the

ability of low threshold stimulation to elicit locomotion (Waller, 1940; Orlovskii, 1969). In fact, these nuclei are often referred to collectively as the subthalamic locomotor region (SLR) because of the impact on behavior they share with the more caudal MLR (Milner and Mogenson, 1988).

This experiment used large drug volumes (1.5-2 μL) to deliver bilateral microinjections of bicuculline (0.37 $\mu\text{g}/\mu\text{L}$) to the STN. The concentration was chosen in order to maximize disinhibition of the STN without producing a depolarization block, which has been reported in previous studies at higher concentrations (P erier et al., 2002). When preliminary research did not reveal a behavioral effect with 0.2 μL of the drug, the volume was increased until an increase in forward progression was observed. At the effective volume, diffusion of the drug brought it into contact with areas adjacent to the STN, raising serious questions regarding what area or areas in and around the subthalamus could have been responsible for the increase in locomotion observed in bicuculline-injected rat pups.

In the present study, both the ZI and the LH of microinjected rat pups showed increased c-Fos immunoreactivity to rival, and in some cases surpass, that of the STN (fig. 3-9). However, this increase in activity was not exclusive to microinjected rat pups. In the LH, which is located mediorostral to the STN, immunoreactivity in decerebrate PD15 rat pups was greater than LH activity of age matched controls, implicating disinhibition of the LH in the production of decerebrate obstinate progression at PD15. Unlike the STN, the LH projects to the LDT. The LDT reciprocates the input, providing a direct excitatory feedback loop between the subthalamus and the tegmentum (Woolf and Butcher, 1986; Hallanger and Wainer, 1988; Semba and Fibiger, 1992). Also in contrast to the STN, the SLR sends more direct projections to the SNc than to the SNr (Kita and Kitai, 1987). This pattern of connectivity between the SLR and the midbrain was reflected by c-Fos activity in microinjected rat pups of the present study, which demonstrated an

increase in LDT activity, but not SNr activity (figs. 4-2 and 4-3), suggesting further that bicuculline-induced forward progression resulted from activation of the SLR, and not the STN.

Perhaps the most relevant contribution the results obtained from microinjected rat pups of the present study made to the understanding of decerebrate obstinate progression is the similar absence of c-Fos reactivity in the SNc of both groups (fig. 4-2). If bicuculline-induced forward progression is the result of SLR-activation, then the SNc would be expected to be more highly activated in microinjected rat pups, not only because of greater excitatory input to the SNc from the SLR (Kita and Kitai, 1987), but also lower SNr activity, resulting in less inhibition of the SNc (Hajós and Greenfield, 1994). That the SNc does not exhibit c-Fos immunoreaction-product in a case where there should clearly be an increase in cellular activity lends further support to the observations made by others that c-Fos immunoreactivity is not a sensitive measure of SNc activity (Kaatz and Albin, 1995; Labiner et al., 1993), and favors the explanation of decerebrate obstinate progression involving activity of the SNc at the center of feed forward circuitry between the STN and the tegmentum.

As demonstrated by the representative sections in figure 4-2, transected PD15 and PD25 rat pups showed little to no activity in the ZI, which is located immediately above the STN. In both decerebrate obstinate progression and L-DOPA-induced locomotion the entire STN is activated in distinct contrast to the immediately adjacent ZI (Staup and Stehouwer, 2006). This may not be a condition that is replicable with direct application of GABA-antagonists in non-pathological animals. The STN is small, which makes it impossible to use large volumes of drug without spreading to the GABA-sensitive surround. Yet, smaller volumes that do not diffuse beyond the STN do not activate the entire nucleus. Unitary activity is uncharacteristic of the STN, which under normal conditions engages in segregated, parallel processing of sensorimotor,

associational and limbic information from the striatopallidal complex (Temel et al., 2005).

Although densely nucleated and without a lot of glial separation between its neurons, the STN is devoid of gap junctions (Chang et al., 1983), and intranuclear glutamatergic collaterals have not been shown to be capable of producing synchronous firing (Wilson et al., 2004).

Microinjected rat pups and rat pups that received microinjections with lesions demonstrated a significant decline in limb alternation at each bin of the testing session (fig. 3-8), and a treatment effect was no longer evident for either measure of duration at any age during the final 15 minutes of the testing session for these groups (figs.3-6 and 3-7). The abbreviated locomotor behavior of microinjected rat pups probably reflects the breakdown and clearance of bicuculline from the injection site. The metabolism of bicuculline may not have provided enough time for accumulation of sufficient levels of c-Fos in every area of the brain involved in bicuculline-induced forward progression to perform an analysis. Given this limitation, it should not be assumed that the ZI, LH, STN and LDT were the only brain areas appreciably activated in microinjected rat pups while they engaged in locomotion.

C-Fos immunoreactivity in PD5 Rat Pups

At PD5, only the LDT of bicuculline-injected rat pups demonstrated an increase in c-Fos expression. Although cell-labeling was evident in the STN and SNr of bicuculline-injected rat pups, it did not reach significance. However, the mere presence of immunoreaction product rules out the possibility that these brain areas were active but not expressing c-Fos during decerebrate obstinate progression at this age. The low levels of c-Fos found in the SNr and STN of microinjected PD5 rat pups may be attributed to the abbreviated expression of forward progression by these animals, who only demonstrated more sector crosses than age-matched controls during the first bin of the testing session (fig 3-5). However, decerebrate PD5 rat pups

demonstrated an increase in sector crosses for all three bins, and still exhibited a complete absence of c-Fos immunoreaction product in the basal ganglia and midbrain.

If the STN and the midbrain are not active during decerebrate obstinate progression at PD5, then the neural activity that drove the behavior at this age is a mystery. It is possible that the SNc of these rat pups was active, based on the inconsistencies between cellular activity and c-Fos-labeling that have been reported (Kaatz and Albin, 1995; Labiner et al., 1993). However, the SNc is intimately associated with the SNr, and projects heavily to the STN and tegmentum (Hajós and Greenfield, 1994; Hassani et al., 1997; Semba and Fibiger, 1992). It would be unusual for the SNc to become active without producing activity in any of these areas.

It is possible that transections of PD5 rat pups resulted in the release of a more caudal, medullar center of motor activity. However, post-nigral transections, which leave the medulla intact, have not revealed an increase in spontaneous locomotion at this age (McCrea, 1994). Additionally, the only other report of decerebrate obstinate progression in rat pups did not produce an increase in locomotion at PD5 using pre-mammillary transections just caudal to the STN (Campbell and Stehouwer, 1979). These results warrant further investigation, utilizing multiple transections at various levels rostral and caudal to the STN in PD5 rat pups.

Conclusion

To review, decerebrate obstinate progression in PD15 and PD25 rat pups appears to result from the liberation of feed-forward, midbrain circuitry from the inhibitory control of subcortical forebrain structures. This is evidenced by dense c-Fos reactivity in the STN, SNr, and LDT, which are all targets of inhibitory projections from the striatopallidal complex (Parent et al., 2000, Smith et al., 1998). Although no c-Fos reactivity was evident in the SNc, other studies of rats exhibiting similar behavioral and immunohistochemical results have reported increases in

SNC rate-of-firing and neurotransmitter release that were not reflected by c-Fos immunoreactivity (Kaatz and Albin, 1995; Labiner et al., 1993).

Microinjections of bicuculline into the subthalamus produced an increase in forward progression at PD5 and PD25, and increased c-Fos immunoreactivity in the tegmentum at these ages. Sensorimotor and limbic arousal of the intact striatopallidal complex may have contributed to the greater variability of behavior in microinjected rat pups. Disinhibition of areas of the SLR, exhibited by greater c-Fos reactivity in the ZI and LH of microinjected rat pups, may have made a more significant contribution to the increase in locomotion and cellular activity of bicuculline-injected rat pups than disinhibition of the STN. In contrast, with the exception of an increase in c-Fos expression in the LH of PD15 rat pups, the only area of the subthalamus to become significantly activated in decerebrate rat pups was the STN. However, rat pups that engaged in decerebrate obstinate progression and rat pups that engaged in bicuculline-induced forward progression both exhibited an increase in c-Fos activity in the LDT, suggesting that activation of the mesopontine tegmentum may be a common mechanism responsible for the similar behavioral results of these two manipulations.

Destruction of the GPe did not sufficiently disinhibit the STN to produce an increase in c-Fos immunoreactivity in any of the nuclei where c-Fos was evident in transected rat pups, and did not result in an increase in forward progression at any of the ages studied. Given these results, it is unlikely that activation of the STN in decerebrate rat pups results solely or even primarily from the removal of the striatopallidal complex, as there is very little evidence of any substantial input to the STN from the striatum in the rat (Cantares, 1990). Rather, it was suggested that decerebration results in disinhibition of not only the STN, but the SN and tegmental motor nuclei, as well. These nuclei are thought to sustain a high level of activity via a

network of positive feedback between one other. This brain circuitry utilizes many glutamatergic projections that may be neurotoxic at prolonged, high levels of activity (Rodriguez et al., 1998). Excitatory projections from tegmental nuclei are thought to be responsible for maintaining just such a level of heightened activity in the STN of PD patients, contributing to secondary degeneration during later stages of the disease. Increased activity of glutamatergic projections from the tegmentum itself may also contribute to the deterioration of the SNc (Pahapill and Lazano, 2000, Wright and Arbuthnott, 2007). The area of the tegmentum that is recruited by excitotoxic, feed forward circuitry appears to vary between models of STN hyperactivity, which may differentially impact the severity and/or form of deterioration associated with later stages of basal ganglia dysfunction (Staup and Stehouwer, 2006). It would be fruitful for future investigations, using more precise measures of cellular activity in the basal ganglia and midbrain, to make direct comparisons of decerebrate obstinate progression with other models of persistent locomotor behavior that demonstrate similar patterns of c-Fos immunoreactivity, such as L-DOPA-induced air-stepping and intrastriatal 1S, 3R-ACPD-induced circling behavior. Longitudinal, within subjects studies of these treatments may reveal what impact, if any, age of onset has upon the effects of different methods of inducing positive feedback between the midbrain and basal ganglia.

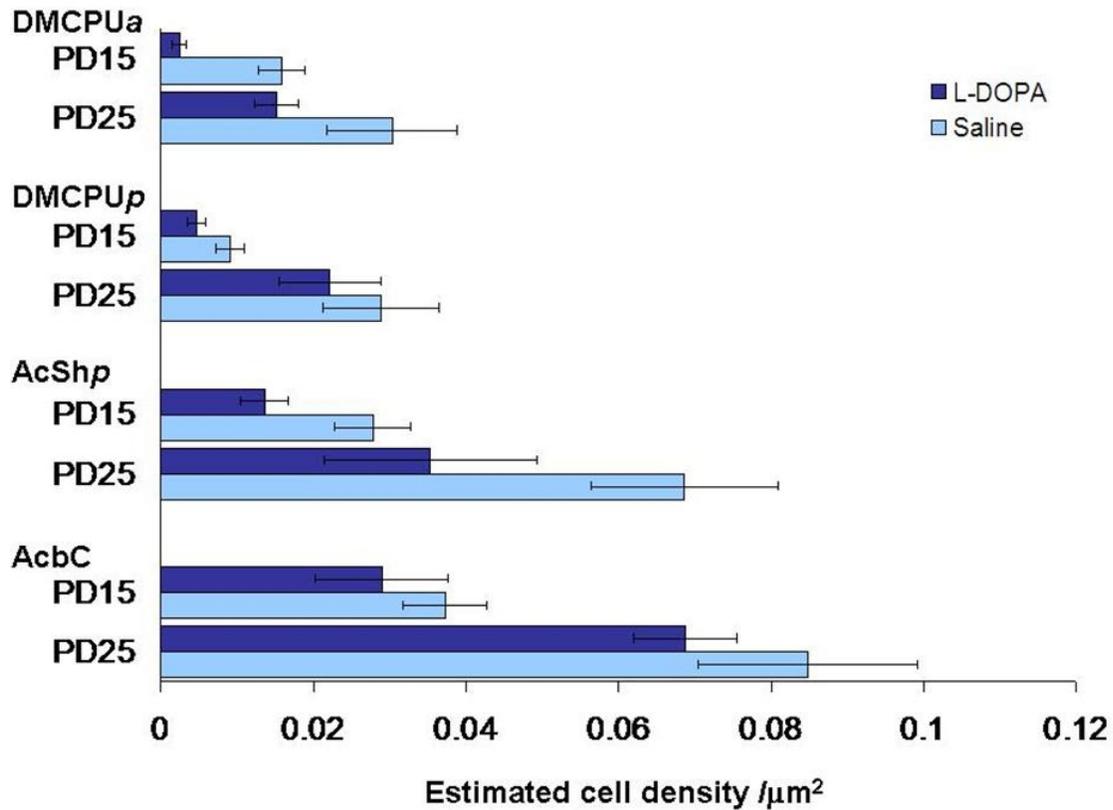
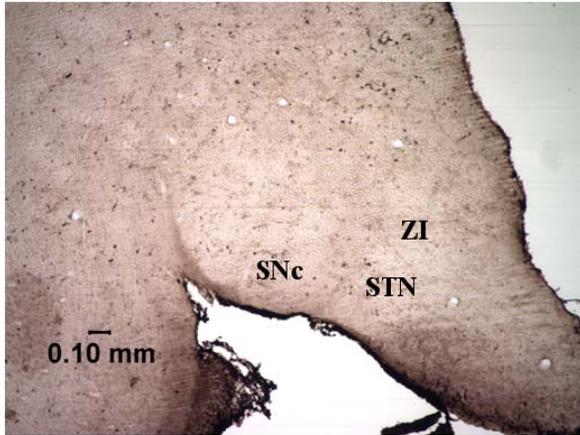
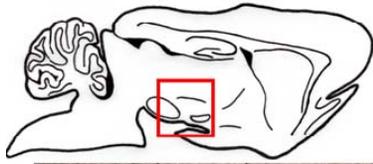
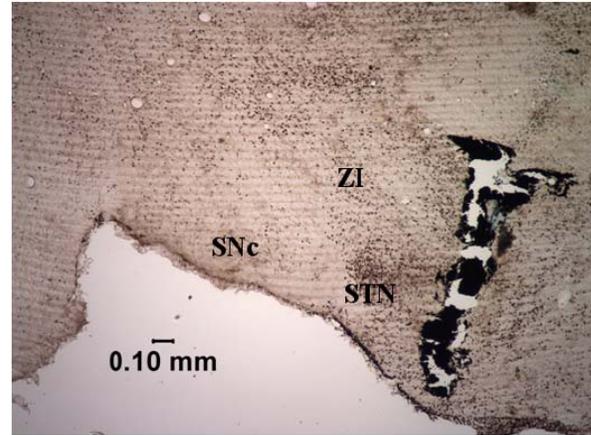


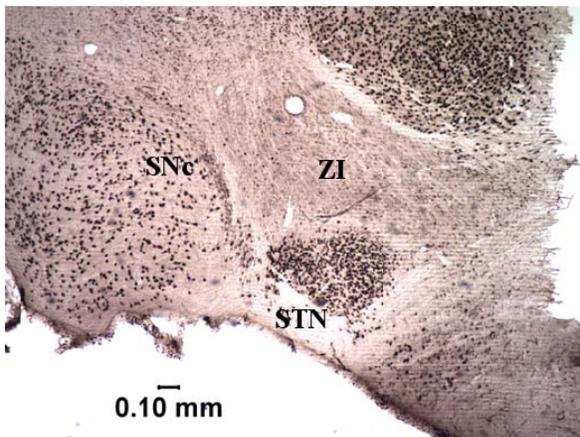
Figure 4-1. C-Fos immunoreactivity in the striatum of L-DOPA-injected rat pups. Greater c-Fos labeling in PD25 rat pups than in PD15 rat pups was evident throughout the striatal complex: anterior (DMCPUa) and posterior (DMCPUp) dorsomedial caudate-putamen, posterior shell of the nucleus accumbens (AcShp), and the accumbal core (AcbC). There was also an inhibitory trend for the administration of L-DOPA, although this effect was only significant in the DMCPUa. (Adapted from Staup, M. A., Robertson, K., Stehouwer, D. J. (2005) Ontogenetic changes in neural and locomotor responses to L-DOPA. *Presented at the 2005 International Society for Developmental Psychobiology conference*)



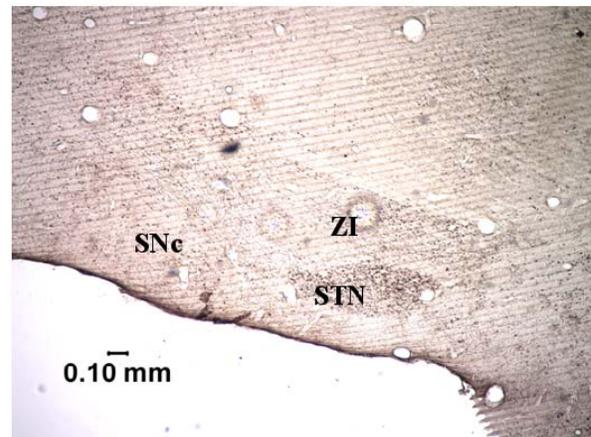
A



B

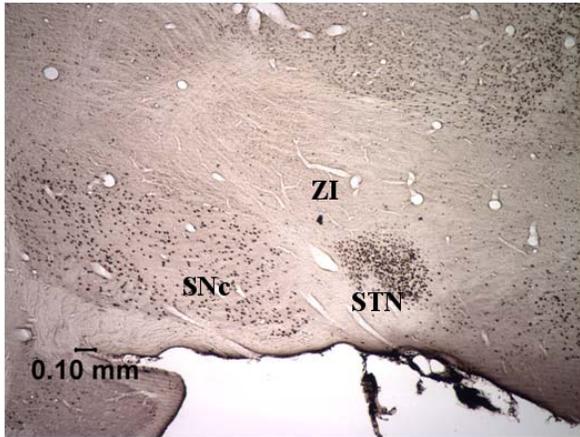


C

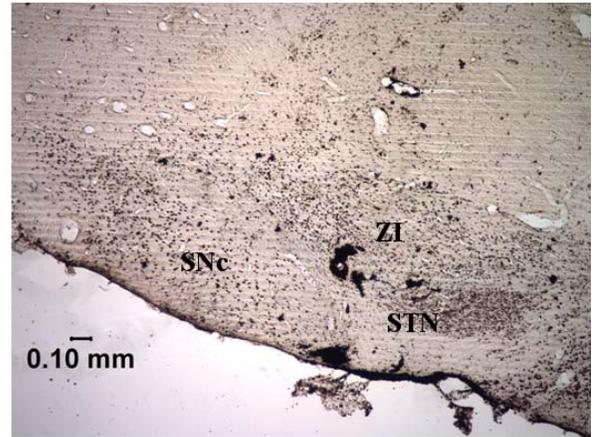


D

Figure 4-2. The subthalamic nucleus of decerebrate and microinjected rat pups. A) C-Fos reactivity was not evident in decerebrate PD5 rat pups, although B) mild expression was often visible in the subthalamus of microinjected PD5 rat pups. Discrete labeling was evident within the STN of C) decerebrate PD15 rat pups and E) decerebrate PD25 rat pups, while D) microinjected PD15 rat pups and F) microinjected PD25 rat pups displayed labeling in the STN and in the region above the STN, the ZI.

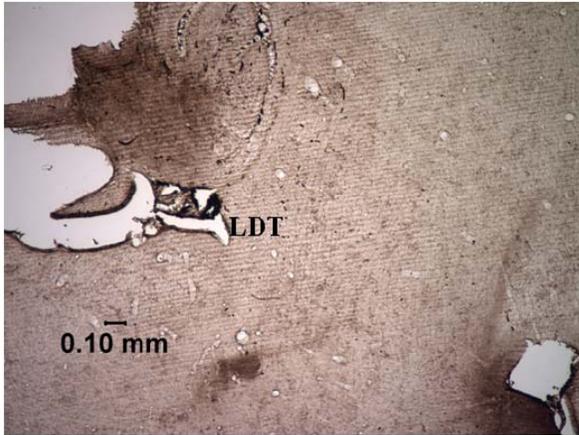
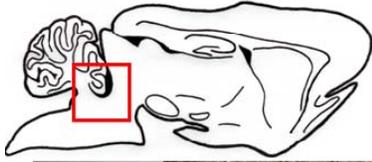


E

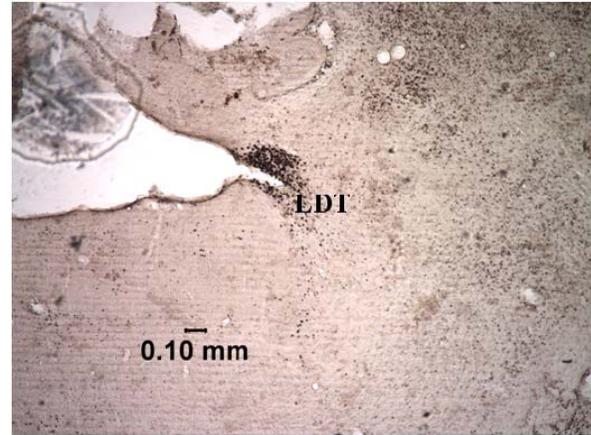


F

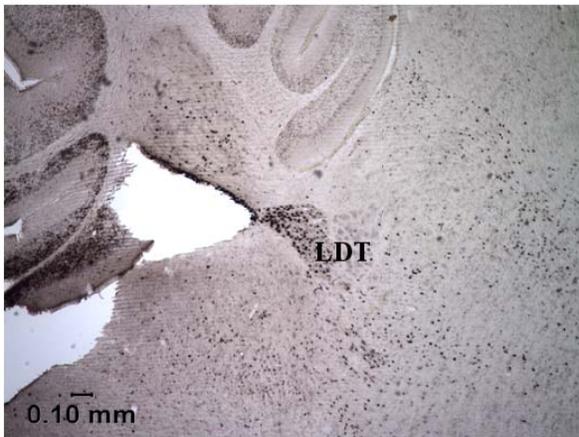
Figure 4-2. Continued



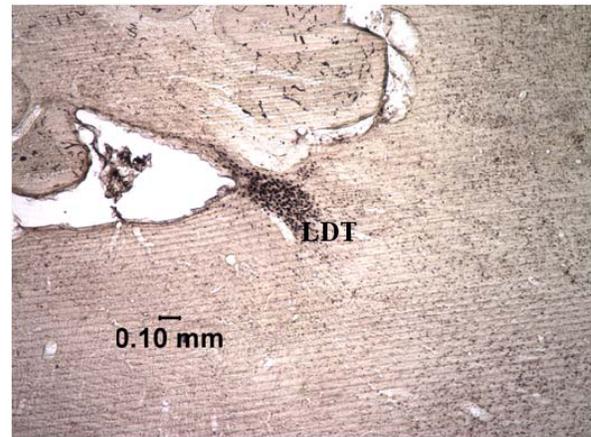
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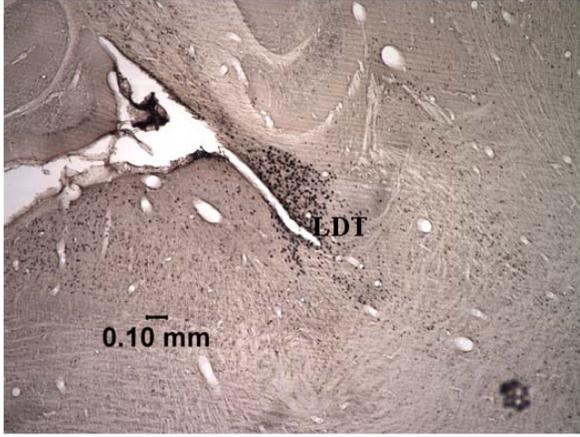


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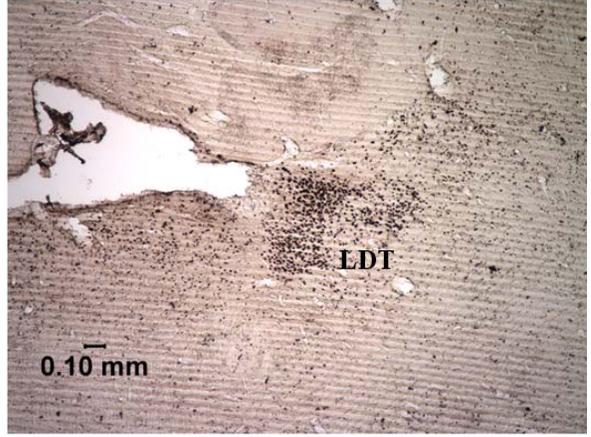


D

Figure 4-3. The lateral dorsal tegmental nucleus of decerebrate and microinjected rat pups. Immunoreactivity was not seen in the LDT of A) transected PD5 rat pups, but was evident in B) microinjected PD5 rat pups. Immunoreactivity was also evident in C) transected PD15 rat pups, D) microinjected PD15 rat pups, E) transected PD25 rat pups, and F) microinjected PD25 rat pups.



E



F

Figure 4-3. Continued

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BIOGRAPHICAL SKETCH

Michael Anthony Staup was born on May 28, 1975 in Houston, Texas. His family moved to Omaha, Nebraska, when he was six years old. Michael graduated from Mount Michael Benedictine High School in 1993. He earned his B.S. in psychology with an emphasis in biology from the University of Nebraska at Omaha (UNO) in August 2002. That same month, Michael entered the graduate program for behavioral neuroscience in the Department of Psychology at the University of Florida. In December 2005, Michael was awarded his M.S. in psychology. One year later, he successfully passed his qualifying examinations for candidacy to earn his Ph.D. in psychology. Upon completion of his Ph.D. program, Michael and his wife, Kathryn Lee Staup, will move to San Diego, California to seek their fortune.