NEUROTRANSMITTER SYSTEMS AND EEG RELATED TO ACUPUNCTURE

by

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This thesis was prepared under the direction of the candidate's dissertation advisor, Dr. Howard M. Prentice, Department of Biomedical Sciences, and has been approved by the members of his supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

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Acupuncture has been used for thousands of years to treat a wide range of diseases, but the mechanisms involved in the process have remained a mystery. The present study measures EEG responses to stimulation of a specific acupuncture point, GB37 (Guang Ming), with two different types of manual needle stimulation. Previous studies stimulated for a maximum of 2 minutes. The present study reflects the normal acupuncture treatment time of 20 minutes, with EEG recordings during and for 10 minutes prior to and after stimulation. Our results show no changes in the global spatial and temporal properties of EEG during and shortly after acupuncture treatment of acupoint GB37.

The second part of this study examines the global protein expression of glutamic acid decarboxylase (GAD) knockout mice. GAD is the rate-limiting enzyme in the synthesis of GABA, the major inhibitory neurotransmitter in the brain. The protein content of wild type, hetero-, and homozygous GAD knockout mice brains were

determined using a LC-MS-based gel-free shotgun profiling of complex protein mixtures. The data was analyzed using the Raculovic algorithm to determine the proteins differences. A short list of 32 proteins was determined with four that have been shown to be significant proteins that influence cell survival and excitotoxicity in the brain and have potential relationships with GABA. These proteins include V-ATPase, Glutamine synthetase, Beta-synuclein, and Micortuble associated protein (MAP). The proteomics results provide a preliminary best guess list of proteins influencing GAD and GABA production.

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GENERAL INTRODUCTION

Acupuncture/EEG

Acupuncture is among the oldest healing practices in the world. The term "acupuncture" describes a family of procedures involving the stimulation of anatomical points on the body using a variety of techniques. The acupuncture technique that has been most often studied scientifically involves penetrating the skin with thin, solid, metallic needles that are manipulated by the hands or by electrical stimulation.

Practiced in China and other Asian countries for thousands of years, acupuncture is one of the key components of traditional Chinese medicine (TCM). In TCM, the body is seen as a delicate balance of two opposing and inseparable forces: yin and yang. Yin represents cold, slow, or passive aspects of the person, while yang represents hot, excited, or active aspects. A major theory is that health is achieved through balancing yin and yang and disease is caused by an imbalance leading to a blockage in the flow of qi (vital force). This imbalance leads to blockage in the flow of qi along pathways known as meridians. Qi can be unblocked, according to TCM, by using acupuncture at certain points on the body that connect with these meridians. A general chart for acupuncture points and meridians is shown in Figure 1. As can be seen, the system of acupuncture meridians and points is a complex web-like maze that appears to form patterns similar to those created by lines of force around a magnetic source. Sources vary on the number of meridians, with numbers ranging from 14 to 20. Similarly, the number of acupuncture points is in the neighborhood of 2,000 points.

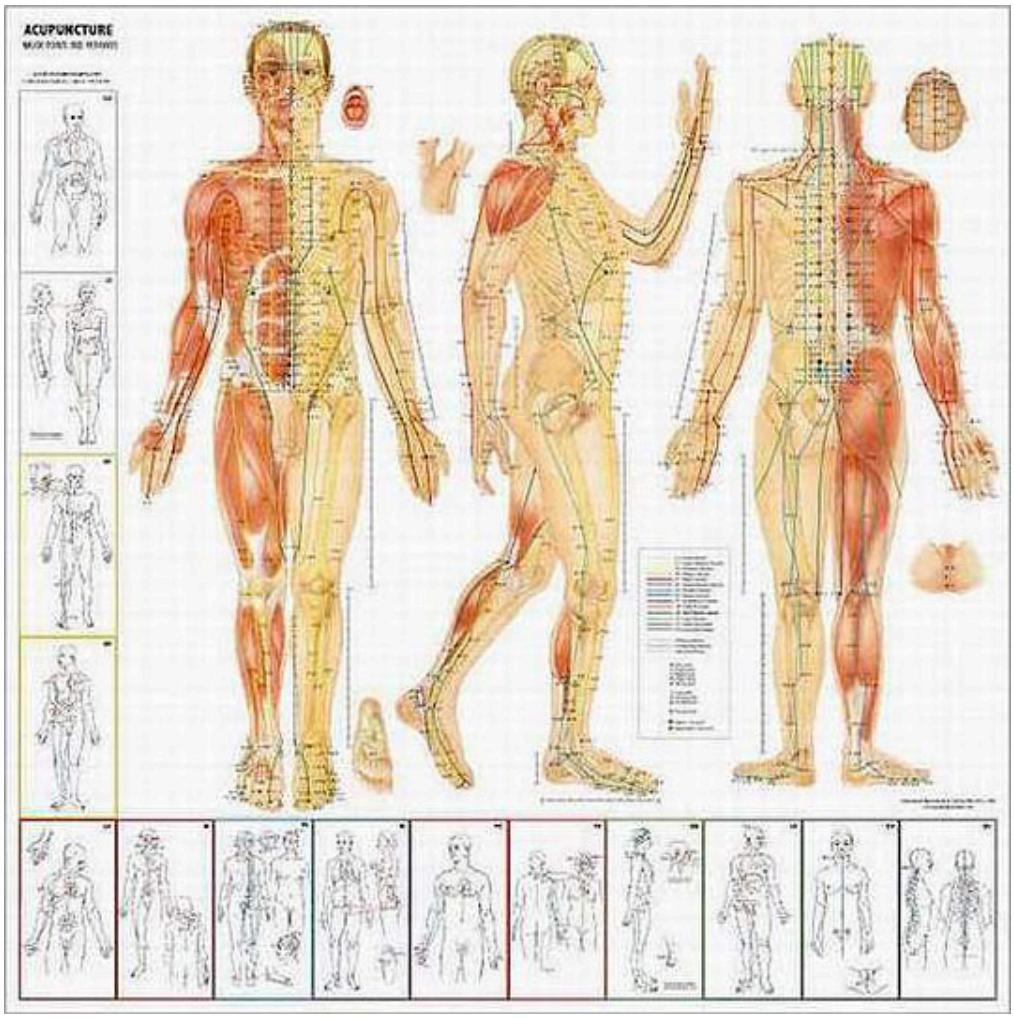


Figure 1. General Chart for Acupuncture Meridians and Points (www.Acupuncturecharts.com)

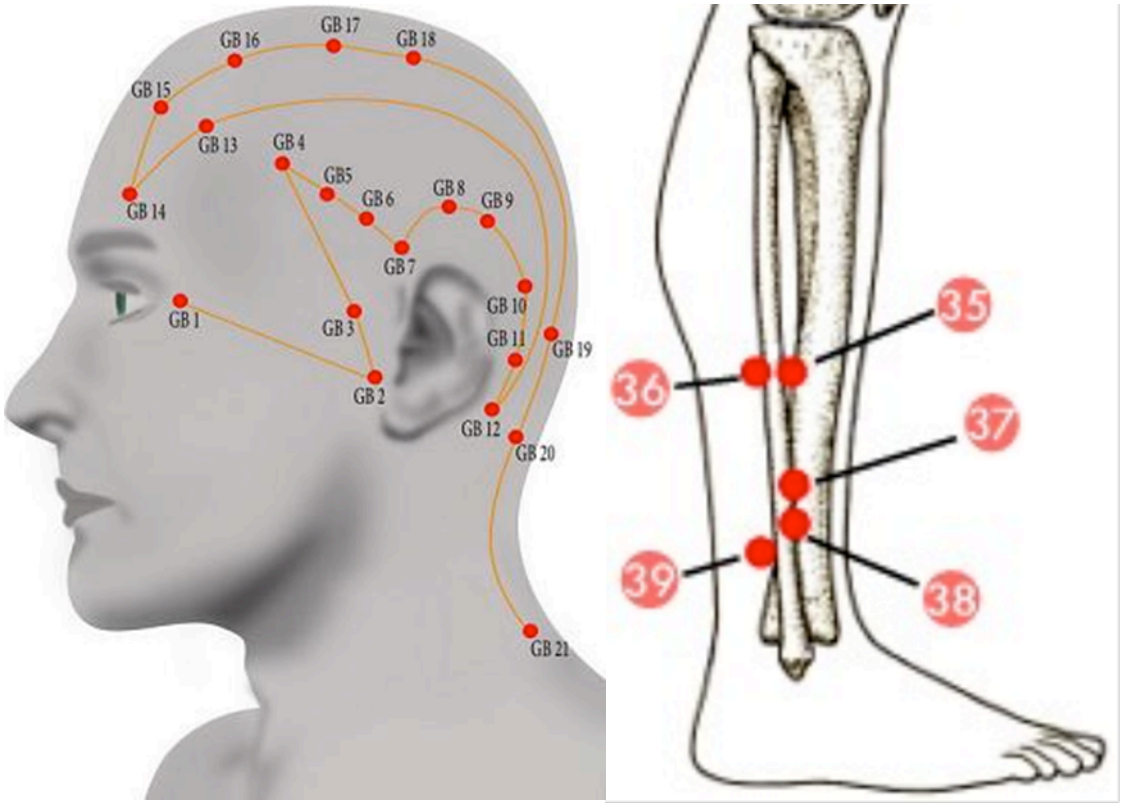
Acupuncture became better known in the United States in 1971, when New York Times reporter James Reston wrote about how doctors in China used needles to ease his pain after surgery. American practices of acupuncture incorporate medical traditions from China, Japan, Korea, and other countries.

According to the 2007 National Health Interview Survey (nnccam, 2011), which included a comprehensive survey of complementary and alternative medicine (CAM)

used by Americans, 1.4 percent of respondents (an estimated 3.1 million Americans) said they had used acupuncture in the past year. A special analysis of acupuncture data from an earlier NHIS found that pain or musculoskeletal complaints accounted for 7 of the top 10 conditions for which people use acupuncture. Back pain was the most common, followed by joint pain, neck pain, severe headache/migraine, and recurring pain.

Acupuncture is being studied for its efficacy in alleviating many kinds of pain. “Positive” findings (evidence that a therapy may work) and "negative" findings (evidence that it probably does not work or that it may be unsafe) have been listed by the National Center for Complementary and Alternative Medicine (NCCAM) for several conditions. A short list of their findings are as follows: carpal tunnel syndrome (positive), fibromyalgia (mixed), headache/migraine (conflicting), low back pain (positive), menstrual cramps (positive), myofascial pain (positive), neck pain (positive), osteoarthritis/knee pain (positive), postoperative dental pain (positive), and tennis elbow (positive). The NCCAM has funded a wide range of acupuncture research projects to advance the scientific understanding of acupuncture and has specifically targeted what happens in the brain during acupuncture treatment along with ways to better identify and understand the potential neurological properties of meridians and acupuncture points.

The acupuncture/EEG study done here at Florida Atlantic University involved the gall bladder (GB) acupuncture meridian. This meridian has 44 points and runs from the lateral canthus of the eyes to the 4th toe, bilaterally. The parts of the meridian involved directly in this study are shown in Figure 2.



(a) (b)

Figure 2. Location of Points on Gall Bladder Meridian on Head (a) and Leg (b). (a) www.all-about-acupuncture.com/acupuncture-free-charts-all-meridian-points.html. (b) www.icanm.com/pages/samples/ch8GallMer.asp.

In traditional acupuncture, the GB meridian is associated with the gall bladder and eyes/vision. As can be seen in Figure 2, the course of the GB meridian begins at the corner of the eye, courses along the lateral side of the skull to the occiput, anteriorly to just above the eye, and back to the occiput before moving to the rest of the body. The path of this meridian courses over the skull and underlying cortical structures. Of all the regular acupuncture meridians, the GB meridian has more points and meridian pathways over the course of the skull and brain than any other meridian. It probably

offers the best opportunity to study the local activity of the brain and the adjacent acupuncture system. Ancient knowledge suggests that the acupuncture systems lies just beneath the skin and EEG measures electrical activity just at the skin surface of the skull, providing a unique construct to study acupuncture and underlying cortical activity. GB 37 was chosen to test because of its ancient reputation for influencing visual conditions. By measuring the EEG activity in the cranial GB system with the stimulation of GB 37, it was hoped that changes in EEG would be reflected in areas known to be involved in visual cortical activity. What we were looking for were changes in the EEG related to the visual cortices (primary, secondary, and/or tertiary) upon the stimulation of the acupuncture point GB 37.

An electroencephalogram, EEG, is a test that examines the electrical activity of the brain. Electrodes are glued to the scalp and electrical measurements are made from different positions reflecting the activity of the cortex and underlying structures. EEGs are most commonly used to identify epilepsies and neurological disorders that involve gross synaptic firing. They are currently used in the field of neuroscience to explore patterns and timing for various neurological events in the brain and generally indicate functional changes in the brain. Factors that create such EEG changes include individual differences, changes in mental states, age, cognitive stimuli and mental activities, physiological changes in the body, and pathological processes in the brain. EEG constitutes an inexpensive, objective, continuous, noninvasive and simple method of evaluating cerebral activities. The objective of the present study is to use this modality to explore the relationship between acupuncture of a particular point and the effect it has on cortical activity.

There have been very few human EEG studies during acupuncture. Changes in EEG were recorded in two of three normal volunteer subjects by Saito et al. (1983). EEG recordings were made during acupuncture-induced analgesia for removal of a thyroid tumor by Starr et al. (1989). EEG measures were unchanged during the operation. The mode of action producing the analgesia was not revealed in this study. Liao et al. (1992) reported changes in EEG with acupuncture stimulation. Unfortunately, these early reports are limited and contradictory, being derived from different protocols. These difficulties and problems associated with this type of study are due to the variable nature of the outcomes of both acupuncture and EEG. The variety in outcomes with both procedures makes reproducibility and comparison very difficult. Also, the magnitude of change brought about by acupuncture may be small and the conventional paper recordings of EEG of that time may have been insufficient to record a difference or change.

Digital EEG recording and brain mapping techniques allow for more quantitative data analysis and may provide better resolution and accuracy. Rosted et al. (2001) provided the first digital EEG study of LI4 (Hegu). In their study, the electrodes were placed on the scalp in a modified 10/20 system using a center line of electrodes from Fpz to Oz and linked to the mastoids as the reference. Baseline eyes closed EEG data were recorded in two "events" each of 3 min duration prior to all acupuncture treatments. Acupuncture stimulation was performed by manual manipulation for event durations of 30 sec, 1 min and 2 min. Following each acupuncture stimulation, subjects rested with their eyes closed for 3 min before opening. The frequency analysis of the EEG data from each acupuncture event was compared to the baseline data to show any

significant changes over the bandwidth 0.3 to 30 Hz. Only changes greater than 2 standard deviations were considered significant. The results were that in 10 subjects the frequency spectra remained unchanged during acupuncture, but in 3 significant increases were recorded in the amplitude of very low frequencies between 0.5 and 2 Hz and in one subject there was an increase in the amplitude of the alpha band during acupuncture. The changes in the delta band of individuals during acupuncture were large but highly variable. They arose at a frequency that is on the limit of the recording equipment and where recording and physiological artifacts were known to occur. The one case where there was an increase in the alpha band is attributed to the suppression of the dominant rhythm brought about by anxiety concerning the procedure which subsequently disappeared during acupuncture as the subject relaxed. Their conclusion was that there are no changes brought about by acupuncture in resting EEG in the frequency range 2-30 Hz and no evidence to attribute changes below 2 Hz to a direct effect of acupuncture. A more recent EEG study using transcutaneous electrical stimulation of LI4 revealed significant changes in theta power during high- and low-frequency stimulation (Chen et al., 2006). The significance of the Rosted et al. (2001) study was that it was the first reported human study using acupuncture and EEG. Unfortunately, the longest time tested in this study was two minutes, whereas, the normal acupuncture treatment time is twenty to thirty minutes, so the results did not reflect a normal treatment period. The present study sought to remedy this deficiency.

Modern EA (acupuncture in which the needles are stimulated electrically) has been shown to be more effective than traditional manual acupuncture for anti-nociception in rats (Wang et al., 1992). EA is now more commonly used than manual

acupuncture in modern studies because of its convenience and high repeatability of stimulus control. The physiological and psychological effects of acupuncture often depend upon stimulus parameters such as site, intensity, mode, and length of stimulation. Of these parameters, stimulus frequency has been proven to be the most important to impact brain activities (Zhang et al., 2003; Napadow et al., 2004). Different acupuncture point stimulations and frequencies can induce different neurochemical effects. Acupuncture can be used as an anesthetic or for analgesia. Stimulation at a frequency of 15-30 Hz was more effective than a lower frequency of 2-3 Hz in triggering peptide release (Racke et al., 1989). Burst stimulation was more effective than constant frequency stimulation on cortical excitation (Cazalis et al., 1985). Both high-frequency and low-frequency stimulations can reduce or increase cortical excitation to induce analgesia, but there were differential effects of low-and high-frequency acupuncture on the types of endorphins released (Shen, 2001). Low-frequency (2 Hz) and high-frequency (100 Hz) EA selectively induced the release of enkephalins and dynorphins (an opiod peptide) in both animals and humans (Ulett et al., 1998). Willer et al. (1982) demonstrated that in human subjects, low-frequency (2Hz) high intensity stimulation could induce a partial naloxone reversible acupuncture effect compared with high-frequency (100 Hz) low-intensity stimulation of the nociceptive R-II component of the blink reflex. Lin et al. (2002) showed that patients required less morphine post-surgically in 24 h in a high-frequency group than a low-frequency group and sham group and vomiting and nausea were lower in the acupuncture group than in sham and control group. Zhang et al. (2003), in a report of EA-induced analgesia using behavioral withdrawal index and fMRI, a positive correlation of analgesic effects was

observed in the contralateral motor area, the supplementary motor area, and the ipsilateral superior temporal gyrus for low-frequency 2 Hz stimulation compared with the contralateral inferior parietal lobe, ipsilateral anterior cingulate cortex, nucleus accumbens, and pons for high-frequency 100 Hz stimulation. The determination that EA is more effective than manual stimulation for research purposes is a significant finding. The observation that different stimulation frequencies stimulate different parts of the brain creating different effects is equally significant. The present study used manual acupuncture stimulation because traditional acupuncture used only manual stimulation and it was felt that it was the best starting point for study.

The Design of Dissertation Project

The acupuncture points most studied were those with general functions and effects. The present study sought to examine an acupuncture point with a more specific response. It was hoped that this narrow scope of outcomes would be reflected in a more focused outcome in the EEG. In traditional acupuncture, there is an acupuncture point, GB 37 (Guang ming), which translates as "bright light", used to treat vision-related disorders such as cataract, night blindness, and optic atrophy (Liu et al., 1997). The effect of acupuncture stimulation of GB37 on the visual cortex has been studied with fMRI by Cho et al. (1998, 1999) and Gareus et al., (2002) with contradictory results. It had not been studied with EEG. Here, we investigate whether effects of manual needle stimulation of acupoint GB 37, as recorded with multi-channel EEG in normal subjects, can be found in the global properties of neural activation, with special attention to the visual cortex. We also tested two traditional manual needle techniques to see if there were differences in neural responses as measured by EEG.

Acupuncture and Neurotransmitters

The studies mentioned previously represent a partial description of studies showing that acupuncture has an effect on EEG. Since EEG is generated by nerve impulses, a logical question to ask is "What neurotransmitters are involved in acupuncture and what is their function?" Many studies are now focused on establishing measurable physiological changes in response to acupuncture and attempting to explain the processes responsible through neurological pathways and mechanisms.

Acupuncture has been shown to have many effects on the central nervous system (CNS) and neurotransmitters. There have been several studies dealing with the CNS and autonomic nervous system control of the cardiovascular system. Carpenter et al. (2010) found that acupuncture restored sympathovagal balance lowering blood pressure in human subjects. Nahas (2008) also found that acupuncture reduced hypertension in human subjects. Tjen-A-Looi SC et al. (2007) found that EA caused prolonged suppression of reflex elevations in blood pressure for 1–2 h in anesthetized preparations in cats. They suggested that a long-loop pathway involving the arcuate nucleus, ventrolateral periaqueductal gray, and rostral ventrolateral medulla was involved in sympathoinhibitory cardiovascular EA effects. The arcuate nucleus, an important component in the long-loop pathway in the EA cardiovascular response, is required for prolonged suppression of reflex cardiovascular excitatory responses by EA. Furthermore, in the rostral ventrolateral medulla, opioids and GABA, but not nociceptin, participate in the long-term EA-related inhibition of sympathoexcitatory cardiovascular responses.

Neurological pathways and neurotransmitter, including GABA, have been suggested to play a role in the balancing effect on blood pressure produced by acupuncture.

In a related study, Fu and Longhurst (2009) tested the hypothesis that EA modulates the release of these neurotransmitters in the ventrolateral periaqueductal gray through a presynaptic CB (1) receptor mechanism. They measured the release of GABA and glutamate simultaneously by using high-performance liquid chromatography to assess samples collected with microdialysis probes inserted unilaterally into the ventrolateral periaqueductal gray of intact anesthetized rats. Twenty-eight min of EA (2 Hz, 2-4 mA, 0.5 ms) at the PC5-6 acupoints reduced the release of GABA by 39% during EA and by 44% 15 min after EA. Thirty-five minutes after EA, GABA concentrations returned to pre-EA levels. In contrast, sham EA did not change the ventrolateral periaqueductal gray GABA concentration. Blockade of CB (1) receptors with AM251, a selective CB (1) receptor antagonist, reversed the EA-modulated changes in GABA concentration, whereas microinjection of vehicle into the ventrolateral periaqueductal gray did not alter EA-modulated GABA changes. In addition, they observed no changes in the ventrolateral periaqueductal gray glutamate concentrations during EA, although the baseline concentration of glutamate was much higher than that of GABA (3,541 ± 373 vs. 33.8 ± 8.7 nM, glutamate vs. GABA). They indicated that their results suggest that EA modulates the sympathoexcitatory reflex responses by decreasing the release of GABA, but not glutamate, in the ventrolateral periaqueductal gray, most likely through a presynaptic CB (1) receptor mechanism.

In a follow-up study, Tjen-A-Looi et al. (2009) studied the roles of endocanabinoids and GABA in the processing of cardiovascular information in the ventrolateral periaqueductal gray during EA. The arcuate nucleus provides excitatory input to the ventrolateral periaqueductal gray, which, in turn, inhibits neuronal activity in the rostral ventrolateral medulla. Previous studies have shown that endocannabinoid CB (1) receptor activation modulates gamma-aminobutyric acid (GABA)-ergic and glutamatergic neurotransmission in the dorsolateral periaqueductal grey in stress-induced analgesia. Their results showed that, in the rostral ventrolateral medulla, the EA-related inhibition from 18 ± 3 to 8 ± 2 mmHg was reversed to 14 ± 2 mmHg by microinjection of the CB (1) receptor antagonist AM251 (2 nmol, 50 nl) into the ventrolateral periaqueductal gray. Pretreatment with gabazine eliminated reversal following CB (1)-receptor blockade. The EA releases endocannabinoids and activates presynaptic CB (1) receptors to inhibit GABA release in the ventrolateral periaqueductal gray. Reduction of GABA release disinhibits ventrolateral periaqueductal gray cells, which, in turn, modulate the activity of rostral ventrolateral medulla neurons to attenuate the sympathoexcitatory reflex responses.

These three studies show that acupuncture-induced endocannabinoid CB (1) receptor activation modulates GABAergic neurotransmission that inhibits sympathoexcitatory cardiovascular responses, modulating blood pressure.

Acupuncture and Disease

Acupuncture has been used as a treatment modality for several pathological conditions and diseases. By examining the responses of patients with these conditions to

acupuncture, more clues as to the nature of the disease/disorder and/or the nature of acupuncture may be elucidated. Several such studies have provided new insights.

Of particular interest are those studies dealing with acupuncture and Parkinson's disease. Huang et al. (2009) used positron emission tomography (PET) and the 18-flourodeoxyglucose (an analogue of glucose that is labeled with fluorine-18) tracer to study cerebral effects of complementary acupuncture in Parkinson's disease. Five patients received scalp-acupuncture and Madopa (a Parkinson's disease drug), while the other five had Madopa only. PET scans before and after 5 weeks of complementary acupuncture treatment showed increased glucose metabolisms in parietal, temporal, occipital lobes, the thalamus, and the cerebellum in the slightly-diseased hemisphere, and in parietal and occipital lobes of the severely-diseased hemisphere. No changes were observed in the Madopa-only group. Their conclusion was that acupuncture in combination with Madopa may improve cerebral glucose metabolism in Parkinson's disease. This study is important because it suggests that acupuncture has an effect on glucose metabolism in various parts of the brain.

Jia et al. (2009) tested the use of EA to alleviate motor symptoms in rat models of Parkinson's disease. The effects of EA stimulation were investigated in a rat hemiparkinsonian model induced by unilateral transection of the medial forebrain bundle (MFB). EA stimulation at a high frequency (100 Hz) significantly reduced apomorphine-induced rotational behavior. Tyrosine hydroxylase immunohistochemistry revealed that EA at 100 Hz protected axotomized dopaminergic neurons from degeneration in the substantia nigra (SN). Moreover, high frequency EA reversed the axotomy-induced decrease in substance P content and increased the glutamate

decarboxylase-67 (GAD 67) mRNA levels in the midbrain; however, it did not affect the axotomy-induced increase in enkephalin content in the globus pallidus. Their results suggested that the effects of high frequency EA on motor symptoms of Parkinsonian rats may involve restoration of the homeostasis of dopaminergic transmission in the basal ganglia circuit. A similar study by Jia et al. (2010) found that EA stimulation at a low frequency (2 Hz) had no effect, however, EA stimulation at a high frequency (100 Hz) significantly improved motor coordination. Neither low nor high EA stimulation significantly enhanced dopamine levels in the striatum. EA stimulation at 100 Hz normalized the MFB lesion-induced increase in midbrain GABA content, but it had no effect on GABA content in the globus pallidus. This study is important because the results suggest that high-frequency EA stimulation improves motor impairment in MFB-lesioned rats by increasing GABAergic inhibition in the output structure of the basal ganglia.

Yoon et al. (2004) investigated the effect of acupuncture on acute ethanol-induced dopamine release in the nucleus accumbens and the potential role of the GABA, specifically the B receptor system, in acupuncture. Male Sprague-Dawley rats were administered with the highly selective GABA (B) antagonist SCH 50911 (3 mg/kg, i.p.) 1h prior to an intraperitoneal injection of ethanol (1 g/kg). Immediately after ethanol treatment, acupuncture was given at bilateral HT7 (Shenmen) points for 1min. Acupuncture at the specific acupoint HT7, but not at control points (PC6 or tail) significantly decreased the dopamine release in the nucleus accumbens. Inhibition of dopamine release by acupuncture was completely prevented by SCH 50911. They indicated that their results suggest that stimulation of specific acupoints inhibits

ethanol-induced dopamine release by modulating GABA (B) activity and implied that acupuncture may be effective in blocking the reinforcing effects of ethanol. This study is important because it relates acupuncture to GABA (B) activity and a subsequent influence on addictive behavior.

Yoon et al. (2010) investigated the effect of acupuncture on morphine self-administration to determine the potential roles of GABA receptors in the efficacy of acupuncture. Male Sprague-Dawley rats were trained to self-administer morphine at 0.1 mg/kg per infusion during daily one hour sessions. Following the stable response of self-administration of morphine, acupuncture was applied to HT7 points bilaterally (1 min) prior to the testing session. Other groups of rats were given the GABA (B) receptor antagonist SCH 50911 (3.0 mg/kg, i.p.), the GABA (A) receptor antagonist bicuculline (1.0 mg/kg, i.p.) or saline 30 min prior to acupuncture treatment. They found that acupuncture at the acupoint HT7, but not at the control point LI5 (Yangxi), significantly decreased morphine self-administration. Moreover, either SCH 50911 or bicuculline blocked the inhibitory effects of acupuncture on morphine self-administration. Their results are important because they suggest that acupuncture specifically at the HT7 point regulates the reinforcing effects of morphine via regulation of GABA receptors.

Park et al. (2010) performed a study to determine whether spinal GABAergic systems mediate the relieving effects of low frequency EA on cold allodynia in a rat tail model of neuropathic pain. For neuropathic surgery, the right superior caudal trunk was resected at the level between the S1 and S2 spinal nerves innervating the tail. Two weeks after the nerve injury, the intrathecal catheter was implanted. Five days after the

catheterization, rats were intrathecally injected with gabazine (GABA (A) receptor antagonist, 0.0003, 0.001 or 0.003 mμg), or saclofen (GABA (B) receptor antagonist, 3, 10 or 30 mμg). Ten minutes after the injection, EA (2Hz) was applied to the ST36 acupoint for 30 min. Cold and warm allodynia was assessed by the tail immersion test (i.e. immersing the tail in cold (4º C) water and measuring the latency of an abrupt tail movement) before and after the EA treatment. EA stimulation at ST36 significantly inhibited the cold allodynia sign, whereas EA at non-acupoint and plain acupuncture at ST36 (without electrical stimulation) did not show antiallodynic effects. Intrathecal administration of gabazine or saclofen blocked the relieving effects of ST36 EA stimulation on cold allodynia. Their results are important because they suggested that spinal GABA (A) and GABA (B) receptors mediate the suppressive effect of low frequency EA on cold allodynia in the tail of neuropathic rats.

Guo et al. (2008) investigated the effect of EA on spontaneous recurrent seizure (SRS) and expression of GAD (67) mRNA in the dentate gyrus (DG) in epileptic rats. EA at bilateral acupoints of ST36 was administered. Two sham EA controls were set: sham EA at bilateral nearby nonacupoints in the hamstring muscles, and sham EA at bilateral ST36 without electrical stimulation. Lithium-pilocarpine injection was performed to establish the rat model of epilepsy on day one. Three time points were set according to the day when the rats were killed (30th, 45th, 60th day). The results showed that EA at ST36 significantly reduced the times of spontaneous recurrent seizure, neither of the two sham EA controls displayed significant effect on spontaneous recurrent seizure. Moreover, EA at ST36 significantly elevated the expression of GAD (67) mRNA in DG granule cell layer (GCL), but not in the hilus; neither of the two

sham controls showed significant effect on the expression of GAD (67) mRNA in the granule cell layer or hilus. Their findings suggest that EA at ST36 possess some curative effect on epileptic rats, related with change of GAD (67) mRNA levels in the DG region. This is the first study to relate acupuncture to GAD (67) mRNA levels.

The first study to relate proteomics and acupuncture is Jeon et al. (2008) where they used proteomics analysis to investigate whether acupuncture alters protein expression in the substantia nigra (SN) as a possible therapy for neuronal degeneration. They stated that acupuncture is frequently used as an alternative therapy for Parkinson's disease (PD), and it attenuates dopaminergic (DA) neurodegeneration in the SN in PD animal models. C57BL/6 mice were treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 30 mg/kg/day), intraperitoneal (i.p.) for 5 days. EA [2 or 100 Hz] was applied at the effective and specific acupoint, GB34 (Yanglingquan), once a day for 12 consecutive days after the first MPTP treatment. Both treatments in MPTP mice led to restoration of behavioral impairment and rescued tyrosine hydroxylase (TH) - positive DA neurodegeneration. Using peptide fingerprinting MS, changes in 22 proteins were identified in the SN following MPTP treatment, and nine of these proteins were normalized by EA. These proteins were involved in cell death regulation, inflammation, or restoration from damage. The levels of cyclophilin A (CypA), which is a neuroprotective agent, were unchanged by MPTP treatment but were increased in MPTP-EA mice. Jeon et al. (2008) stated that their results suggest that acupoint GB34-specific EA changes protein expression profiles in the SN in favor of DA neuronal survival in MPTP-treated mice, and that EA treatment may be an effective therapy for PD patients.

THE ROLE OF GABA AND GAD IN THE CENTRAL NERVOUS SYSTEM

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the mammalian central nervous system. It regulates and modulates the excitatory processes that drive the central nervous system. Disturbances in GABA neurotransmission have been linked to many neurological diseases: Epilepsy (Freichel et al., 2006; Lloyd et al., 1986), Huntington's Disease (Gourfinkel et al., 2003; Spokes, 1980), Parkinson's Disease (de Jong, 1984), General Anxiety Disorder (Kosel, 2004), implicated as an autoantigen in Insulin-Dependent Diabetes Mellitus (IDDM) (Tian, 1999), Stiff-Person Syndrome (Burbelo et al.,2008; Butler, 1993), and Batten Disease (Chattopadhyay et al., 2002). Many of these diseases are the result of excitotoxicity caused by excessive release of glutamate, the major excitatory neurotransmitter. The conversion of glutamate to GABA may hold the key in the quest for healing and ameliorating the symptoms of GABA-related diseases and disorders in the sense that an ability to up-regulate GABA production may replete GABA deficiencies that result in disease.

GAD, L-glutamic acid decarboxylase (EC 4.1.1.15), is the rate-limiting enzyme in controlling GABA synthesis. GAD gene therapy and GAD vaccines are reported to reverse the physical expression of Parkinson's disease (Dass et al., 2006; Luo et al., 2002), IDDM (Balasa et al., 2001; Li and Escher, 2003), and spinal cord injury (Liu et al., 2008). Research into the factors involved in the production and regulation of GAD

are potentially important in the down-regulation of excitotoxic events in that enhanced GABA production may inhibit target neurons that otherwise would have been activated by the glutamate system.

Mechanisms of GAD Functions in Neurons

As mentioned above, GAD is the rate-limiting enzyme for GABA synthesis. GABA is the major inhibitory neurotransmitter in the central nervous system and serves as the primary inhibitory neurotransmitter at 20- 44% of cortical neurons (DeFelipe 1993; Ribak and Yan, 2000). In the process of excitotoxicity, GAD may have a key role in the control of GABA production and the prevention and treatment of neurodegenerative diseases. A study providing a global account of the proteins involved in GAD production may provide new clues to the signaling pathways that regulate the production of GABA and the development of neurodegenerative diseases.

GABA is synthesized by the α-decarboxylation of glutamate, catalyzed by GAD (Petroff, 2002). It has been demonstrated that biosynthesis of neurotransmitter GABA from glutamine largely proceeds through the Krebs cycle (Waagepetersen et al., 2001) in the GABA shunt (Figure 3).

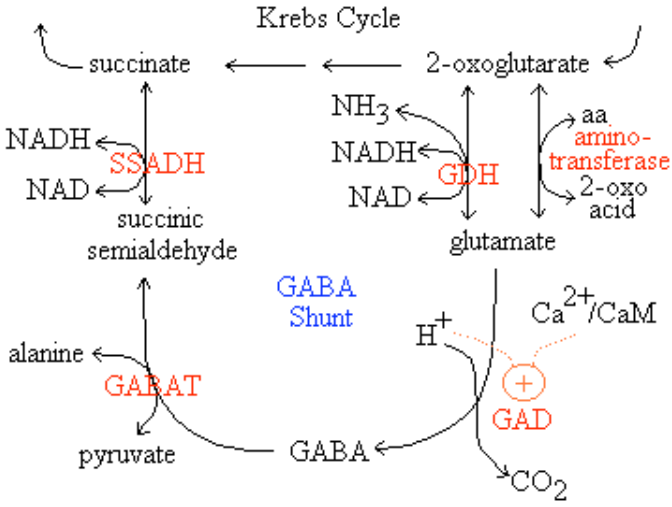


Figure 3. GABA Shunt (Rhodes, 2009).

GAD has two isoforms, GAD 65 and GAD 67, based upon their respective molecular weights of 65 and 67 kDa. GAD 65 and GAD 67 are encoded by different genes located in humans on chromosomes 2 and 10 (Erlander and Tobin 1991; Bu et al., 1992) and have different subcellular localizations, functions, regulatory properties and co-factor interactions (Kaufman et al., 1991). GAD 67 is thought to be found throughout the cytoplasm being utilized for purposes other than neurotransmission such as functioning as a trophic factor for synaptogenesis during early development, protection after neuronal injury, source of energy via the GABA shunt and regulator of redox potential during oxidative stress (Lamigeon et al., 2001; Pinal and Tobin, 1998; Waagepetersen et al., 1999). GAD 65 is concentrated in nerve terminals where it synthesizes GABA for the purpose of neurotransmission (Martin and Rimvall, 1993). Structural studies of GAD 65 and GAD 67 indicate that GAD 67 is constitutively active and responsible for the basal GABA production while GAD 65 is transiently activated

in response to the extra demand of GABA in neurotransmission (Fenalti et al., 2007). Studies of transgenic mice have shown that genetic knockout of GAD 67 is lethal because of cleft palate (Asada et al., 1997), whereas, GAD 65 knockout mice could survive but are more susceptible to seizures, anxiety and epilepsy, indicating an impairment in GABA neurotransmission (Asada et al., 1996). It has been shown that GAD 65, but not GAD 67, forms a complex with heat shock cognate 70 (HSC 70), then cysteine string protein (CSP) and finally vesicular GABA transporter (VGAT) in the nerve terminal (Jin et al, 2003). Furthermore, it appears that this complex is necessary for efficient GABA synthesis and packaging into synaptic vesicles (SV) through VGAT (Jin et al., 2003). It was shown that SV-associated GAD 65 and VGAT are functionally coupled and that VGAT preferentially transports into SV newly synthesized GABA generated predominantly by GAD 65 over pre-existing GABA generated predominantly by GAD 67 (Jin et al., 2003) (Figure 4). In GAD 65 -/-mice this preference for VGAT was abolished (Wu et al., 2007). In other studies, GAD 65-/- mice were shown to be more susceptible to seizures, anxiety and epilepsy due to the inability to synthesize GABA to suppress over excitatory input, supporting the premise that GAD 65 is directly involved in GABA neurotransmission (Heldt et al., 2004; Kash et al., 1997; Stork et al., 2003, Wu et al., 2007).

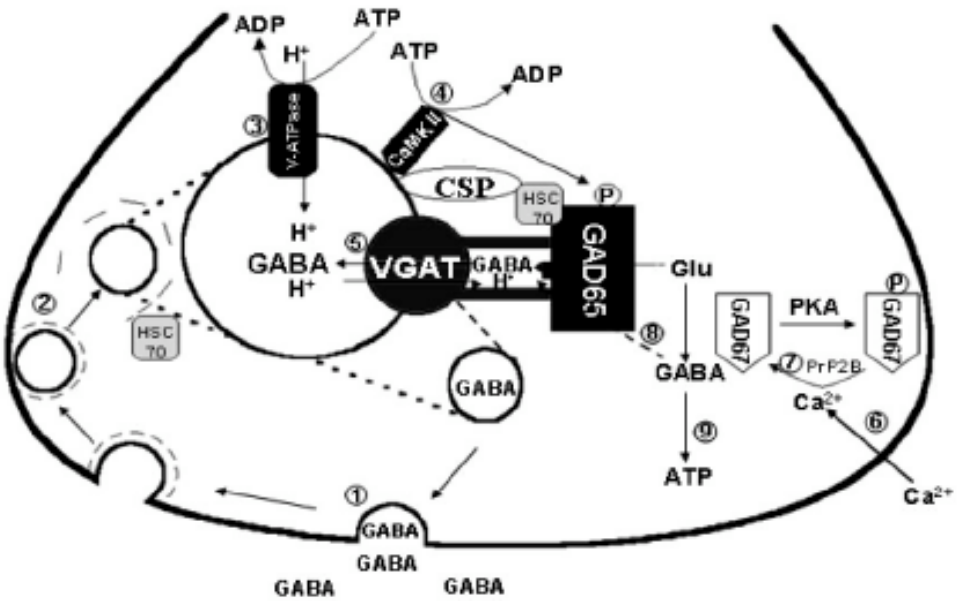


Figure 4. A model depicting a structural and functional coupling between GABA synthesis and vesicular GABA transport into SV. GAD 65 is anchored to SVs first through forming a protein complex with the chaperone protein, HSC70, followed by association of HSC70_GAD 65 complex to CSP, VGAT, and CaMKII on SVs (Jin et al., 2003).

GAD exists in vivo in two isoforms: an active form, holoGAD and an inactive form, apoGAD. The steady state of GABA is thought to be regulated by the relative concentrations of holo- and apoGAD through the pyridoxal-5'-phosphate-dependent interconversion of active (holo-GAD) and inactive forms (apo-GAD) (Martin 1993; Martin and Rimvall 1993; Martin and Tobin, 2000) (Figure 5).

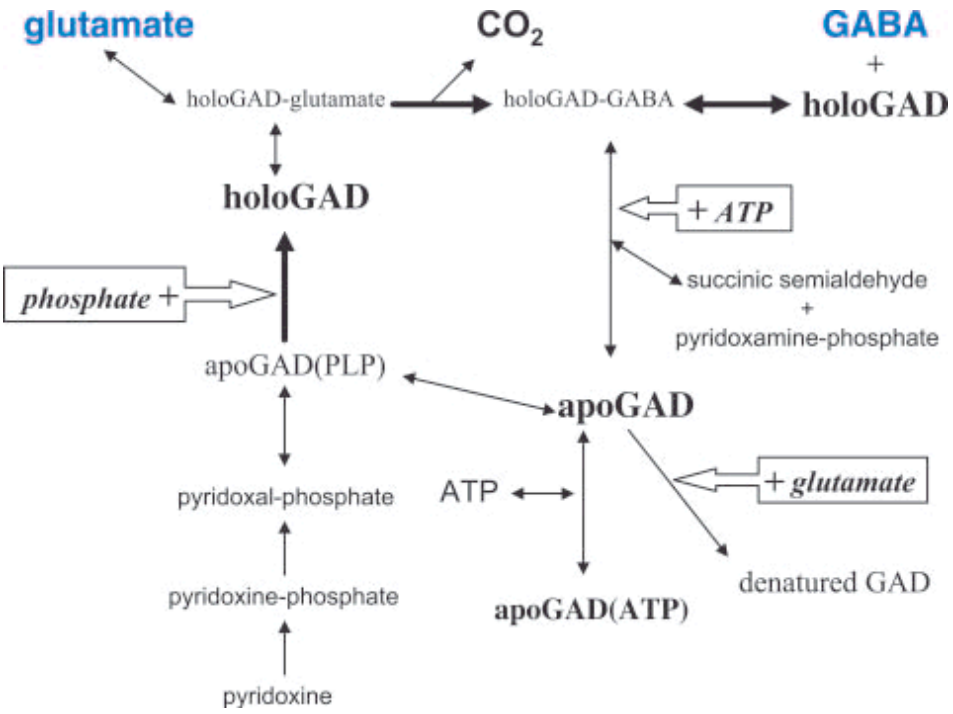


Figure 5. Schematic of the regulation of GABA synthesis. GABA = gamma-aminobutyric acid; ATP = adenosine triphosphate; PLP = pyridoxal phosphate; CO2 = carbon dioxide; holoGAD = holo-enzyme (active) isoform of glutamic acid decarboxylase; apoGAD = apoenzyme (inactive) isoform of glutamic acid decarboxylase (Petroff, 2002).

At least 50% of the total GAD in the brain is apoGAD (Petroff, 2002). The activation of GAD is stimulated by inorganic phosphate and inhibited by ATP, GABA, and aspartate. ATP promotes the formation of apo-GAD and stabilizes it. Without ATP, apo-GAD has a half-life of a few minutes at 37 °C. The conversion of apo-GAD to holo-GAD by pyridoxal-phosphate is a two-step process. The reversible association of apo-GAD with activated pyridoxine is rapid (ATP inhibits binding of apo-GAD with pyridoxal-phosphate). Inorganic phosphate antagonizes the inhibitory effects of ATP and accelerates the formation of holo-GAD from the apo-GAD/pyridoxyl-phosphate intermediate. GAD is activated by changes in energy states: depolarization, acidosis,

increased carbon dioxide, low bicarbonate, low phosphocreatine, increased magnesium, increased ADP, and decreased ATP.

It has been demonstrated that biosynthesis of neurotransmitter GABA from glutamine largely proceeds through the Krebs cycle (Waagepetersen et al., 2001). There is evidence of GAD 65 being associated with mitochondria (McLaughlin et al., 1975). The synthesis of GABA and packaging into SV by SV membrane-bound GAD 65 is proton-gradient dependent (Hsu et al., 1999). It has been hypothesized that mitochondria-associated GAD 65 may be involved in providing energy to drive GABA synthesis and packaging at the SV membrane (Buddhala et al, 2009) (Figure 6). It is suggested that there could be crosstalk between mitochondria-associated GAD 65, GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSDH), with GABA-T and SSDH being mitochondrial enzymes involved in the breakdown of GABA into component that enter the Krebs cycle (Tillarkaratne et al., 1995).

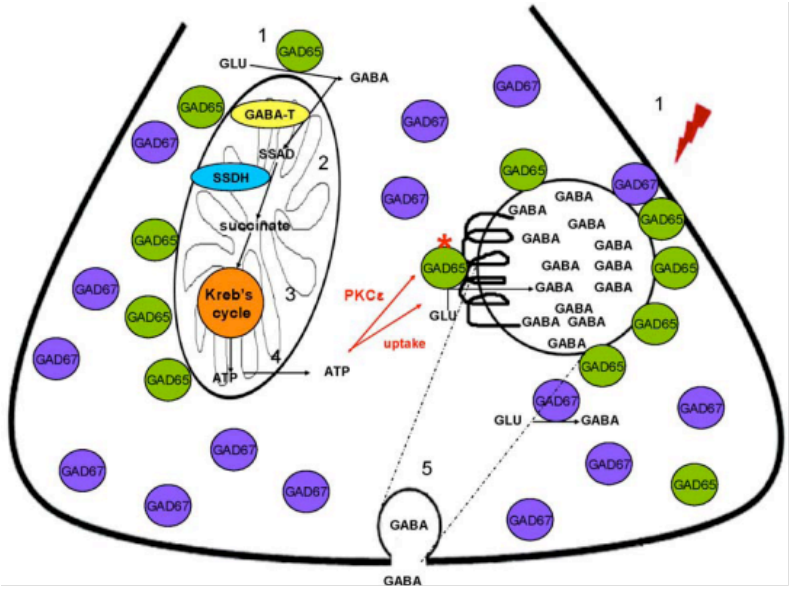


Figure 6. Proposed model of the role of mitochondrial GAD 65 in providing energy to drive GABA biosynthesis at GABAergic synaptic vesicles (Buddhala, et al,. 2009).

Regulation of GADs

The regulation of GAD has been the subject of much research and only recently have the regulatory mechanisms been ascertained. Regulation of GAD occurs in three ways: transcription, translation, and post-translation. GAD 65 and GAD 67 genes and their proteins are regulated differently during development (Somogyi et al., 1995). Evidence exists at the mRNA level that GAD 67 has spliced variants, GAD 44 and GAD 25 during embryonic development and only GAD 44 is known to be enzymatically active (Behar et al., 1993). No such splice variants are reported with GAD 65. GAD 67 is expressed early on during development and GAD 65 occurs only later consistent with the timing of expression of fulfilling their roles in synaptogenesis and as a source for neurotransmitter (Pinal and Tobin, 1998).

GADs are known to undergo post-translational modifications including palmitoylation, proteolytic cleavage, and phosphorylation. Palmitoylation is found only in GAD 65. Synthesized primarily as a soluble cytosolice protein, GAD 65 undergoes palmytoylation at cysteine residues 30 and 45, which is crucial for post-Golgi trafficking to presynaptic terminals (Kanaani et al., 2004). It has more recently been found that a depalmitoylation-repalmitoylation cycle serves to cycle GAD 65 between Golgi and post-Golgi membranes and dynamically regulates the levels of the enzyme directed to the synapse (Kanaani et al., 2008). Proteolytic cleavage of GAD to generate truncated GAD has been shown in vivo (Sha et al., 2005, Wei et al., 2003) and in vitro (Chu and Metzler, 1994; Sha et al., 2008; Wei et al., 2006). The truncated GAD 65 was found to be 2-3 times more active and stable when compared to the full-length GAD 65

(Wei et al., 2003). Full-length GAD 67 was found to be more active compared to the truncated forms (Sha et al., 2005).

Reversible protein phosphorylation is a key regulatory post-translational mechanism by which most signaling pathways including cell growth, differentiation, migration, and apoptosis in eukaryotes operate (deGraauw et al., 2006). GAD 65 and GAD 67 have been shown to be regulated by phosphorylation and dephosphorylation (Wei et al., 2004). GAD 65 was activated in vitro by phosphorylation mediated by protein kinase Cε, whereas, GAD 67 was inhibited by phosphorylation mediated by PKA (Wei et al., 2004). It was also found that T91 was an important phosphorylation site for GAD 67 by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Wei et al., 2004). It has been reported that phosphorylation of serine residues 3,6,10, and 13 in GAD 65 is involved in its anchoring to the SV membrane and has no effect on GAD activity (Namchuk et al., 1997). **Determining what kinases and phosphatases are involved in vivo in the regulation of GAD is crucial to understand how GABA biosynthesis is regulated** (Buddhala et al., 2009). The importance of cyteine residues in the function of brain GAD activity has been investigated (Wu and Roberts, 1974). Cysteine residues 455 for GAD 67 and 446 for GAD 65 have been reported to be very important structural residues in regulating GAD activity (Wei and Wu, 2005). It was found recently that in the crystallographic structure of GADs these cysteine residues reside in the co-factor pyridoxal 5'-phosphate (PLP) binding domain (Fenalti et al., 2007).

Another means of short-term regulation of GADs is the interaction with PLP. As discussed previously, there is a shuttling between active PLP-bound holoGAD and

an inactive PLP-unbound apoGAD form. About 93% of GAD 65 is largely apoGAD, whereas, 73% of GAD 67 is in its holoGAD form, with demand for GABA neurotransmission regulating the conversion from apo- to holo GAD (Battaglioli et al., 2003). There have also been reports that GADs are influenced by hormones (Grattan et al., 1996), cytokines (Schmidli et al., 1996), neurotransmitters, e.g. dopamine (Laprade and Soghomonian, 1995a) and excitatory amino acids (Laprade and Soghomonian, 1995b). The level of GAD 65 and GAD 67 protein is also regulated differently by GABA (Rimvall and Martin, 1994).

Disturbances in GABA or GAD in the CNS have been linked to many neurological diseases: Epilepsy (Freichel et al., 2006; Lloyd et al., 1986), Huntington's Disease (Gourfinkel et al., 2003; Spokes, 1980), Parkinson's Disease (de Jong, 1984), General Anxiety Disorder (Kosel, 2004), Implicated as an autoantigen in Insulin-Dependent Diabetes Mellitus (IDDM) (Tian,1999), Stiff-Person Syndrome (Burbelo et al.,2008; Butler, 1993), and Batten Disease (Chattopadhyay et al., 2002). GAD 65 and GAD 67 are differentially altered following a pathological stimulus. In monkey rendered Parkinsonian by systemic MPTP administration, significant increases in the number of GAD 67-immmunoreactive neurons in the external and internal segments of the globus pallidus while no significant differences in the number of GAD 65-immunoreactive neurons was observed (Stephenson et al., 2005). In a separate rat model with seizures induced by kainite, an up-regulation of GAD 67 mRNA, but not GAD 65 mRNA, was observed in dentate granule cells following seizures (Freichel et al., 2006). In schizophrenia, decreased GAD 67 expression was observed (Guidotti et al., 2000; Impagnatiello et al., 1998).

PROTEOMIC STRATEGIES FOR ANALYSIS OF TISSUE FUNCTION

Proteomics is the study of the proteins expressed in a specific cell, tissue or organism (Wilkins et al., 1996). "Proteomics" was a term coined to make an analogy with genomics, the study of genes. The term "proteome" is a blend of "protein" and "genome" and was first coined by Marc Wilkins in Wasinger et al. (1995). A proteome is a complete and total expression of protein found in a cell, tissue, or organism at a specific point in time under specific conditions. Protein expression is tightly regulated and specific sets of proteins are expressed in different tissue and cell types (Falk et al., 2007). The protein composition in every cell is very dynamic and adapts to factors with cell cycle progression and environmental fluctuations. Proteins form the backbone of metabolism and control virtually every process in the body. The impact of proteins as targets in drug development is reflected by the fact that more than 80% of all available pharmaceutical drugs act through proteins (Drews, 2000).

Drabik et al. (2007) defined the questions that might be answered by proteomic studies: What are the functions of proteins in the context of their presence in a particular tissue? What is the role of post-translational modifications (PTMs) and how do they influence the activity of proteins? In what way a protein function can be changed because of interaction with other molecules, in particular with other proteins? What is the relationship between a protein function and its three-dimensional structure? What is the influence of transcriptional and translational regulation, RNA alternative splicing, compartmentalization, proteolysis, and the PTMs on the final protein expression and

activity? They point out that these questions cannot be answered by genomic studies. They go on to point out that proteins, not genes, play a key role in various neurodegenerative disorders; for example, Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis (ALS), or prion diseases. Proteomics might help not only in diagnostics or treatment of diseases, but also in the treatment of various non-physiological states such as drug dependence. They point out that we might treat drug or alcohol dependence as a process initiated and developed by abnormal protein balance or activity, especially in brain tissue. The results of experiments that compared proteomes of healthy and alcohol-addicted organisms might point out that some proteins are up- or down-regulated in the addicted population.

The discrepancies observed when trying to correlate gene (mRNA) and protein expression are vast. The reason that the phenotype of a cell cannot be predicted solely on the basis of the genome is that post-transcriptional and post-translational modifications create outcomes that cannot be predicted from the genome. Alternative mRNA splicing, differential turn-over of mRNA and protein can affect the relation in between transcript and protein levels at the steady state. Posttranslational modifications (PTMs), such as proteolysis, phosphorylation, glycosylation, etc. all contribute to the diversity of the proteome.

The relationship between genomics and proteomics is significant. The DNA information elucidated by genomic studies is useful in the study of proteomics. The DNA sequences provide a theoretical possibility as to what proteins may be found in a sample. Furthermore, a known gene sequence is commonly used to identify and verify proteins detected by various proteomics methods (Falk et al., 2007). In the human

genome, 23,000 protein coding genes have been found, but the number of protein species has still not been determined (Falk et al., 2007). Several protein species may be generated from one gene, with two or three transcripts generated from one human gene (Nakao et al., 2005; Stamm et al., 2005). There are a larger number of transcripts from the human genome that are not apparently translated and estimates on the total number of proteins vary widely from about 400,000 to several million, reflecting the importance of post-translational modifications and alternative splicing of transcripts. The human body consists of roughly 250 different cell types with different temporal and spatial protein expression patterns, alternatively spliced variants, post-translational modifications (PTMs), and transient protein-protein interactions (Liao, 2009). The situation with gene transcripts and protein is complicated because, unlike the genome that is relatively constant from cell to cell within an organism, transcription profiles and protein composition vary widely among cell types and tissues, and protein composition varies widely among body fluids (Montine et al., 2006).

The study of proteomics has blossomed into a wide variety of sub-specialties (Kislinger and Gramolini, 2010): expression proteomics (identification of all or many proteins expressed in a sample of interest), functional proteomics (mapping of protein–protein interactions), post-translation modification proteomics (aimed at the identification of an ever growing body of enzymatic or nonenzymatic protein modifications), chemical proteomics (interaction of proteins with specific chemical structures), structural proteomics (the large-scale identification of 3D protein structures), and quantitative proteomics (the relative or absolute quantification of proteins).

The size and complexity of the dynamic total protein complement is enormous. Speciation of the proteome is a concept developed by Jungblut et al. (2008). It states that each and every polypeptide is defined by the sum of its covalent chemical bonds meaning its primary structure and in addition any covalently bonded moieties. Therefore, every protein, every expressed member of a multigene family, i.e. protein isoform, and PTM modified form of a protein is understood as a unique protein species. It is not clear if every protein species has its own function. Zhang et al. (2009) has suggested that one primary structure can result in multiple secondary structures and conformations and thus possible multiple functions due to bias towards synonymous, slowly translated codons.

The proteomes of eukaryote organisms are quite complex and present many challenges. There is a huge dynamic range in a biological sample which spans over 10 orders of magnitude in relative abundance of different proteins (Anderson and Anderson, 2002). This range makes it complicated to detect rare protein species without extensive preparatory work as fractionation or depletion of several abundant protein species. Other demanding factors are, alternative promoter usage and a number of post-transcriptional alterations of proteins including, alternative splicing and posttranslational modifications (PTMs) (Godovac-Zimmermann et al., 2005). The three-dimensional structure and the range of physiological and chemical properties among proteins also have to be considered as this makes various proteins less suitable to study using certain approaches. Moreover, a great part of proteins are more or less fragile and subjected to degradation and thus, sample handling is a crucial part in many proteomics methods (Falk et al, 2007). The proteomics community has created and employed a

broad range of methods to address and study the vastly different areas including, protein localization, protein-protein interactions, PTMs, and altered protein composition in tissues and body fluids related to environmental and genetic conditions.

Protein expression is tightly regulated and specific sets of proteins are expressed in different tissue and cell types (Falk et al., 2007). In each cell, the protein composition is very dynamic and varies with the cell cycle progression and environmental fluctuations. Mass spectrometry (MS) is the tool of choice in modern exploration of proteomes (Falk et al., 2007). Mass spectrometry is a generic term for various combinations of ionization techniques, mass analyzers and detectors. Therefore, the choice of instrument is of the highest importance for the study. Initially, MS-analysis was restricted to thermostable, smaller molecules. The independent development of two milder ionization techniques, electrospray ionization (ESI) (Yamashita and Fenn, 1984) and matrix assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988), made investigation of macromolecules possible and thereby revolutionized the field of biological mass spectrometry. This triggered the development of new mass analyzers and other supportive equipment and techniques. Currently, mass accuracy on the ppm-level using time-of-flight (Stoeckli et al., 2001) (TOF) MS or even sub-ppm level using Fourier transform ion cyclotron resonance (FTICR) MS is routinely achieved (Falk et al., 2007). Limits of detection on the zeptomole-level (10^{-21}) have been reported for peptides and smaller proteins (Belov et al., 2000). However, in case of biological samples, the possibility to detect low-abundant proteins is restricted by the presence of high-abundant components and the dynamic range of the mass spectrometer.

Ever since its introduction in the mid-1970s, two-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) has been a workhorse for separation of proteins in complex mixtures (Klose, 1975; O'Farrell, 1975). Proteins are separated according to isoelectrical point (pI) and size, after which they are visualized as spots on the gel by different staining methods. In combination with MALDI-TOF MS, 2D-PAGE constitutes the standard method in expression proteomics. Prior to MS-analysis, the proteins are enzymatically digested in the gel and thereafter extracted into liquid phase. The masses of the resulting peptides are determined by MS. As the genomes of many organisms are known, theoretical masses of translated proteins can be calculated. Bioinformatics tools are used to match the measured masses to theoretical masses from protein databases, and to estimate the probability that the ''peptide mass fingerprint'' originates from a certain protein. Bioinformatics tools are then used to interpret the peptide masses. This is a critical point in the analysis because reliable statistical methods are crucial to distinguish true protein hits from false positives. 2D-PAGE is a robust method that separates intact proteins, and reflects the presence of isoforms, posttranslational modifications and the expression levels. At present, over 5000 protein spots can be resolved on gel in the same experiment, 2000 proteins routinely, and protein spots containing <1 ng protein can be detected (Gorg et al., 2004). There are problems with the technique in that proteins of extreme sizes and hydrophobicities are discriminated against, and the method is rather labor intensive, difficult to automate and time consuming (Falk et al., 2007).

An alternative approach, shotgun proteomics (MacCoss et al., 2002), involves the simultaneous digestion of all proteins in the sample. Shotgun proteomics is the most popular application for unbiased, discovery type analysis of the proteome (Hoehenwater et al., 2010). It is a powerful approach for identifying and quantifying proteins. In this technique, protein mixtures are converted to peptides by proteolytic digestion, and these peptides are used as surrogates for identification and quantitation of the proteins present in the original mixture (Liao et al., 2009). First, peptides are fractionated to reduce sample complexity and then ionized for tandem mass spectrometry analysis (MS/MS). The ions are mass-selected in the first stage of analysis (MS1), fragmented, and the fragmentation ions are analyzed in the second stage of mass analysis (MS2). The MS2 spectra are searched against a protein sequence database to identify the amino acid sequence of the peptides and extrapolate the identities of the proteins in the sample. With the shotgun approach, around 100,000 tryptic peptides are resolved and detected in one single experiment (Falk et al., 2007). This is a fast method that requires little sample handling and manual work. The major advantage of shotgun proteomics is its ability to identify and quantify thousands of proteins in a single analysis. The one drawback to the shotgun approach is that information on the intact proteins and isoforms are lost in the digestion step (Falk et al., 2007).

Currently, fourier-transform ion cyclotron (FT-ICR) mass spectrometry (MS) delivers the highest mass resolving power and mass measurement accuracy (MMA) (Tolmachev et al., 2009). FT-ICR MS is uniquely suited for proteomics analysis (Smith, 2000, Bohac et al., 2005). The characterization of biological systems through the analysis of intact proteins (Hofstadler et al., 1996; Kelleher, 2004) and protein

complexes (Benesch and Robinson, 2006) with FT-ICR MS, e.g. top-down proteomics, continues to reveal the complex and dynamic nature of biological systems (Sharma et al., 2007; Smallwood et al., 2007). Characterization of post-translational modification plays an important role in understanding the regulation and control of biological systems. Top-down proteomic analysis effectively characterizes the number of post-translational modifications, how post-translational modifications change between biological system states, and correlations between PTMs.

Post-translational modified proteins probably represent the most post-translational modification important phenomenon that multiply the number of gene-products from a single transcript (Pandey and Man, 2000; Neverova and Van Eyk, 2005). Post-translational modifications have an enormous array of function in cells that are commonly different from their unmodified counterparts. Protein conformation is altered by covalent modification of primary structures that create new recognition motifs, hide existing motifs or making previously hidden motifs accessible (Hoehenwater et al., 2010). Post-translational modifications can have allosteric effects, controlling protein function via modification of non-active sites. Initial modification can recruit subsequent additional modifications by either the same or different chemical moieties which may act in concert. Two or more different post-translational modifications can compete for modification of the same residue in protein primary structure. All of these mechanisms affect protein interaction via specific post-translational modification-binding domains which defines function (Seet et al., 2006).

Murray and Capaldi (2008) used a proteomic approach to screen for the metabolic basis of neurodegeneration. They used antibody-based capture arrays to

analyze the proteomic differences in oxidative phosphorylation enzymes between human heart and liver tissues, cells grown in media promoting aerobic versus anaerobic metabolism, and the catalytic/proteomic effects of mitochondria exposed to oxidative stress. They state that neurodegenerative diseases are a symptom of a deficiency in regulation or execution of metabolic reactions. They go on to say that mitochondria, as the central organelles in metabolic regulation as well as the chief generators of reactive species, clearly have a role to play in the etiology of neurodegenerative conditions. They are developing antibody-based capture arrays to determine multiple parameters of key mitochondrial proteins. Parameters include enzyme activity, quantity, oxidative modification (including nitrative and oxidative stress), and regulation (phosphorylation and acetylation). This study focused on the array of oxidative phosphorylation enzymes.

Proteomics is invaluable in determining the basic protein structures associated with normal and abnormal conditions. The global protein matrix of proteomics reveals, in exquisite detail, the effects of perturbations of the cell. Monitoring the proteomics of subcellular structures provides clues as to the signaling pathways and interactions that occur as the result of variations in genetics or environmental influences. For example, two specific members of the Src family of tyrosine kinases, Src and Lck, were found to play a previously unknown important role in the genesis of late preconditioning as a result of studying the proteomics of an induced myocardial ischemia (Ping et al., 1999), identifying a new component in the signaling mechanism of ischemic preconditioning. This led to a further study in which the results suggested a heretofore-unrecognized function of PKCε and provided an integral framework for the understanding of PKCε –

dependent signaling architecture and cardioprotection (Ping et al., 2001). In another proteomics study related to cardioprotection, it was found that ischemic preconditioning and pharmacological preconditioning similarly altered mitochondrial signaling complexes (Wong, 2010). These studies show that proteomics provides new ways of studying individual enzymes or chemical compounds as well as the intricate pathways associated with signaling, environmental stressors.

Guina et al. (2007) studied the MglA transcriptional regulator of genes that contributes to the virulence of Francisella tularensis using the exact same methodology used in the present study. They used a label-free shotgun proteomics method to determine the F. tularensis subsp. novicida (F. novicida) proteins that are regulated by MglA. This is an excellent example of using proteomics to study the protein response of a particular species to environmental stressors. Through proteomics differences in relative protein amounts between wild-type F. novicida and the mglA mutant were derived directly from the average peptide precursor ion intensity values measured with the mass spectrometer by using a suite of mathematical algorithms. Among the proteins whose relative amounts changed in a F. novicida mglA mutant were homologs of oxidative and general stress response proteins. The F. novicida mglA mutant exhibited decreased survival during stationary-phase growth and increased susceptibility to killing by superoxide generated by the redox-cycling agent paraquat. The F. novicida mglA mutant also showed increased survival upon exposure to hydrogen peroxide, likely due to increased amounts of the catalase KatG. They suggested that MglA coordinates the stress response of F. tularensis and is likely essential for bacterial survival in harsh environments.

Montine et al. (2007) used the LC–MS–MS techniques to study the proteomics of aging and Alzheimer's disease (AD). Alzheimer's disease is characterized by the accumulation of abnormal proteins amyloid- β (Aβ) in senile plaques (SPs) and tau in neurofibrillary tangles (NFTs) (Hardy and Selkoe, 2002). Wang et al. (2005) undertook a proteomic analysis of laser-captured microdissected (LCM) NFTs. They identified a total of 155 proteins in laser captured NFTs, 72 of which were identified by multiple unique peptides. Of the 72, 63 proteins had no previously known association with NFTs. Expected proteins, such as tau and apo E, were identified in their proteomic screen, confirming the ability of this approach to identify NFT-associated proteins. They validated by immunohistochemistry that glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the proteins identified in their investigation that had not been previously associated with NFTs, colocalized with the majority of NFTs as well as plaque-like structures in AD brain and coimmunoprecipitated by antibodies to PHFtau, but not tau, in temporal cortex. They further characterized the pathological association of GAPDH with AD by showing that it, along with P-tau and Aβ peptides, was present in detergent-insoluble fractions from AD temporal cortex but not from age-matched controls. They reported that these data were the first proteomic investigation of NFTs at that time.

Kovacech and Zilka (2009) used four proteomics approaches to uncover the key steps leading to neurofibrillary degeneration and thus to identify therapeutic targets for AD. They used functional neuroproteomics to generate the first transgenic rat model of AD by expressing a truncated misordered form of tau, ''Alzheimer's tau''. The rat model showed that Alzheimer's tau toxic gain of function is responsible for the

induction of abnormal tau cascade and is the driving force in the development of neurofibrillary degeneration. Structural neuroproteomics allowed them to determine partial 3D structure of the Alzheimer's filament core at a resolution of 1.6 A. Signaling neuroproteomics data lead to the identification and characterization of relevant phosphosites (the tau phosphosignalome) contributing to neurodegeneration. Interaction neuroproteomics revealed links to a new group of proteins interacting with Alzheimer's tau (tau interactome) under normal and pathological conditions, which could provide novel drug targets and novel biomarkers for treatment of AD and other tauopathies.

McFarland et al. (2008) used a targeted comparative proteomics approach for the unbiased identification of phosphorylation-dependent α-Synuclein protein interactions. The carboxyl terminus of α-synuclein can be phosphorylated at tyrosine 125 and serine 129, although only a small fraction of the protein is phosphorylated under normal conditions (Okochi et al., 2000). Under pathological conditions, such as in Parkinson's disease, α-synuclein is a major component of Lewy bodies, a pathological hallmark of Parkinson disease, and is mostly phosphorylated at Ser-129 (Anderson et al., 2006). They report that a controversy exists over the extent to which phosphorylation of α-synuclein and/or the visible protein aggregation in Lewy bodies are steps in disease pathogenesis, are protective, or are neutral markers for the disease process. In this study they used the combination of peptide pulldown assays and mass spectrometry to identify and compare protein-protein interactions of phosphorylated and nonphosphorylated α-synuclein. They showed that non-phosphorylated α-synuclein carboxyl terminus pulled down protein complexes that were highly enriched for mitochondrial electron transport proteins, whereas, α-synuclein carboxyl terminus

phosphorylated on either Ser-129 or Tyr-125 did not. Instead the set of proteins pulled down by phosphorylated α-synuclein was highly enriched in certain cytoskeletal proteins, in vesicular trafficking proteins, and in a small number of enzymes involved in protein serine phosphorylation. Abdi et al. (2006) studied the detection of biomarkers with a multiplex quantitative proteomic platform in cerebrospinal fluid of patients with neurodegenerative disorders. Biomarkers are needed to assist in the diagnosis and medical management of various neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), and dementia with Lewy body (DLB). They employed a multiplex quantitative proteomics method, iTRAQ (isobaric Tagging for Relative and Absolute protein Quantification), in conjunction with multidimensional chromatography, followed by tandem mass spectrometry (MS/MS), to simultaneously measure relative changes in the proteome of cerebrospinal fluid (CSF) obtained from patients with AD, PD, and DLB compared to healthy controls. The diagnosis of AD and DLB was confirmed by autopsy, whereas the diagnosis of PD was based on clinical criteria. Their proteomic findings showed quantitative changes in AD, PD, and DLB as compared to controls; among more than 1,500 identified CSF proteins, 136, 72, and 101 of the proteins displayed quantitative changes unique to AD, PD, and DLB, respectively. Eight unique proteins were confirmed by Western blot analysis, and the sensitivity at 95% specificity was calculated for each marker alone and in combination. Several panels of unique makers were capable of distinguishing AD, PD and DLB patients from each other as well as from controls with high sensitivity at 95% specificity. They suggest that their preliminary findings must be validated in a larger and different population of patients, and that a roster of proteins may be generated and

developed into specific biomarkers that could eventually assist in clinical diagnosis and monitoring disease progression of AD, PD and DLB.

Zhang et al. (2008), in a follow-up study discussed the CSF multianalyte profile that distinguishes Alzheimer and Parkinson diseases. Increased cerebrospinal fluid (CSF) tau and decreased amyloid (A) beta42 have been validated as biomarkers of AD. In contrast, there is no validated CSF biomarker for PD. They validated their proteomics-discovered multianalyte profile (MAP) in CSF from 95 control subjects, 48 patients with probable AD, and 40 patients with probable PD. An optimal 8-member MAP agreed with expert diagnosis for 90 control subjects (95%), 36 patients with probable AD (75%), and 38 patients with probable PD (95%). This MAP consisted of the following (in decreasing order of contribution): τ, brain-derived neurotrophic factor, interleukin 8, Aβ42, β2-microglobulin, vitamin D binding protein, apolipoprotein (apo) AII, and apoE. They reported that this first large-scale validation of a proteomic-discovered MAP suggests a panel of 8 CSF proteins that are highly effective at identifying PD and moderately effective at identifying AD.

Hwang et al. (2010) examined glycoproteomics in neurodegenerative diseases. They point out that among thousands of proteins identified by proteomics in human CSF thus far (Pan et al., 2007; Zougman et al., 2008), only a small portion are related to the CNS structurally or functionally. This deficit in identifying CNS-specific proteins is mainly due to the fact that most of the CNS-specific proteins are low in abundance, and the current proteomic techniques are biased towards abundant proteins in a sample with a large dynamic range (Gulcicek et al., 2005). One of the approaches they suggest to get around this difficulty is to focus on a subproteome(s) that can be isolated readily (e.g.,

proteins with glycosylation, phosphorylation, or oxidation) before proteomic profiling, thereby effectively reducing the dynamic range of a given complex sample (Korolainen et al., 2002; Bahl et al., 2008; Kubota et al., 2008). To this end, they suggest that characterizing glycoproteins is especially appealing because they are intimately related to the health of cells, and in addition, are relatively enriched in body fluids like CSF and plasma (Ohtsubo & Marth, 2006). They report that among various post-translational modifications, glycosylation represents the most common and complicated form. It is estimated that 50–60% of proteins in the human body are modified by glycosylation (Apweiler et al., 1999; Hagglund et al., 2004; Kameyama et al., 2006). Alterations in protein glycosylation have been related to human neurodegenerative disease states, such as Creutzfeldt-Jakob disease (CJD), AD, and PD (Saez-Valero et al., 2003; Silveyra et al., 2006). This study investigated the glycoproteins in human CSF in four groups: control subjects, AD, and PD patients at two different stages. Their MALDI-TOF-TOF analysis revealed a total of 283 nonredundant glycoproteins in human CSF. In comparison with the existing publicly accessible database, 243 of these proteins were annotated in UniProtKB/Swiss-Prot and the ISB database as glycoproteins with known glycosylation sites or probable/potential glycosylation sites. The specificity of this approach was approximately 86% (243/283). Their study on human brain tissues revealed 394 non-redundant glycoproteins. In comparison with the existing database, 343 of these proteins were annotated in the UniProtKB/Swiss- Prot and ISB databases as glycoproteins with known glycosylation sites or probable/potential glycosylation sites. The specificity was approximately 87% (343/394). Their study opened the field to new potential biomarkers that may be critical to disease diagnosis and disease

progression monitoring as well as providing new clues as to the metabolic components involved in the disease process.

Martyniuk et al. (2010) used proteomics to study the response in the brain of the largemouth bass to subchronic dietary exposures to Dieldrin, a persistent organochlorine pesticide. They found that functional enrichment analysis revealed that the biological gene ontology categories of stress response, nucleotide base excision repair, response to toxin, and metabolic processes were significantly impacted by dieldrin. Using isobaric tagging for relative and absolute quantitation, 90 proteins in the male hypothalamus were statistically evaluated for changes in protein abundance. They reported that several proteins altered by dieldrin are known to be associated with human neurodegenerative diseases, including apolipoprotein E, microtubule-associated tau protein, enolase 1, stathmin 1a, myelin basic protein, and parvalbumin. Proteins altered by dieldrin were involved in oxidative phosphorylation, differentiation, proliferation, and cell survival. They reported that this study demonstrates that a subchronic exposure to dieldrin alters the abundance of messenger RNAs and proteins in the hypothalamus that are associated with cell metabolism, cell stability and integrity, stress, and DNA repair.

Katagiri et al. (2010) did a proteomic analysis of proteins expressing in regions of rat brain. They carried out comparative proteomics of six regions of the adult rat brain: thalamus, hippocampus, frontal cortex, parietal cortex, occipital cortex, and amygdala using semi-quantitative analysis by Mascot Score of the identified proteins. In order to identify efficiently the proteins that are present in the brain, the proteins were separated by a combination of SDS-PAGE on a C18 column-equipped nano-liquid

chromatograph, and analyzed by quadrupole-time of flight-tandem-mass spectrometry. Their proteomic data show 2,909 peptides in the rat brain, with more than 200 identified as region-abundant proteins by semi-quantitative analysis. The regions containing the identified proteins are membrane (20.0%), cytoplasm (19.5%), mitochondrion (17.1%), cytoskeleton (8.2%), nucleus (4.7%), extracellular region (3.3%), and other (18.0%). Of the identified proteins, the expressions of glial fibrillary acidic protein, GABA transporter 3, Septin 5, heat shock protein 90, synaptotagmin, heat shock protein 70, and pyruvate kinase were confirmed by immunoblotting. They examined the distributions in rat brain of GABA transporter 3, glial fibrillary acidic protein, and heat shock protein 70 by immunohistochemistry, and found that the proteins are localized around the regions observed by proteomic analysis and immunoblotting. IPA software analysis indicates that pathways closely related to the biological functions of each region may be activated in rat brain. They concluded that these observations indicate that proteomics in each region of adult rat brain may provide a novel way to elucidate biological actions associated with the activation of regions of the brain.

Finally, it is of use to know the present state of proteomics study for the CNS. Zhang (2010) provides an excellent summary article detailing the current state of proteomics, what is being studied and with what techniques, with an eye to future experiments. He described the current proteomics studies on prenatal and postnatal stages of brain development. The description includes studies of the subproteomics of different brain regions and specific tissues or cells, including the visual cortex, retina and cerebellar cortex, the hippocampus, and the CSF. Also described were the current proteomic studies of neural stem cells, synapse and synaptic proteomics, mitochondrial

proteomics in the brain, brain plasma membrane proteins, and blood-brain barrier proteomics. Reviews of the studies dealing with chromosome proteomics related to brain functions for special purpose, proteomics of the brain in different animals, functional proteomics on special proteins and neuroproteomics applied to the study of neurodegenerative and neuropsychiatric disorders were included. Descriptions of the technical requirements for quantitative neuroproteomics analysis including overall proteomic methods, quantitative neuroproteomics, modification of proteomics for the special purpose of proteomic studies, and the comparative analysis and integration of transcriptomic and proteomic data were added. The article ended with a description of the international efforts for deciphering the brain complexity from the proteomic viewpoint: the Human Proteome Organization (HUPO) Brain Proteome Project (BPP) events. Overall, this article provided the greatest review of proteomics efforts of any article surveyed.

MATERIALS AND METHODS

Acupuncture/EEG

Subjects

The subjects were recruited from the student pool at Florida Atlantic University (FAU) and the general public in Boca Raton, Florida, US in 2003. Eight healthy subjects were studied: 2 males and 6 females; age range 20-55 years old (average 30 years). Four had been treated with acupuncture previously and four had not. Subjects were free of known neurological disease and regular medications. Written consent was obtained from each subject and the study was approved by the Independent Review Board at FAU.

Participating Experimenters

A list of the participating experimenters is shown in Table 1.

Table 1

List of Participating Experimenters in Acupuncture/EEG Experiment

Experimental Design	EEG Paste-up	Acupuncture	Data Analysis	Result/ Discussion/ Conclusion
M. Marshall	M. Marshall	M. Marshall	M. Marshall	M. Marshall
A. Fuchs	T. Zanto		A. Fuchs	A. Fuchs

Procedure

EEG was recorded using a cap from Electro-Cap International, Inc. (Eaton, OH) with a montage of 84 electrodes. The cap was properly placed and secured with impedance within acceptable limits (less than 20 kΩ). EEG signals were fed into a Dell Dimension XPS T450 using Windows NT and the software MANSCAN 4.1. Horizontal and vertical EOG (electro-oculograms) was measured simultaneously to allow removal of eye movement artifacts. The raw EEG signals were recorded at a sampling rate of 256 Hz.

After electrode placement, subjects were laid down in a supine position on a mattress in a sound-attenuated room (Industrial Acoustics Company). They were blindfolded and instructed to keep their eyes closed during the whole experiment until told to open them.

The recordings were performed in three blocks: 10 minutes of rest before needles were inserted (Pre), 30 minutes with needle insertion and stimulation (TX, In/Out), and another 10 minutes of rest after needle withdrawal (Post), leading to a total recording time of 50 minutes. EEG recordings were collected for the three blocks using MANSCAN (4.1 software). There were two different treatments performed on separate occasions: in one case, the needle was inserted and twisted until the de qui (needle sensation) was felt (tingling sensation) and retained for 30 minutes (Tx); in the other, the needle was inserted, twirled until the de qui was felt and then removed (In/Out).

All subjects were needled bilaterally at Guang Ming (GB37), a point 5 cun (Chinese unit of measure) superior to the lateral malleolus, anterior to the fibula. J type SEIRIN sterilized disposable stainless steel needles (No.2 [0.18] X 30 mm)

(AcuMarket, Miami, FL) were used on all subjects. They were inserted approximately ½ - 1 cun and twirled until the acupuncturist felt the de qi.

The experiment was designed to have the subject experience, as closely as possible, what would happen in a normal clinical treatment.

Data Analysis

The EEG data was manually inspected and movement artifacts and bad channels were removed. The data was analyzed using the MANSCAN (SAM Technology, Inc., San Francisco, CA) software package and the data was bandpass filtered in the range of 1-50 Hz. The discrete Fourier transform was applied to the averaged waveshape to obtain power spectra for each block. The power spectrum allows the determination of how much signal occurs at a specific frequency. The spatial distribution of power for pre-treatment (Pre), treatment (Tx), and post-treatment (post) was determined for five frequency bands: delta = 1-4 Hz, theta = 4-8 Hz, alpha = 8-12 Hz, beta = 12-20 Hz, gamma = 20-50 Hz, as well as global power. The spatial patterns for Pre, Tx, and Post were obtained from a principal component analysis (PCA). PCA involves a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible

The power spectra of the time series corresponding to the first five PCA components with frequency bands using a moving window of two minutes (or 30720 data points) were determined for the Pre, Tx, and Post.

GAD

Triplicate peptide digests of the whole brains of three mice (genetically distinct) from wild type and hetero- and homozygous GAD knockout mice (12 weeks old) are analyzed for protein content. Each sample is run four times resulting in a total of 12 runs. A list of participating experimenters is shown in Table 2.

Table 2

List of Participating Experimenters in Protemics Experiment

Experimental Design and Sample Preparation	Sample Analysis	Data Analysis	Result/ Discussion/ Conclusion
H. Prentice	J. Busby	D. Radulovic	M. Marshall
J. Wu			H. Prentice
M. Marshall			J. Wu
C. Buddhala			D. Radulovic
D. Radulovic			

Samples are analyzed using microcapillary high-pressure liquid chromatography (LC) electrospray ionization (ESI) tandem mass spectrometry on a linear ion trap Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) (Guina et al., 2004). The MS/MS protocol is given in Table 3.

Table 3

MS/MS Protocol

Step 1
Protein samples were carbamidomethylated on cysteines using DTT and iodoacetamide. Ammonium bicarbonate buffer (100mM) was added (50uL) and the pH was checked to ensure pH=8. Trypsin (250 ng) was added and the sample digested overnight at 37C. An aliquot of each sample (40 µg) was cleaned using Stage Tips (Proxeon) following manufacturer's instructions. Cleaned samples were taken to near dryness and reconstituted in 20uL of 0.1% acetic acid for mass spectrometric analysis.
Step 2
One quarter of the reconstituted sample (5 µL) was injected for each mass spectrometric analysis. All columns were packed in house using 360x 100 fused silica (Polymicro) and Jupiter 4µm C18 packing material (Phenomenex). The precolumn contained 5 cm of packing material and the analytical column contained 30 cm of packing material. Briefly, the sample was loaded onto the precolumn and washed to waste with solvent A (0.1 M HOAc in 1% MeCN) for 5 minutes. A valve switch aligned the precolumn and the analytical column and the peptides were gradient eluted to 90% MeCN in 120 minutes with a flow rate of ~300nl/min. Electrospray ionization was performed using a NanoMate (Advion) with a voltage of 1.7kV throughout the gradient. Ions were detected using an Orbitrap mass spectrometer (Thermo) operated in data dependent mode with the top three most intense ions in each scan being selected for fragmentation in CAD mode. After selection, masses were added to the dynamic exclusion list with a repeat count of 1 and an exclusion time of 30 sec.
Step 3
MS/MS spectra were extracted from the RAW file using DTA select and merged to a standard mgf file. This file was searched using Mascot and the Mouse IPI protein database using trypsin as the cleavage enzyme. The error allowed for the precursor mass was 10 ppm and the fragment masses were within one Da. Two missed cleavages were allowed as well as static modification to cysteine (+57) and variable modification to methione (+16). Search results were validated using Scaffold (Proteome Software).

Note: This table outlines the 3 major steps in the MS/MS analysis employed in this study: 1) Digestion 2) Running the column and 3) Extraction of MS/MS spectra.

Mass spectrometry (MS) is an analytical technique used in the determination of elemental composition and structure of a sample and has emerged as a primary

technology for determining protein expression (Kislinger and Emili, 2003; Aebersold and Mann, 2003).

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), also known as Fourier transform mass spectrometry, is a type of mass analyzer (or mass spectrometer) for determining the mass-to-charge ratio (m/z) of ions based on the cyclotron frequency of the ions in a fixed magnetic field (Marshall et al., 1998). Fourier transform ion cyclotron resonance (FTICR) mass spectrometry is a very high resolution technique in that masses can be determined with very high accuracy.

The average relative amount of each peptide for wild type and GAD knockout mice will be determined by using an improved version of the DRAGON algorithm (Radulovic et al., 2004). This program automatically detects and quantifies large numbers of peptide peaks in feature-rich ion mass chromatograms, compensates for spurious fluctuations in peptide signal intensities and retention times, and reliably matches related peaks across many different data sets. This program facilitates pattern recognition and biomarker discovery in global comparative proteomic studies. The differences in peptide identities, abundances, and post-translational states between will be determined for wild type and GAD knockout mice.

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recognition and biomarker discovery in global comparative proteomic studies. The differences in peptide identities, abundances, and post-translational states between will be determined for wild type and GAD knockout mice.

The Radulovic protocol includes the following: Step 1: Data Filtering and Signal Extraction: Radulovic et al. (2004) developed a procedure that would reliably extract genuine signals, representing individual peptides from large collections of interpolated MS full scans. Figure 7 is an example of data filtering and signal extraction from Radulovic et al. (2004) and is similar to that found in the present sample.

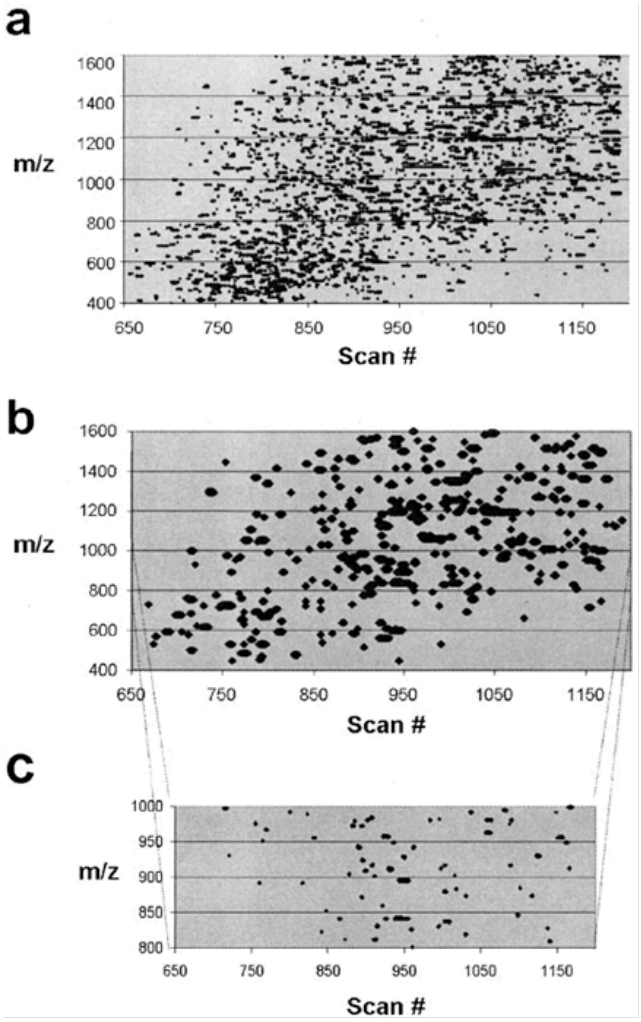

Figure 7. Feature detection in LC-MS-based proteomic profiles.

Representative examples of (a) Level 5 and (b) Level 2 data pamphlets, harboring 16,000 and 2,000 peptide features (black pixels),respectively. The datasets were generated by extracting ion peak signal above a specified threshold from an LC-MS dataset obtained for a yeast tryptic digest. c, higher resolution "zoom in" of the Level 2 pamphlet reveals good peak dispersion. x-axis, scan number; y-axis, m/z ratio. (Radulovic et al, 2004).

They reduced noise by creating a filtering algorithm to pre-process the spectra, binning and smoothing the data using moving averages. They then used a feature extraction algorithm to select an optimal set of rules to acquire a predefined series of features for a geometrically increasing sequence. Their algorithm starts conservatively, extracting the most prominent ion features first, and then progressively adding features until the cutoff is met.

Step 2: Peak Definition: Radulovic et al. (2004) point out that global proteomic studies depend on the comprehensive accounting of peptide patterns. They developed a contour detection algorithm, based on established boundary detection and integration techniques to automate peak definition. To test the reliability of peak detection, they mapped high-confidence (p value < 0.05) peptide sequences, derived by searching CID spectra against a comprehensive protein-sequence database using the SEQUEST (Eng et al., 1994) and STATQUEST (Kislinger et al., 2003) algorithms, on to a data pamphlet. They reported that as expected, peak-to-peptide overlap was extensive, with few outliers. They also reported that this analysis also affirms a well-known fact that considerably more peptide peaks are detectable in full scan MS mode than can be identified in the same time frame using the quasi-stochastic CID process. An example

of this from Rudulovic et al. (2004) is shown in Figure 8. The same methods of Radulovic et al. (2004) were employed in our current study.

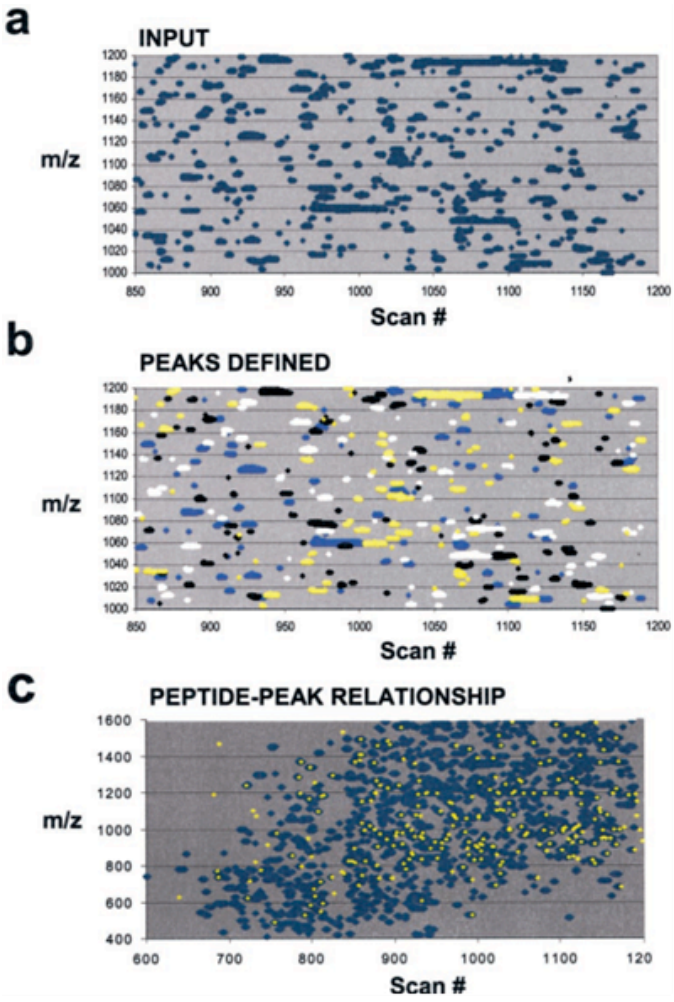

Figure 8. Automated Peak Detection. Automated detection of discrete peaks using boundary detection and integration (contour mapping) techniques. Examples of an (a) input pamphlet and (b) post-analysis of this same profile, with individual peaks highlighted in alternating colors to enhance visual discrimination, (c) example of the close correspondence of high-confidence peptide sequences identified by CID (yellow) to pamphlet peaks detected by the software (blue) (Radulovic et al., 2004).

Step 3: Correction of Peak Drift and Distortion and Peak Alignment: Etzioni et al. (2003) and Diamandis (2004) stated that biomarker discovery depends on the careful

examination of protein abundance across multiple samples. The repeated LC-MS analyses are generally highly similar, but, there is an obvious alignment problem following attempts to overlay related data pamphlets. To surmount this problem, Radulovic et al. (2004) devised an efficient pamphlet alignment algorithm that uses self-optimizing 2D smooth spline transformation to correct for nonlinear deviation in peak patterns. Their algorithm optimizes feature overlap between an input pamphlet and a second reference pamphlet. Exhaustive pair-wise alignments were performed to find a global optimum with larger sample sets. To compensate for residual random (nonsmooth) variation, each of the peaks detected by contour mapping was "wobbled" to maximize peak overlap. Although this local optimization was limited in scope (±1% total scans), it provided an added measure of peak matching. They noted that just as data normalization is often used to correct for systemic signal discrepancies in microarray studies (Quackenbush, 2002), global peak intensities of different datasets can likewise first be normalized by adjusting median feature intensities to unity prior to matching. They go on to point out that many substantive issues are raised by normalization procedures. Radulovic et al. (2004) report that, in their experience, well-controlled sample preparation and LC-MS procedures serve sufficiently well in most instances such that data normalization is not a major concern. An example of this is shown in Figure 9.

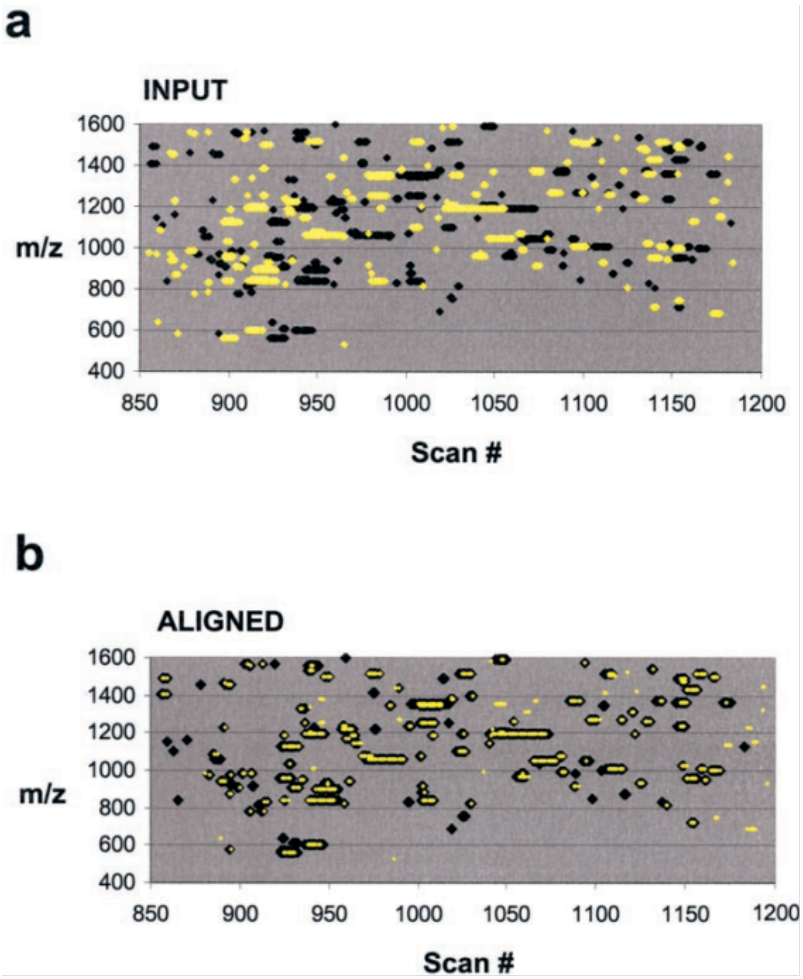

Figure 9. Profile alignment and peak mapping. Nonlinear deviation in peak patterns recorded in repeat LC-MS datasets. a, a misalignment problem presents itself following attempts tooverlay two related data pamphlets. Peak drift and distortion are more pronouncedat the beginning of the analyses. b, data overlap is considerably improved after application of the peak alignment algorithm (Radulovic et al., 2004).

Step 4: Quantitative and Qualitative Proteomic Comparisons: Once peaks were aligned, the LC-MS datasets were directly compared in a systematic manner. A simple first-pass measure of similarity, feature overlap, was calculated. The more closely related two data pamphlets were, the higher their ratio. For quantitative peak comparisons, grouped feature intensities were summed. Radulovic et al. (2004)

suggested that their platform is relatively robust to artifacts stemming from standard sample handling procedures.

RESULTS

Acupuncture/EEG

The spatial distribution of power for pre-treatment (Pre), treatment (Tx), and post-treatment (Post) was determined for five frequency bands: Delta = 1-4 Hz, Theta = 4-8 Hz, Alpha = 8-12 Hz, Beta = 12-20 Hz, Gamma = 20-50 Hz, as well as Total Global Power. The power distributions for subject one (S1) (30 minute [30m] and in/out[I/O]) and subject (S2) [30m] for the three treatments is shown in Figure 10. Only the powers for Alpha, Beta, and Total Power are shown in Figure 10. The differences in the color distribution are apparent, reflecting the differences in power over the scalp. In all sessions, the power was highest for the Alpha and lowest for the Gamma band. The spatial distribution of power during the treatment and post-treatment blocks did not change from the Pre baseline (Figure 10). All three measures were highly reproducible within single subjects for all phases and sessions. Alpha, Beta and Total Power are shown in Figure 10. This result suggests that there was no change created by either acupuncture treatment or the change was not measurable using EEG.

The similarity between normalized spatial patterns of different frequencies for Tx and Post (30 m and I/O) treatments is shown in Figure 11, for two subjects.

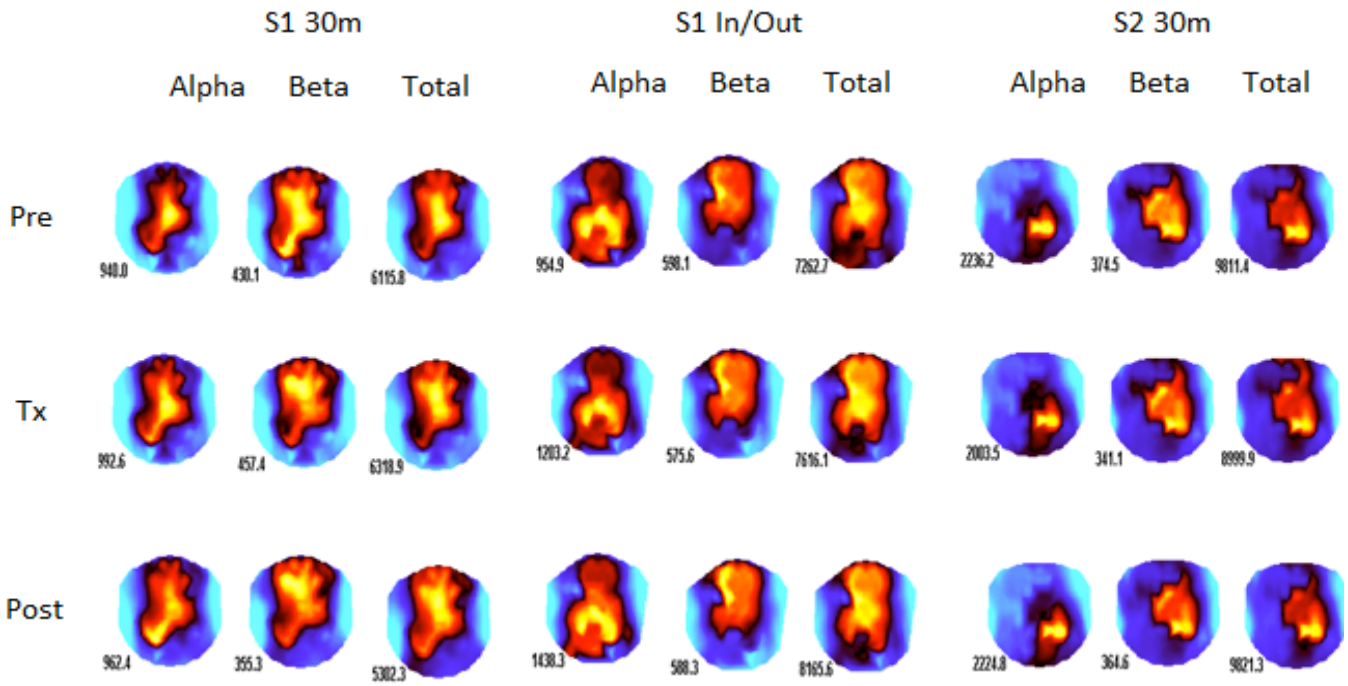

Figure 10. The spatial power distribution (alpha, beta, and total) for two 30 min and one I/O treatment of Pre-acupuncture, Tx (during acupuncture), and Post-acupuncture on two subjects (S1 and S2).

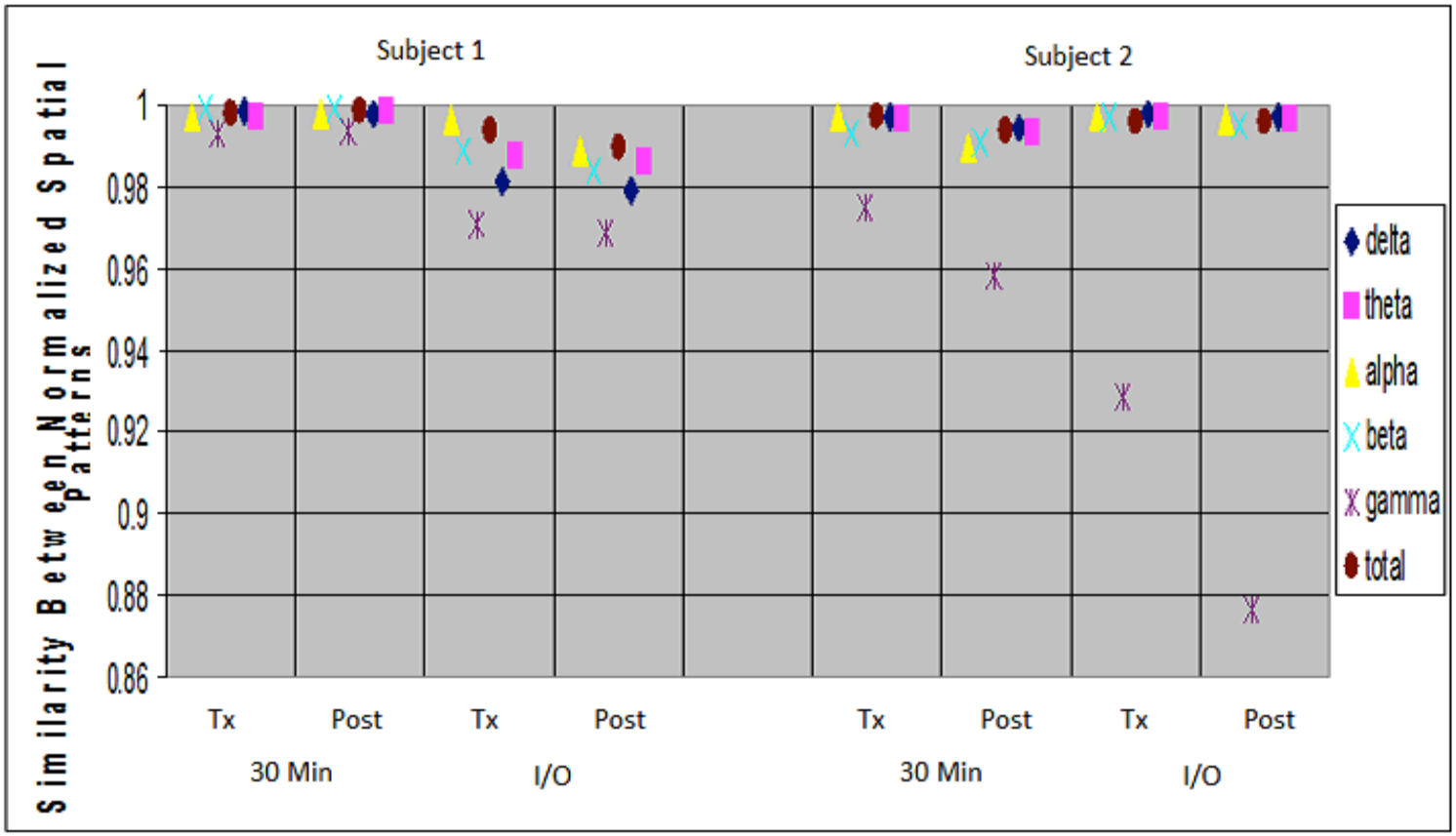

Figure 11. The similarity between normalized spatial patterns for two subjects where the baseline (pre-treatment) is compared to 30 min and I/O treatment periods.

Every spatial pattern for each frequency was highly correlated to the baseline. The difference in the gamma frequency was due to the small sample size. This result suggests that there are no measurable differences for the various wavelengths between the baseline (Pre) and the treatments 30 m or I/O.

Principle component analysis (PCA) was performed for the Pre, Tx, and Post with the resultant spatial patterns for one subject found in Figure 12. PCA is an important tool for spatial pattern recognition. It is a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the data as possible and each succeeding component accounts for as much of the remaining variability as possible. The spatial principal component analysis in Figure 12 shows no difference between Pre, Tx, and Post acupuncture. The spatial shape of PCA patterns was the same up to a mode (the ninth) that contributed only about 1% to the signal variance.

The power spectra of the time series corresponding to the first five PCA components are shown in Figure 13.

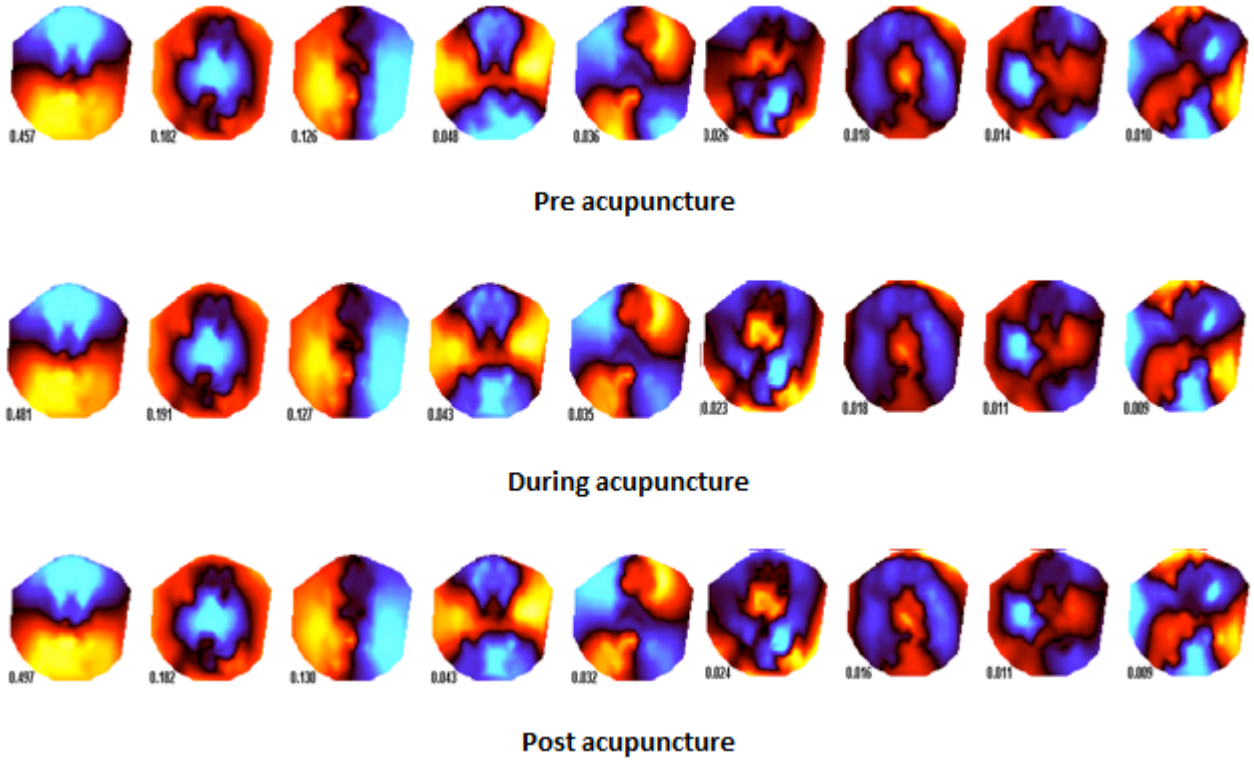

Figure 12. The spatial principal component analysis patterns for one subject for pre-acupuncture, during acupuncture, and post-acupuncture.

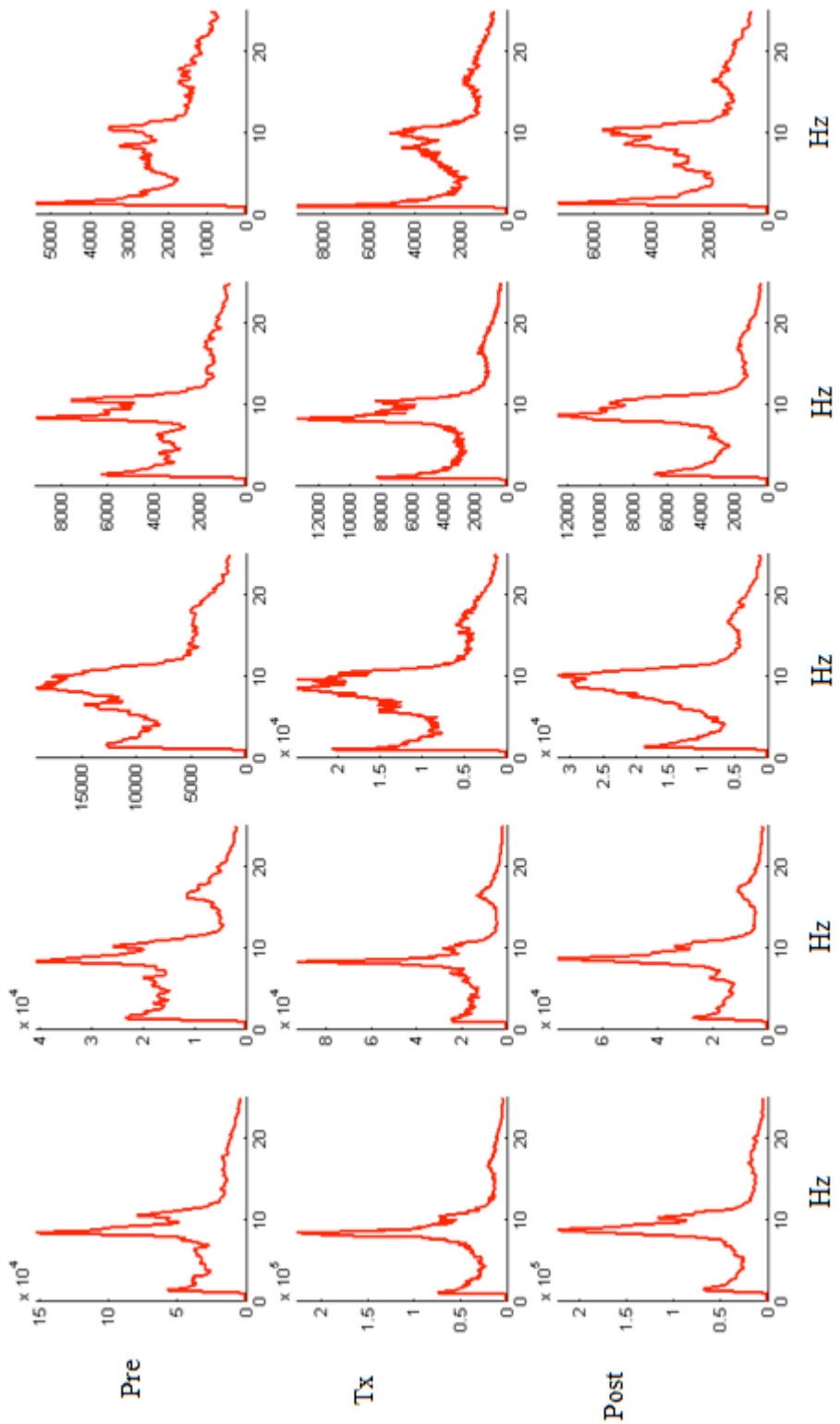

Figure 13. The power spectra of the time series corresponding to the first five PCA components for a single subject.

The highest peaks were seen in the Alpha frequency (8-12 Hz) with a secondary peak in the Delta frequency (1-4 Hz) for the first four modes. Similar characteristic shapes were found in each treatment block, but were less similar across treatment blocks. No differences were observed between treatment and post-treatment blocks compared to the baseline.

Proteomics

The results of this study are defined by the procedures and analysis of the raw data, so it is helpful to discuss them in detail. Radulovic et al. (2004) developed the protocol used in this study and describe succinctly the details and results derived from their procedures. They point out that peptide mixtures derived from protein digests are fractionated using a chromatography column packed with reverse-phase media and electrosprayed into an online MS/MS instrument in typical LC-MS-based profiling experiments. They report that in data-dependent experiments, the instrument alternatively records the signal intensities, m/z ratios, and retention times of all of the detectable eluting peptides, as well as the fragmentation pattern of individual peptides subject to collision-induced dissociation (CID). Wysocki et al. (2000) reported that the recorded ion peak intensities reflect the intrinsic electrochemical properties of a peptide. This peak intensity also reflects its relative concentration (Wang et al., 2004; Bondarenko et al., 2002). A peptide sequence can often be deduced from a search of the database for the daughter on spectra (Kislinger and Emili, 2003; Aebersold and Mann, 2003).

We have employed LC-MS to obtain raw MS peak data for wild type (WT), heterozygous knockout (HZ) and homozygous knockout (KO) mice at the Proteomics

Facility at Scripps, Florida—(Collaboration with Dr. Jennifer Busby). Representative examples of raw MS peak data are shown in Figure 14.

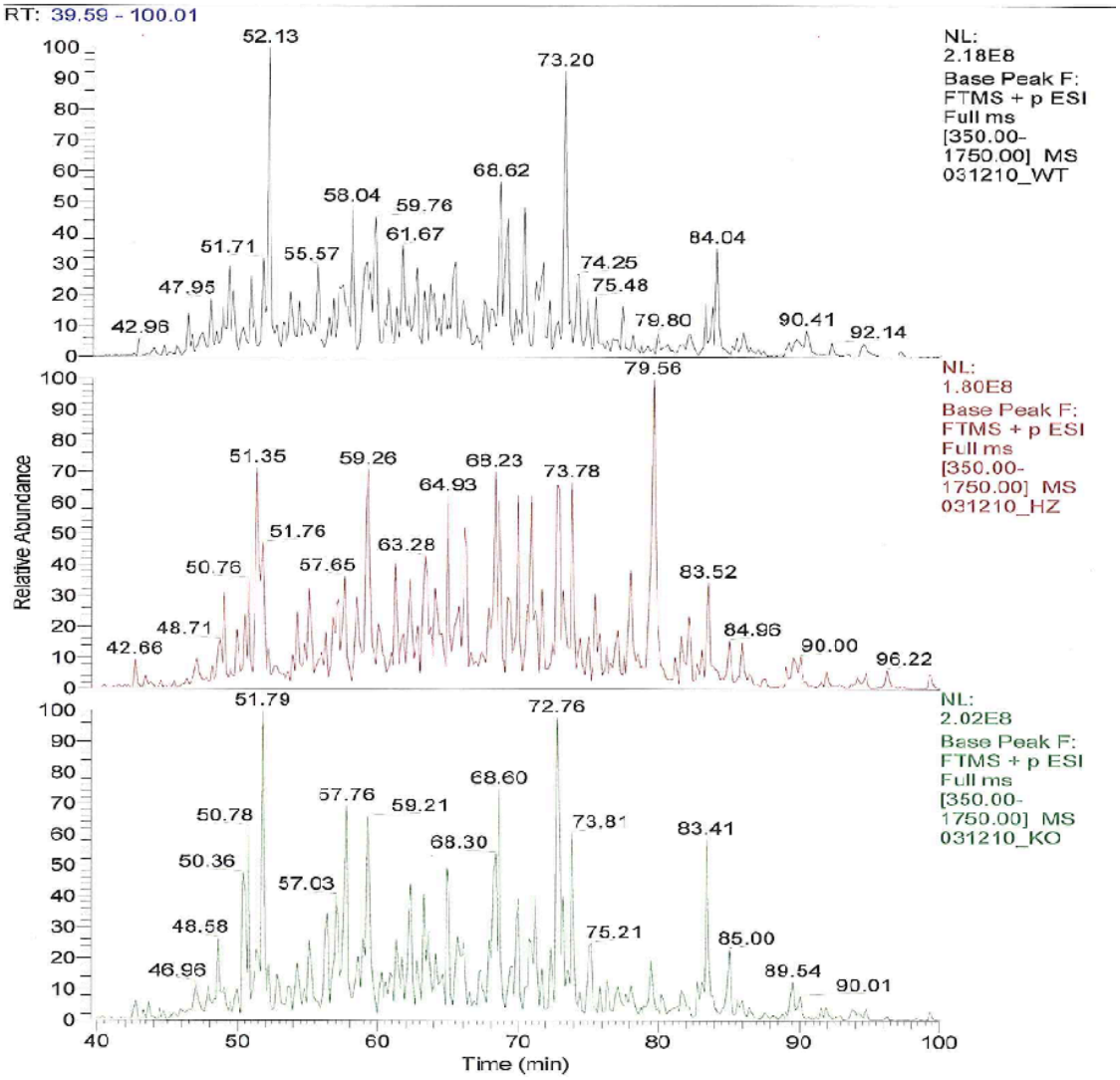

Figure 14. Raw MS Peak Data (Representative Examples).

With the advent of modern LC-MS systems, hundreds of peptides can be resolved (Kislinger and Emili, 2003; Aebersold and Mann, 2003). Typical datasets consist of a multiplexed stream of co-eluting ion peaks (or ion map) acquired on a quadrupole ion-

trap (Radulovic et al., 2004). They refer to such data as an empirical profile. Examination of the total ion chromatograms reveals the general reproducibility of LC-MS. However, both stochastic system performance variation and chemical and electronic noise can affect the relative position, width, amplitude, and shape of individual peaks, even under controlled conditions. Radulovic et al. (2004) refer to this peak artifact as drift and distortion.

There are many difficulties in extracting quantitative information from LC-MS datasets. Repeated analysis is an effective way to enhance the signal-to-noise ratio (Han and Kamber, 2000). The challenge in profiling experiments is then to detect related peaks across different datasets, despite peak drift and distortion (Radulovic et al., 2004). Sophisticated filtering techniques, such as time series, Fourier transform, expectation-maximization, and certain pattern recognition algorithms (Han and Kamber, 2000) can be challenging to implement in an effective and practical manner. Radulovic et al. (2004) developed a four step procedure that has robust, assumption-free, threshold-like data filtering algorithms for detecting real differences in peak number and intensity that would not be overly sensitive to the effects of spurious noise.

Annesley (2003) stated that theory and empirical evidence suggests that peak intensities are not independent, but rather can be negatively correlated due to ion-ion interactions leading to signal suppression. It was suggested that this effect was often pronounced with contaminants such as detergents or polymers that perturb ionization efficiency and are manifested by prominent vertical “drop-out” strips.

In our present study, Radulovic compared reproducibility between samples. Four different MS analyses of the same trypsinized mixture sample were made from

knockout mice brains. The values for each peptide/protein were averaged for samples (1 and 2) and (3 and 4) and plotted on a Log scale, Figure 15.

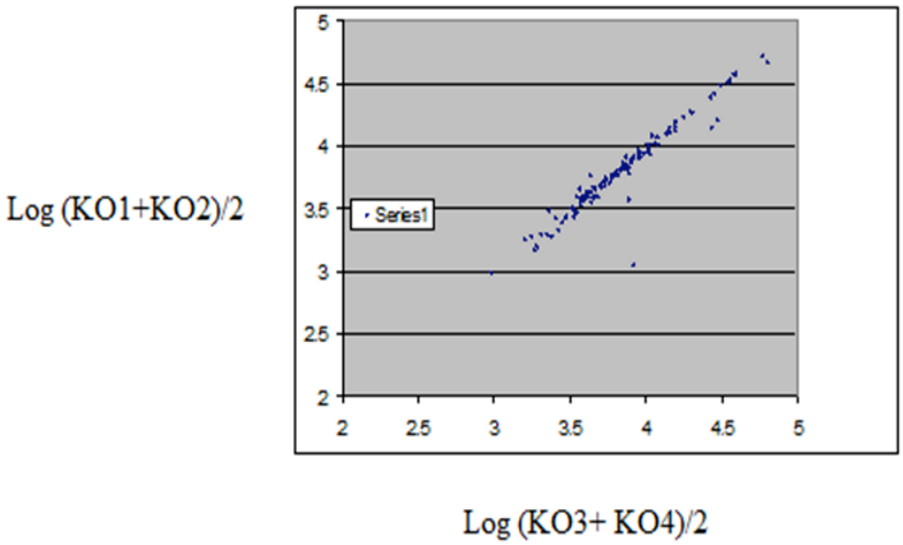

Figure 15. Total Ion Current for Knockout Groups (1 and 2) and (3 and 4) Averages.

The numbers in Figure 15 represent the "Total Ion Current" values derived by aggregating all the MS peaks belonging to a peptide/protein for the averaged samples. They are used for relative quantifications. They are considered relative because different peptides/proteins ionize differently so one can only use this measurement for the comparisons of identical peptides/proteins before and after treatments. They must not be used to compare different peptides/proteins. The plot shows vary little variability in total ion current between samples, suggesting that the data is reliable and reproducible.

In Figure 16, the log of the total current average for the protein/peptides of the knockout mice is compared to that of the average wild type mice.

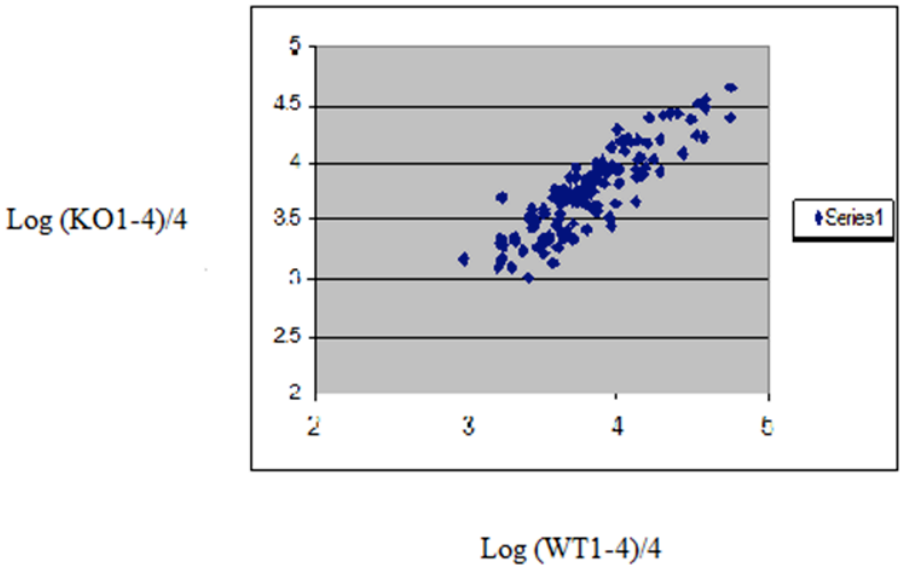

Figure 16. Total Ion Current for Average Knockout Values versus Average Wild Type Values for each particular peptide/protein.

The variance in total ion current is greater between the knockout mice and wild type than within the knockout mice sample variance.

An overlay of Figures 15 and 16 is shown in Figure 17.

Figure 17 highlights the observation that the difference between knockout and wild type total ion currents deviates extensively from the linear relationship obtained by comparing knockout values from multiple runs. Knockout/wild type comparisons show differences resulting from biological mechanisms. The distribution between knockout and wild type graphs illustrate clear biologically derived differences.

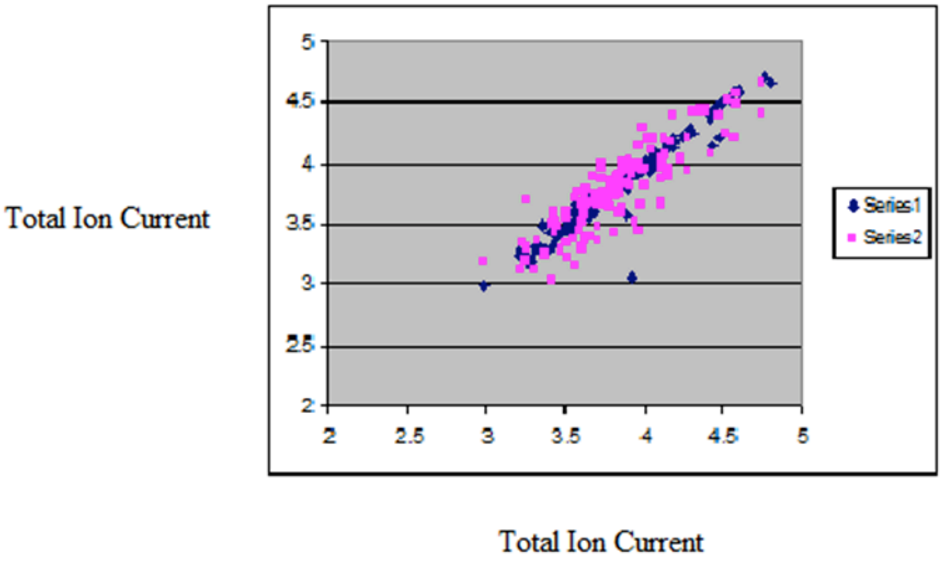

Figure 17. Overlay of Total Ion Current for Figure 13 and 14.

The results of the present study are of a very high quality as verified by Dr. Radulovic (personal communication). The laboratory analyses done in the Radulovic et al. (2004) study were carried out at the Fred Hutchinson Cancer Research Center in Seattle, WA. The laboratory analysis for the present study was done at The Scripps Research Institute, Scripps Florida in Jupiter, Florida.

The strength of the current study is the internal consistency of multiple MS analyses on the same protein stamples.

An example of the short list of proteins found in wild type and heterozygous GAD mice in the present study is shown in Table 4.

Table 4

List of Selected Proteins

peptide	Protein	MS1	MS2	WT_1	WT_2	WT_3	WT_4	HZ_1	HZ_2	HZ_3	HZ_4		Confidence	Pval
NTGIIcTIGPASR	Isoform_M1_of_Pyruvate_kinase_isozymes_M1_M2	9	9	11753	12349.3	11842	12328.2	3043.38	3019.23	2992.56	3377.94	WT>HZ	100.00%	1.14E-07
NLDIERPTYTNLNR	Tubulin_alpha_4A_chain	12	12	14689.4	14846.5	15701	15481.1	6797.94	6772.35	8339.16	7698.91	WT>HZ	100.00%	9.91E-06
VNADEVGGEALGR	Beta_globin	4	4	8300.01	8796.15	9368.82	9006.82	5261.51	5346.91	6144.1	5595.69	WT>HZ	100.00%	3.71E-05
VTVAGLAGKDPVQcSR	Protein_DJ_1	4	4	9971.95	8810.54	8619.83	9122.87	5565.75	5198.72	5826.13	6046.38	WT>HZ	99.98%	0.000186
AVTELNEPLSNEDR	14_3_3_protein_eta	10	10	5931.52	5439.17	5805.41	5239.77	3166.03	2960.76	3175.91	3208.38	WT>HZ	99.98%	0.000199
AKEILQEEEDLAEIVQLVGK	Isoform_1_of_V_type_proton_ATPase_catalytic_subunit_A	12	12	6011.77	5928.13	5674.46	5882.68	3690.85	4281.36	3625.94	3614.44	WT>HZ	99.98%	0.000238
FSAVALcK	Ubiquitin_carboxyl_terminal_hydrolase_isozyme_L1	11	10	6677.46	6988.23	6439.33	6587.2	4862.19	5130.17	5146.58	5594.2	WT>HZ	99.97%	0.000323
TSGNQDEILVIR	Isoform_1_of_Dynamin_1	10	10	8716.71	8198.31	7874.81	8129.76	4573.16	3894.25	5410.71	5072.12	WT>HZ	99.96%	0.00037
LTGFHETSNINDFSAGVANR	Glutamine_synthetase	10	10	11388.7	10755.1	10860.8	10925.3	6379.6	6405.4	7748.08	7377.63	WT>HZ	99.95%	0.000455
AcAETIDPASLWEY	Fascin	7	7	4732.87	4463.31	4429.45	4653.35	3616.69	3901.31	3415.67	3786.98	WT>HZ	99.93%	0.000742
KITISDcGQL	Peptidyl_prolyl_cis_trans_isomerase	12	12	15646.3	16048.4	16940.1	17955.1	12339.9	11713	12526.7	12658	WT>HZ	99.85%	0.001478
DIcNDVLSLLEK	14_3_3_protein_zeta_delta	12	12	6085.85	6197.27	7016.69	7040.11	4286.98	4522.29	4737.58	4312.04	WT>HZ	99.84%	0.001619
QLEALGLAEKPQLVTR	synaptojanin_1_isoform_a	2	2	24015.2	23737.2	25631.3	25859.9	17630	16708.1	19861.9	20263.2	WT>HZ	99.84%	0.001647
AVGKDNFTLIPEGTNGTEER	Dihydropyrimidinase_related_protein_2	12	12	16701.9	15571.8	15685.1	15748.1	12107	12541.6	13714	14294.2	WT>HZ	99.38%	0.006245
SQGGEPTYNVAVGR	Isoform_1_of_Profilin_2	5	5	6844.14	6993.37	6572.26	6532.77	5221.58	4588.82	3338.74	4698.78	WT>HZ	99.19%	0.008113
LAVSQVPR	Transketolase	8	8	9022.29	8861.9	8661.49	8732.61	2330.31	552.433	543.635	5409.76	WT>HZ	98.98%	0.010173
DcVGPEVENAcANPAAGTVILLENLR	Phosphoglycerate_kinase_1	12	12	7990.32	7691.88	8246.82	7837.06	11725.7	11829.9	12108.7	12211.8	WT<HZ	100.00%	3.13E-07
EQASHLGGAVFSGAGNIAAATGLVK	Beta_synuclein	8	8	4046.6	4559.87	4121.92	4084.39	10043.8	10543.6	10675.3	11120	WT<HZ	100.00%	3.86E-06
VIHDNFGIVEGLMTTVHAITATQK	similar_to_Glyceraldehyde_3_phosphate_dehydrogenase__	12	12	10248.7	9953.84	8807.32	8577.53	28581.8	28438.2	31325.7	30644.4	WT<HZ	100.00%	5.38E-06
EGNASGVSLLEALDTILPPTRPTDKPLR	Elongation_factor_1_alpha_2	5	5	3925.2	4048.31	3798.52	3915.17	8168.93	8374.2	9327.7	8579.47	WT<HZ	100.00%	1.45E-05
FWEVISDEHGIDPSGNYVGDSDLQLER	Tubulin_beta_3_chain	4	4	5729.86	5143.33	5463.16	4784.81	8950.46	8998.21	9482.84	8143.65	WT<HZ	99.99%	7.54E-05
TEFIPLLDGDEK	Isoform_1_of_Microtubule_associated_protein_4	4	4	2551.63	2533.14	2895.76	2706.15	6460.42	5918.49	6956.99	6513.43	WT<HZ	99.99%	8.99E-05
GLVGEIIKR	Nucleoside_diphosphate_kinase_B	6	6	5358.43	5489.51	5225.07	6039.81	7474.88	7175.58	7210.86	7742.05	WT<HZ	99.98%	0.000236
WGEAGAEYVVESTGVFTTMEK	Glyceraldehyde_3_phosphate_dehydrogenase	6	6	3569.2	2746.81	2997.94	3105.66	9234.74	8391.34	10322.7	10270.7	WT<HZ	99.97%	0.00026
DLFDPIIEER	Creatine_kinase_B_type	12	12	28726.5	25663.2	27815.8	27959.1	32951.5	34376.9	36015.8	36456.8	WT<HZ	99.96%	0.00045
AAVPSGASTGIYEALELR	Gamma_enolase	12	12	10356.8	10589.7	10522.6	11617.5	13389.6	13865.3	13315.2	13553.4	WT<HZ	99.92%	0.000837
ADLINNLGTIAK	MCG18238	12	12	8698.19	8570.16	8927.36	9840.75	11965.3	12184.8	11962.5	11679.3	WT<HZ	99.91%	0.000863
LSLESEGANEGATAAPELSALEEAFR	Tubulin_polymerization_promoting_protein	12	12	4892.35	4746.82	4939.36	4320.6	5392.71	6002.17	6330.02	5875.33	WT<HZ	99.65%	0.003515
DATNVGDEGGFAPNILENKEALELLK	Alpha_enolase	12	12	5664.01	5480.26	5542.52	5962.78	6250.3	6152.37	6482.59	6175.79	WT<HZ	99.52%	0.004845
TVATPLNQVANPNSAIFGGARPR	Isoform_Long_of_Eukaryotic_translation_initiation_factor_4	1	1	2189.47	2228.06	2107.09	2286.11	4029.32	5225.94	5218.38	6171.33	WT<HZ	99.36%	0.006424
NLISPDLGVVFLNVPENLKDPEPNIK	Microtubule_associated_protein_1B	9	9	2099.25	1833.6	2085.27	2081.74	2398.77	2866	3175.91	2898.99	WT<HZ	99.02%	0.009759

Table 4 is a short list of the top 32 proteins showing significant alterations in expression levels across wild type and homozygous knockout mice. The confidence level was high: 98.98-100.00 %. The full wild type, hetero- and homozygous GAD knockout mice data is not presented here because these results are tentative and require the analysis of 18 more samples before adequate comparisons and definitive interpretations can be made. None-the-less, it is useful to examine the list for potentially significant proteins that influence cell survival and excitotoxicity in the brain and have potential relationships with GABA. The four proteins are chosen here for further discussion on the basis of relevance to the biochemistry and metabolism of neurons in the brain:

(1) V-proton ATPase (V-ATPase) is involved in maintaining the pH gradient of the synaptic vesicle and is involved in the packaging of GABA into synaptic vesicles.

(2) Glutamine synthetase converts glutamate to glutamine, a part of the metabolic pathway involving GABA.

(3) Beta-synuclein is a protein associated with Parkinson's disease.

(4) Microtubule associated protein (MAP) promotes the stability of microtubules and make dendrites and axons more stable. Decreases in MAP are found in immature neurons of several neurological diseases.

DISCUSSION

Acupuncture/EEG

Prior to 2003, when this study was done, only one study (Rosted, 2001) had been published using manual acupuncture stimulation and EEG. In that study, the maximal stimulation was for 2 minutes. The present study used treatment times of 30 minutes, reflecting the common treatment time used in normal clinical practice. The results show no change in spatial distribution or power during or immediately after acupuncture stimulation as compared to baseline. These results were the same as Rosted (2001) suggesting that the effect of manual needle stimulation probably is not adequate to elicit changes measurable by EEG. Napadow et al. (2005) found that electroacupuncture (particularly at low frequency) produced more widespread fMRI signal increase in the brain than did manual acupuncture. Using transcutaneous electrical stimulation, Chen et al. (2006) found that high frequency (100 Hz), but not low-frequency (2Hz) stimulation at HeGu (LI4) point induced short term specific EEG modulation of Theta activity in the midline frontal area. Cho et al. (1999), using fMRI, reported activation of the visual cortex with manual acupuncture stimulation of GB 37. Gareus et al. (2002) found no significant BOLD-effect in the visual cortex using fMRI with manual stimulation of GB 37. The present study failed to clarify the relationship between manual acupuncture stimulation of GB 37 and the visual cortex.

The present study also tested two needle stimulation techniques commonly used in clinical practice: 1) manual stimulation until de qi with needle retention 2) manual

stimulation until de qi is achieved and then needle removal. Due to the lack of detectable change in EEG, no differences in these techniques were observed.

An incidental finding of the study was the high stability in EEG spatial and power distributions for a 50 min time period was an unexpected outcome suggesting constant activity, as measured by EEG, for that period under specified conditions.

Proteomics

The field of proteomics is ever-expanding and the search for biomarkers is ongoing. The present study sought to examine the differences in global protein output in genetically different mice with respect to GAD. The methodology employed was crucial to the results and is important when comparing results of this experiment with others. It is important to review the aspects of this methodology that influenced the results.

Gel-free LC-MS-based profiling methods, such as those employed here, offer remarkable analytical speed and sensitivity (Wu and MacCross, 2002), however, its variability has limited its general suitability for biomarker discovery (Boguski and Mc Intosh, 2003). In our present study, we employed Radulovic et al. (2004), a complementary informatics strategy Radulovic designed to derive reliable qualitative and quantitative protein profiling data using established, broadly applicable LC-MS procedures. The software used corrects for spurious deviation between experiments, permitting meaningful comparisons of proteomic datasets for the purpose of identifying differential protein expression between samples. It also automates large-scale pattern recognition and mining of proteomic datasets for the purpose of sample classification and biomarker discovery. The Mother Pamphlet strategy, a feature detection

methodology, in particular, allows detection of reproducible differences in proteomic patterns, improving proteome coverage and dynamic range.

In order to validate their peak matching procedures, Radulovic et al. (2004) evaluated the peptide sequence identities deduced for the peaks matching across different datasets. This was an ultimate test of their methodology, because failure of any step (e.g. peak detection, alignment, matching, or even the database search itself) would result in nonuniform identifications. In their study, 647 peaks matched across the mouse samples (Level 2 pamphlets), ~ 200 were sequence identified ($p < 0.05$) in more than half the samples. They found that the vast majority (>93%) of the matched peaks were assigned the same sequence identity across the different datasets, confirming the reliability of the software to accurately track identical peaks between samples. The few exceptions consisted of either an occasional mismatch (< 2%), possibly due to errors by the database search algorithm, or to dual sequence assignments (~5%), most likely because the peak detection or alignment algorithms had artifactually fused two adjacent but distinct peaks.

Radulovic et al. (2004) pointed out that a lack of reproducibility represents a barrier to biomarker discovery, because it seems unlikely that one could distinguish between closely related samples (e.g. 95% peak overlap), when experimental reproducibility is 80%. They tested their protocol and found that virtually all of the peaks detected in one pamphlet were likewise found in at least one of the other datasets. Hence, even limited repetition reveals essentially all of the principle peptide peaks that define a sample. They conclude that repetition improves data consistency.

The goal of many proteomic studies is biomarker identification. Radulovic et al. (2004) pointed out that one should be able to detect peptides that differ reproducibly between sample groups by comparing Mother Pamphlets. They tested this and found that their profiling procedures revealed biologically relevant changes in the abundance of physiological significant biomarkers (albeit relatively abundant enzymes) with absolute specificity and sensitivity.

Pan et al. (2008) used a similar technique to the one used in the present study to do a targeted quantitative proteomics study of human CSF. This study demonstrated the use of MS/MS analysis with a different separation process than our current study. They used a matrix-assisted laser desorption/ ionization time-of-flight tandem mass spectrometer (MALDI TOF/TOF)-based platform, whereas, we used the ESI-based instrument. They pointed out that, in comparison to the ESI-based instrument, the MALDI tandem mass spectrometer offers several unique features, including (1) offline separation to include all of the peptides eluted from LC on a MALDI plate, (2) decoupling of MS and MS/MS acquisition that allows for a selective MS/MS analysis, and (3) an ease to interpret data structure because of the generation of predominantly singly charged ions. They reported that their process of verifying/ validating novel and brain-specific protein markers unique to neurodegeneration has been hampered because of the lack of specific antibodies, a common problem in modern protein studies. They used 14 unique peptides, chemically synthesized with stable isotope labeling as signature references for targeted analysis of the corresponding native peptides in CSF. These peptides were previously observed in their profiling analysis. Because of the complexity of a biological system at the protein level, the selection of appropriate

signature peptides to represent a targeted protein was critical for quantitative analysis. Signature peptides were selected to represent a specific protein or a group of proteins. In general, their criteria to be considered included the uniqueness of a signature peptide to the corresponding targeted protein or protein group, known post-translational modifications or amino acid variants on the peptide, adequate sensitivity and appropriate mass for the mass spectrometry platform applied (e.g., 800–4000 Da for MALDI TOF/TOF), and good chromatographic characteristics. They deliberately avoided depletion of abundant proteins, such as albumin and immunoglobulins, prior to proteomics analysis for the concern that other proteins could be depleted along with abundant proteins under nondenaturing conditions. To reduce the analytical difficulties associated with the enormous complexity and dynamic range of protein concentrations of human CSF, the tryptic digest of CSF proteins was fractionated with SCX and combined into six fractions. Each fraction was spiked with a known amount of reference peptides and subjected to LC separation/spotting and MALDI TOF/TOF-targeted analysis. They reported that after MS acquisition, the mass of the reference peptide was searched against the MS data to locate and identify the precursor ions that have a mass within the mass search window (0.3 Da). The identification of the reference peptide using accurate mass led to the preliminary identification of the corresponding native peptide, which was defined by a distinct mass shift from the reference peptide because of stable isotope labeling. Notably, for complex samples, the search for the mass of a peptide might result in multiple candidate precursor ions with similar mass but located at different spots on the MALDI plate; therefore, a MS/MS analysis was needed to further distinguish and identify the targeted peptide. The precursor ions

selected (including reference and native peptides) were then subjected to MS/MS analysis for peptide sequencing and identification, which provides additional identification confidence on peptide validation in addition to the mass matching.

In comparison, the present study used a microcapillary high-pressure liquid chromatography (LC) electrospray ionization (ESI) tandem mass spectrometry. The full complement of the resident proteins is analyzed at one time, without the use of antibodies or isotopes.

Guina et al. (2007) studied the MglA transcriptional regulator of genes that contributes to the virulence of Francisella tularensis using the exact same methodology (MS/MS and algorithm) used in the present study. They used a label-free shotgun proteomics method to determine the F. tularensis subsp. novicida (F. novicida) proteins that are regulated by MglA. This is an excellent example of using proteomics to study the protein response of a particular species under two environmental conditions. It demonstrates that protein changes occur with variations in environmental stressors. Through proteomics, differences in relative protein amounts between wild-type F. novicida and the mglA mutant were derived directly from the average peptide precursor ion intensity values measured with the mass spectrometer by using a suite of mathematical algorithms. Among the proteins whose relative amounts changed in an F. novicida mglA mutant were homologs of oxidative and general stress response proteins. The F. novicida mglA mutant exhibited decreased survival during stationary-phase growth and increased susceptibility to killing by superoxide generated by the redox-cycling agent paraquat. The F. novicida mglA mutant also showed increased survival upon exposure to hydrogen peroxide, likely due to increased amounts of the catalase

KatG. They suggested that MglA coordinates the stress response of F. tularensis and is likely essential for bacterial survival in harsh environments.

Some recent proteomics studies have shed light on important aspects of neuronal dysfunction and neurodegerative disease. Montine et al. (2006) used the LC–MS–MS techniques to study the proteomics of aging and Alzheimer's disease (AD). Alzheimer's disease is characterized by the accumulation of abnormal proteins amyloid-β (Aβ) in senile plaques (SPs) and tau in neurofibrillary tangles (NFTs) (Hardy and Selkoe, 2002). Wang et al. (2005) undertook a proteomic analysis of laser-captured microdissected (LCM) NFTs. They identified a total of 155 proteins in laser captured NFTs, 72 of which were identified by multiple unique peptides. Of the 72, 63 proteins had no previously known association with NFTs. Expected proteins, such as tau and apo E, were identified in their proteomic screen, confirming the ability of this approach to identify NFT-associated proteins. They validated by immunohistochemistry that glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the proteins identified in their investigation that had not been previously associated with NFTs, colocalized with the majority of NFTs as well as plaque-like structures in AD brain and coimmunoprecipitated by antibodies to PHFtau, but not tau, in temporal cortex. They further characterized the pathological association of GAPDH with AD by showing that it, along with P-tau and Aβ peptides, was present in detergent-insoluble fractions from AD temporal cortex but not from age-matched controls. They reported that these data were the first proteomic investigation of NFTs at that time. The importance of this study is that proteomics revealed a relationship that was previously unknown between a

protein and a pathological biomarker. It demonstrates that proteomics can be used to find potential protein relationships that have previously not been found.

The Main Result: Key Proteins from the Short List

The goal of the present study was to identify proteins associated with GAD that ultimately influence the production of GABA. Like the previous two studies mentioned, we obtained information on a potential novel involvement for several proteins in degenerative aspects of neuronal dysfunction and/or in neurodegeneration. A short list of 32 proteins was obtained, of which, at least four showed remarkable relevance to neural processes. The four selected proteins presented here (V-ATPase, Glutamine synthestase, Beta-synuclein, MAP) are known to be involved in neurodegenerative diseases.

(1) V-ATPases. Sun-Wada and Wada (2010) report that vacuolar-type H+-ATPases (V-ATPases) are a family of multi-subunit ATP-dependent proton pumps involved in diverse cellular processes, including acid/base homeostasis, receptor-mediated endocytosis, processing of proteins and signaling molecules, targeting of lysosomal enzymes, and activation of various degradation enzymes and that it is important in neurotransmitter accumulation. Giovanni et al. (2010) report that acidification of synaptic vesicles by the vacuolar proton ATPase is essential for loading with neurotransmitter. V-ATPase may be related to GABA packaging and transport into synaptic vesicles. A deficiency or excess of V-ATPase may lead to an under- or overproduction of GABA influencing neurodegeneration.

(2) Glutamine synthetase. Glutamine synthetase (GS) forms glutamine from glutamate and has an important role in the metabolism of glutamate and GABA (Figure 18).

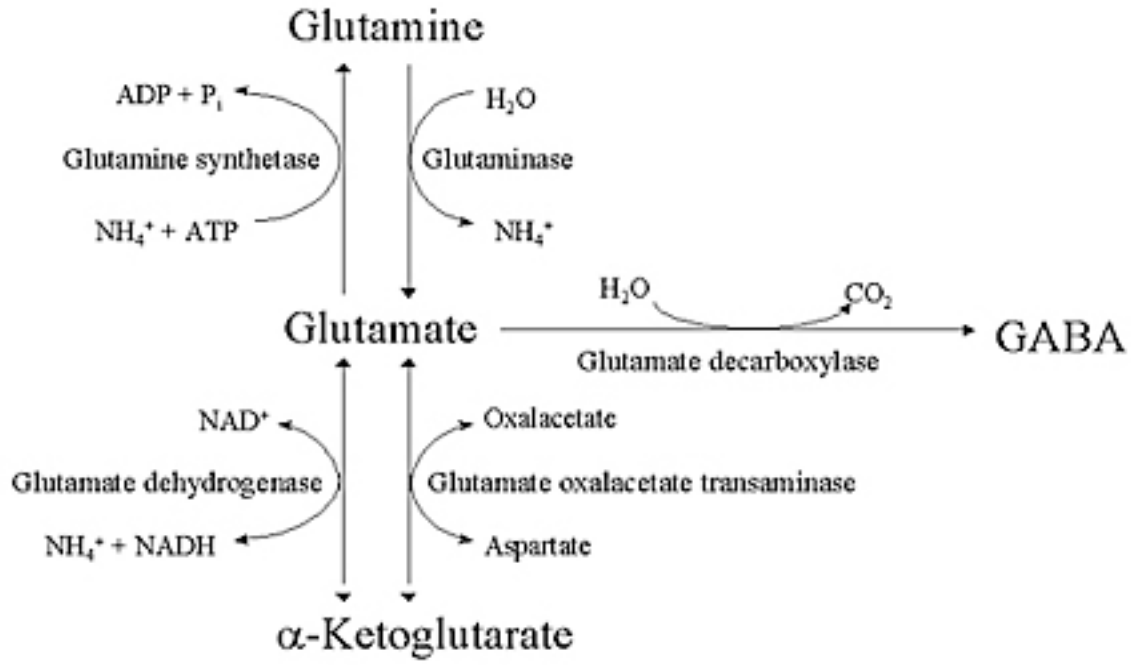

Figure 18. Glutamate – GABA Imbalance =>Excitotoxicity (Comprehensive Heart Care, Inc, 2011)

GS is an integral link in the production or lack of production of GABA and may be involved in neurodegeneration. The balance between glutamate and GABA is critical in the prevention and treatment of neurodegenerative disorders. Up-regulation of GAD has two important consequences: 1) The removal of the excitotoxic substrate, glutamate, by converting it to GABA. 2) The increase in the concentration of GABA which opposes the neuro-excitatory action of glutamate. In healthy brains, GS is restricted to astrocytes but in Alzheimer's disease and cell culture, GS has been detected in neurons (Fernandes et al., 2010). Swamy et al. (2010) report that the decreased activity of glutamine synthetase may favor the prolonged availability of glutamic acid

causing excitotoxicity leading to neuronal damage in anoxia. One of the goals of current research is to find ways to decrease glutamate production and increasing GABA production to limit excitotoxicity.

(3) Beta-synuclein. Beta-synuclein (βS) and alpha-synuclein (αS) are homologous proteins implicated in Parkinson's disease and related synucleinopathies (Israeli and Sharon, 2009). They suggest that while αS is neurotoxic and its aggregation and deposition in Lewy bodies is related to neurodegeneration, βS is considered as a potent inhibitor of αS aggregation and toxicity. Since at least 70% of the afferents to substantia nigra dopaminergic neurons are GABAergic (Tepper and Lee, 2007), the role of GAD may be of great importance to the treatment of Parkinson's disease as well as other neurodengerative diseases.

(4) Microtubule associated protein. Microtubles undergo rapid cycles of polymerization and depolymerization in dividing cells, but they are much more stable in mature dendrites and axons. This stability is due to Microtubule associated protein (MAP)s which promote the oriented polymerization and assembly of the microtubules (Kandel et al., 2000). Kris et al. (2010) state that the regulation of microtubule growing and shortening dynamics is essential for proper cell function and viability, and that MAPs such as the neural protein tau are critical regulators of these dynamic processes. They go on to propose that misregulation of microtubule dynamics may contribute to tau-mediated neuronal cell death and dementia in Alzheimer's and related diseases. This is a reasonable proposal since neurofibrillary tangles found in Alzheimer's disease are primarily composed of microtubule-associated protein tau that is aberrantly

hyperphosphorylated (Xia et al., 2008). MAPs may be found to be important in neurodegeneration through its involvement in GAD and GABA production.

CONCLUSION

Acupuncture/EEG

Our results show no changes in the global spatial and temporal properties of EEG during and shortly after acupuncture treatment of acupoint GB37. This result does not exclude the possibility that there is a relationship that can be shown experimentally between acupuncture and EEG. Changes in technique and updated equipment may yield a completely different result. Electro-acupuncture has been shown to produce more widespread fMRI signal increase than manual acupuncture in the brain and would be the next natural step in the present type of study. Studies of low (2 Hz) and high (100 Hz) frequency electro-acupuncture stimulation resulted in differences in fMRI signal intensities and would be added to the future EEG study. The use of 124-ch EEG, instead of 84-ch, with updated processing equipment may yield more detailed and precise data and would certainly be part of any further acupuncture/EEG study. Lastly, there may be no measurable EEG result from acupuncture or there may be a relationship that has yet to be discovered.

Proteomics

The purpose of this study was to begin to understand the protein dynamics involved in the production of GAD, the rate-limiting enzyme in the production of GABA. The protein profiles of wild type, heterozygous and homozygous GAD knockout mice provide metabolic snapshots of the dynamic changes that occur in the brain as it regulates GABA. The best analytical procedures and data processing

algorithms available were used and evaluated for further testing. Both produced highly reproducible and reliable data, establishing a worthwhile technique that produces high quality results that justify the high cost of this particular type of experimentation. This study contains a small number of samples and is seen as a necessary "first step" to establish reliable and efficacious protocols and procedures. This study has accomplished this goal and further studies are planned with larger numbers of samples, so that more definitive results and conclusions can be made. An additional 48 runs (3x4x4 =48) will be needed for the next phase of the study.

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