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Protein Expression in the Cortex Following Motor Skill Learning

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Protein Expression in the Cortex Following Motor Skill Learning

BY

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

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MASTER OF ARTS

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Abstract

Learning a new motor skill can induce plastic changes in neural networks in rats. Within the motor cortex, learning-induced plastic changes include dendritic reorganization, synaptogenesis, and changes in synapse morphology. Behavioural studies have demonstrated that learning requires protein synthesis. It is likely that some of the proteins synthesized during learning are involved in, or the result of, learning-induced structural plasticity. It was predicted that the expression of proteins involved in neural plasticity are altered in a learning dependent fashion. Rats were trained on a series of motor tasks that vary in complexity, so that the effects of activity could be teased apart from the effects of learning. The motor cortices from these animals were examined for MAP2 and synaptophysin protein using Western blotting and immunohistochemistry. Western blotting revealed that expression of MAP2 was not detectably influenced by this behavioural manipulation, whereas synaptophysin expression increased on day 1, 3 and 5 of motor skill learning. Although expected to reveal where protein expression differed, immunohistochemistry did not reveal any changes in protein expression in either the motor or sensory cortices for MAP2 or synaptophysin. Therefore, expression of MAP2 does not seem to indicate difficultly of task or duration of training time, whereas increases in synaptophysin expression seem to be correlated with the first 5 days of motor skill learning, possibly coinciding with synaptogenesis.

PROTEIN EXPRESSION IN THE CORTEX FOLLOWING MOTOR SKILL LEARNING

Chapter 1

Neuronal Plasticity

It is becoming increasingly evident that the ability of the mammalian brain to change in order to meet the demands of the environment is not limited to development. This capacity for change, referred to as plasticity, extends well into maturity and can occur in response to hormones, drugs, brain damage and learning new behaviours (Kolb & Whishaw, 1998). Neurons and synapses change to form reorganized neural networks of communication. These neuronal changes are thought to form the neurobiological substrate of long-term memory. Although poorly understood at present, revealing the cellular mechanisms underlying adult brain plasticity will help elucidate the relationship between neuronal structure changes, memory formation and maintenance of long-term memory.

Complex Environments

Early investigations of experience-induced plasticity looked at the effects of rearing rats in complex environments compared to rearing rats in standard laboratory cages. Hebb (1947) was the first to use this type of manipulation, with his own home as the complex environment. After keeping the rats at home, much like pets, these rats performed better on learning tasks than rats that had remained in the laboratory in standard cages. Thus, rearing rats in an environment that promoted exploration and novel experiences seemed to have an impact on their behaviour and abilities. Subsequent to this

observation, researchers sought to explore this phenomenon and created complex environments in laboratories. Typically, a complex environment is a larger than standard cage that can house several animals, and contains objects and toys. Rosenzweig, Bennett, Hebert, and Morimoto (1978) describe a complex environment as "a combination of complex inanimate and social stimulation", which provide increased opportunities for activity, exploration and social contacts. If raising a rat in this type of environment can influence behaviour, then it is likely that there are biological events occurring within the brain to support the modified behaviour. Examinations of structural properties of brains and neurons from complex environment-exposed rats (EC) have supported this hypothesis.

Rearing rats in complex environments influences the structure of their brains. Looking at the gross anatomical level, the brains of EC rats compared to those of rats kept in standard laboratory cages had thicker and heavier cortices (Rosenzweig, Krech, Bennett, & Zolman, 1962; Rosenzweig, 1966). A cellular level of analysis may provide an explanation for this apparent, experience-induced malleability of the cortex. Diamond (1967) found increases in the size of neuronal cell bodies in the visual cortex of EC rats. Greenough and Volkmar (1973) used the Golgi method, which randomly stains 2-3% of cells, and found increased dendrite branching complexity in neurons of the occipital cortex. These types of cell growth may partially account for the increases in cortical weight and thickness, which would result from the addition of cellular material and physical separation of cortical tissue. Also, if there is a relationship between altered brain structure and improved learning ability, it could be suggested that new or strengthened neural connections may be part of the structural changes responsible for the phenomenon.

As the synapse is the site of neural communication, it has long been hypothesized that changes in behaviour, especially learning and memory, may be represented by changes in the synapses themselves. Accordingly, increases in dendritic material may serve to house more synapses (Sirevaag & Greenough, 1987, 1988).

Two of the most interesting experience-induced plastic changes are spine (the post-synaptic element of a synapse) proliferation and synaptogenesis. Globus, Rosenzweig, Bennett, and Diamond (1973) found an increase in the number of spines per basilar dendrite of pyramidal neurons in the occipital cortex. An increase in the number of spines raises the possibility of an increase in synaptic contacts. Although Golgi staining is a useful tool for examining neuron morphology, this technique does not allow for the examination of neuron ultrastructure, or synapses. In order to examine synapse morphology, electron microscopy (EM) techniques are needed to look at ultrastructural alterations that occur in response to the complex environments. West and Greenough (1972) found that EC rats had longer post-synaptic densities in basilar dendrite spines of the occipital cortex. Indeed, Turner and Greenough (1985) found an increase in the number of synapses per neuron in the occipital cortex. As synapses are the principle sites of neuron-to-neuron communication, the addition of new, experience-induced synapses may contribute to a new memory. Increased numbers of synaptic spines with discontinuities, called perforated synapses, were also found in the occipital cortex (Greenough, West, & DeVoogd, 1978). Therefore, not only does exposure to complex environments add new synapses, but it also alters the shape of the new or existing synapses. Complex environments seem to affect the brain on many levels, from its basic size to synaptic ultrastructure.

The previously discussed studies demonstrate experience has an effect on the structure of cortical neurons. The rats in these studies, however, were approximately 25 days old when they were first exposed to the complex environments. This observation leaves open the possibility that the structural changes observed were due to a combination of the complex environment and properties unique to the still developing brain. An important question, therefore, is whether or not environment can affect the fully developed brain in a similar manner as the developing brain. A variety of research indicates similar effects do occur in the adult brain. Greenough, Juraska, and Volkmar (1979), for example, found increases in dendrite branching in the occipital cortex of rats exposed to a complex environment at 80 days of age that were qualitatively similar to those exposed to the environment at 25 days of age. Riege (1971) also found similar effects in rats exposed to a complex environment at 285 days of age. Whereas plastic changes were not as robust as those observed in the weanling rats, the adult brain seems to retain the capacity to change, and these changes are driven by experience.

A widely accepted hypothesis concerning experience-induced structural modifications is that they rely on the same processes that mediate learning and memory (Rosenzweig & Bennett, 1996). This statement also implies that the structural changes are the biological substrate of learning and memory. Consistent with this hypothesis is the observation that neural reorganization does not occur ubiquitously throughout the brain in response to the environment (reviewed in Grossman, Churchill, Bates, Kleim, & Greenough, 2002). If structural changes are related to learning and memory, they should be observed in regions of the brain associated with the behaviour(s). For example, the hippocampus is involved in spatial navigation, and damage to the hippocampus results in

difficulty learning spatial tasks (Aggleton, Hunt, & Rawlins, 1986). Faherty, Kerley, and Smeyne (2003) found that a complex environment increased neural growth in the CA1 and dentate gyrus pyramidal cells in the hippocampus of adult rats, but not in pyramidal cells within the motor cortex or spiny neurons within the striatum. These observations suggest that morphological plasticity is limited to regions in the brain involved in the neural processing related to the experience. Therefore, it can be argued that the plastic changes observed in the hippocampus occur because of its involvement in the spatial navigation required for the animal to make its way around a changing environment. In contrast, the absence of these plastic changes in the motor cortex or striatum may reflect the less critical role of these structures in exploring a novel environment, suggesting a functional relationship between the observed neuronal changes and behaviour.

As previously mentioned, complex environments provide rats with opportunities for many different experiences compared to control rats, making it difficult to attribute a morphological change to a specific element of the complex environment associated with learning. One avenue of research that attempts to minimize influencing factors that influence neural plasticity, and focuses on the process of learning, involves teaching rats new motor skills. This type of manipulation allows for a more focused assessment of the types of effects a specific behaviour has on plastic changes in the brain.

Motor Skill Learning

Although it is thought that the structural changes seen in EC animals are the biological substrate of learning and memory, this hypothesis is difficult to test directly. A complex environment is vastly different than a standard laboratory cage, making it

difficult to specify what aspect(s) of the manipulation is/are inducing plastic changes. An approach experimenters have used in an attempt to address this issue takes advantage of rats' adeptness at learning new motor behaviours. Training rats on a novel motor task can be an effective means of studying learning and memory due to the specificity of the manipulation. By teaching a rat a new motor skill, it is possible to change the type of motor behaviour the rat exhibits, while keeping other variables constant. Therefore, it is possible for two rats to experience very similar sensory stimuli, but execute different motor behaviours. By manipulating motor behaviour in this manner, it is possible to tease apart the effects of motor learning from motor activity by comparing the brains of trained versus non-trained animals. Brain structures associated with motor behaviour, including the primary motor cortex, cerebellum, striatum and brain-stem, as well as the spinal cord, can be examined for differences in gross anatomy, cellular anatomy and molecular characteristics. Furthermore, the motor cortex is organized into discrete and well characterized lamina, lending itself well to studying function-specific plastic changes. For example, layer II/III receives input from other cortical areas, and layer V sends output down the corticospinal tract. Therefore, changes observed in one layer but not the other may reflect plasticity associated with laminar specific processing.

When using motor skill learning as an experimental manipulation, it is important to characterize what motor behaviour qualifies as motor learning. Sanes (2003) describes complex motor learning as "involving multiple joints and across-limb co-ordination" (p. 225). Two paradigms used with rats that seem to match these descriptions are reach training and acrobat training. Reach training involves teaching a rat to reach through a narrow slot in order to retrieve a food pellet, an action that is repeated and requires a

stereotypical series of movements involving the shoulder, elbow, wrist and digits of the rats (Whishaw & Pellis, 1990). Animals improve the speed and accuracy of their reaching, indicative of learning this motor skill. Control animals are placed in the same environment, but are not given the opportunity to reach for food, and consequently, do not learn the reaching behaviour. Acrobat training involves teaching a rat to traverse a runway containing obstacles it must balance on or climb over. It is thought that improvement in traversing the acrobat course reflects the acquisition of a motor skill. A control group for this procedure involves having rats perform a motor activity that is similar in length to the acrobat course, but does not require the acquisition of a learned behaviour (eg. Kleim et al., 1998). A similar apparatus used by Dr. T. L. Ivanco's lab uses only the most complex aspect of the acrobat courses used by other labs (eg. Dr. W. T. Greenough) as a motor skill task for training rats. This apparatus has two potential advantages. First, the motor control animals in Dr. Greenough's apparatus run along a runway that have walls along the side, but the acrobat course does not. None of the motor tasks used in the present experiment have walls along the side, and are therefore more similar to each other. Second, the apparatus used in the present experiment uses motor tasks of varying degrees of difficulty, not simply one motor skill condition and a different motor control condition. The apparatus is further described in the method section of this paper.

Like exposure to a complex environment, motor skill learning affects the gross anatomy of the brain. Anderson, Eckburg & Relucio (2002) trained rats on an acrobat course and measured cortical thickness of four coronal planes. An increase in cortical thickness was found in the medial region of the two most anterior coronal planes of the

motor cortex compared to motor control animals, who had free access to a running wheel. Similarly, Kleim et al. (1998) found a thicker molecular layer of the cerebellum after acrobat training. Motor learning-induced changes in neuronal structure have also been reported. Reach training induces increased dendritic branching in layer II/III and layer V motor cortex pyramidal neurons (Greenough, Larson, & Withers, 1985; Withers & Greenough, 1989). This dendritic branching, however, differs between the two neural populations. The dendritic reorganization in layer V was limited to apical dendrites in the hemisphere contralateral to the reaching arm. Conversely, the reorganization in layer II/III was limited to basilar dendrites of pyramidal neurons, but occurred in both hemispheres. Just as exposure to a complex environment does not induce plastic changes throughout the brain, the effects of reach training appear to be selective within the motor cortex. Therefore, not only do neuronal alterations occur in specific brain areas, but they are also limited within an area. This finding may reflect functional differences between these two neuron populations and their role in motor skill learning. Withers and Greenough hypothesize that layer II/III may be less discriminating when it comes to processing incoming information, whereas layer V is more selective in processing information for execution of the behaviour.

Motor skill learning can also induce synaptogenesis and changes in synapse morphology. Animals trained on an acrobat course had an increase in the number of synapses per pyramidal neuron in the motor cortex (Kleim, Lussnig, Schwarz, Comery, & Greenough, 1996) and per Purkinje neuron in the cerebellum (Black, Isaacs, Anderson, Alcantara, & Greenough, 1990). Control animals that ran the same distance as the acrobats, but on a flat runway, did not exhibit synaptic changes. Acrobat animals also had

an increase in the number of perforated synapses on dendrites of layer V neurons in the motor cortex compared to controls (Jones, 1999). The same training paradigm also results in more multiple synapses (more than one post-synaptic element in contact with a pre-synaptic terminal) in parallel fibers of the cerebellar cortex (Federmeier, Kleim, & Greenough, 2002). The fact that these structural changes are not occurring in animals that experience activity, without the motor challenge, suggests that the structural changes are involved in the acquisition of new motor skills.

Persistence of motor learning-induced structural alterations has also been investigated. Kleim, Vij, Ballard, and Greenough (1997) found that increases in synapse per Purkinje cell in the cerebellum seen in acrobat animals persist for at least 28 days after the last of 10 training days. Using an unbiased stereological technique (physical dissector method), which allows for a more precise estimation of volume density, a decrease in Purkinje cell density was also observed after 10 training days. Furthermore, unlike the increase in synapse per neuron, this effect did not persist over the subsequent 28 days. Morales, Pinto-Hamuy, Fernandez, and Diaz (1999), however, found that a decrease in cell density in the motor cortex did persist for a comparable length of time after 10 days of reach training. These findings suggest that the cerebellum maintains its new synaptic connections once a skill has been learned, whereas the cortex may be in a constant state of flux, requiring continuous activity to maintain its synaptic connections. Decreases in neuronal density, however, appear to follow the opposite pattern. The cerebellum does not maintain this change in neuronal density, whereas the cortex does. If decreases in neuronal density are due to dendritic growth pushing cell bodies further apart from each other, then perhaps the function of cerebellar plasticity is to maintain

specific connections, whereas the function of motor cortex plasticity is to maintain dendrites, possibly for housing temporary synaptic connections. In contrast to this hypothesis, however, is the finding that rats exposed to a complex environment for 1 month do maintain an increase in synapse per neuron in the visual cortex once removed from the complex environment for one month (Briones, Klintsova, & Greenough, 2004). Perhaps the persistence of experience-dependent neural modifications differs between brain structures and according to the type of experience that induced the change.

Although examining motor skill learning-induced neural changes in the motor cortex and cerebellum is useful to determine the types of cellular modifications that may be important in the process of learning and memory, another effect of motor skill learning appears to be alterations in representational topography in the motor cortex. Changes in somatosensory topography, which is a representational cortical map of the peripheral sensory system, occur in response to disruption of input via nerve transection or digit amputation in primates. Such dramatic losses of normal inputs to the cortex result in an increase in representation of adjacent skin or digits with intact cortical innervation (Merzenich, Kaas, Sur, & Lin, 1978; Merzenich, et al., 1984). Similar effects also result from behavioural manipulation in primates. Adult squirrel monkeys were trained on a motor task requiring skilled digit movements. Following a period of training, digit representation increased at the expense of the wrist/forearm area (Nudo, Milliken, Jenkins, & Merzenich, 1996). Conversely, in the same study monkeys trained on a task requiring wrist/forearm movements had increased wrist/forearm representation at the expense of the digit areas. In a parallel study, monkeys performed similar tasks that did not require any skill learning. Unlike the motor maps of trained animals, the motor maps

of these animals did not show any systematic changes (Plautz, Milliken, & Nudo, 2000). These finding indicate that, like structural changes in neurons, representational changes in motor maps occur in response to motor learning, but not to motor activity.

Learning-dependant changes in motor maps also occur in rodents. Reach training in rats resulted in the expansion of wrist/digit representation into non-trained areas (Kleim, Barbay, & Nudo, 1998; Kleim, Cooper & VandenBerg, 2002; Remple, Bruneau, VandenBerg, Goertzen, & Kleim, 2001), whereas neither simple bar pressing (Kleim et al., 1998; Kleim et al., 2002), nor strength training (Remple et al., 2001) altered motor map representations. Specifically, reach training altered the caudal forelimb area, but not the rostral forelimb area, or the hindlimb area (Kleim, et al., 1998). Additionally, layer V pyramidal cells had more synapses per neuron in the caudal forelimb area compared to motor activity control animals (Kleim et al., 2002). Thus, reach training not only induced synaptogenesis, but also altered motor map representation by increasing the cortical forelimb area.

It appears that disrupting motor cortex topography plasticity also disrupts motor learning, suggesting that motor learning is supported by changes in motor map representation. Conner, Culberson, Packowski, Chiba, and Tuszynski (2003) induced lesions to the basal forebrain cholinergic system, which is thought to be involved in mediating learning-induced cortical plasticity. Disrupting this system blocked the motor map reorganization normally seen with motor learning, as well as the acquisition of the skilled reaching task. Kleim et al. (2003) also injected one of the protein synthesis inhibitors, anisomycin or cycloheximide, in the motor map area immediately after reach training. Similar to the basal forebrain lesions, inhibiting protein synthesis also disrupted

motor map reorganization. Additionally, acquisition of the motor skill was also interrupted by this drug manipulation. Therefore, motor map plasticity seems to be required for motor learning, and the co-occurrence of synaptic modification and cortical representation change reported by Kleim et al. (2002) suggests that both cortical map and synaptic alterations are associated with motor skill learning.

In further describing the nature of motor learning, researchers have pointed out at least two distinct phases: an initial brief period of rapid improvement in the behaviour (early phase), followed by a longer period of more gradual gains (late phase) (Nudo et al., 1996; Kleim et al., 1996). These two phases have been identified in non-human primates (Nudo et al., 1996), humans (Karni et al., 1998), and rodents (Kleim et al., 1996; Kleim et al. 2004). This description is useful in further characterizing the anatomical changes that occur with motor learning over time. For example, synaptogenesis in the motor cortex of acrobat rats occurs in the late phase of learning (Kleim et al., 1996) as does motor map reorganization (Kleim et al., 2003). This temporally defined distinction may reflect a difference in the molecular requirements for early and late phase learning, the later requiring neuronal structure changes.

Chapter 2

Molecular Relationships in Neuronal Plasticity

Much research is aimed at describing the morphological characteristics of neuronal plasticity. Another goal of plasticity research, however, is to reveal the cellular mechanisms that underlie learning and memory, and morphological alterations. Although these mechanisms are undoubtedly a complex series of cellular events and cascades (Bailey, Bartsch, & Kandel, 1996) researchers made significant gains as to the inner workings of neural cells that contribute to plasticity over the last 40 years. This information has sent research in various directions, including identifying the need for, and location of, protein synthesis in learning and memory.

In an early study indicating experience-induced alterations in brain chemistry, Krech, Rosenzweig, and Bennett (1960) found increased levels of acetylcholinesterase (AChE) activity in the cortex of EC animals. Bennett, Diamond, Rosenzweig, and Krech (1964) similarly reported an increase in overall protein levels in the cortex of EC rats. One of the first indications that protein synthesis was necessary for memory retention came from Flexner et al. (1962), who demonstrated that rats receiving a protein synthesis inhibitor treatment did not learn a task that was easily learned by rats receiving a vehicle treatment. The protein synthesis inhibitor in this case was puromycin, which is toxic and therefore confounded the finding. These results, however, have been replicated with less toxic inhibitors, such as anisomycin (Flood, Rosenzweig, Bennett, & Orme, 1973) demonstrating that the observed learning deficit was linked to the lack of new protein and not an effect induced by the toxicity of the treatment.

Building on the work by Flexner and others, researchers have highlighted the need for protein synthesis in the process underlying learning and memory (Stanton & Sarvey, 1984; Montarolo et al., 1986; Frey, Krug, Reymann, & Matthies, 1988). One approach of this research has been to use a reductionist model of long-term memory, using an organism with a relatively simple nervous system. The Aplysia is a mollusk with such a nervous system. Electrically stimulating the tail of the Aplysia results in a behavioural reflexive gill- and siphon-withdrawal response. This simple behaviour is subject to sensitization, an implicit form of memory, resulting from an increase in the efficacy of synaptic connections. A single pulse of electrical stimulation to the tail of the Aplysia can lead to an increase in the strength of the gill- and siphon-withdrawal response for minutes to hours, referred to as short-term facilitation (STF). Repeated pulses of electrical stimulation increase sensitization of this reflex for days to weeks, referred to as long-term facilitation (LTF) (Castellucci, Blumenfeld, Goelet, & Kandel, 1989). This system can be simplified by isolating the monosynaptic contact between a sensory and motor neuron in culture. This preparation uses serotonin application to the sensory neuron in place of electrical stimulation to the tail. Similar to the electrical stimulation in the intact animal, a single application of serotonin results in STF, and multiple applications result in LTF (Montarolo et al., 1986). Protein synthesis inhibitors applied to either the intact animal or the culture preparation blocked the formation of LTF, without disrupting STF. This effect indicates a protein synthesis-dependent phase of learning in this simplified model, corresponding to the establishment of long-term memory, and separate from the acquisition of short-term memory. Additionally, LTF is associated with an increase in the number of sensory neuron varicosities (synaptic

boutons or spines) and protein synthesis inhibitors also block this structural change (Bailey, Montarolo, Chen, Kandel, & Schacher, 1992). Thus, protein synthesis seems to be required for structural changes that accompany long-term enhancement of synaptic transmission.

Inhibiting protein synthesis in a mammalian has similar effects to those seen in the aplysia. Due to the difficulty of studying cellular mechanisms within complex neural networks, many researchers use long-term potentiation (LTP) as a model of learning and memory. LTP is an NMDA receptor-dependent strengthening of a synaptic connection that can be induced by electrical stimulation, which requires protein synthesis for the late stages to occur (Steward & Worley, 2002). Stanton and Sarvey (1984) attempted to induce LTP in the CA1 region of rat hippocampal slices that had been incubated in protein synthesis inhibitors. Incubating the preparation for 30 minutes disrupted the establishment of LTP normally seen in these slices in a dose dependent manner. This finding suggests that like LTF in the Aplysia, hippocampal LTP is dependent on protein synthesis. It has also been observed that neurons in hippocampal slice preparations have more dendritic spines than neurons from perfused hippocampi (Kirov, Sorra, & Harris, 1999). This naturally occurring phenomenon is also blocked by protein synthesis inhibitors (Johnson & Ouimet, 2004). Thus, within the mammalian system, protein synthesis seems to be required for long-term synaptic transmission enhancement, as well as spine proliferation.

That protein synthesis is a necessary component of long-term memory is widely accepted (eg. Bailey, Bartsch, & Kandel, 1996). Where these proteins are being synthesized, however, has become the subject of much research. The traditional view of

activity-induced protein synthesis is that the cell body manufactures proteins that are then transported out into neuronal processes. More recently, evidence has accumulated that shows that protein synthesis also occurs locally within dendrites and dendritic spines (Torre & Steward, 1992; Van Minnen et al., 1997). One of the first indications that synthesis occurs in dendrites came from Steward and Levy (1982), who observed polyribosomes, which contain necessary machinery for protein synthesis, in dendrite spines of hippocampal neurons in the rat. The presence of other protein synthetic machinery, including endoplasmic reticulum and Golgi apparatus, has also been observed in dendrites (Gardiol, Racca, & Triller, 1999). Indeed, Rao and Steward (1991) have shown that protein synthesis occurs within isolated synaptosomes, a preparation that contains dendritic synapses. A criticism of this approach, however, is that these types of preparations are easily contaminated with fragments of neuronal and glial cell bodies (Jiang & Schuman, 2002). A more convincing approach used by Aakalu, Smith, Nguyen, Jiang, and Schuman (2001) used green fluorescent protein (GFP)-based synthesis reporters to demonstrate brain derived neurotrophic factor (BDNF)-induced protein synthesis in isolated dendrites. Healthy, transected dendrites infected with a GFP-synthesis based reporter showed an increase in fluorescence following BDNF treatment that was not seen in dendrites that did not receive this treatment. Further, the increase in fluorescence was blocked by anisomycin, demonstrating that the fluorescence was due to protein synthesis.

The observation that proteins are synthesized at synaptic sites suggests that these proteins likely support synaptic functioning and plasticity. *In situ* hybridization has identified numerous mRNAs in rodent dendrites, some of which are known to be

important for LTP. With mRNA in the presence of the machinery needed for protein synthesis, it is likely that the mRNAs found in dendrites will influence neural plasticity based on the function of their protein products. Among the different mRNAs found, is the mRNA for a subunit of the NMDA receptor (NR1), which is essential for the induction of LTP (Jiang & Shuman, 2002). Also present are the mRNAs for BDNF and its receptor TrkB (Tongiorgi, Righi, & Cattaneo, 1997). BDNF has also been shown to play an important role in the induction of LTP (Korte et al., 1995; Kang & Schuman, 1995). If these locally synthesized proteins are involved in synaptic activity plasticity, they are likely regulated by synaptic activity.

As previously mentioned, the late stages of LTP require protein synthesis to occur (Steward & Worley, 2002). Also, LTP studies use neural circuitry with known termination areas corresponding to a population of neurons. These features of LTP make it suitable for studying the process of activity-induced protein synthesis. In order for polyribosomal complexes to synthesize proteins, mRNA would need to be present in dendritic spines, or transported from the cell body via activity-induced signaling. LTP studies indicate that neuronal activity does increase mRNA within dendrites selectively at sites of synaptic activation. Stimulation of projections to the dentate gyrus resulted in an increase of the immediate-early gene (IEG) activity-regulated cytoskeleton-associated protein (Arc) within activated dendritic segments (Steward, Wallace, Lyford, & Worley, 1998). Arc protein also accumulated in the same segments. Recently, Moga et al. (2004) found that Arc upregulation is restricted to segments of a dendritic arbor receiving direct, plasticity-inducing stimulation. Electrophysiological stimulation of this pathway also altered levels of mRNA for microtubule-associated protein (MAP2) (Steward & Halpain,

1999). Therefore, accumulation of mRNA and protein in dendritic segments seems to accompany activation, and these mRNA may locally translate proteins required to support activity-induced learning and memory. If this hypothesis is true, similar changes in mRNA and proteins should be learning-induced as well.

Rearing rats in a complex environment resulted in a greater percentage of spines associated with polyribosomes (Greenough, Hwang, & Gorman, 1985). This manipulation also induced dendritic growth in the visual cortex. Therefore, the same behaviourally-induced increase of protein synthetic machinery in spines also induced neuronal structure changes. This observation is important because it demonstrates that the findings from in vitro experiments can be generalized to behaviour. Just as electrophysiological stimulation can change mRNA and protein levels, behaviourally-induced experience-dependant changes in mRNA and protein levels also occur. RNA concentrations are elevated in the cortex of rats reared in a complex environment (Ferchmin & Eterovic, 1986). Alterations in the expression of tissue plasminogen activator (tPA) (Seeds, Williams, & Bickford, 1995), BDNF (Ishibashi et al., 2002), FMRP (Fragile-X Mental Retardation Protein) (Irwin et al., 2000) and Fos (Kleim et al., 1996) proteins have occurred in response to behavioural learning. All of these proteins have been implicated in synaptic plasticity, and evidence suggests that the mechanism by which they are upregulated may be through local translation of mRNA.

MAP2

One way newly synthesized proteins may function to support long-term memory is through their involvement in the structural changes in neurons associated with learning.

Microtubules are tubular structures that originate in the cell body and extend out into dendrites and axons. There are at least 10 proteins associated with microtubules, referred to as microtubule associated proteins (MAPs), that play various roles including maintaining neuronal structure (Feldman, Meyer & Quezer, 1997). Whereas some MAPs are found specifically within axons (eg. tau), and others within both axons and dendrites (eg. MAP1B), MAP2 is specific to dendrites (Kaech, Parmar, Roelandse, Bornmann & Matus, 2001), and functions to regulate dendritic stability (Hering & Sheng, 2001). The observation that dendrites reorganize in response to reach training (Greenough, Larson, & Withers, 1985; Withers & Greenough, 1989) suggests that the microtubules within dendrites also restructure.

MAP2 appears to be required for neurite growth and stabilization (Sanchez, Diaz-Nido, & Avila, 2000). Suppression of MAP2 via MAP2 antisense oligonucleotides suppressed neurite outgrowth and dendritic microtubule stabilization in cultured cerebellar (Caceres, Mautino, & Kosik, 1992) and cortical (Sharma, Kress, & Shafit-Zagardo, 1994) neurons. It is likely that the need for MAP2 for dendrite elongation in a cultured preparation has parallels in learning-induced dendrite reorganizing. The presence of MAP2 mRNA within dendrites (Steward & Schuman, 2001) supports this notion, and suggests that it may be locally synthesized and regulated by neuronal activity. Indeed, MAP2 mRNA is altered by electrophysiological stimulation within the dentate gyrus (Steward & Halpain, 1999). Further, reach training increases MAP2 immunostaining in the trained somatosensory cortex of rats receiving unilateral somatosensory cortex lesion in the opposite hemisphere, indicative of neuronal cytoskeletal changes (Bury & Jones, 2002). These observations suggest that MAP2 expression may alter in order to

accommodate dendritic structural changes associated with learning-induced plasticity. Miller and Kaplan (2003) suggest a regulatory mechanism whereby neural activity induces MAP2 to interact with microtubules during dendrite formation or stabilization through the CaMKII and MEK-ERK pathways. This mechanism may provide a means for synaptic activity produced by behaviour to alter dendrites, which may then function to support the behaviour. Thus, motor skill learning would likely result in MAP2 expression changes in the motor cortex.

Synaptophysin

Synaptophysin is the major integral membrane protein in pre-synaptic vesicles (Thiel, 1993) and is thought to be involved in vesicle formation and exocytosis (Valtorta, Pennuto, Bonanomi, & Benfenati, 2004). Synaptophysin has been used to study pre-synaptic terminals in a number of contexts, including synapse formation during rodent development (Knaus, Betz, & Rehm, 1986). Expression of synaptophysin has also been used to investigate kindling-induced axon sprouting. Kindling is a model of neuronal hyperexcitability induced either chemically or electrically. Synaptophysin immunoreactivity increases in the hippocampus and layer II/III of the piriform cortex following electrical stimulation-induced kindling, indicative of neural activity induced axon sprouting (Li, Reinprecht, Fahnestock, & Racine, 2002).

Synaptophysin is also used to indicate the formation of new synapses. Reinprecht, Bschanes, Windisch and Fachbach (1999) investigated the effects of the drug Cerebrolysin on synapse density in the hippocampus and entorhinal cortex. Cerebrolysin, a peptidergic drug demonstrated to improve memory function, has previously been

demonstrated to promote resistance to glutamate-induced toxicity in cultured telencephalic neurons (Hutter-Paier, Grygar & Windisch, 1996), as well as improve cognitive performance in young and old rats on a spatial learning task (Gschanes & Windisch, 1996; 1998). The authors reason that the pharmacologically induced neuro-protection and improved behavioural performance are likely accompanied by synaptogenesis, and used synaptophysin as a means of quantifying the measure. Indeed, Cerebrolysin did result in increased immunostaining for synaptophysin, and presumably, synapse density.

Given that synaptophysin is used to indicate the effects of electrical stimulation on axon sprouting and pharmacologically induced synaptogenesis, expression of synaptophysin may also change in response to behavioural stimulation. Indeed, Nithianantharajah, Levis and Murphy (2004) reported an increase in synaptophysin levels in multiple brain regions, including the motor cortex, in mice exposed to a complex environment. Learning-induced synaptogenesis (as per Kleim et al., 1996) and/or synaptic modification (as per Jones, 1999) may also alter synaptophysin expression. Increased expression may reflect increased synapse number and/or increased synaptic activity associated with motor learning. Thus, motor skill learning may produce changes in synaptophysin expression in the motor cortex.

Chapter 3

Experiment 1

The cellular events that take place during learning-induced neuronal plasticity are complex. Understanding these processes, however, is a worthwhile challenge. The present experiment examines the influence of motor skill learning on levels of MAP2 and synaptophysin protein in the motor cortex. Animals were trained on motor tasks that vary in complexity, thereby separating the effects of motor skill learning from motor activity. The motor cortex from one hemisphere from these animals were examined for overall differences in protein levels. Layer II/III and layer V within both the motor and sensory cortex from the contralateral hemisphere will also be examined for differences in amount of MAP2 and synaptophysin using immunohistochemistry. Additionally, animals were trained on the different motor tasks for different numbers of days in order to assess influences of motor skill learning on protein changes over time.

Method

Animals in this study were 75 female, Long-Evans rats 4-6 weeks of age upon arrival from Charles River Laboratory, housed 2-3 per cage. Cages were kept in an animal room with a 12-hour light/dark schedule (lights on at 06:00). Food and water were available *ad lib* up to 3 days prior to pre-training, at which point the animals were placed on restricted food diet (15g food/200g). Weights of all rats were recorded daily starting the day before they were placed on the restricted food diet and continued for the duration of the experiment.

Behavioural Training

Motor tasks.

The motor tasks consisted of 4 different runways (184 cm length x 5 cm width): a flat beam, which is the no learning task (NLT), an easy learning task (ELT) which is a series of rungs spaced 3 cm apart, a harder learning task (HLT) which is a series of unevenly spaced rungs, and difficult learning task (DLT) which is a series of upright dowels (0.6 cm diameter) spaced 2 cm apart and situated at varying angles (see Figures 1a-1d). Three different stands support the runways such that they are elevated approximately 3 feet from the ground and can be readily interchanged. The stands that supported the ends of the runways also double as the starting and finishing platforms, the latter of which also houses a goal box for treat pellets. The different tasks are designed to require varying levels of overall coordination from the rats to traverse, thereby separating the effects of motor learning from motor activity.

Pre-training.

Rats were approximately 10 weeks old at the start of pre-training. All animals but the inactive control (IC) group (n = 3) were pre-trained on the NLT task for 3 days. Exposure to this task was intended to give the rats time to become familiar with the new environment, the location of the goal box and the general nature of the task. On each day, animals were initially placed on the finishing platform until they ate 7-10, 20mg pellets from the goal box or for up to 15 min. After the initial exposure to the finishing platform, the animals traversed the runway from the starting to finishing platform 3, 5, and 8 times on days 1, 2, and 3 respectively. If the animals did not immediately eat from the goal box

upon completing the task, they were allowed up to 5 min to eat some pellets at the end of the pre-training session.

Motor Skill Training.

Following pre-training, animals were randomly assigned to one of the other 3 tasks and to 1, 3, 5, or 7 days of motor training (12 groups; n = 6/group). Each training day consisted of traversing the task 8 times. If the animal stopped on the runway before reaching the finishing platform, it was gently prodded on the hindquarters to re-initiate forward movement. Times to run the task were measured using a motion-sensitive timer. A switch located underneath the starting platform starts the timer when the animal places its front paws on the runway and initiates forward movement. The timer was stopped when a beam of light perpendicular to the runway was broken when the animal placed its paws on the finishing platform. The time was shown on a LED display, recorded and the 8 trials were averaged as an indication of that animal's performance for that day. These data were analyzed using an ANOVA with day as a within-subjects factor.

Western Blot Analysis

Approximately 24 hrs after the last training day, rats were deeply anesthetized with an i.p. sodium pentobarbital injection (100mg/kg) and placed in a stereotaxic apparatus. The skull was exposed and an area of the skull and dura mater above the left motor cortex was removed. Incisions approximately 2mm deep were made around the motor cortex at midline, ~1.5mm lateral to midline, 1mm caudal and 1mm rostral to bregma. A wire loop, flattened at the bottom, was inserted into one of the incisions and

used to cut underneath the motor cortex. The tissue was immediately put on ice and stored at -80°C.

Cortical samples were homogenized in PLC++ buffer (vol/10ml: 0.5ml HEPES pH 7.5, 0.3 ml 5M NaCl, 0.15ml 0.1M MgCl2, 0.02ml 0.5M EGTA, 1ml 0.1M NaPPi, 2ml 0.5M NaF, 1ml glycerol, 0.1ml Triton x-100), 0.1ml sodium orthovanadate and 1 mini-protease tablet. Homogenates were centrifuged at 13,000 x G for 20 minutes at 4°C and the supernatant was removed. Protein in the supernatant was quantified using a Bradford Assay. Equal amounts of protein from each sample from a group (n=6) were combined and mixed with 2xSDS buffer. Combined samples were electrophoresed in SDS buffer (vol/1l: 3.02g Tris, 14.4g glycine, lg SDS) at 170V. Proteins were transferred onto a PVDF membrane using a semi-dry transfer cell and blocked with 5% nonfat dry milk in TBS-T (vol/1l: 20ml 1M Tris; pH 7.5, 30ml 5M NaCl, 1ml Tween-20). Blots were incubated in MAP-2 antibody (1:1000, Sigma) overnight at 4°C, washed with TBS-T, and incubated in HRP goat anti-mouse IgG. Protein bands were detected using an ECL detection kit and then stripped and re-probed using synaptophysin antibody (1:2000, Sigma) overnight at 4°C and HRP goat anti-mouse IgG secondary (1:20 000) for 1 hour at room temperature.

Digital images of the blots were created and optical density measurements of the bands were taken with Image-Pro software. These measurements were interpreted as a percentage change from control levels.

Immunohistochemistry

Following removal of the left motor cortex for Western analysis, rats were transcardially perfused with 200ml 0.1M PBS followed by 150ml 4% paraformaldehyde in 0.1M PB. Coronal sections were cut at 50μm with a freezing microtome. Sections were sequentially blocked in 3% normal horse serum with 0.1% Triton-X for 30 minutes followed by an avidin and biotin blocking step (Vector labs), and then incubated in monoclonal MAP-2 (Sigma) or monoclonal synaptophysin (Sigma) antibody with 1% normal horse serum for 1 hour. A biotinylated horse anti-mouse secondary was used for both antibodies for 30 minutes. Subsequently, sections were incubated in ABC reagent for 30 minutes. Sections were washed with PB between each step. Visualization was done using DAB as the chromogen and sections were then mounted on slides, dehydrated and coverslipped.

Digital images from six sections per animal were captured with a 4x objective using a Coolsnap-Pro monochrome camera mounted on an Olympus BX51 microscope and converted into grayscales. Neutral density filters with known relative optical densities (ROD) were used to create an internal calibration for all images. ROD measurements from six cursors placed within motor and sensory cortex layers II/III and V for each section were obtained using Image-Pro software (see Figure 2). ROD measurements were also taken from the corpus callosum of section, and a cortex ROD/callosum ROD ratio for each of the 6 cursors was obtained and used in the analysis.

Statistical Analysis

To determine if time to complete the motor tasks were significantly different between groups, a one-way analysis of variance (ANOVA) was completed for each time point (1-, 3-, 5-, 7-day). Due to similarities in time to run the task, 7-day data are reported and relationships between time points are discussed using these data. A one-way ANOVA was also used to examine differences between groups in the Western analysis and the immunohistochemical analysis. For the Western analysis, the optical densities of bands from four blots were averaged for statistical purposes. For the immunohistochemical analysis, the average ROD of the six cursors was used for statistical purposes. Tests were two-tailed, and statistical significance was set at $p < .05$.

Results

Behaviour

A one-way ANOVA revealed a significant main effect for group for all days ($F(2, 14) = 30.08, p < 0.0001$). There was also an interaction effect of group by day ($F(12, 84) = 10.2, p < 0.0001$). Post hoc tests (Tukey's LSD; $p < 0.05$) showed that for days 1, 3 and 5, times for the DLT animals were higher than all other groups. 7-day DLT animals, however, were not significantly different from 1-, and 3-day ELT animals, nor were they different from 1-, 3-, or 5-day HLT animals (see Figure 3). Further post hoc (Tukey's LSD; $p < 0.05$) inspection of the day 7 analysis show that for animals than ran the ELT task, times were faster on day 7 than on day 1. HLT animals' times were faster on day 7 compared to days 1 and 2. DLT animals' times were faster on day 7 compared to days 1, 3 and 5.

Western Blot Analysis

MAP2.

A one-way ANOVA was performed on all Western and immunohistochemistry data. A main effect for group was found ($F(12, 39) = 2.16, p < 0.05$). Post hoc tests (Tukey's LSD; $p < 0.05$) indicated that bands from 1- and 5-day ELT animals, 5-day HLT animals, and 7-day DLT animals were darker than the bands from the control animals (see Figures 4a and 4b).

Synaptophysin.

A main effect for group was found ($F(12, 39) = 2.89, p < 0.01$). Post hoc tests (Tukey's LSD; $p < 0.05$) indicated that bands from the 1-, 3- and 5-day DLT animals were significantly darker than the bands from the control animals (see Figures 5a and 5b).

Immunohistochemistry

MAP2.

A significant effect of group was found for layer II in both motor ($F_{(12, 62)} = 2.06$, $p < 0.05$) and sensory ($F_{(12, 62)} = 2.44, p < 0.05$) cortex. Post hoc tests (Tukey's LSD; $p < 0.05$) revealed that the RODs for the 7-day DLT animals were darker than control levels. An effect of group was also found for layer V sensory cortex ($F_{(12, 62)} = 2.26, p < 0.05$), with post hoc tests revealing RODs for the 7-day ELT and DLT groups significantly darker than control levels.

Synaptophysin.

No significant differences in any of the synaptophysin-stained sections were found.

Discussion

The present experiment demonstrates that proteins involved in neuronal structure and function are dynamically altered during the course of motor skill learning. The area of interest for the Western blot analysis was the motor cortex. For the immunohistochemical analysis, the motor and sensory cortices were investigated. In addition to motor activity, the motor tasks that the animals were trained on likely required a significant amount of tactile information processing as well; there is reason to suspect experience-induced changes in the sensory cortex in addition to the motor cortex.

Motor Skill Learning

For days 1, 3, and 5, all DLT animals took significantly longer to complete their motor task, indicating that these animals experienced a greater challenge in navigating their way across the runway than the ELT or HLT groups. This finding was not quite the same for day 7, as the animals that ran the task for 6 and 7 days were not significantly slower than the animals that ran the ELT or HLT tasks on the first few days, demonstrating that the DLT rats' times began to approach the times of the ELT and HLT rats. This pattern of behaviour indicates that the DLT task allowed for continued improvement over the 7 days of training, suggesting that this task presents a motor challenge and a motor learning scenario to the rats. That the DLT animals showed continued and more dramatic improvement over the seven training days suggests that they experienced a significant degree of motor skill learning.

Changes in MAP2 Expression

Data from Western blots indicated that MAP2 is upregulated at different stages of learning a motor task, and these different stages vary according to the difficulty of the task. The motor cortex from the ELT animals had more MAP2 on days 1 and 5 of training. HLT animals had more MAP2 on day 5, and DLT animals on day 7 of training. Data from immunohistochemistry indicates that MAP2 increases in layer II/III of motor and sensory cortices, and in layer V of the sensory cortex in the 7-day DLT animals.

Steward and Halpain (1999) observed two distinct stages of MAP2 immunostaining: a period of downregulation in the target region within 5 minutes of electrical stimulation, followed by upregulation in the two layers adjacent to the target layer following 1 to 2 hours of stimulation. Assuming that learning-induced morphological changes in dendrites occur as a result of behaviour driven activation, one would expect changes in MAP2 expression in the DLT animals. The pattern of expression seen in the motor cortex, as a whole, may not follow that reported by Steward and Halpain because neuronal activation in the motor cortex triggered by behaviour is much more complex than that induced by electrophysiological activation. Whereas behaviourally-induced neural activation is diffuse and not confined to a specific brain region, electrophysiology studies generally examine smaller brain regions with more defined neural pathways. Although not all were statistically significant, MAP2 bands for all groups were darker than controls, suggesting that the experience common to all, but the control rats may be sufficient to induce MAP2 expression. Although the tasks were designed to separate motor learning from motor activity, the novelty of the situation may have masked any learning effects. The pattern of MAP2 expression that did occur,

however, was difficult to interpret, and may have been influenced by other factors. Alternatively, a learning-dependent effect may only be detectable at an earlier stage of learning.

The pattern of MAP2 staining reported by Steward and Halpain (1999) coupled with the observation that skilled reaching induced dendritic growth in layer II/III and V neurons (Greenough, Larson and Withers, 1985; Withers and Greenough, 1989) suggests that a pattern of downregulation followed by upregulation of MAP2 might be found in these layers. Our immunostaining, however, did not reveal this pattern, and it was also inconsistent with Western blot data. Western blots indicated MAP2 expression increased in 1- and 5-day ELT animals, 5-day HLT animals, and 7-day DLT animals. Immunohistochemistry suggested that layer II staining in both the motor and sensory cortex were darker for 7-day DLT animals, and layer V staining was darker for 7-day ELT and DLT animals. These seemingly contradictory results may have resulted because of the difference in the amount of tissue examined between the two techniques. Our Western blots looked at the entire cortex, whereas our immunostaining only looked at the area of cortex corresponding to the location of dendrites within layer II/III and V. Expression changes in these areas alone may not have been detectable. Alternatively, they may not have occurred in the dendritic areas, suggesting that the changes observed in the Western blot analysis occurred closer to cell bodies.

Changes in Synaptophysin Expression

Levels of synaptophysin were significantly higher in the animals that experienced the most motor skill learning. Furthermore, this effect occurred during the

first five days of training. Considering that synaptogenesis in the motor cortex is detectable at the EM level following five days of motor learning (Kleim et al., 1996), synaptophysin may be upregulated before the new synapses completely form, and return to normal levels once the synaptic connection has been established. This upregulation may also reflect an increase in synaptic activity, possibly the result of motor skill acquisition. Non-significant differences between groups from the immuno-staining may indicate that, given the restricted area of inspection, differences may have occurred closer cell bodies than dendritic layers.

In summary, the improvements in time to complete the motor tasks suggests that the extent of motor skill learning by the DLT group far surpassed the HLT and ELT groups. Although differences in MAP2 expression were expected between these groups, and to follow a distinct time course, the results indicated a more complex picture. Changes in synaptophysin expression, however, did meet expectations in that levels of the protein were elevated in the DLT group on days 1, 3, and 5.

Chapter 4

Experiment 2

The previous experiment examined changes in protein expression over 7 days of motor skill learning. Greenough and Withers (1989) reported dendrite growth following 10 days of reach training. Previous work in Dr. Ivanco's lab indicated that there is no change in MAP2 expression after 10 days of training on the DLT task (Derksen & Ivanco, unpublished data). Although Experiment 1 aimed to examine MAP2 and synaptophysin at time points within 10 days, there were no significant differences between groups with regard to MAP2 expression. Therefore, a detectable change in MAP2 expression may occur within a narrower time frame yet. The current experiment tests this hypothesis by training rats on a motor skill task at different time points within 24 hours of motor training. Examination of the 8 trials of the DLT animals on the first day of training from Experiment 1 suggested that the times to run trials 4-8 were much more consistent than times to run trials 1-3, indicating a much steeper learning curve. In light of this observation, rats were trained for 1, 3, or 8 trials. 1-trial training was selected to observe any expression changes that may be occurring immediately upon motor learning. Looking for expression changes after 3 trials of training at a point that seemed to indicate the beginning of consolidation of the motor skill. 8 trial training was chosen to examine any possible expression changes at the end of a training day.

Methods

Animals were 29 female Long Evens rats, 9-11 weeks of age, bred at the University of Manitoba, and housed 2-3 per cage. Cages were kept in an animal room

with a 12-hour light/dark schedule (lights on at 06:00). Food and water were available *ad lib* up to 3 days prior to pre-training, at which point the animals were placed on restricted food diet (15g food/200g). Weights of all rats were recorded daily starting the day before placed on the restricted food diet and continued for the duration of the experiment.

Motor Learning

The NLT and DLT tasks previously described were the only tasks used in this experiment. All rats, but the IC group (n = 5), were pre-trained as described in Experiment 1. Following pre-training, animals were randomly assigned to 1 of 4 groups (n = 6/group): 1-trial, 3-trial, 8-trial or 24-hour. For the 1, 3, and 8 trial groups, the animals were trained on the DLT task for the given number of trials, and immediately anesthetized with an i.p. sodium pentobarbital injection (100mg/kg). Animals in the 24-hour group were trained on the DLT task for 8 trials, and then anesthetized approximately 24 hours after training.

Western Blot Analysis

Procedures for the tissue collection and Western blot analysis were as described in Experiment 1.

Statistical Analysis

A one-way ANOVA was also used to examine differences between groups in the Western analysis. The optical densities of bands from three blots were averaged for statistical purposes.

Results

Behaviour

Times for the rats trained on the DLT task for 8 trials were similar to those of the rats trained for Experiment 1 (see Figure 6).

Western Blots

No significant difference in Western blot density was found for MAP2 ($F(4, 15) = 0.658$, $p = 0.63$) (see Figures 7a and 7b), or synaptophysin ($F(4, 15) = 0.839$, $p = 0.53$) (see Figures 8a and 8b).

Discussion

Behaviour

Rats in the 8-trial and 24 hour group improved in time to complete the DLT task. Further, the rats improved much more dramatically over the first 3 trials than the last 5 trials. Of the total time the rats spent traversing the apparatus during the 8 trials, 68% was used during the first three trials. In an effort to gain a very specific time course for protein expression changes during motor learning, the decision to look at the brain after the first, third and eighth trials seems justified.

Western blots

MAP2.

Whereas the previous experiment demonstrates that MAP2 is dynamic and fluctuates over 7 days of behavioural training, this effect does not seem to occur within

one day of training. In keeping with the same predicted pattern according to Steward and Halpain (1999), a possible scenario for the current study would have seen MAP2 expression downregulated after one and three trails, and then upregulated after 8 trials. Neural activity induced by electrical stimulation is much more invasive and direct than neural activity induced by behavioural learning. It is possible that learning does not have the same effects electrophysiology does, or at least not as profound or rapid. Further, the hippocampus is a brain region known for fast and large plastic changes. Although LTP can be induced in the cortex, it is slower to occur than in the hippocampus (Ivanco, Racine & Kolb, 2000). Changes in MAP2 expression may also be more difficult to induce in the cortex than in the hippocampus.

Synaptophysin.

Experiment 1 demonstrated that during motor skill learning, synaptophysin expression is upregulated during the first 5 days of training. The current experiment demonstrates that synaptophysin expression does not change within a shorter time frame. Mullany and Lynch (1997), found increases in synaptophysin expression in the entorhinal cortex 40 minutes after LTP induction in the dentate gyrus, suggesting that synaptophysin expression can be rapidly altered. Again, LTP in the hippocampus is a technique that is likely to generate more plastic changes than behavioural training on the cortex and synaptic changes in the cortex may take longer.

In summary, further examination of protein expression changes during the first 24 hours of motor skill learning did not reveal any differences. Whereas LTP is able to

induce rapid changes in protein expression, these effects may occur over a longer period of time when behaviourally induced.

Chapter 5

General Discussion

Expression alterations of MAP2 do not follow a distinct time course during 7 days of motor learning, whereas synaptophysin exhibits a pattern of increased expression that is induced by motor learning. Experiment 1 demonstrated this finding, and also that the apparatus used is appropriate, in that it allows for the comparison between animals that have learned a motor skill and those that did not. Experiment 2 demonstrated that behaviourally induced expression of MAP2 and synaptophysin do not change rapidly following motor skill learning.

Changes in MAP2 expression

The observed changes in MAP2 expression were not as predicted. Examining MAP2 or MAP2 mRNA has been used in to indicate cytoskeletal reorganization following brain injury (Martinez et al., 1997), behavioural training following injury (Bury and Jones, 1999), and electrophysiological stimulation (Steward and Halpain, 1999). These paradigms involve a manipulation that creates an artificial environment within the brain. Although they are useful tools in their own right, such invasive measures may not always be indicative of what occurs during more natural reorganization. MAP2 is very prevalent in the cortex, and changes in MAP2 expression sufficient to accommodate learning-induced dendrite changes may not have been detectable through Western blot analysis, given the more subtle nature of manipulation used in these experiments. With the immunohistochemical analysis, perhaps looking closer to the cell bodies would reveal differences in expression within these layers.

Another explanation for the lack of a clear time course of MAP2 expression is that the MAP2 changes needed for learning-induced dendrite reorganization may not be related to new MAP2 synthesis, but to altered phosphorylation states. Phosphorylation is a reversible, post-translational modification that alters the function of a protein. There is evidence that suggests phosphorylated, as opposed to non-phosphorylated, MAP2 is an important factor in regulating dendrite growth (Sanchez, Diaz-Nido, & Avila, 2000). The phosphorylation state of MAP2 is developmentally regulated (Riederer, Draberova, Viklicky, & Draber, 1995), and is also correlated with dendritic branching. Diez-Guerra and Avila (1993) prepared hippocampal neurons in culture and observed a correlation between the level of phosphorylated MAP2 and the number of neurites with more than one branch per dendrite over 3 days. Total amount of MAP2 was also increased, but modestly compared to the level of phosphorylation. Diez-Guerra and Avila (1995) also observed a correlation between MAP2 phosphorylation and extension of dendrite-like processes in embryonic neurons. Also, Diaz-Nido, Montoro, Lopez-Barneo and Avila (1993) found that extracellular potassium-induced synaptic activity in hippocampal neurons is also correlated with increased phosphorylation of MAP2. These findings suggest that motor learning may also alter the phosphorylation state of MAP2, which may be sufficient for dendritic plasticity. A future study to investigate this hypothesis could use the same methods, but use antibodies specific to phosphorylated and non-phosphorylated MAP2.

Another avenue for further investigation is to use male rats. Females are desirable for such behavioural manipulations because they are smaller, more agile, and generally more willing to participate in the motor activities. Females also, however, experience

cycles of hormonal fluctuations that males do not. Reyna-Neyra, Camacho-Arroyo, Ferrera and Arias (2002) studied the effects of the hormones estradiol and progesterone on MAP2 expression. MAP2 expression in the hippocampus was affected by both hormonal treatments. Estrogen receptors are also found in high number is the cortex, including the association and primary motor cortex (Kritzer, 2002). Therefore, it is possible that the estrous cycles of the rats used in these studies impacted MAP2 expression. This situation may have been sufficient to mask the effects of motor learning versus motor activity.

Changes in synaptophysin expression

Synaptophysin is used to study synaptic activity (eg. Li et al., 2002) and synaptogenesis (eg. Reinprecht et al., 1999). The pattern of synaptophysin expression during motor skill learning in this study may reflect both of these phenomena. The neural activity within the motor cortex of animals learning a motor skill may exceed that of the animals that only experience motor activity. In this case, the increase in synaptophysin expression could be the direct result of this elevated activation. It is also possible that as the animals became more familiar with the motor learning task, and the initial acquisition phase of learning has passed, neural activation returned to normal levels, thereby reducing the level of synaptophysin present in the motor cortex.

The pattern of synaptophysin expression may also reflect the presence of new synapses. Kleim et al. (1996) found that motor skill learning induced synaptogenesis, as measured by EM, was detectable after the first 5 days of training. It is possible that during this time period, synaptic activity is causing pre- and post-synaptic elements of

dendrites to seek out new connections. More connections may be made than are necessary, and only subsets of new synapses remain established. This would result in a decrease of synapses after 5 days, which is reflected by the return of synaptophysin levels closer to controls. This model of synaptogenesis is similar to the natural process of pruning of synapses that occurs during development.

In summary, the relevant literature suggests that MAP2 and synaptophysin may play a role in learning-induced plastic changes, and therefore may undergo expression changes during learning. MAP2 expression does change, but not in a clear, predictable manner. Other studies looking at MAP2 expression changes have used LTP and in the context of the damaged brain. These differences between other studies looking a MAP2 expression changes and the present experiments may explain the lack of a clear pattern in our studies. The changes in synaptophysin expression observed in Experiment 1 are consistent with the literature. During a time of synaptogenesis, synaptophysin is increased in the motor cortex, likely in response to increased neural activity that is specific to motor learning, not motor activity.

LTP is a good model for synaptic plasticity and learning and memory that is frequently used. Results presented in this paper, however, seem to indicate that LTP is not always representative of the molecular events that occur during behavioural learning. MAP2 levels did not match expectations, perhaps due to a number of factors that exist in behavioural paradigms, but are not represented in the LTP model. Our motor tasks, however, appear to be appropriate for behavioural testing. Synaptophysin levels were consistent with results found in studies using similar tasks. This result suggests that

synaptophysin can be used as a marker to indicate the presence of new synapses, and that our motor tasks are useful for studying the effects of motor skill learning.

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Appendix

List of Abbreviations

AchE	acetylcholinesterase
Arc	activity-regulated cytoskeleton-associated protein
BDNF	brain-derived neurotrophic factor
CAMK-II	calcium/calmodulin dependent kinase
DLT	difficult learning task
EC	complex environment
EM	electron microscopy
ERK	extracellular signal-regulated kinase
ELT	easy learning task
FMRP	Fragile-X Mental Retardation Protein
HLT	hard learning task
IEG	immediate-early gene
LTF	long-term facilitation
LTP	long-term potentiation
MAP2	microtubule-associated protein
MEK	MAPK kinase
mRNA	messenger ribonucleic acid
NLT	no learning task
NMDA	N-methyl-D-aspartate
ROD	relative optical density
STF	short-term facilitation

Figure Captions

Figure 1a. Final portion of the no learning task, finishing platform and goal box.

Figure 1b. Final portion of the easy learning task, finishing platform and goal box.

Figure 1c. Final portion of the hard learning task, finishing platform and goal box.

Figure 1d. Final portion of the difficult learning task, finishing platform and goal box.

Figure 2. Representative coronal section of motor cortex immunostained with anti-MAP2, with cursors.

Figure 3. Behavioural data depicting average time ($M \pm SEM$) to complete the different motor tasks over 7 days.

Figure 4a. Representative Western blot from Experiment 1 with MAP2-stained bands.

Figure 4b. Graphic depiction of average band densities ($M + SEM$) from MAP2-stained Western blots.

Figure 5a. Representative Western blot from Experiment 1 with synaptophysin-stained bands.

Figure 5b. Graphic depiction of average band densities ($M + SEM$) from synaptophysin-stained Western blots.

Figure 6. Behavioural data depicting average time ($M \pm SEM$) to complete the DLT task over 8 trails during one day of training.

Figure 7a. Representative Western blot from Experiment 2 with MAP2-stained bands.

Figure 7b. Graphic depiction of average band densities ($M + SEM$) from MAP2-stained Western blots.

Figure 8a. Representative Western blot from Experiment 2 with synaptophysin-stained bands.

Figure 8b. Graphic depiction of average band densities ($M + SEM$) from synaptophysin-stained Western blots.

Figure 1a

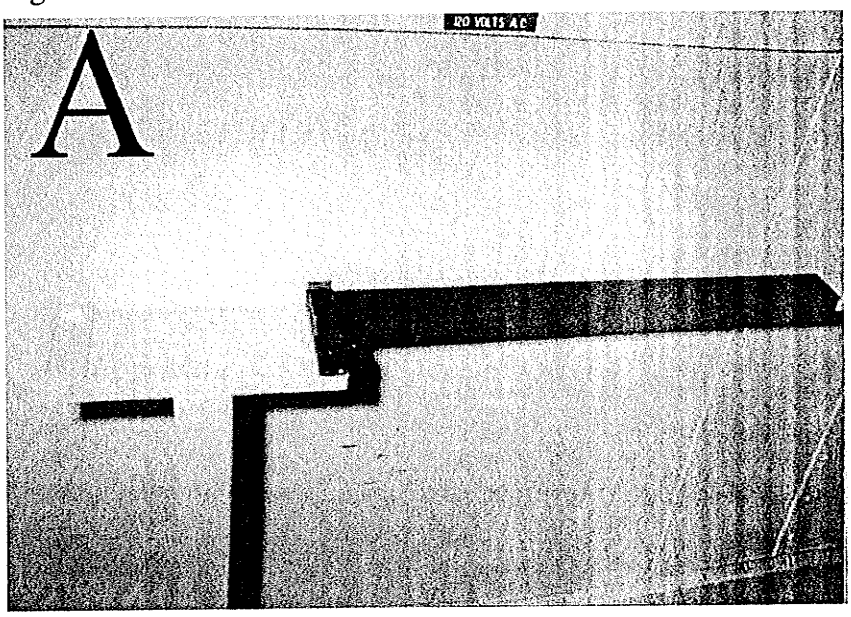


Figure 1b

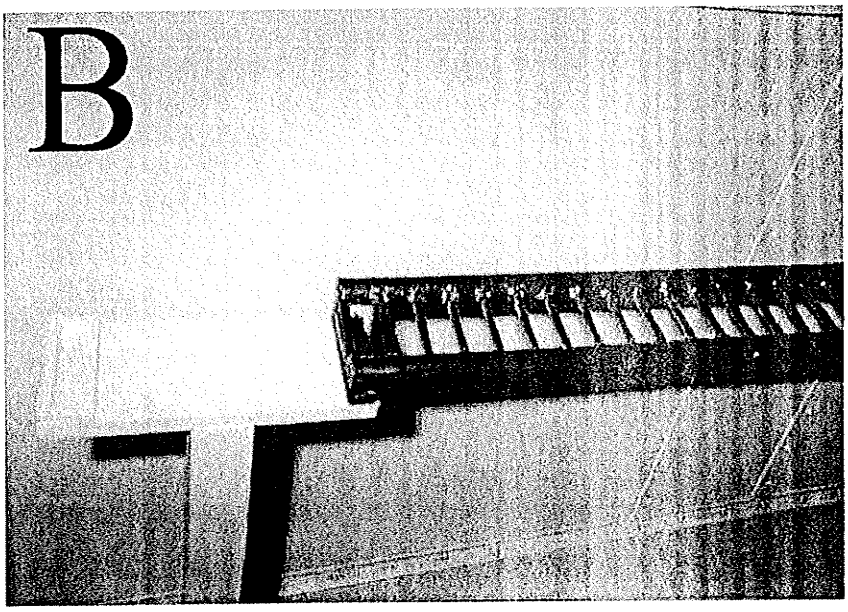
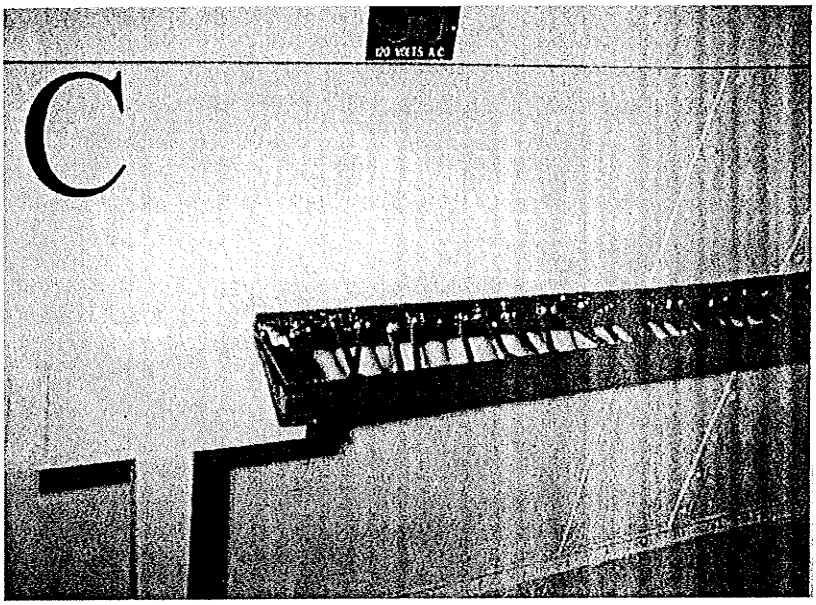


Figure 1c



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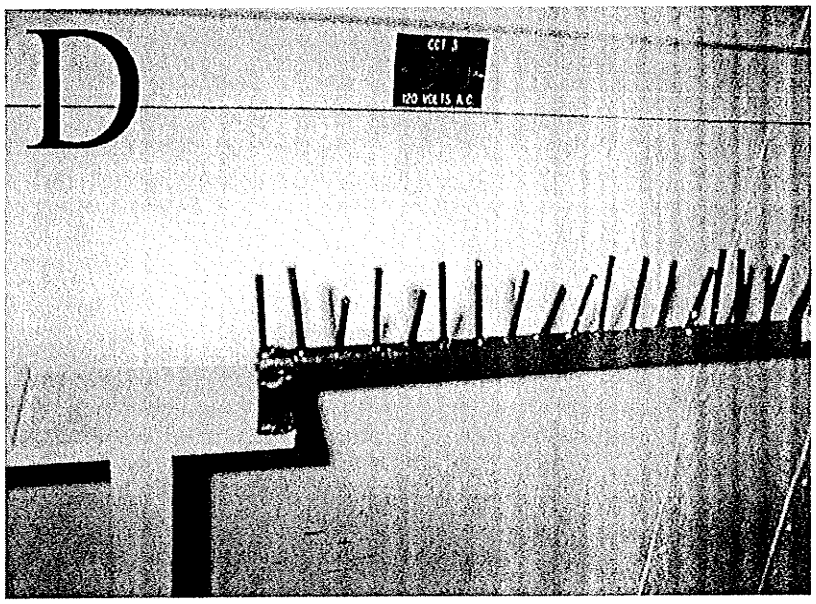

Figure 2

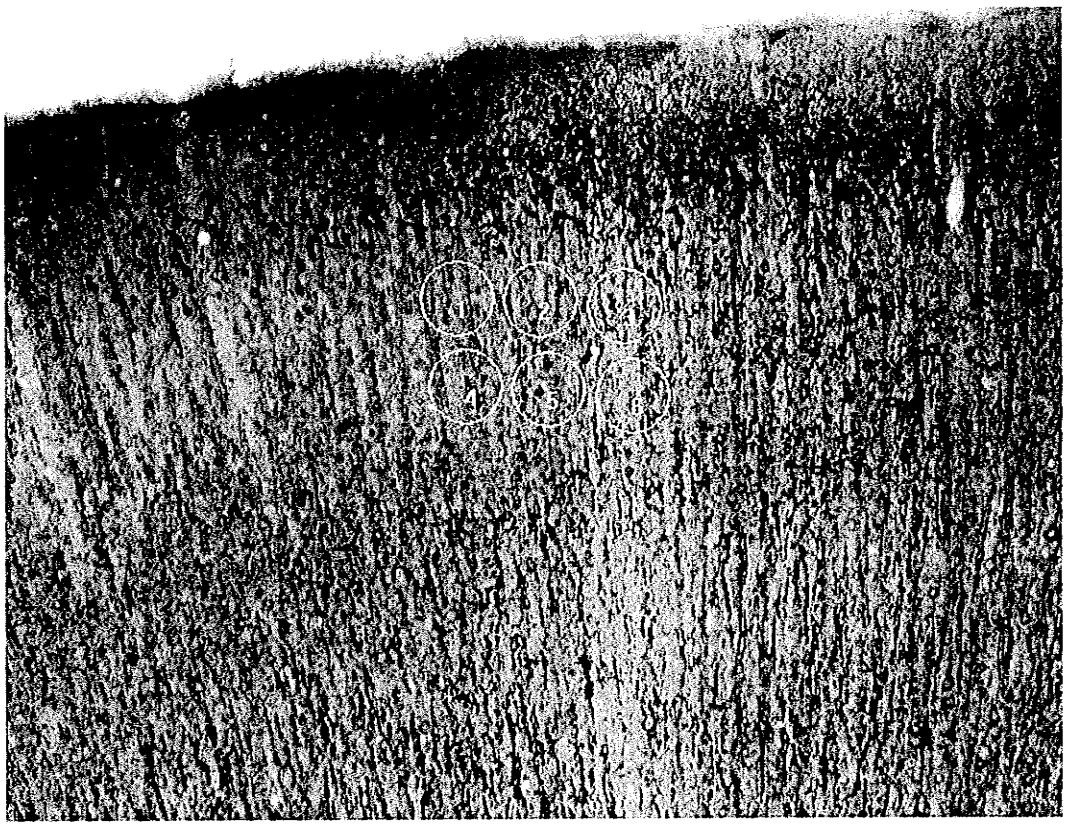

Figure 3

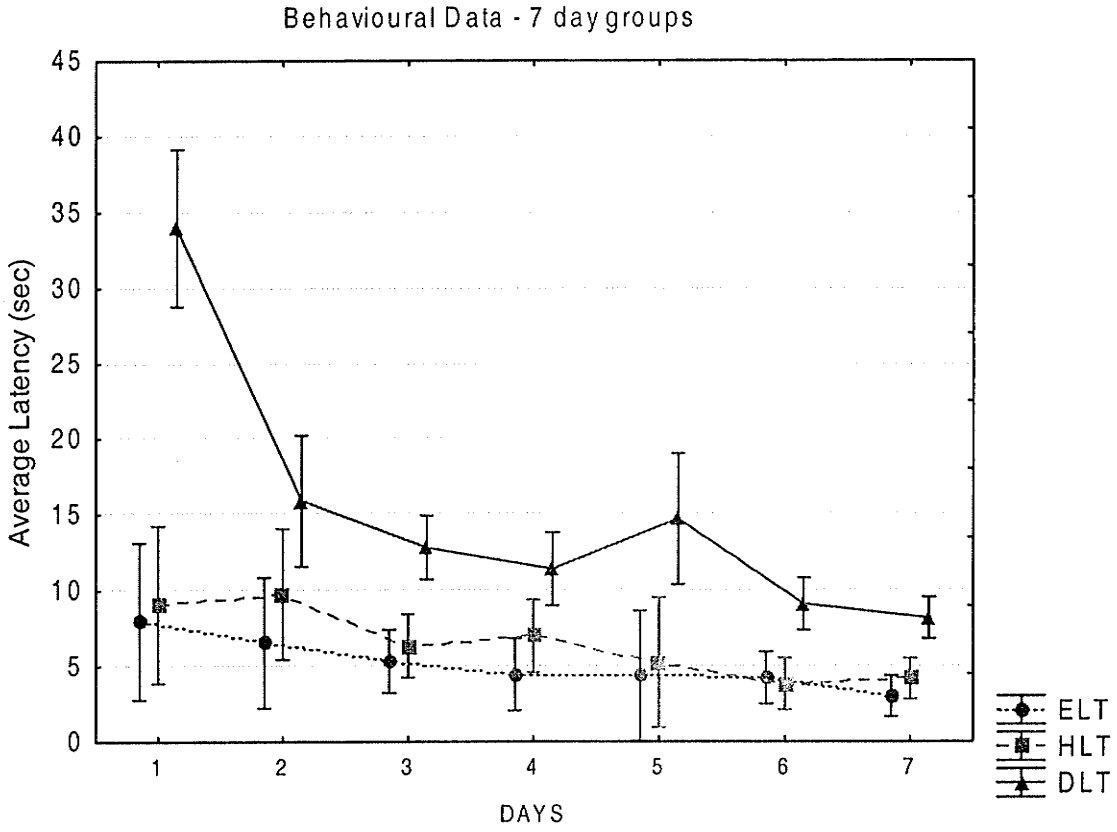
Behavioural Data - 7 day groups
Average Latency (sec)
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
DAYS
ELT
HLT
DLT

Figure 4a

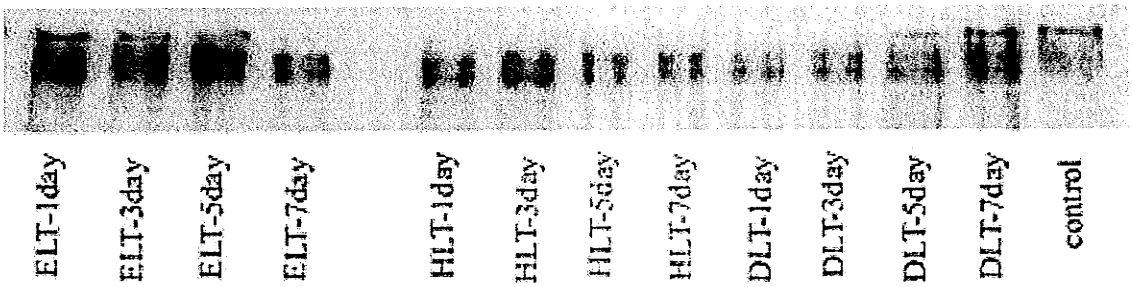

Figure 4b

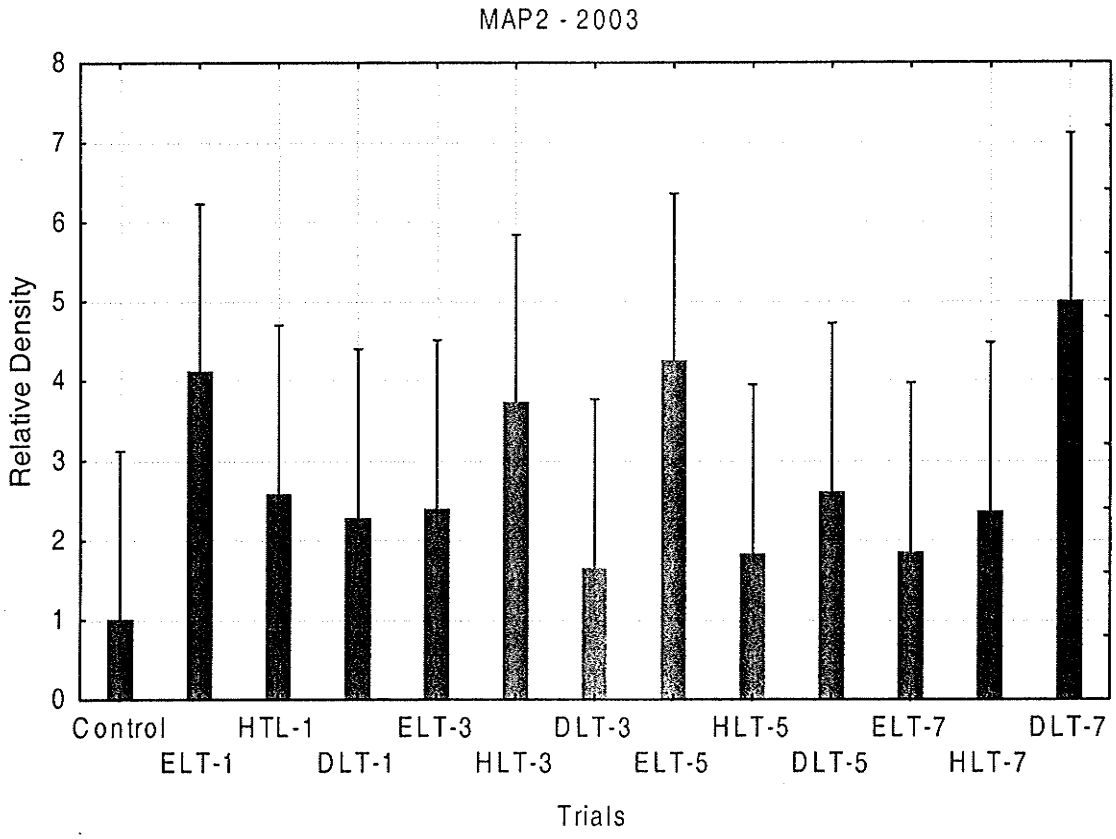

Figure 5a

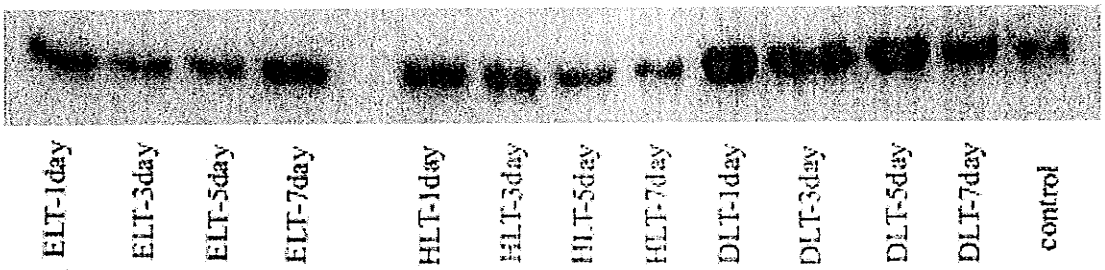

Figure 5b

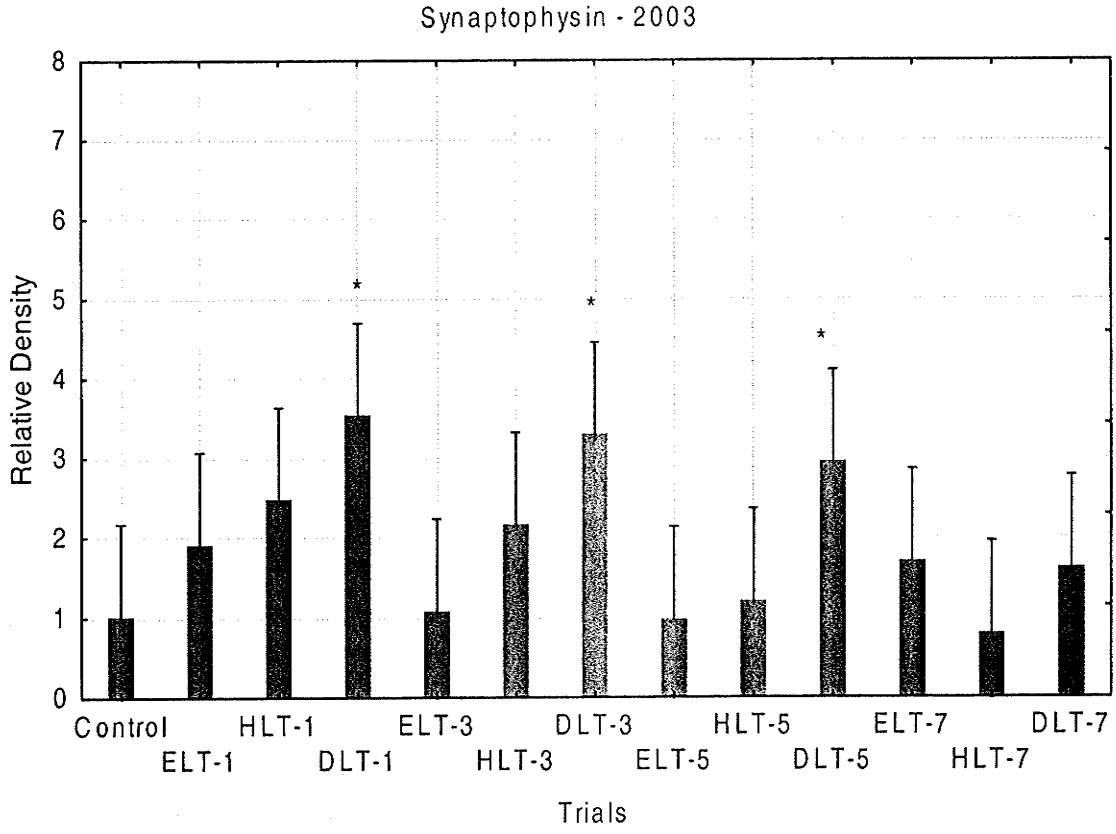

Figure 6

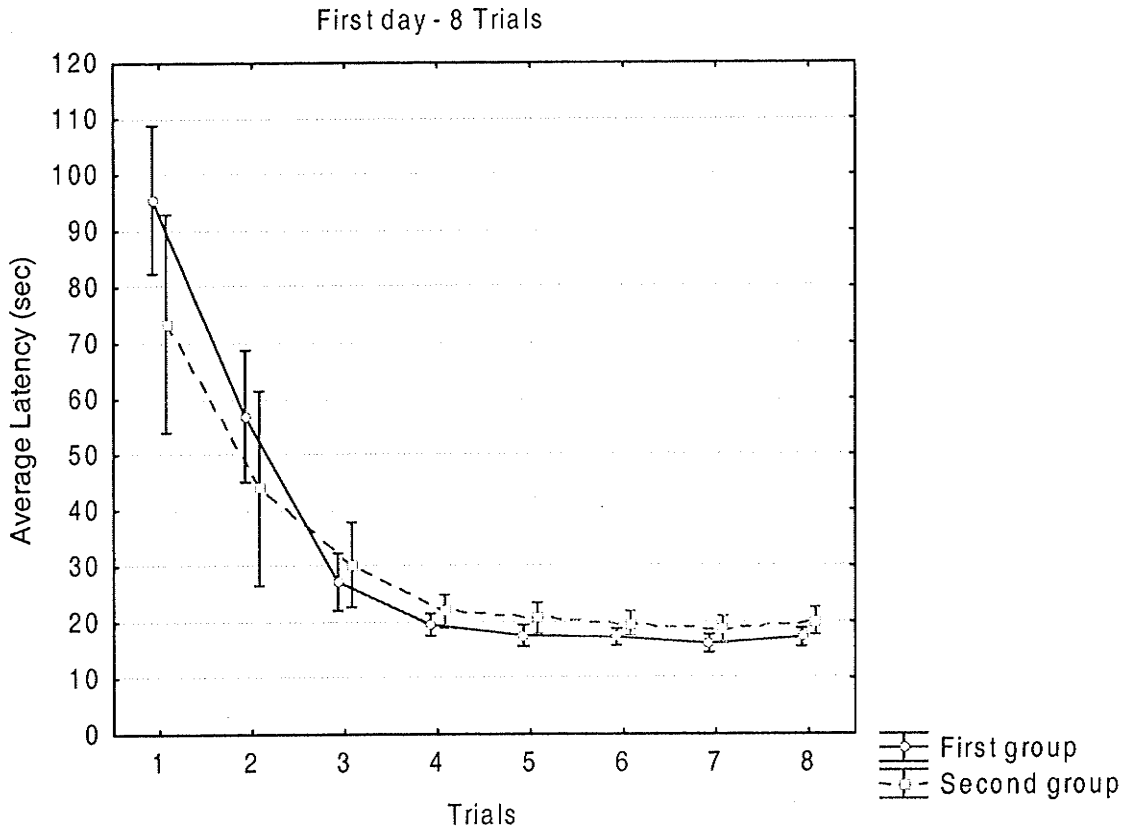
First day - 8 Trials
Average Latency (sec)
120
110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
Trials
First group
Second group

Figure 7a

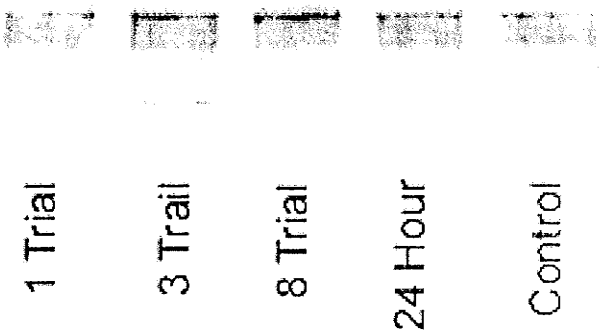

Figure 7b

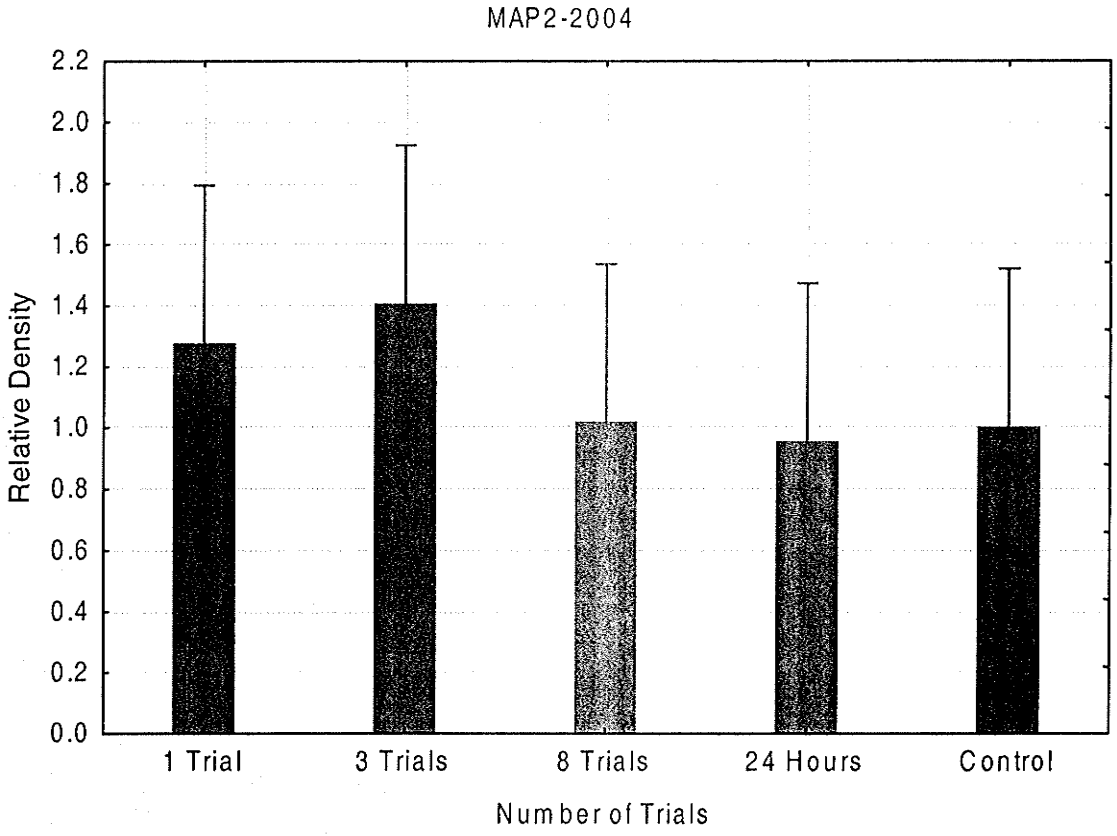

Figure 8a

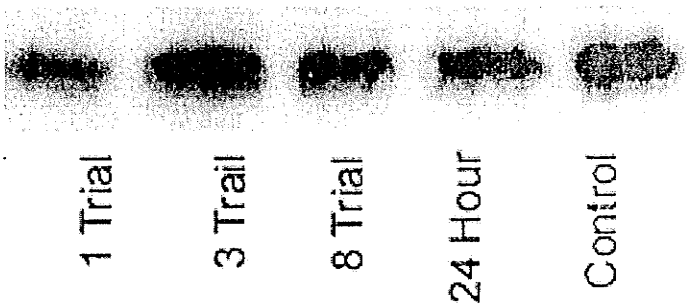

Figure 8b