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Motor Learning and Stroke Dynamics: A MicroPET Study

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Abstract

Ischemic stroke is the third leading cause of death, being responsible for over 200,000 deaths per year in the United States, and is the leading neurological cause of disability. The treatment of chronic behavioral loss following stroke is considered a major problem in the field of medicine. One way to develop new treatments for stroke patients is to use animal models, which have been developed principally in rodents, to mimic the pathology of stroke and to try to understand the basic mechanisms which might underlie functional improvement. Among the many animal models of stroke, the intraluminal middle cerebral artery occlusion is one of the most consistent and reliable. It is important, given the difficulty of treating individuals during the post-stroke period to look for possible pre-stroke interventions that may be neuroprotective, and may be crucial in helping the individual by either mediating the stroke damage or facilitating stroke recovery. Among these interventions, specific fine motor skills look promising in that they have been shown to cause enhanced neurogenesis, specifically dendritic branching, in the motor cortex, an area very often damaged in stroke patients. In the study proposed in this paper, it is hypothesized that that pre-stroke motor learning will reduce severity of stroke in rats and will enable them to rehabilitate affected body areas more quickly than those of control rats who do not learn the motor task.

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Stroke

Ischemic stroke is the third leading cause of death (200,000 deaths per year in the United States) and is the leading neurological cause of disability (Mattson, Duan, Wan, & Guo, 2004). The treatment of chronic behavioral loss following stroke is a major problem in the field of medicine. In fact, it would not be an overexaggeration to say that, in clinical cases, the treatment of brain ischemia has been extremely unsuccessful. One way to develop new treatments is to use animal models, which have been developed principally in rodents, to mimic the pathology of stroke and to try to understand the basic mechanisms which might underlie functional improvement (Gonzalez & Kolb, 2003). Further establishment of microPET methods for measurement of stroke damage and recovery can be used to test and evaluate a number of types of therapeutic approaches for stroke treatment. These studies will provide important new information on the potential therapy in the experimental stroke rodent model, as well as on fundamental understanding of mechanisms how motor skill training mediates functional recovery from stroke (Hossman, 1998).

The damage from stroke results from insurmontable cellular stress. Damage generally starts before the stroke even happens. These antecedent events include damage to cerebrovascular endothelial cells, atherosclerosis, and hypertension. During the stroke, the occlusion or rupture of a cerebral blood vessel causes hypoperfusion of the brain tissue supplied by the affected vessel, subjecting those neurons to hypoxia and hypoglycemia. Under these conditions, neurons die rapidly as result of energy failure and disruption of cellular ion homeostasis. The neurons undergo apoptosis (form of cell death in which a programmed sequence of events leads to the destruction of cells without releasing harmful substances into the

surrounding area) or related forms of cell death. There is a large amount of research being done in order to investigate the involvement of free radicals, energy impairment, and perturbed calcium regulation in stroke-induced neuronal death (Mattson et al., 2004).

There is more than one type of stroke, or cessation of blood to the brain. In fact, there are three different categories of blood flow reduction. The first of these categories is transient global ischemia, which is the complete cessation of cerebral blood flow, followed by more or less efficient recirculation. The blood flow decline is incomplete and heterogeneous. Clinically, the main cause of transient global ischemia is cardiac arrest, although it can also result from strangulation, severe shock, or intracranial hypertension. The second category is permanent/transient focal ischemia, which is most often caused by thrombotic (blood clot) occlusion of the middle cerebral artery (MCA). The transient forms of this stroke category are produced by vascular clipping in the course of neurosurgical interventions or by severe vasospasms (exaggerated, persistent contraction of the walls of a blood vessel) (Hossman, 1998). In the laboratory, MCA occlusion can be produced in a number of ways, including occlusion by cauterization, occlusion by clip, occlusion by thread, endothelium-induced lesions, and pial stripping (see Szele, Alexander, & Chesselet, 1995 for a full review), just to name a few.

The third category of stroke is microembolism which, clinically, is caused by fat microemboli after bone fractures, by release of platelet aggregates and thrombotic materials from ulcerating atherosclerotic plaques, or by air bubbles during cardiac surgery. This type of stroke leads to multiple ischemic microfoci (areas of ischemic damage) in the brain.

There is evidence that these different models of stroke may not be equivalent in their postinjury pathology. The ischemia time that is tolerated by the brain depends on a multitude of factors, including the density of ischemia, the tissue concentration of primary and secondary energy

stores, and the rate of energy consumption. This rate, in turn, depends on temperature, degree of functional activity and the absence or presence of other drugs and ischemias. Furthermore, there is a major difference between ischemic vulnerability of different regions of the brain. During stroke, the severity of cell injury is modulated by numerous indirect consequences of the primary ischemic impact, including the large number of hemodynamic and molecular responses which determine the final outcome (Hossman, 1998). As a specific example, ischemic damage to the brain caused by thermocoagulation of the motor cortex produces very different changes in the striatum than that of the damage produced by the aspiration of equivalent areas (Gonzalez & Kolb, 2003). These differences include those of axonal sprouting as well as the expression of molecules associated with neuronal plasticity, including bFGF (basic fibroblast growth factor) (Szele et al., 1995). It is because of these considerations that the ideal model of stroke should be relevant to the clinical situation, highly reproducible, easy to perform, and should avoid complicating side effects.

Global Cerebral Ischemia

There are four main methods that researchers use to mimic global cerebral ischemia in the laboratory. The first of these is cardiac arrest, which is usually produced by ventricular fibrillation or intracardiac injection of cardioplegic agents (drugs that cause elective, temporary stopping of cardiac activity). Researchers also induce complete brain ischemia through the compression of the blood vessels of the neck by strangulation, intra-thoracic occlusion of the innominate and left subclavian arteries, increase of intracranial pressure above normal blood pressure by infusion of fluids under high pressure into the cisterna manga, and, finally, through isolated head or brain preparations.

Global ischemia can also be investigated *in vitro*, using primary neuron cultures, organotype cultures, or brain tissue slices. Brain slices can also be prepared *ex vivo* from decapitated animals in order to study the post-ischemic recovery process. *In vitro* investigation of stroke has major disadvantages, however. The preparation of the slice is associated with severe tissue trauma and the period of ischemia before the tissue is brought into the incubation medium is also a factor. Also, the incubation medium used is different from the surrounding brain medium. Because of these factors, the control recordings of such preparations represent a post-traumatic, post-ischemic recovery state which may be basically different from the normal situation (Hossman, 1998).

Focal Cerebral Ischemia

There are three main ways laboratories model focal cerebral ischemia. The first of these is through intracranial vascular occlusions, the most popular of which is the occlusion of the middle cerebral artery (MCA), because this is the most clinically relevant model. In the laboratory, the MCA can be exposed by transcranial (through the cranium), postorbital (behind the eye socket), or transorbital (through the eye socket) approaches. The transorbital approach requires removal of eyeball but does not cause any trauma to the brain, unlike the transcranial and postorbital approaches. Once exposed, the MCA can be clipped, ligated, or occluded through the local application of the potent vasoconstrictive agent endothelin-1. These methods are not always preferable, however, because the brain always has to be exposed, and often brain tissue proximal to the MCA is damaged due to the surgery, confounding the experimental results.

Incomplete brain ischemia (aka oligemia) can be produced in the laboratory by extracranial ligation of the carotid and vertebral arteries or by lowering arterial blood pressure in combination with bilateral carotid artery occlusion. However, this technique does not result in an

even reduction of blood flow at the microcirculatory level. Also, without appropriate imaging techniques, the ischemic injury in a particular brain region is difficult to assess with this method (Hossman, 1998).

In recent years, surgical exposure of the MCA has been replaced by intraluminal (within the inner space of an artery) placement of flow-obstructing devices, usually microfilaments (threads). The dominant theory of the mechanism of blood flow during an acute ischemic stroke explains that there is a gradual decline of blood flow from the periphery to the center of the supplying territory of the occluded artery. According to this threshold concept of cerebral ischemia, during the initial few hours of the stroke episode blood flow changes little; therefore the most appropriate animal experimental model for the evaluation of early treatment would seem to be a permanent type of vascular occlusion. However, the temporary occlusion of the MCA for 1-3 hours has found increasing popularity because the size of the infarct can be varied as a function of the time of vascular occlusion.

Among the many ways used to induce middle cerebral artery occlusion (MCAO), the use of intraluminal microfilament placement is most advantageous because it avoids the need of craniotomy due to the fact that the microfilament can be inserted from the external carotid artery (ECA), which is accessible through the neck of the animal. Furthermore, the microfilament can be easily withdrawn to produce transient focal ischemia by returning blood flow to the area of infarction. However, the intraluminal placement of microfilaments has its difficulties as well. These include precise placement of the thread, the risk of vascular puncture, and the large infarct size invariably produced. This large infarct size occurs because the microfilament, while occluding the MCA, also occludes the origin of the anterior cerebral artery. In order to overcome this particular difficulty, researchers insert the thread for a limited amount of time (usually 1-3

hours) in order to restrict the size of infarcts. This is an effective technique, however, this reversible type of focal ischemia produces a pathophysiology which is different from that of clinical stroke. Thus there is disputable relevance of this method for treatment studies because blood flow reduction during clinical stroke rarely resolves during 1-3 hours and, when it does (such as during a transischemic attack), clinical symptoms are likely to disappear spontaneously. Also, sudden recirculation of blood after short periods of focal ischemia leads to the generation of oxygen radicals. Free radicals have been implicated in the pathophysiology of reoxygenation injury, which doesn't always occur during clinical stroke recovery. Finally, it can be difficult to exactly standardize microfilaments during their production, which involves cutting them to a specified length, dipping them in poly-L-lysine, and placing a dot of super glue on the end to act as a "bulb" to completely occlude the MCA (Hossman, 1998).

In an attempt to produce lesions of constant size, a microcirculatory occlusion model, such as focal cortical photothrombosis is used. Photothrombosis is done by injecting a photosensitizing chemical (e.g. Rose Bengal). A device is used for activating the photosensitive chemical (such as a xenon arc lamp) that can produce the most sensitive light for the injected dye. However, this produces a ring lesion, which does not show up in human patients. Models of extracranial vascular occlusions via the common carotid artery are also used, but this procedure doesn't produce cerebral ischemia in rats because of collateral blood supply given by the Circle of Willis. Therefore this procedure can only be used in gerbils, whose Circle of Willis is incomplete (Hossman, 1998).

In rat MCAO, regardless of how it was produced, a number of structures including the lateral frontal neocortex, the striatum, and the medial forebrain bundle are concurrently damaged, while the rostral and caudal representation of the forelimb regions in the motor cortex

are spared. This is an extremely surprising finding because the major behavioral deficit found after transient MCAO is contralateral (on the opposite side of the body than the side of the brain that is damaged) paw usage. Over and again it has been found that MCAO produces severe motor deficits impairing performance on sensorimotor tests such as paw placing, forelimb use for postural support, staircase reaching, tray reaching, and limb coordination. However, the cortical area of damage is the lateral neocortex, sparing the motor cortex. Thus, it would seem that even though the motor cortex is largely spared in MCAO stroke, the functions thought to be supported by motor cortex are not. This anatomical finding raises the question of which cortical or subcortical structures are compromised by MCAO stroke which could contribute to MCA-related motor impairments.

Gharbawie, Gonzalez, & Whishaw (2005) attempted to study damage to the lateral frontal neocortex (LFN) in MCAO stroke. The LFN includes the face and barrel (vibrissae) region of the sensory neocortex and is the primary neocortical zone of MCAO stroke damage. The clinical relevance of this study comes from the fact that MCA ischemia in humans can concurrently damage a number of cortical and subcortical areas that are the same areas that are damaged in rat models of MCAO stroke. The findings of this study led the authors to conclude that while some aspects of movement may be localized, skilled movements (such as forepaw use) are likely to involve many different brain regions.

Microembolism

In order to induce microembolisms in the laboratory, calibrated microspheres are injected into the MCA. This has the obvious advantage of the fact that the number and the diameter of the injected spheres can be standardized much more precisely than that of microfilaments. Alternatively, intracarotid injection of air bubbles and infusion of adenosine diphosphate,

arachidonic acid, or phorbol-myristate-acetate, in order to induce platelet aggregation, can be used to induce microembolisms. Following microembolism induction, the blood-brain barrier instantaneously breaks down, while under pure ischemic conditions, the barrier breaks down only after several hours. This is a major disadvantage of this model, and thus limits its clinical relevance. Also, the development of multifocal reactive hyperemia (increased blood flow in many small areas) in the surrounding occluded microvessels often leads to sudden swelling of the brain and inhomogeneous distribution of microcirculation. The outcome of this is that the net blood flow of the total brain may remain normal, making the actual stroke very difficult to detect (Hossman, 1998).

Motor Skill Learning Task: Skilled Reaching

The Task

Skilled Reaching is an action pattern in which a rat must reach through a slit in order to obtain a food reward. This action is not naturally performed by rats, however it can be taught to them. The teaching process requires a box known as a reaching cage (see explanation below), a food reward, and a lot of patience.

Skilled reaching as performed by the rat has been adopted as a model to analyze the development and lateralization of motor control, the neural control of skilled movement, functional recovery after brain damage. It is known that rat reaching differs from human reaching in two ways: First, it is guided by olfaction rather than vision. The implication of this is that motor action in the rat almost certainly necessitates the use of brain areas other than the motor cortex. Second, it has been described in the literature as stereotyped because it has much the same form in many different test situations (Whishaw & Pellis, 1990). This stereotypy is explained by the fact that some movements used by animals are distinctive, easily recognizable

from instance to instance, and minimally modified by experience. These movements are referred to as action patterns. The inflexibility of an action patterns is taken to mean that these movements are innate and the neural circuitry responsible for producing them is highly organized. Skilled reaching by the rat is thus suggested to be an action pattern. This suggestion supports the hypothesis that this type of skilled reaching is produced by a complex and relatively fixed neural circuitry (Whishaw & Pellis, 1990).

Skilled reaching in the rodent requires a complex sequence of movements that involve the entire body. First, the rat must locate pellet through olfaction. It then uses its vibrissae (the stiff hairs that are located around the nostrils or on other parts of the face that serve as tactile organs) and other tactile information to locate the aperture through which it must reach, estimate the height of shelf, and to "know" that its reach has been successful. Finally, it must approach the food anew for each reach, which means it must organize its approach as well as adjust its body posture throughout the reach and retrieval of the food pellet (Gharbawie et al., 2005).

Whishaw & Pellis (1990) analyze the skilled reaching sequence as follows. First, the rat lifts and aims the paw to align it and the forearm with midline of body. During most of this first phase of the skilled reaching, the movement is produced proximally, with the limb lifted and then advanced from the shoulder. Following this, the limb is carried to a parasagittal position so that the long axis of the forearm is aligned along the midline of the body (Whishaw & Pellis, 1990). While the rat advances its paw it opens its digits. Next, there is a pronating (turning downward) of its paw with abduction (movement away from the body) of its elbow and rotation around its wrist. The rat then grasps the food, which is followed by supination (turning upward) of the paw with adduction (movement in towards the midline of the body) of the elbow and rotation and flexion of the wrist in order to place food in its mouth. In Metz & Whishaw (2000),

it was found that reaching success was stable across smaller pellet size but dropped sharply for larger ones.

Neuronal Plasticity

So, what's going on in the brain that makes this task so intriguing to researchers studying brain injury recovery? Learning of any type is associated with the addition of synapses in the brain and also with changes in the synaptic structure itself. Kleim, Lussnig, Schwarz, Comery, & Greenough (1996) showed that the learning of motor skills specifically increased the number of synapses per neuron in the motor cerebral cortex of humans. The qualitative changes in the structure of synapses that have been reported include an increase in the number of axonal boutons and an increase in post-synaptic contacts (Stroemer, Kent, & Hulsebosch, 1998) In fact, there are quite a few studies that find that complex motor skills training improves the functional performance of rodents and enhances synpatogenesis in comparison to simple repetitive exercise (Jones, Chu, Grande, & Gregory, 1999b). Motor skill learning has also been shown to induce long term neuronal changes which include increased expression of GAP-43 and synaptophysin, two proteins which are associated with axonal growth and synapse formation immediately after the injury (Stroemer et al., 1998).

Throughout the last few decades, experiments have shown that redistribution of movement representations (motor maps) in the brain occurs in response to a variety of manipulations (Sanes & Donoghue, 2000). Transcranial magnetic stimulation (TMS) imaging has shown that motor skill learning enhances the reorganization of the human primary motor cortex (Tegenthoff, Cornelius, Pleger, Malin, & Schwenkries, 2004). However, a review of the literature shows that very little is known about how the motor cortical system is organized to produce such skilled movements (Metz & Whishaw, 2000).

The literature is clear, however, that motor skill training induces reorganization of motor maps and synaptogenesis within the adult motor cortex. The actual learning of the task does not develop by the same amount across training sessions. The motor learning is characterized by two phases: an initial phase in which there are rapid improvements in performance, followed by a second phase in which there are slower, much more moderate gains (Kleim et al., 1996). Imaging studies of the brain suggest these two phases are supported by different patterns of activity across the motor system. The initial, fast phase of learning is associated with activation of the striatum and cerebellum while the later, slower phase engages the motor cortex directly (Ungerleider, Doyen, & Karni, 2002). The changes in cortical synapse number have been shown to be both localized to regions of motor cortex that undergo reorganization (Kleim, Cooper, & VandenBerg, 2002b) and to be only detectable during the later phase. This reorganization is characterized by an areal expansion and also by an increase in the number of representations corresponding to the trained movements (Kleim, Barbay, & Nudo, 1998). These changes are learning specific, and there is no analogous motor map reorganization associated with unskilled movements, strength training, or exercise training (Kleim et al., 2002a).

Researchers using intracortical microstimulation of layer V within the caudal forelimb area of the motor cortex found that both motor map reorganization and synapse formation occur during the late phase of skill learning. It was also found that synaptogenesis precedes motor map reorganization. From these findings, Kleim et al. (2004) proposed that motor map reorganization and synapse formation do not contribute to initial acquisition of motor skills but represent the consolidation of motor skills that occur during the late stages of training. Thus, this study found that the changes in motor map reorganization and synaptogenesis are not linearly related to improvements in motor performance.

The reason that Kleim et al. (2004) used stimulation of layer V of the motor cortex is because it has been shown that in layer V of the motor cortex contralateral to any lesion, there are time-dependent increases in dendritic arborization (Jones & Schallert, 1992), neuropil volume, synapse to neuron ratios perforated synapses, and multiple synaptic boutons (Jones, 1999a). It has also been shown that the time course of neuronal growth results from an interaction between lesion-induced central changes and behavioral changes. In (6), researchers attempted to assess the effects of complex motor skills training after unilateral forelimb representation area of the sensorimotor cortex (FLsmc) lesions. The main area of focus was on the synaptic changes in layer V of the contralateral motor cortex. Their finding was that synaptogenesis in layer V of the motor cortex opposite FLsmc lesions was enhanced by postinjury training.

Motor skill training has also been found to result in an increase in synapse number per neuron in layer II/III of the motor cortex of intact adult female rats in comparison to motor controls (Kleim et al., 1996). It has also been shown that there is a transient reduction in axonal processes found in the motor cortex at time points preceding neuronal growth. This degradation has been found to result in increases in neurotrophic (proteins responsible for growth and maintenance of neurons) factors, cytoskeletal restructuring, and reactive changes in glial cells (non-neuronal cells that provide support and nutrition, maintain homeostasis, form myelin, and participate in signal transmission in the nervous system) (Ridet, Malhotra, Privat, & Gage, 1997). These changes are likely to support the structural reorganization associated with synaptogenesis, possibly making a brain region malleable in response to the behaviorally induced changes in neuronal activity. This supports the hypothesis that motor cortical structural plasticity can be enhanced using appropriate postoperative behavioral training (Jones et al., 1999). Because of

this, it should be possible to capitalize on this sensitivity by using behavioral manipulations to enhance the structural changes.

Intriguingly, although forelimb impairments could result from MCAO damage to the striatum and lateral hypothalamus, which are the brain regions typically associated with forelimb impairments, to date there has been no evidence of whether the region of the lateral frontal neocortex damaged by MCAO stroke contributes to forelimb use. It seems the best explanation for this is that the cortical area responsible for the organization of skilled reaching may encompass more than just the forelimb motor cortex area. Furthermore, it is likely that it is the integration of these various brain area contributions that makes a movement distinctively "skilled". This idea has led to a model of motor function in which the elements necessary for successful skilled reaching are olfaction, sensory input via vibrassae, and forelimb motor control. These elements are represented in the brain by a distributed network including the olfactory bulb, the medial frontal cortex, the forelimb motor cortex, the lateral frontal cortex, and the contralateral forelimb motor cortex (Whishaw, 2003).

Clinical studies have shown that spontaneous functional recovery after brain injury, though it tends to be short-term in nature, is often associated with neuronal plasticity and reorganization of the cortex around the lesion site and also in the undamaged, contralateral cortex (Cauraugh & Summers, 2005). It is hypothesized, then, that because these pathways accompany both motor skill learning and stroke recovery (Ramic et al., 2006), it should be possible to manipulate the pathways into motion that will help with brain recovery even before the brain is damaged.

Dietary Restriction

When training rats on a skilled reaching task, it is more common than not for researchers to enforce dietary restrictions (DR) on the animals in order to motivate them to want the food reward for which the task is performed. The general practice is to restrict the food the animals receive until they are maintained at 85-90% of their body weight (personal observation). There is an intrinsic problem with this practice, however, as some research shows that DR can be an intervention with powerful neuroprotective and neuro-restorative actions. For one, the lifespan of all mammals studied so far can be increased by reducing their caloric intake and/or meal frequency. Specifically the lives of rats and mice can be increased by up to 50% using DR (Weindruch & Sohal, 1997). It has also been shown that DR can increase neurogenesis in rats and mice (Lee, Duan, Long, Ingram, & Mattson, 2000; Lee, Duan, & Mattson, 2002). Furthermore, DR promotes the survival of newly generated neural cells and it increases the expression of the genes that encode the proteins that promote neuronal survival and plasticity. Finally, it elicits a cellular stress response, the exact nature of which is unclear (Lee et al. 2002).

There are striking similarities in the effects of DR, physical exercise, and mental exercise (eg motor skill learning) on neurotrophic factor expression, cellular stress resistance, and neuronal plasticity, suggest a shared mechanism underlying their beneficial effects in the brain. It is hypothesized that these activities activate stress response pathways akin to those induced during ischemic preconditiong (Prolla & Mattson, 2001; Guo, Ersoz, Butterfield, & Mattson, 2000). The mild stresses of DR, exercise, and mental gymnastics may stimulate calcium influx in the neurons, which activates kinases and transcription factors that induce the expression of genes encoding proteins that promote cell survival, synaptic plasticity, and neurogenesis (Rumajogee, Madeira, Verge, Hamon, & Miquel, 2002; Goggi, Pullar, Carney, & Bradford, 2002).

Also, the ability of DR to improve the outcome after a stroke has been demonstrated in a rat model in which the MCA was transiently occluded, resulting in damage to the cerebral cortex and striatum supplied by that artery and unilateral motor dysfunction. The ability of DR to increase the resistance of neurons to oxidative, metabolic, excitotoxic, and apoptotic insults in animal models makes it a clear confound in any study looking at the effects of a certain manipulation on stroke outcome (Yu & Mattson, 1999).

Rehabilitation

The two impairments associated with stoke damage which are the most common and the most resistant to rehabilitation are those that affect the forelimb and digit movement. Environmental Enrichment (EE) before or after an ischemic injury results in improved behavioral outcome on several sensorimotor tasks, including forelimb and digit movement (Ohlsson & Johansson, 1995; Johansson & Ohlsson, 1996). An enriched environment is one which consists of different objects and/or toys designed to encourage usage of the limb which is contralateral to the side of the brain which is damaged. It is basically the laboratory equivalent of the physical therapy given to brain injured humans). When rodents are housed in complex environments, it is found that there are increases in the complexity of the dendrites in cortical neurons and an increase in the number of synapses in many different neurons in the brain. Since EE has also been shown to enhance recovery from brain damage, this means it may involve changes in neurotrophic factors, neurotransmitter concentrations and transmission, hormonal signaling pathways and to alter the expression of several neurotrophic growth factors, including nerve growth factor (Dahlqvist et al., 1999) and basic fibroblast growth factor (Rowntree & Kolb, 1997). Falkenberg et al., (1992) has shown that EE specifically stimulates the production of brain derived neurotrophic factor in the hippocampus. EE has also been shown to foster motor

map reorganization (thus making it highly compatible with motor learning tasks (Liepert, Bauder, Miltner, Taub, & Weitller, 2000) suggests that rehabilitative therapies may remodel neuronal circuits within the surrounding tissue of the infarct, and that this reorganization contributes to recovery of the motor function that is compromised by the injury.

In order to answer the question of how EE facilitates these changes in the brain, one study investigated whether new anatomical pathways would be developed in association with improved motor function after brain damage and, further, if that development would be linked specifically to rehabilitation. Rehabilitation consisted of EE (via exposure to an environmentally enriched cage) for at least one hour each day. Cortical aspiration (rather than a stroke) of the motor cortex was used to induce the lesion. Following surgery, rehabilitation was conducted at days two and five, and continued two times per day for 3 weeks, and one time per day for the remaining three weeks. The results of this study showed that the animals which underwent rehabilitation had significant improvement in both the skilled reaching and the ladder rung walking tasks (described below). Also, neuroanatomical tracings of efferent pathways from the contralateral, non-damaged cortex resulted in the finding that rehabilitation significantly increased axonal growth in the deafferented basilar pontine nuclei. This supports the idea that rehabilitation can enhance neuronal plasticity and improve functional recovery after central nervous system injury. Therefore, this study demonstrates that rehabilitation following brain injury results in significant improvement in the skilled forelimb reaching test and in the skilled ladder rung walking test (Ramic et al., 2006).

Example Study

The study conducted by Biernaskie & Corbett (2001) combines both EE and skilled reaching training in an effort to provide the necessary situation in which use of the affected limb

would be increased, thereby stimulating the compromised sensorimotor system. Importantly, EE treatment was delayed until fifteen days after the injury was induced in order to establish the efficacy of such therapy if introduced at a more clinically relevant time point, as well as to exclude possible deleterious effects of early behavioral overuse on recovery (Risedal, Zeng, & Johansson, 1999). The reason for this is that it has been found that some types of behavioral experiences which occur early after damage may be detrimental to functional outcome. These negative effects can be blocked by an NMDA antagonist, suggesting that they may result from a use-related exaggeration of excitotoxicity (the pathological process by which nerve cells are damaged and killed by glutamate and similar substances.) This finding raises the issue of a possible sensitivity window for enhancing functional recovery in the brain (Kozlowski, James, & Schallert, 1996).

It has been theorized that there may be an ipsilateral contribution to functional recovery after stroke (Kopp et al., 1999), so another objective of Biernaski & Corbett's (2001) study was to assess the effect of enriched rehabilitation on dendritic reorganization within the intact hemisphere and to establish its potential importance to functional recovery after stroke.

It is instructive to go into the details of this study, as it is a good example of the studies like it which are found in the literature. Furthermore, it uses specific techniques not used by the study proposed in this paper, thus showing the myriad of methods which can be used in an attempt to answer the same question. This study used 57 male Sprague Dawley rats weighing 290-320 grams. All behavioral assessments were done during the dark cycle of the rats biological rhythm, which is when the rats are most active (Biernaskie & Corbett, 2001). However, many studies choose to do away with this convention, using the assumption that, during any repeated

training, the rats become entrained to be awake during the daily (or, nightly for them) behavioral assessment.

The MCAO was induced by stereotaxic microinjection of endothelin-1 (a vasoconstrictor) to the distal portion of the MCA with a methodology introduced by Sharkey, Ritchie, & Kelly (1993). The method chosen for stroke induction is especially important in that it has been shown that post-stroke interventions can have different effects depending on the method used to produce the stroke.

Enriched rehabilitation consisted of EE cages containing several different objects designed to encourage exploration which were changed twice weekly in order to encourage consistent exploration due to curiosity. Also, previous to the surgery, the animals were trained on the staircase reaching task. This task differs from the skilled reaching task because, in it, animals are trained to reach for pellets in a staircase cage. This particular cage consisted of seven steps, with three food pellets situated on each step. Animals were required to climb on a central platform to successfully retrieve and eat pellets from stairs located on either side of the animal. The number of pellets eaten per side was used as a measure of forelimb reaching ability. The design of the apparatus prevents any dropped food pellets from being retrieved. The food reward was mini-M&Ms and the rats were food deprived.

After the stroke, three behavioral testing measures were used to measure forelimb use. The first was the Staircase Skilled Reaching Test, done at days 10-15 after surgery and again at 4 and 9 weeks. Second was the Asymmetrical Forelimb Use Test. For this test, the animals were placed into a clear plexiglass cylinder with the number of forelimb wall contacts used for postural support being recorded. The percentage of contacts by the limbs both ipsilateral and contralateral to the injury are then calculated (Jones & Schallert, 1994). Finally, the beam

traversing task was used. In this task, animals were required to rapidly cross an elevated wooden beam into a darkened plastic tube. Latency to cross the beam and the number of foot faults were recorded (Kolb and Whishaw, 1983).

After the final behavioral assessment, the animals were sacrificed using an overdose of sodium pentobarbital and were then transcardially perfused with 10% formalin. The brains were then harvested, sectioned, and stained with hematoxylin and cosin, cresyl violet, or Golgi-Cox (explained below), depending on the thickness of the section.

In this study, all animals tested showed vigorous contralateral forelimb retraction, as well as exaggerated rotation and twisting in the direction contralateral to the lesioned hemisphere when held by the tail. This showed that the lesion was effective by using behavioral analysis. The infarct volumes as shown by histochemical analysis included the lateral regions of the cortex and the lateral striatum (caudate- putamen area).

The study further showed significant effects for the skilled reaching treatment condition. In the anatomical analysis, the skilled reaching group which was kept in the enriched environment had significantly greater dendritic length compared with the controls, and there was a similar pattern for the total number of dendritic branch segments per neuron.

In summary, this study showed that enriched rehabilitation after MCAO resulted in dramatic improvement of skilled use of impaired limbs as measured by the Skilled Reaching and Beam Walking Tasks. The behavioral recovery was long term (greater than three months) and occurred despite ischemic injury to both the cortex and the striatum. Furthermore, ischemic animals exposed to enriched rehabilitation had significantly greater dendritic arbors in the undamaged contralateral motor cortex compared with controls and this was also associated with improved functional outcome (Biernaskie & Corbett, 2001)..

Post-Stroke Behavioral Assessment

Most behavioral scoring tests which try to assess the extent of stroke damage within 24 hours after surgery are based on the Bederson postural reflex scoring system (Bederson et al., 1986), which has been correlated with the size and location of areas of infarction. In this system, the neurological status of each rat is evaluated carefully at 24 hours after stroke surgery (hopefully by a blind observer). The tests described below are conducted sequentially. If the rat exhibits the appropriate behavior at one step but not at the subsequent step, it is graded at the former level. For the first test, the rats are held gently by the tail, suspended one meter above the floor, and observed for forelimb flexion. Normal rats extend both forelimbs toward the floor. Rats that extend both forelimbs toward the floor and that have no other neurological deficit are assigned a grade of 0 (no deficit). Rats with infarction will consistently flex the forelimb contralateral to the injured hemisphere; posture can vary from mild wrist flexion and shoulder adduction with extension at the elbow to severe posturing with full flexion of wrist, elbow, and adduction with internal rotation of the shoulder. Rats with any amount of consistent forelimb flexion and no other abnormality are graded 1 (moderate deficit). For the third test, rats are placed on a large sheet of soft, plastic coated paper that can be gripped firmly by their claws. With the tail held by the researcher's hand, gentle lateral pressure is applied behind the rat's shoulder until the forelimbs slide several inches. This maneuver is repeated several times in each direction. Normal or mildly dysfunctional rats resist sliding equally in both directions. Severely dysfunctional rats consistently reduce resistance to a lateral push toward the contralateral to the injury side, and are graded 2 (severe deficit). Finally, rats are then allowed to move about freely and are observed for circling behavior. Rats that circled toward the contralateral side consistently were graded 3 (very severe deficit). Forelimb flexion is always observed in rats with decreased

resistance to lateral push; both forelimb flexion and decreased resistance to lateral push is always observed in rats that display circling behavior.

A major benefit to the Bederson postural reflex scoring system is that the neurologic examination can be performed in 3 to 5 minutes, and is based on behaviors that are obvious. It is also highly correlated with histological examinations in size and severity of stroke damage. Also, it gives an immediate idea of if the stroke procedure was successful, and the severity of the stroke. However, in a laboratory experiment, it needs to be confirmed with either histological or imaging techniques.

The Ladder Rung Walking task is different from the Bederson tasks in that it tests for the accuracy of limb placement and coordination, which is mainly a consideration of recovery, not one of damage measurement. Generally, rats are able to cross a horizontal ladder runway (roughly 1m in length and 10 cm in width) with wooden rungs distance 1-2 cm apart with less than one foot slip per every ten steps (Ramic et al., 2006). Stroked rats present with many more foot slips. One advantage of this assessment method is that there is no pre-surgical training necessary. It is also a quick assessment procedure, with animals only needing two separate ten minute sessions in order to be acclimated to the ladder apparatus. Also, the animals can be videotaped as they walk across the ladder in order to watch the tape frame by frame for accurate analysis of foot placement.

However, there are some disadvantages to this assessment. First, the foot slips can be very difficult to count accurately, as rats walk very quickly. It can be difficult to operationalize exactly what constitutes a "foot slip", as this concept is defined differently from paper to paper. Also, if the observer is not blind to the condition of the rat (trained or enriched vs. control), it can

be very difficult to count accurately as more foot slips will be expected in one group over another (Ramic et al., 2006).

Pet Scanning

Radioactive substances are the key to Positron Emission Tomography (PET) scanning, which is a medical imaging technique that produces a three-dimensional image or map of functional processes in the brain (or body). The images of the brain are detected from the radiation given off from these substances. The substances aren't radioactive by nature, but are usually a metabolically active molecule that is tagged with a radioactive atom, such as ^{11}Carbon, ^{18}Fluorine, ^{15}Oxygen, or ^{13}Nitrogen. The forming of these radioactive atoms includes bombardment of normal chemicals with neutrons. The radionucleotides are injected directly into the laboratory subject (or patient), but the radiation isn't a problem, because these radioactive atoms have extremely short half lives (^{15}Oxygen's half-life is just two minutes). The radionucleotides decay, emitting a positron (among other things) in the process. Once the radioactive substance is injected, if one of its positrons collides with an electron in the brain tissue, they will annihilate each other, producing a pair of gamma rays which fly off in exact opposite directions (Phelps, Hoffman, Mullani, & Ter-Pogossian, 1975).

In a microPET scan, an anesthetized and stereotaxed rodent is injected with the radioactive substance and placed on a flat table that moves in increments through a "donut" shaped housing (the bore). The stereotaxing and placement of the subject is extremely important so that successive scans of the same animals can be compared. In this way, the animal can actually act as its own control, being scanned before treatment, after treatment, etc.

The bore of the PET machine contains a circular gamma ray detector array, which has a series of scintillation crystals, each connected to a photomultiplier tube. The crystals convert the

gamma rays emitted from the subject to photons of light, and the photomultiplier tubes convert and amplify the photons to electrical signals. This technique depends on simultaneous or coincident detection of the pair of photons; photons which do not arrive in pairs (i.e., within a few nanoseconds) are ignored. These electrical signals are then processed by the computer controlling the machine in order to generate images. The table with the patient or subject is then moved, and the process is repeated, resulting in a series of thinly sliced images of the body over the region of interest in the brain.

Most electron-positron decays result in two 511 keV gamma photons being emitted at almost 180 degrees to each other; hence it is possible to localize their source along a straight line of coincidence (also called formally the "line of response" or LOR). Using statistics collected from thousands of coincidence events, a set of simultaneous equations for the total activity of each parcel of tissue along many LORs can be solved by a number of techniques, and thus a map of radioactivities as a function of location for parcels or bits of tissue (voxels) may be constructed and plotted. The resulting map shows the tissues in which the molecular probe has become concentrated, and can be interpreted by a researcher in the context of stroke research in order to investigate stroke dynamics and extent of stroke damage.

There are some limitations to the use of PET scanning. Most of these arise from both the high costs of cyclotrons, which are machines needed to produce the short-lived radionuclides which are used for PET scanning, and the need for specially adapted on-site chemical synthesis apparatus to produce the radiopharmaceuticals. Few hospitals and universities are capable of maintaining such systems, and most clinical PET is supported by third-party suppliers of radiotracers which can supply many sites simultaneously. This limitation restricts clinical PET

primarily to the use of tracers labeled with F-18, which has a half life of 110 minutes and can be transported a reasonable distance before use.

Despite this, PET scanning is extremely versatile. It can provide images of a myriad of biochemical functions, including blood flow (which is vital in studying when considering stroke damage) and glucose metabolism, all depending on the type of radioactive molecule injected. It can also show rapid changes in brain activity. Compared to other imaging techniques available today, it is unmatched in being able to give a temporal picture of the brain (Young, Baum, & Cremerius, 1999).

Histochemical Methods

Histochemical methods consist of staining the brain in such a way that the area of interest shows up as either separate or more clearly than the rest of the brain. In stroke studies, it is instructive to be able to see a delineation between dead cells and living ones. After death, the soft brain tissue is destroyed by autolytic (cell destroying) enzymes, so before staining the brain must be preserved. To do this the brain is placed in a fixative, the most common of which being formalin. Fixatives harden the brain tissue and kill micro-organisms. Once fixed, a microtome or vibratome is then used to slice neural tissue for viewing under a microscope. Special histological stains have been developed so that cell bodies, nerve fibers and nerve cell membranes can be selectively viewed. There are three main types of stains used in neuro-histochemistry. The first of these is the cell body stain, also called the Nissl stain. A very common type of Nissl stain is the use of cresyl violet which, when applied to neural tissue, can selectively reveal cell bodies. The second major class of stain is the myelin stain. This stain colors the myelin sheath that surrounds nerve cells so that fiber bundles are easily revealed. The third major class of stain is the membrane stain. This stain contains salts of various heavy metals which interact with the

axon membranes. The Golgi-Cox stain is one of these, and it uses silver to enable the researcher to see the branching of individual neurons and trace the connectivity of neurons.

In the Golgi-Cox staining method, animals are transcardially perfused with 0.9% saline and the brains are then removed. The whole brains are then immersed in a Golgi-Cox solution for 2 weeks before being placed in a 30% sucrose solution for 2-5 days. The Golgi-Cox solution is made up of potassim dichromate, mercuric chloride, and potassium chromate, all distilled in water and stored in the dark for five days. The brains are sectioned with a vibratome, slide mounted, and stained using a long, complicated set of processes, the basics of which are as follows: The slides are dipped into the following solutions in the following order: 1) Distilled water for 1 minute; 2) Ammonium Hydroxide for 30 minutes (in the dark); 3) Distilled water for 1 minute; 4) Kodak Fix solution for 30 minutes (in the dark); 5) Distilled water for 1 minute; 6) 50% alcohol for 1 minute; 7) 70% alcohol for 1 minute; 8) 95% alcohol for 1 minute; 9) 100% alcohol for 5 minutes; 10) 100% alcohol for 5 minutes; 11) 100% alcohol for 5 minutes; 12) CXA (solution consisting of chloroform, xylene, and alcohol) for 15 minutes (Glaser & Van der Loos, 1981).

The main drawback of the Golgi-Cox staining method is that it is long, involved, and it is easy to make errors along the way (Gibb & Kolb, 1998). One advantage of this method of staining, especially in regards to MCA stroke and motor learning, is that both apical and basilar dendrites of layer V pyramidal cells within the forelimb motor cortex of both hemispheres can be located and drawn. This staining method allows for computer-assisted three-dimensional reconstruction of the dendritic arbor, thus providing accurate measurements of dendritic length (Biernaskie & Corbett, 2001).

2,3,5-triphenyltetrazolium chloride (TTC) is an oxidation-reduction indicator which has been used for years for early histochemical diagnosis of myocardial infarction. The staining action of the salt is based on the presence or absence of a dehydrogenase, with tissue with normal levels of dehydrogenase stained red, while ischemic and infarcted tissue remains unstained. In normal tissue, dehydrogenase reduces TTC to formazan, which stains red. Dehydrogenase activity is reduced or eliminated in ischemic or infarcted tissue. It is commonly observed that TTC immersion gives smaller infarct sizes than those shown by other staining methods. This may be attributed to macrophage and glial cells, as both of these contain either mitochondria or enzymes that reduce TTC, causing the tissue to be stained red (Bederson et al., 1986).

Hypothesis

The experimental procedure I have designed and plan to carry out (with two other laboratory workers) is described below. The hypothesis, or purpose, of this experiment is to combine environmental enrichment with daily skilled-reach training to assess the effect of intensive task-specific rehabilitation on long-term behavioral outcome.

Methods

Note: From here on out the usage of "we" refers to the laboratory staff under the supervision of Dr. M. Duff Davis at the University of Texas Health Science Center in San Antonio. These include myself, Erica S. Crooke, Sean Buckley, and David Lewis.

Animal models of focal cerebral ischemia provide the opportunity to investigate the pathophysiological mechanisms of this clinically most common form of stroke. Focal ischemia can be broadly categorized into two types, permanent and transient. As mentioned before, the transient MCAO model most closely mimics the clinical situation, in which the MCA is embolized and reperfusion occurs as a result of recanalization during surgery or after

recombinant tissue plasminogen activator administration, as well as from spontaneous recanalization. MCA intraluminal filament occlusion has been well accepted as an experimental stroke model in mice and in rats, since it was developed two decades ago. There is a consensus in the literature that the rat is the standard mammal for brain ischemia modeling because it is inexpensive, well studied in many biological contexts, and physiologically a very durable animal. Strokes caused by the procedure encompassed in this model are due to occlusion of the origin of the MCA, rather than through craniotomy, which causes endothelial injury and artifactual elevation in intracerebral thrombosis which is induced by the filament. Studies suggest that 2 hour MCAO in rats leads to consistent and extensive cerebral infarction, which has also been seen in previous studies in this laboratory. Additionally, the MCAO model allows for long-term recovery, while many other models include death as an immediate endpoint after the surgery. Therefore this model allows for the study of short and long-term behavioral recovery after stroke damage. In this particular case, it is good for studying the long-term effects of motor skill training.

The purpose of our study is (1) to determine whether preconditioned motor learning-induced synaptogenesis causes neuroprotective changes in stroke dynamics or is helpful in recovery after stroke damage is induced; (2) to use *in vivo* PET imaging to investigate the dynamics of stroke. In vivo PET imaging will be conducted with the injection of ^{15}O labeled tracers such as $H_2{}^{15}O$, $O^{15}O$ gas and ^{15}O-labeled blood as well as ^{18}F-FDG labeled radioactive tracers to measure cerebral blood flow (CBF), cerebral blood volume (CBV), oxygen exchange fraction (OEF), cerebral metabolic rate of oxygen ($CMRO_2$), and neuronal metabolism in the rat brain; and, (3), to apply these imaging techniques to assess stroke dynamics and stroke therapeutics using experimentally induced strokes in rats.

Animals Used

The measurement of stroke damage and recovery via microPET requires the use of live animals. The pathophysiological mechanism of neuronal injury due to ischemia and reperfusion in stroke, as well as the neuroprotective mechanisms of skilled motor learning described in this proposal are multifactorial and involve integrated effects on multiple organ systems. Investigation of these mechanisms thus requires a "whole animal" model. The cerebrovascular anatomy and physiology of rats is similar enough to humans to provide meaningful experimental results, and we have previously demonstrated that stroke studies can be successfully performed in rats. Sprague Dawley rats are used because this is the strain which are the most sensitive to ischemia/reperfusion injury and they have also been widely used in experimental stroke studies, including intraluminal filament models. Furthermore, they have a specific vascular anatomy, not found in other strains of laboratory rats, that make them the best choice for our particular model of stroke induction.

Rats are also used widely in stroke studies because of the extensive literature availability of stroke models, dynamics, and recovery in this animal and its minimal intersubject variability. In addition, for imaging studies using microPET, rodents are the most appropriate subjects due to the small size of the scanner bore. There is consensus in the literature that the rat is a standard mammal for brain ischemia modeling because it is inexpensive, well studied in many biological contexts, and physiologically a very durable animal.

One of the major purposes of this study is to conduct a skilled motor training procedure and to determine the neuroprotective effects of the procedure on motor behavior in rats with ischemic brain injury. The most common and economic animal model for this purpose (the

Single Pellet Reaching Task) has been developed in rats. Yet another advantage of this species is that considerable information exists on the structure and function of their brain, as well as the pathophysiological effects of ischemia on different brain regions. Finally, the ability to use routine anesthesia, the rat's relative resistance to infection, and the availability of many specific antibodies for immunohistochemistry make this animal superior to other alternatives

A total of 50 male Sprague-Dawley rats (150-225g) will be randomly assigned to three groups: an experimental group consisting of 15 animals that will be used to practice and train laboratory staff in the use of the MCAO filament stroke procedure. These animals will be terminated without post-surgery recovery from anesthesia. They will undergo surgery and PET imaging while under anesthesia before and after stroke induction, followed immediately by euthanasia. Another group of 35 animals will then be used to expand the utility of the procedure by using PET to evaluate the evolution and extent of stroke damage during a recovery phase. This group of 35 animals will be further split into an experimental and a control group for the motor learning behavioral study. ~17 animals will be trained on the Single Pellet Reaching Task, while the control group of ~17 animals will not learn the task. All 35 animals will undergo the MCAO procedure. After full recovery from the stroke, all animals will undergo rehabilitation in the form of an enriched environment and will be tested repeatedly on the Ladder Rung Walking task.

Motor Learning

In order to facilitate learning induced synaptogenesis in the rat before the stroke surgery, we will be teaching the rat a skilled motor task called the Single Pellet Reaching Task. This task will be performed as follows: the rats will be placed in a specially designed Plexiglas cage with a slit in the front and a shelf for placing a reward food. The experimental animals will be

habituated to being handled and to the reaching-cage environment, and then will be encouraged to reach for the extremely palatable treat through the slit. In order to facilitate the learning of the task, there will first be a trough in front of the slit filled with the food reward, and the reward will be offered for successive approximations of the task. For example, if the rat moves the correct paw off of the ground, he is rewarded with the treat. During the next training session, if the animal moves his paw off the ground and in the direction of the slit, he is rewarded, and etc. until the animal is reaching through the slit to the trough. When the animal is consistently retrieving 20 food pieces per session, the trough will be replaced by a tray on which a single food piece will be placed, at first directly in front of the slit, and then further to the side contralateral to the side of the brain to be stroked in the animal. The shelf allows for forcing the rat into using one particular paw over another once the task is learned. Once the shelf is introduced, each training session will last approximately one hour, or until the animal has retrieved 20 food items. The animals will be scored according to the following equation: hit % = number of hits (divided by) number of reaches (multiplied by) 100. Animals will be required to score an average of 65% to be included in the study. Control animals will be handled, introduced to the reaching cage, and given the highly palatable food reward, but they will not learn the reaching procedure.

For post-stroke rehabilitative training, we will introduce the rats into a rehabilitative environment in order to facilitate recovery. The rehabilitative environment will consist of objects designed to encourage the rats to climb and use their forelimbs, the areas of the body damaged the most after MCAO. These objects will consist of an angled ladder, a cylinder for climbing, and varied other “toys” designed to passively force the rats to explore and move their forelimbs. Objects will be rearranged each week in order to capitalize on the rat’s curiosity. The recovery progress will be tested using a simple motor task called the Ladder Rung Walking Task which

does not require previous motor training. In this task the rat simply walks across a ladder into a darkened box. The action is videotaped and reviewed frame by frame to look for foot slips and paw placement errors. It is our hypothesize that the animals exposed to prior skilled motor learning will perform better on the Ladder Rung Walking Task than the control animals.

Stroke Induction

The induction of the stroke will be carried out using an intraluminal microfilament method to occlude the MCA. Simple therapeutic interventions that will be used for the stroke experiments will include hypothermia and hypercapnia, since hypothermia reduces metabolic demand in the brain and hypercapnia increases oxygen supply to the brain. The evolution of the induced stroke, whether therapeutically intervented or not, will be dynamically detected using the *in vivo* PET imaging technique.

In this experiment, unilateral MCAO will be implemented to monitor the evolution of physiological changes that occur during and following induction of stroke using PET imaging. MCAO will also be used track the short-term and long-term normalization of brain function as assessed with imaging techniques. It is hypothesized that as immediate response to the stroke, CBF will decrease, $CMRO_2$ will be unchanged, Oxygen Extraction Fraction (OEF) will increase, and neuronal metabolism will decrease. The OEF of the brain measures how much oxyhemoglobin has been converted to deoxyhemoglobin, which basically is just a measure of metabolism in that specific brain area. This is the phase of reversible ischemia, as the tissue maintains metabolism through increasing OEF despite the fall in blood flow. As ischemia converts to infarction, $CMRO_2$ will fall to match CBF and OEF will return to a normal level or go below normal level (< 40%).

There are a wide variety of models of stroke (Hossman, 1998), and even within the realm

of MCAO stroke specifically, there are many methods used to model the pathology of clinical situations. The microfilament method appears to produce the most reliably reproducible pathophysiological changes (Watson, Dietrich, Busto, Wachtel, & Ginsberg, 1985; (Hu, Wester, Brannstrom, Watson, & Gu, 2001). The MCAO procedure we have chosen is an intraluminal (within the empty spaces of an artery) filament MCAO procedure. It requires a surgery to expose the carotid artery, during which a piece of surgical thread (aka a microfilament) of specific length is placed in the artery to cause the stroke, and then is removed two hours later to allow for reperfusion. This particular procedure was chosen because of its reliability in effectively producing an MCAO, its low inter-animal variability, and its compatibility with microPET and MRI procedures.

All survival surgery will be conducted using aseptic technique. Surgical instruments and materials will be sterilized at the beginning of each day using Cidex solution. When multiple surgeries are conducted in one day, dry sterilization will be used on the instruments. Also, sterilized disposable materials will be used, such as drapes, gloves, and masks. All experimental animals will be marked with permanent ink on their tails for identification. Surgical procedures are required to place intra-venous and intra-arterial catheters and to induce stroke. For these procedures anesthesia will be established using isofluorane (5 % for induction, in a mixture with oxygen, 1-2 % for maintenance). Anesthesia will be maintained with a facemask by the same mixture delivered from a precise and calibrated vaporizer during the entire surgical procedure. Rodents will not need to be orally intubated or mechanically ventilated in using MCAO model. A temperature probe will be inserted into the rectum, with a heating pad being used to maintain body temperature at 37^{o}C to 38^{o}C (which is normal body temperature for rats).

The procedure for MCAO stroke induction will be as follows: Animals will be prepped by shaving and swabbing of the surgical site using Betadine or Povidine. Fur from rodent skin will be removed by a blade allowing at least 1/2 inch margin around the intended incision site to prevent contamination of the site by unclipped hair. The incision site will be scrubbed using an iodine based scrub and rinsed with alcohol. This combination will be repeated three times. We will not saturate the animals with prep solution or remove too much fur in order to avoid hypothermia. Investigators will wear sterile surgical gloves and the surgical site will be covered in a sterile drape to keep the field sterile and avoid contamination of sterile instruments and supplies. Anesthesia will be induced and maintained using isofluorane and O_2 (5% for induction and 1-2% for maintenance). Temperature will be maintained using a T-Pump warming blanket and a temperature probe inserted into the rectum. Under an operating microscope, the right common carotid artery (CCA) will be exposed through a midline neck incision and will be carefully dissected free from surrounding nerves and fascia from its bifurcation to the base of the skull. The occipital artery branches of the external carotid artery (ECA) will then be isolated, and these branches will be dissected and coagulated. During this procedure, the right common carotid artery lumen will be occluded with the microfilament and a clamp will be positioned to occlude the common and internal carotid arteries. The right CCA will be occluded with a suture loop and a clamp will be positioned to occlude the common and internal carotid arteries. The (ECA) will then be severed. The occluding filament will be dipped in Poly-L-Lysine solution, introduced into the internal carotid artery (ICA) via the ECA stump, and advanced 18 to 19 mm. The silk suture around the ECA stump will be tightened around the intraluminal nylon suture to prevent bleeding. After the ventral midline neck incision is closed with sutures, the rats will be awakened from anesthesia and monitored carefully. Waking the animal before the filament is

removed is a standard procedure used widely in other laboratoriess to reduce total anesthesia time. This reduction improves post-surgical latency to recovery. After 120 minutes of MCAO, rodents will be re-anesthetized for filament withdrawal using the same method as described above. The intraluminal suture will be carefully removed by reopening the closed incision and the CCA and ICA will be inspected to ensure the return of good pulsations. The neck incision will be closed with synthetic suture, and the animals will be returned to the home cage for recovery. Protection from malnutrition and dehydration will be addressed by administering a 20 ml subcutaneous injection of 5% dextrose in Lactacted Ringers solution.

This procedure will eliminate blood flow to one side of the motor cortex, along with other brain areas, causing impaired use of the contralateral limb in the animals. The animals will then be allowed 1-2 weeks for recovery. Cerebral Blood Flow (CBF) monitoring during surgery by laser doppler imaging will assist in verifying success of the stroke. The LDF probes (Transonic systems, Inc.) will be stereotaxically placed on exposed intact dura 1mm anterior and 5mm lateral to bregma as a peripheral ischemic area recording, as well as 1 posterior to bregma and 2 mm above the rhinal fissure for central ischemic area recording. Blunted ear bars will be used in the stereotaxic device to prevent injury to the tympanic membrane. Large blood vessels will be avoided. Regional CBF will be measured with LDF, before (baseline), during, and after MCA occlusion. The measurement of cerebral blood flow will be measured only for the length of surgery, and the LDF probes will be removed before recovery.

Simple neurological examinations will be used to determine the sensorimotor effects of the brain ischemia and reperfusion, including assessment of tremor, relative strength, balance, and locomotion. The experimental animals will be closely monitored immediately following the procedure and will be kept warm using a T-pump warming blanket until internal recumbency is

regained. General health will be assessed by observing body weights, hydration (skin "tenting"), nasal and oral discharge, and bowel function. Any signs of initial malnutrition or dehydration will be treated with subcutaneous injections of fluids and glucose. At 1-5 days post-MCAO, animals will be expected to have mostly intact mental alertness, such as eye opening, locomotion (though with some stereotypy), and eating. If the animal can eat but cannot reach the food provided at the top of the cage, a mush will be prepared from dry pellets and placed in an open container within reach of the animal. Animals experiencing difficulty in reaching the sipper tube of the water bottle will be provided with long sipper tubes to make reaching easier. Any signs of infection will be treated accordingly with Penicillin-G. Animals will be given a dose of Butorphanol (1-2mg/kg) immediately following recovery from anesthesia, and once every 4-6 hours as needed. For animals that will be included in the long-term cohort, surgical sutures will be removed 10 days after surgery. All animals will be observed and weighed daily by the laboratory staff. Animals will be euthanized if discomfort cannot be controlled, such as if they are unable to eat, remain active, or perform purposeful movements. If animals lose more than 10% of their body weight during the first 3 post-surgical days, they will be sacrificed rather than receiving fluid administration. A Laboratory Animal Resources veterinarian will be consulted if the animals lose more than 10% of their body weight. Animals will be euthanized if infection with an extensive discharge of pus persists for more than 3 days after treatment with antibiotics.

PET Imaging

PET scans will be acquired before and after behavioral training; before, during, and after filament insertion; and at regular intervals during the learning, recovery and rehabilitation period. Quantitative PET measurements of CBF, $CMRO_2$ and OEF in rat brain using bolus, intravenous administration of ^{15}O tracers ($H_2{}^{15}O$ and $O^{15}O$), and ^{18}F-FDG in the setting of a stroke model

will be used in this experiment. This will be the first use of PET to study stroke in a rat and, we believe, to study the dynamics of stroke in any species.

Further development and validation of the ^{15}O and ^{18}F-FDG PET techniques for rat brains will be done by employing well-known physiological challenges such as graded hypercapnia (established by increasing carbon dioxide concentration stepwise up to 12 %, supplied only for 3 min; carbon dioxide concentration in room air is ~0.3 %); hyperoxia (100% oxygen) and hypothermia (body temperature measured by rectal temperature down to 32 ˚C; normal rat temperature is ~37 ˚C). Hypercapnia is a widely used challenge for animal imaging as well as for human imaging to modify blood flow without changing oxygen metabolic rate in the brain (Hoge et al., 1999; Davis, Kwong, Weisskoff, & Rosen, 1998). Hyperoxia is a challenge to decrease blood flow significantly (Floyd et al., 2003). Hypothermia is a simple way to lower oxygen metabolic rate, as well as blood flow, in the brain. These three measures will show that PET scanning is effective in measuring both quantitative and qualitative changes in cerebral blood flow.

The PET scanning procedure will consist of anesthetizing the animals as described above and then placing an intravenous tail vein line in order to administer radiopharmaceuticals using either a 25 or 27 gauge winged infusion set. After radiopharmaceutical injection, the line will be flushed with ~ 0.3 ml of sterile saline. The surgical procedures also include catheterization of the external carotid artery and femoral vein for the injection of radiolabeled tracers such as $H_2{}^{15}O$ for blood flow measurement, $O^{15}O$ (either as a gas state or as a labeled hemoglobin state) for MRO_2 and OEF measurements. If $O^{15}O$ is injected as a gas (not as labeled hemoglobin or red blood cells), the surgical procedure will require a tracheotomy for bolus gas injection into the lung. Additional femoral vein catheterization may be required for anesthetic intraveneous infusion, if

the tail vein is not secured properly. For withdrawing some blood for labeling the hemoglobin or red blood cell with $O^{15}O$, femoral artery catherization may be required. However, if the external carotid artery catheterization is present, the femoral artery catheterization will be avoided. For PET imaging for blood flow measurement, ^{15}O labeled water ($H_2{}^{15}O$, 3-4 mCi, 0.1-0.2 ml saline) will be injected through the surgically inserted catheters (into the external carotid artery or the femoral vein) and PET imaging will be initiated slightly before the injection of radiolabeled tracers. For PET imaging for MRO_2 and OEF measurements, $O^{15}O$, either in gas form (2-3 mCi, 0.5 ml gas) or in labeled blood form (1-2 mCi, 0.1-0.2 ml blood), will be injected through surgically inserted catheters (into external carotid artery or femoral vein, when the oxygen is injected in the form of labeled blood) or through cannula (16 G) inserted by tracheotomy (when the oxygen is injected in the gas form) and PET imaging will be initiated slightly before the injection of radiolabeled tracers. PET imaging will be performed for 3 min for each tracer injection.

PET scan sessions will be initiated 24 hours before first behavioral intervention, 24 hours pre-surgery, then repeated at 24 hours, 1 week, 2 weeks, 3 weeks and 6 weeks post-surgery. At the end of the 6-week scan, the animals will be decapitated under the isoflurane anesthetic.

For some PET scans, the animal will be allowed to recover for an amount of time appropriate to its half-life during tracer uptake and equilibrium. At the end of the uptake period, the animal will again be anesthetized with 1-2% isofluorane/oxygen mixture using a nose-mask, then positioned onto an acrylic stereotaxic frame. The frame will be placed in the gantry of a Concorde R4 rodent microPET scanner and the rodent maintained at 37^oC as during the scan session as regulated with a thermal blanket and rectal-feedback probe. The head will be positioned in the PET field of view for a 20 minute imaging acquisition scan. At the end of the

scan session, the animal will be allowed to recover for a period of time, and then returned to its home cage.

Histochemical Analysis

In order to validate our PET data, we will be taking the brains of the rats and staining the slices in order to document the infarcts.

We will freeze the brain for 1-2 hours. After freezing, the brains will be sliced from between 0.5-3.0mm slices. The slices will be submerged in a preparation of 2% TTC/phosphate buffer solution (PBS). The slices will be incubated at 37 degrees Fahrenheit for 30 minutes. Then, the TTC is removed and replaced with buffered formalin. The sections will be mounted on slides and photographed within 3-7 days

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