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EFFECT OF OPTOGENETIC STIMULATION ON NEUROPLASTICITY OF THE EMBRYONIC CHICK MOTOR SYSTEM

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By

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AN ABSTRACT OF THE THESIS OF

ERNEST KWESI OFORI, for the Master of Science degree in BIOMEDICAL ENGINEERING, presented on May 21, 2014 at Southern Illinois University Carbondale.

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MAJOR PPROFESSOR: Dr. Andrew Sharp

There is growing knowledge that neuronal circuitry undergoes alteration throughout development. Experience plays a key role in the reorganization of neuronal circuitry through the various mechanisms of learning. For example, when an animal is deprived of sensory input such as light in one or both sides of the eye, it can result in blindness on that side. In a study of rats placed in either isolated or enriched environments, those placed in enriched environments performed better on learning tests (maze test) than those placed in isolated environment. There was increased neurogenesis, synaptogenesis, myelination and angiogenesis in rats placed in enriched environments. These were all as a result of learning, which induces neuroplasticity in the nervous system.

The goals of this study were to determine how evoked movement is altered by changes in key parameters of light stimulation: intensity and period and to determine if one hour of light (optogenetic) stimulation could give rise to plastic changes in the nervous system as indicated by alterations in spontaneous motility.

To ascertain how evoked motor activity influences neuronal activity through learning and experience, optogenetics was employed to evoke movement in an embryonic chick at embryonic day nine (E9) after electroporation of a channelrhodopsin variant, ChIEF, into the neural tube. I first attempted to determine the optimal intensity needed to cause neuroplasticity in an embryonic chick by varying current to a LED light to produce three different light intensities. A protocol of 5 pulses of light with a period of 2 seconds was used to illuminate the right leg of 5

embryonic chicks with each intensity. To determine the optimal period of stimulation, I varied the period to 3 s and 4 s with one animal. Stimulation for an hour with a training protocol of 1800 pulses/hour (with a period of 2 s) of blue light (470 nm) was then used to illuminate the right thigh of the embryonic chick.

There were varied responses to light of all intensities used for stimulation, but high light intensity (maximum – 100%) seemed to have produced the best responses in terms of producing the largest joint angle changes and shortest latencies of movement in all joints of the leg of embryonic chick. Movements of the hip and ankle joints were the most robust. This was closely followed by those of the mid (83.33%) intensity. Therefore, it can be inferred that the greater the intensity of light, the better the response. The training protocol did not produce significant changes in embryonic activity. There were some decreases in joint angles and variable spontaneous movement duration in all animals used but there could be some changes going on at the neuronal or muscular level which were beyond the scope of this study to investigate.

It is my hope that this study will provide some knowledge pertinent to the treatment or management of neurodevelopmental disorders that may result in paraplegia or Erb's palsy.

DEDICATION

I would like to dedicate this thesis to all those who have helped in diverse ways to bring this to a successful conclusion.

I would like to dedicate this thesis to my sister, Angela Ofori (Mrs. Adu-Prah), her husband, Dr. Samuel Adu-Prah, and to my family members for all their prayers, love and support throughout my stay in Carbondale.

ACKNOWLEDGEMENTS

I would like to thank Dr. Andrew Sharp for his invaluable guidance, assistance and insights leading to writing this paper. My sincere thanks also go to the other three distinguishable members of my graduate committee for their patience and understanding during the two years of effort that went into the writing of this thesis.

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CHAPTER 1

INTRODUCTION AND BACKGROUND

Until the 20th century the brain and its neurons were believed to be immutable after a critical period in development. Before then, neuroscientists, Ramon y Cajal (1893) and Tanzi (1893) had suggested that developmental plasticity is still present in adulthood, but this was not proven until one hundred years later (reviewed in Markham et al., 2007). It is becoming common knowledge that experience actually influences the behavior of an organism. This has been shown in the gill withdrawal reflex in a marine snail (Aplysia). After constant excitation of the gill, the snail learns to reduce its response or to completely ignore the stimulus (reviewed in Kandel et al., 2000). It is possible that the snail may have trained its system to respond better or to ignore the stimulus as a result of plastic changes occurring in the nervous system. It has now been postulated that the brain and neurons remain malleable into adulthood. This was especially evident in a study where neuronal cells were labeled with bromodeoxyuridine (BrdU) to track neurogenesis in these cells. It was established that there was formation of new neurons in the neocortex of primates such as macaque monkeys as well as in the dentate gyrus and olfactory bulb of mammals in adulthood (e.g. Gould et al., 1999; Kornack & Rakic, 2001; reviewed in Rakic, 2002). Neurons in the brain continue to adjust and develop many more synaptic connections in order to meet the body's demands for sensorimotor activity (Clifford, 1999). For example, studies of sensory system such as the ocular system of cats, monkeys and frogs have shown that complex sensory experiences at early stages of development cause changes in neuronal circuitry (reviewed in Kandel et al., 2000).

Despite extensive studies showing how sensory experience causes neuroplasticity and affect behavior in various animals, there have been few studies on how motor experience influences neonatal or fetal plasticity. In particular, it is unclear how motor experience affects the neuronal circuitry of mammals and birds at the fetal stage of development. Studies have shown that cataract removal in infancy does not result in any visual impairment in relation to those removed in later life. Studies done on newborn monkeys nurtured in the dark for first 3-6 months of their lives resulted in visual impairment of differentiating between simple shapes when brought to normal visual world. These monkeys took weeks or months to distinguish between shapes whereas normal monkeys took only days. In a monocular deprivation study done by Hubel and Wiesel (1977), in which monkeys were raised from birth to 6 months with one sutured eye, there was blindness in the sutured eye when exposed to light six months later. There were changes in the visual cortex such that it did not respond to inputs from the deprived eye (reviewed in Kandel et al., 2000). For effect of motor experience on brain plasticity, Pons et al., (1991) also demonstrated this, in a deafferentation study, in which either left or right hand of monkeys were severed and the adjacent representation area of somatosensory cortex were examined few years later. It was then established that there was a significant plasticity of at least 10-14 mm encroachment (i.e. at least one order magnitude greater than what had been previously reported) of representation area of the intact hand into the representation area of the severed hand. There was some compensation by the representation area of severed upper limb of the cortex that it responded to inputs from the normal limb. This implied that the representation area of the severed upper limbs responded to inputs from adjacent representation area (reviewed in

Clifford, 1999). In rat embryo at embryonic day 17-21 (E17-21) (Galli & Maffei, 1988), there was segregation of retinal inputs in the thalamus that was determined by spontaneous, coordinated neural activity of retinal neurons before eye opening at birth. The spontaneous and coordinated firing of groups of retinal afferent fibers may cause plasticity by strengthening synapses (reviewed in Kandel et al., 2000). Therefore, this suggests that experience or environment plays a key role in influencing plasticity or reorganization of neural pathway of the nervous system. There is the need to show if motor experience also influences spontaneous movement of an embryonic chick. It is believed, this would cause some changes in the neuronal circuitry that modulates spontaneous movement of an embryonic chick.

Neuroplasticity

Neuroplasticity is defined as brain, synaptic or neuronal changes that occur as a result of experience, changes in behavior or after bodily injury (Davidson & Lutz, 2008; Pascual-Leone, 2011; Pearson-Fuhrhop & Cramer, 2010). Research indicates that experience can actually change both the brain's physical structure and functional organization (Chaney, 2007). This suggests that the use of a body part causes an increase in brain size and responsiveness. In a study of trained and untrained owl monkeys using a frequency-discrimination task, the representation map of the Area 3b of somatosensory cortex of trained monkey’s hands were found to have increased significantly in size (by 1.5 to >3 times) over that of the untrained owl monkeys. Additionally, the trained monkeys responded better to frequency change than the untrained monkey (Recanzone et

al., 1992). The alteration in neuronal circuitry is depicted in changes in synaptic number and/or strength (Pearson-Fuhrhop & Cramer, 2010).

As a result of the malleability of the nervous system, we can retain information and learn to adapt to different kinds of new experiences in life. For example, early sensory experience plays a critical role in the development of ocular dominance of cats, monkeys and frogs by causing changes in neuronal circuitry of the ocular system (reviewed in Kandel et al., 2000). Genetic factors may also play a role in normal brain development of mammals by altering neuronal circuitry through single nucleotide polymorphism (SNP) on the gene for human brain-derived neurotrophic factor (BDNF) may result in decreased neuronal repair and reduced brain plasticity. BDNF plays critical role of plasticity through both short- and long-term memories. Also a pair of SNPs on the gene for apolipoprotein E (ApoE) can result in gene variants ApoE2-4, which is linked to accelerated decline in cognitive function and decreased plastic changes. ApoE plays a major role in controlling repair, remodeling and protection of neurons (Kandel et al., 2000; Pearson-Fuhrhop & Cramer, 2010).

Synaptic plasticity, which occurs at the cellular level, is manifested as alteration in the number and/or strength of connections between neurons and results in a reorganization of representational maps. The strength of synapse is then shown in increases in the number and size of each synapse (reviewed in Markham et al., 2007). A mechanism of neuroplasticity is long-term potentiation (LTP), which is defined as continuous increase in synaptic strength or efficiency of two neurons as result of excitatory stimulation of the presynaptic neuron with a high-frequency stimulus (Pearson-Fuhrhop et al., 2010).

Types of Neuroplasticity

Three main types of neuroplasticity occur during regular maturity, namely; experience-independent (e.g. Shatz, 1992; reviewed in Clifford, 1999), experience-expectant and experience-dependent (e.g. Black & Greenough, 1998; reviewed in Clifford, 1999).

The first type of neuroplasticity is experience-independent plasticity, which is described as changes that occur in the brain without any influence from external environment or experience but through spontaneous activity. This type of neuroplasticity plays a critical role in the normal development of the brain. A typical example of this plasticity is the growth of the layers of the lateral geniculate nucleus of the thalamus, which transports signals to the visual cortex (e.g. Shatz, 1992; reviewed in Clifford, 1999).

According to Hebbian plasticity, synapses are reinforced or weakened depending on similarities or differences in the timing of their presynaptic or postsynaptic activity. Some of these synapses (Hebbian synapses) become stronger when presynaptic and postsynaptic neurons are stimulated to fire action potentials simultaneously (e.g. Buonomano & Greenough, 1998; Hebb, 1949; reviewed in Clifford, 1999). Other synapses are weakened or lost when their neurons do not fire simultaneously (Shatz, 1992; reviewed in Clifford, 1999). Humans and other mammals produce neurons and synapse in abundance and spontaneous activity initiates continual sprouting of neuronal projections from the brain, and therefore all neurons that are not used during spontaneous activity lose their responsiveness and eventually die (reviewed in Clifford, 1999).

The next type of neuroplasticity is experience-expectant plasticity, which occurs when developmental changes in the brain are influenced by feedback from the exterior environment (Clifford, 1999). In this type of plasticity, the genome for neuronal growth and reorganization is preset to react to inputs from the environment and henceforth to drive the development of the neuronal circuitry. The genome is then instructed to expect particular experiences for proper development to take place. The issue of sensitive or critical period (a short time) of development comes to play in this type of plasticity, and it is species and system specific, when an organism is ideally trained to react to a specific type of input from the environment (Markham et al., 2007). In the sensitive period of the development of neuronal circuitry, the absence of a heuristic feedback can result in a very destructive damage than in an adult (Clifford, 1999; Shatz, 1992). For example in humans, cataracts in children can result in blindness if not treated properly, while cataracts in adults only cause visual impairment until surgical removal (reviewed in Clifford, 1999).

A perfect example of experience-expectant plasticity is the formation of ocular dominance columns in the visual cortex. The development of the ocular dominance column depends on inputs from the left and right visual fields in order to fully develop and organize itself in alternating stripes, with each receiving input from the left and right visual fields. For instance, if there is visual deprivation to a cat at birth, its visual cortex does not develop properly into the normal left-right striped pattern; instead, there is overlap of axons projecting to the visual cortex from separate fields (e.g. Hubel & Wiesel, 1962, reviewed in Clifford, 1999).

The last type of neuroplasticity is experience-dependent plasticity, which occurs in features of the brain or nervous system that can be changed by experience (internal or external environment). These features of the brain therefore need experience to develop (Clifford, 1999). The experience-dependent plasticity is not influenced by the genome of the organism and it is characteristic of a particular individual. Learning is a form of this type of plasticity, which causes changes in the brain and can occur anytime across the lifetime (Clifford, 1999; Markham et al., 2007). These changes caused by learning occur at the neuronal level by strengthening or weakening of synapses depending on relative timing of neuronal activity (Hebbian plasticity). The changes in neuronal circuit can lead to changes in the structure of the cortex. For example, Pons et al. (1991) demonstrated through a deafferentation experiment (in which one limb is severed) in macaque monkeys, that there was an increase in the order magnitude of about 10-14 mm of neuronal projections form representation maps of cortex, greater than previously reported (1-2mm) (reviewed in Clifford, 1999).

Features of neuroplasticity

In the developing nervous system, which undergoes experience-independent plasticity, there is interplay between excessive formation of neurons and synapses (controlled by the genome) and their subsequent pruning when they are not used. This falls into the notion of nature that cells that are called into action grow and those that are not used eventually die. The excessive production of neurons and synapses falls in line with the expectation for internal or external stimulation to accurately mould the brain or nervous system and therefore neurons or synapses die when they are not used. By Hebb's plasticity theory,

synapses are strengthened or weakened when their neurons fire at the same time (e.g. Hebb, 1949; reviewed in Clifford, 199)

It has been shown in monkeys (e.g. Booth et al, 1979) and in humans (e.g. Huttenlocher, 1979; Huttenlocher & Dabholkar, 1997) that experience-expectant and experience-dependent plasticities occur during their lifespan and that human's nervous system reacts the same way as that of an animal to premature sensory deprivation, treatments of environmental complexities and skill training (reviewed in Markham et al.,2007). There is a similar synaptic production followed by synaptic pruning characterized by cellular pattern, increased volume of grey matter and subsequent postpubertal decline in adult values in pediatric neuroimaging studies (e.g. Giedd et al., 1999; reviewed in Markham et al., 2007). There have not been further studies with humans due to ethical consideration, but information from animal studies could have direct implications to humans due to the similar processes of plasticity occurring in both humans and animals.

Another feature of neuroplasticity is the increase in size of astrocytes in the cerebellar cortex. Astrocytes provide biochemical support to the brain. The hypertrophy of the astrocytes (although transient) followed by synaptogenesis, which occurs as result of learning, may be due to a response to increased metabolic activities of neurons in skill training and to some extent physical activity. This was evident in a study in rats with motor skilled tasks such as acrobats and motor control (e.g. Kleim et al., 1997; reviewed in Markham et al., 2007). Astrocytic changes as a result of experience are necessary to improve and stimulate adaptive changes in the neuronal circuitry of the brain.

Alterations in the cerebrovascular system are stimulated by repeated motor activity. Black et al. (1990) showed in a study in active and inactive rats that, both motor skill learning and regular physical activity also stimulates angiogenesis in the cerebellar cortex of rats.

Although, experience-induced changes of myelination have not been extensively studied, it is believed that increased axonal activity that results in motor activity leads to increased myelination of axons during the critical period of development as a result of learning. This would therefore results in increased conductivity of action potentials by the axons (reviewed in Markham et al., 2007) and lead to decreased latencies and increased joint angles.

Mechanism of Neuroplasticity

Habituation is a mechanism of learning which results in decrease in responses to a stimulus after prolonged exposure. It is involved in motor-dependent implicit learning. Habituation always follows sensitization, which is the initial increase in response when exposed to stimuli. The decreased response in habituation may be due to fatigue or as a result of reduced synaptic efficiency within the pathways of the motor system which had been continually stimulated. There is also a reduction in synaptic strength between excitatory interneurons and motor neurons. A typical example of habituation was depicted in the gill withdrawal reflex in marine snail (Aplysia) and limb-withdrawal reflex studies in human, which observed a decrease in reflex response after repeated activation. Habituation is homosynapitc and could either be short or long-term. To produce long term memory, spaced training with rest is more effective than mass training

with no rest (reviewed in Kandel et al., 2000). Habituation is therefore as a result of decreased synaptic strength from reduced transmitter release leading to a decreased response to stimulation. Figure 63-2 in Kandel et al. (2000) depicts decreased synaptic connections with habituation in Aplysia.

Facilitation or sensitization is the enhancement of postsynaptic potentials (PSPs) which are evoked by stimuli that closely follow each other. This mechanism occurs in synaptic plasticity that is brief, lasting from tens to hundreds of milliseconds and involved in implicit learning. The mechanism underpinning facilitation is the concentration of calcium ions at the presynaptic terminal and greater release of neurotransmitter at the synaptic vesicle as a result of firing of action potentials that are close to each other. It occurs in multiple synapses and therefore increased synaptic strength is stimulated by interneurons, as depicted in the gill withdrawal reflex of marine snail. Sensitization therefore results in an increase in synaptic strength due to presynaptic calcium ion concentration. Figure 63-6 in Kandel et al. (2000) gives a descriptive summary of decreased synapses with habituation and increased synapses with sensitization in Aplysia.

Long-term potentiation (LTP) is the one mechanism that underlies learning and memory. It is the long-term increase in the transmission of impulse between neuron and leads to the simultaneous firing of action potentials by these neurons. As a result, there is increased synaptic strength that underpins learning and memory. LTP is a phenomenon necessary for storage of explicit memories in the hippocampus. Two main properties come to play here; one of the properties is cooperativity, which refers to the simultaneous activation of a critical number of presynaptic neurons. These presynaptic neurons interact

with each other in order to initiate LTP. In order to elicit LTP, the frequency of stimulation and the strength of stimulation must be inversely related. The postsynaptic cell must therefore be adequately depolarized to conduct current (mainly calcium ions) via the NMDA receptor channel (Malenka, 2003). Another property is associativity; which requires both pre- and postsynaptic neurons to be active simultaneously (Hebbs' law). The early phase of LTP includes glutamatergic conduction and postsynaptic processes that results in increased sensitivity of receptors to glutamate and enhancement of release of transmitter substance. In the latter phase of LTP, there is protein synthesis that leads to cellular changes and formation of new synapses. Figures 63-8 and 63-9 in Kandel et al. (2000) shows LTP resulting in increased synaptic responses in mossy fiber pathway to CA3 and Shaffer collateral pathway to the CA1, respectively of the hippocampus.

Embryonic chick

The embryonic chick has been preferred to rat and mouse fetuses for numerous studies due to its advantages. Some studies have shown a close resemblance of the development of the embryonic chick and that of human fetus. For example the growth of sensorimotor circuitry in the chicken embryo, in contrast to rats or mice, is closely similar to that of a human.

Early movements in the neck of embryonic human and chick occur much earlier than in that of rat. In humans, neck movements are observed between 7 and 8 weeks of gestation, in rats at E16, and in chicks (gestation – 21days) at E3.5 (Hamburger, 1963; reviewed in Sharp & Bekoff, 2001). It can, therefore, be deduced that the development of

the human fetus is relatively more similar to the development of the chick embryo than to that of other animal models due to the fact that major events occur during each trimester of development. At embryonic day nine, E9 of the chick, motility is characterized by an established half-center alternation of extensor and flexor muscle synergies, which form the foundation for mature motor patterns such as walking (Bekoff, 1992; Sharp & Bekoff, 2001). Extension and flexion of leg joints has been shown to be coordinated from E9 to E13. Electromyography recordings have shown a high level of coactivity of the leg muscle synergies and the half-center type alternation of extensors and flexors (Sharp & Bekoff, 2001).

The fact that embryonic chicks grow outside the mother in an egg provides us with easy access and with the ability to easily perform various experimental treatments without any injury to the mother. Compared to other animal models, the chick has two legs for walking or bearing its weight. The number of spinal nerves in chickens is relatively similar to the number in humans. Humans have 31 pairs of spinal nerves whereas chickens have 29 pairs of spinal nerves.

Optogenetics

Optogenetics is the use of light and genetics (light-activated ions channels) to modulate neuronal activity in an organism (Deisseroth, 2011). It is a non-invasive technique with the advantage of reduced fatigue over microelectrode stimulation (Li et al., 2011; Llewellyn et al, 2010). These light activated ion channels are mostly derived from unicellular microorganisms such as algae by genetic engineering techniques. The light-gated ion channels are activated by light of a particular wavelength in the

electromagnetic light spectrum. Examples of these ion-gated ion channels are channelrhodopsin and halorhodopsin.

ChIEF, a variant of channelrhodopsin-2 (ChR-2), is a light-gated ion channel that has an excitatory effect on neurons because it is a mixed cation conductance channel that allows predominantly sodium ions into neurons when it is activated by blue light in the region of 470nm. This depolarizes the neurons and can cause them to fire action potentials. It has been artificially engineered to possess better conductibility of ions and therefore, stimulates neurons optimally to fire action potentials. Even under constant light only 33% of its active sites are inactivated while other variants like native ChR-2 have 77% of their active sites inactivated (Lin et al, 2009).

Optogenetic approach has been used to stimulate oocytes from the African clawed frog (Xenopus laevis) and cause depolarization of cell membranes by incorporation of channelrhodopsin-2 (CHR-2) into the cells (Nagel et al., 2003). It has been used to modulate activities of the central nervous system such as breathing and vision and the study of Parkinson disease, fear and pain (reviewed in Boyden, 2011). Also, it has been employed to demonstrate organized recruitment of motor neurons in mice *in vivo* by use of ChR-2 and an optical cuff around the sciatic nerve (Llewellyn et al., 2010). More importantly, it has been used to evoke movements in an embryonic chick by the integration of ChIEF into the neural tube (Sharp & Fromherz, 2011). Finally, it has been employed to show plasticity in the somatosensory cortex of rats by reducing inhibition of deprived cortex (Li et al., 2011).

Previously, electroporation was used to deliver into the neural tube of the embryonic chick, a special fusion construct of pPB-ChIEF-Tom and CAGPBase. (Sharp

&Fromherz, 2011). Electroporation is a minimally invasive technique of introducing DNA into an organism (Neumann et al., 1982; Sharp and Fromherz, 20011). Even though there are various methods of introducing genes into an organism, the PiggyBac (PB) transposon system is a preferred system, due to its advantages over other forms of transfection such as the recombinant lentiviral system. The PB transposon system allows for stable incorporation of DNA into the genome of the chicken embryo and it also provides rapid transgenic expression (Lu et al., 2009), without the use of any extensive viral control mechanism in the laboratory.

To verify the expression of ChIEF in the neural tube, Tomato (Tom) is excited with green light (~550nm) without exciting ChIEF at the same time. Due to ChIEF-Tom being a fusion construct, an expression of the reporter molecule (Tom) will signify the presence of ion channel (ChIEF) in the target tissues (neural tube).

With growth of the nervous system of the embryo, the DNA gets incorporated into transformed nervous tissues and therefore, stimulation with blue light (~470nm) positioned around the thigh could be used to excite motor neurons (fused with ChIEF) of the leg at E9 in order to cause movement. Excitation of ChIEF causes the ion channels to open for sodium ions to enter neuronal cells and result in action potential. The release of neurotransmitter (acetycholine) at neuromuscular junction will result in chemical changes in muscles for contraction to occur. The contraction of the muscles will then result in movement of the leg.

Specific Aims

Hypothesis

It is general knowledge that spontaneous motor activity contributes to the establishment of the nervous system. We hypothesized that the embryonic neurological system is plastic and that driving motor behavior optogenetically could affect the neuronal circuitry that controls behavior and that movement would change.

We therefore developed a strategy to optogenetically manipulate the motor activity of embryonic chick in order to understand how the embryo uses motor experience to affect its normal movement and additionally provide us the ability to affect the development of embryonic chicks.

The main goal was to use optogenetic manipulations of leg movement of an embryonic chick to alter subsequent movement as a result of altered neural circuitry. In order to determine if embryonic neural system is plastic and that activity plays a role in neuronal reorganization, we used electroporation of ChIEF-Tom into the neural tube of an embryonic chick and employed optogenetic manipulation with different parameters of light to cause a change in movement. We anticipated that change in movement would result in neuronal changes that would affect subsequent movement pattern.

Specific Aim 1

To determine how evoked movement is altered by changes in key parameters of light stimulation: intensity and period

To determine if evoked movement is influenced by the intensity of light and period of stimulation**,** a range of light intensity obtained by adjusting the current to the

blue LED was used to illuminate the leg of embryonic chicks with 1s pulse duration for a period of 2s to adhere to normal frequency stimulation of embryonic chick. It was expected that there would be a direct input and output relationship between intensity of light and response as more of the ChIEF molecules are activated. Since 50% current capacity did not evoke any response, stimulations were performed with high (100% current capacity), mid (83.33%) and low (66.66%) light intensities. Stimulation was then performed with intensity that produced maximal behavioral response for long periods of 3s and 4s. To verify if increased intensity and period of stimulation have any effect on movement, joint amplitudes, onset latency, peak latency and relaxation times of evoked movement were determined.

Specific Aim 2

To demonstrate the ability to alter spontaneous movement after one hour of light (optogenetic) stimulation

We postulate that the embryonic neurological system is plastic and that driving motor behavior optogenetically would affect the neuronal circuitry that controls behavior. It was expected that, there would be an increased strengthening of synapses observable in increases in joint excursions due to saturation of all active sites of ion channels (ChIEF) that would evoke changes in subsequent spontaneous activity. It was also expected that after an hour of training, there would be increase in movement time or duration due to increased excitability of motor neurons and increased synaptic strength. This would lead to increased muscle strength and increased movement duration. There would be decreased inter-movement intervals (IMI) and an unchanged period of motility, due to increased spinal or motor excitability and therefore, animal tends to move more than rest.

Since the period is the sum of the movement time and IMI, it was expected to remain the same.

To test this hypothesis, the intensity of light that produced a maximal behavioral response was used. Periodic stimulation with the high intensity was used to stimulate the animal for an hour (total of 1800pulses/hour), for short pulse durations of 1s for a period of 2s. This would constitute a training paradigm such that the embryo would then replicate the same maximal joint angle when stimulation is taken off after an hour as a result of learning and experience. To verify if there was any change in spontaneous movement, the maximum, minimum joint angles, joint excursion and duration of episodes of spontaneous motility were determined.

CHAPTER 2

METHODOLOGY

General Methods

All procedures using embryos were in compliance with Southern Illinois University Institutional Animal Care requirements. While I learned all of the procedure described here, the electroporation and stimulation protocols that generated these data were performed by Dr. Sharp and I performed all the analysis of these data.

DNA construct and Electroporation

A rotating forced-air incubator was used to incubate fertile leghorn chicken eggs until embryonic day (E) 3. This corresponds to Hamburger-Hamilton stages 16-18 (Hamburger, 1963). A 10µL syringe was used to draw 3-4 ml of albumin from the tapered end of the egg. This allowed the embryo to drop down away from the shell and provided enough space to perform electroporation. Each side of the marked center line was taped to provide support. With the help of a pair of scissors or forceps, a puncture was made at the top side of the egg and a round hole about 1.5 cm in diameter was carefully created in the shell to expose the embryo. The embryo was then placed on a stage under a stereomicroscope (Wild M5A) to locate the neural tube. A micropipette, made from borosilicate glass tubing (10 cm in length with filament, O.D.=1.0 mm, I.D.= 0.50 mm, Sutter Instrument Co., Novato, CA) with a P-97 micropipette puller (Sutter Instrument Co., Novato, CA), was used to deliver DNA into the embryo. DNA, a mixture of pPB-ChIEF-Tom and CAGPBase (Lu et al., 2009; Sharp & Fromherz, 2011), was drawn into the tip of the pipette with suction. The pipette was then mounted on a

micromanipulator and the tip of the micropipette was lowered into the embryonic neural tube. The DNA was pressure-injected into the lumbosacral neural tube (20 ms pulses, 20 PSI) using a microinjection system (Picospritzer III, Parker Hannifin Corporation, Fairfield, NJ). Next, 0.5 ml of L-15 medium was placed over the embryo to provide a conductive path for electroporation. The electrodes (Pt-Ir, 0.25-mm diameter, 2-mm length) were placed on both sides of the embryo with a second micromanipulator. Voltage pulses (30-40 V, 7 pulses, 50 ms duration and 500 ms interval) were generated with an electroporation system (Gene Pulser Xcell, BIO-RAD, Hercules, CA). The electrodes were oriented such that the DNA was drawn into cells on the right side of the lumbosacral spine. Since chick embryos usually lie on their left side, the right side of the embryo is available for observation and optical stimulation (Sharp & Fromherz, 2011). A cotton swab was used to remove residue resulting from electroporation from the embryonic environment and electrodes. Lastly, the hole in the shell was taped shut and the embryo was returned to the incubator.

Verification of ChIEF Expression

In order to determine if electroporation resulted in successful transformation, embryos were removed from the incubator on E4-E6 for brief observation. Observation was performed with a fluorescence stereomicroscope (Leica MZ10 F) equipped with filter sets for GFP and Texas Red and a metal-halide illuminator. PPB-ChIEF-Tom incorporation gives rise to ChIEF expression as a fusion protein with the fluorescent reporter molecule, Tomato. Tomato is activated by green light (~550nm) and emits red light (~700nm). Therefore, the presence of red fluorescence was taken to indicate the

expression of ChIEF. By E6, successful transformation resulted in visible innervation of the developing leg.

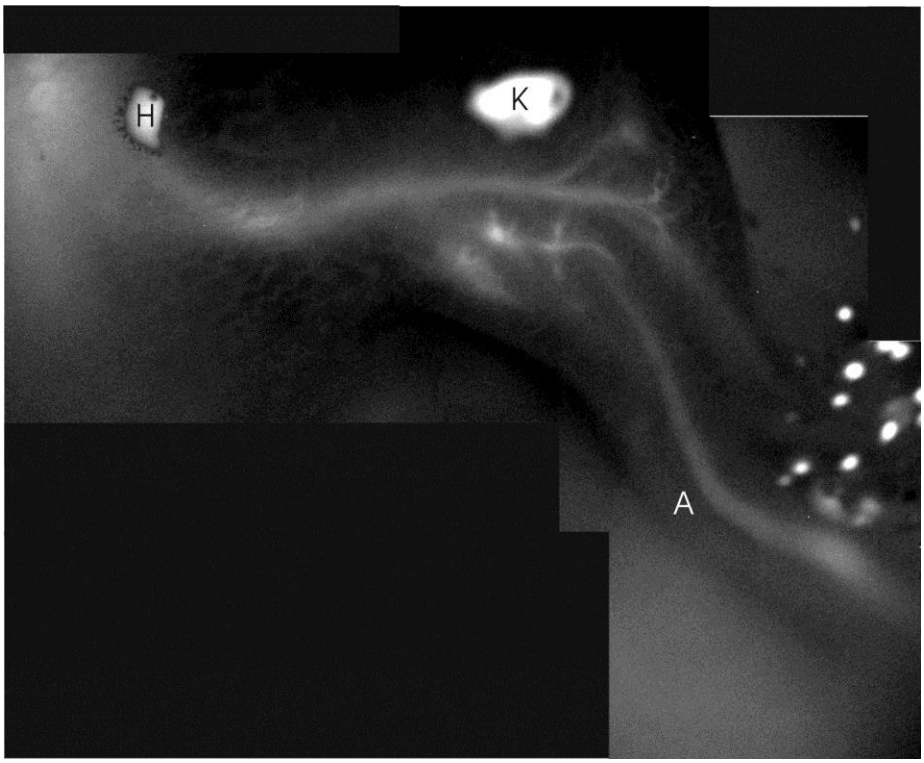

Figure 1. ChIEF expression in motor axons of the leg of a living embryonic chick at E9. H = Hip, K = Knee and A = Ankle. This was taken with a fluorescence stereomicroscope. ChIEF could be visualized as fluorescence in the axons to the lateral side of the right leg of an embryonic chick at E9.

Kinematic Recordings

Embryos were prepared at E9 for kinematic recordings (Sharp et al., 1999) and optogenetic manipulation (Sharp & Fromherz, 2011). Eggs were removed from the incubator and the hole was enlarged with a pair of curved scissors. The hole was made as large as possible without cutting into the extra-embryonic membranes. Each egg was then placed in a heated and humidified chamber under a stereo microscope (M3Z, WILD, Heerbrugg, Switzerland) that is fitted with a color CCTV camera (WV-CL 700, Panasonic, Edmonton, AB). To enable direct access to the embryo, a microsurgery was performed with dissecting scissors and forceps to remove the chorioallantoic and amniotic membranes. Caution was taken during this process in order not to cut any major blood vessels.

With the exposure of the embryo, retractors (made from fine wires) anchored to rubberized pads were glued to the back and tail in order to prevent the embryo from rolling out of plane during experimentation. It was sometimes necessary to push back the blood vessels and membranes with additional retractors in order to prevent them from obscuring the view of the embryo. Kinematic markers, small dots of nail polish were then applied to the lateral surface of the back, hip, knee, ankle and toes in order to delineate joint positions for kinematics. Movements were recorded digitally to a computer at 60 frames per second via a pyro A/V link (Part No: API-557 by ADS Tech; www. adstech.com, Cerritos, CA) with Adobe Premier Elements 4.0 software. The complete video recording was reviewed in Premier Elements and regions for kinematic analysis were selected and imported into a custom MATLAB routine developed in the Sharp laboratory. This routine was then used to capture the joint positions and to calculate the

joint angles in the x-y plane. Data for Aim 1 were analyzed at 30 frames/s and those for Aim 2 were analyzed at 10 frames/s. Further analyses of the data were performed in Excel (Microsoft Excel 2003).

Activation of ChIEF

In order to activate ChIEF, a focused blue (470 nm) LED (M470L2, Thorlabs, Newton, NJ) was used to illuminate about a one cm^2 area of the right thigh. The LED has a power output ranging from 830mW to 1560mW, with a maximum current of 1600mA. Current was delivered to the light-emitting diode (LED) using an LED driver or controller (LEDD1B, Thorlabs, Newton, NJ). The LED driver has an LED current limit set point ranging from 200mA to 1200mA. The LED driver was set to trigger mode to allow three current intensities: low - 66.67 % of current capacity; mid - 83.33% of current capacity; and high - 100% of current capacity. The number and duration of pulses were controlled with pCLAMP 10 software (Molecular Devices, Sunnyvale, CA). Blue illumination caused leg movement by excitation of the motor axons in the thigh which in turn activated the flexors and extensors of the legs. Also, embryos were constantly illuminated with a red LED (627nm, Super Bright LEDs; http://superbrightleds.com) via a power converter (MPU-250BT, MaxPower Corporation, Denver, CO) in order to provide sufficient illumination for constant video recording without activating ChIEF. A long pass filter (550 nm cut off, Thorlabs, Newton, NJ) was placed over the front objective of the microscope to prevent saturation of the camera's sensor during blue light activation. A small amount of green light was visible during the pulses of blue light which allowed visualization of the activation pulses.

Experimental Design

Specific Aim 1

To determine how evoked movement is altered by changes in key parameters of light stimulation: intensity and period

In order to determine if optogenetics can be used to affect the plasticity of neuronal circuitry of an embryonic chick, there was the need to first determine how changes in key parameters of light alter evoked movement of an embryonic chick. As embryonic motility is periodic, with episodes of movement separated by a period of quiescence, which varies from 10seconds to 2minutes (Sharp & Bekoff, 1999), stimulation with blue light was performed after 11s into the quiescence. This was done in order to separate an evoked behavior from the spontaneous activity of the embryo. Anytime an embryo moved spontaneously in the course of the experiment, the data was discarded and the experiment was repeated. After initial test of various light intensities, it was determined that light intensities lower than 2/3rd of the full current capacities were not able to evoke any observable movement. Also, it was difficult to see the lower light intensities in the video for proper digitizing with MATLAB. The lowest stimulation was then started with 2/3rd (66.67%) current capacity or low light intensity.

For this part of the experiment, 5 animals (A-E) were used for a 2-period protocol with 5 pulses blue LED light (~470 nm) for a duration of 1s each. Stimulation with all animals except one (A), started with the high intensity (full current) and regulate downward to the mid and low intensities. For Animal A, stimulation began with low light intensity and graduated to mid and high intensities. Illumination of the right leg was then performed using light intensity (high) that activated all the active sites of the ChIEF and

produced a maximum joint angle with different periods. Stimulation was performed with 5 periodic bursts of light, each with pulse duration of 1s for periods of 3s and 4s with one of the animals (D).

All experiments were video recorded. Analysis was then performed to determine the amplitudes (difference between peak joint angle and baseline of each joint) onset latency, peak latency and relaxation or fall times of evoked movement for each joint. To determine the baseline or resting joint angles, a 30s video prior to stimulation was collected, digitized and then a 95% confidence interval was calculated by using the formula $x \pm 2SD$ (where x = mean joint angle for each joint and SD = Standard deviation). To ascertain if evoked movement was significant, a joint would have to move beyond either the upper or lower confidence interval. Onset latency was determined by calculating the time difference between the start of an evoked movement and when the light pulse was turned on. Peak latency was calculated by finding the difference between time for a joint to reach its peak angle and the start of a light pulse. Relaxation time was determined by finding the difference between the time it took for a joint to return to its resting angle or baseline and the time for its peak angle. These values were then plotted in Microsoft excel.

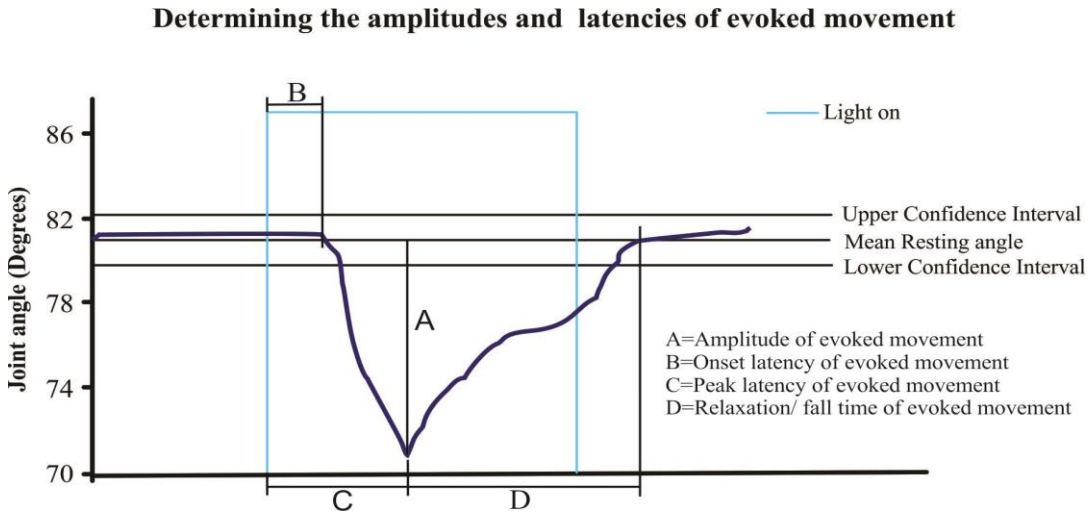

Figure 2. A diagram showing the determination of amplitudes and latencies of an evoked movement.

Specific Aim 2

To demonstrate the ability to alter neuronal circuitry that modulates spontaneous movement after one (1) hour of light stimulation

To determine if optogenetics can affect plasticity of an embryonic chick, the intensity of light that activated all ion channels and gave a reproducible maximum joint angle in the first experiment (for the first aim) was used for stimulation for an hour. This formed a kind of training program that I postulate would strengthen the synapses and would lead to an increased maximum joint angle and a change in the pattern of movement after stimulation.

Five (5) animals were stimulated for an hour with high light intensity, which reproduced maximum joint angles across pulses in the first part of the experiment.

Animals B to D from the first part of the experiment in addition to one extra animal were used for this training protocol. These animals were reassigned letters A to E, to avoid any confusion. Animals were stimulated for a period of 2s with pulse duration of 1s for an hour (a total of 1800 pulses/hour). To determine any change in joint angle and spontaneous motility, a 10-minute video recording was taken before the stimulation (pre-training), three 10-minute videos at specific times during the stimulation were recorded (1^{st} 10-minute, mid 10-minute and last 10-minute) and a 10-minute video after the stimulation (post-training) for each animal.

Analyses were done for maximum and minimum joints angles and joint excursion before, during and after stimulation to determine any change. This was done by collecting three videos of normal spontaneous movement at the start, midway and last part for each of pre-training, specific times during training and post-training. These videos were digitized in MATLAB and the maximum and minimum joint angles were determined in Microsoft excel. The mean and standard deviations were then determined for the three maximum and minimum joint angles calculated for each joint of each animal and plotted. Durations of movement, inter-movement interval and period of motility were determined by obtaining the start and stop times from the 10-minute video. Inter-movement interval was at least 10s before it was used; otherwise, it was still regarded as part of movement time. This is consistent with quiescence period of at least 10s for an embryonic chick at E9 (Sharp & Bekoff, 1999). The means and standard deviations were then calculated and plotted in Microsoft excel. The mean numbers and standard deviations of evoked movement or responses during stimulation for some of the animals (A and B) were also calculated and plotted in Microsoft excel.

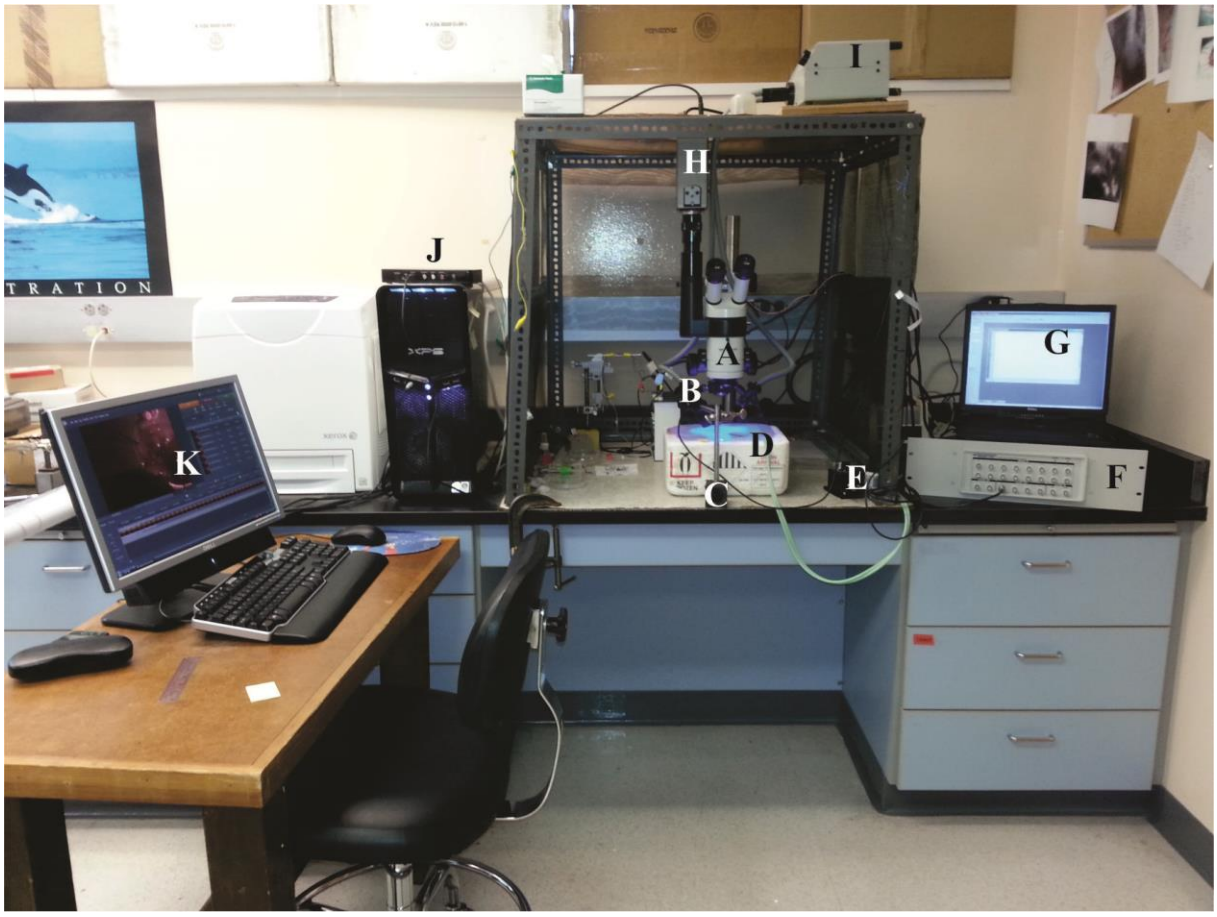

Figure 3. The Image of the experimental set up. Labeling: A = stereomicroscope, B = blue LED, C = LED stand, D = recording chamber, E = LED driver/controller, F = data acquisition system, G = p-clamp computer/controller, H = camera, I = Light source to microscope, J = pyro digital A/V converter/digitizer and K = video recording computer

CHAPTER 3

RESULTS

Optimal light intensity for stimulation

Effect of intensity on joint amplitudes

It was expected that with an increase in light intensity that there would be increase in the amplitudes of evoked joint movement. This follows from the fact that as the intensity of light increases the probability that ChIEF molecules will be activated increases. This should then result in increased activation of motor neurons of the leg, increased muscle tension and increased amplitude of movements. However the animals for this experiment showed variable results, which will be described in subsequent paragraphs.

Previous experiments described light evoking leg flexion (Sharp & Fromherz, 2011). However, in these experiments a combination of flexions and extensions was observed. For the five animals (embryonic chicks) used for Aim 1, there were light evoked hip flexions for all intensities across all pulses in 2 of the animals. The high and mid light intensities evoked hip extensions followed by flexions for several pulses in 2/5 of the animals (see Figure 4). In contrast, low intensity light evoked only extensions. In one animal that evoked both flexions and extensions with different intensities, amplitudes were generally smaller. In the knee, all intensities evoked flexions in 3/5 of the animals and both flexions and extensions in the others. High intensity light evoked mostly either flexions (2/5) or extensions (2/5) with all pulses. In the ankle, all intensities evoked only flexions in 2/5 of the animals. In the other 3/5, a combination of flexions and extensions

was observed. In 2 of these animals, extensions (increase in joint angle) occurred before flexions (reduction in joint angle).

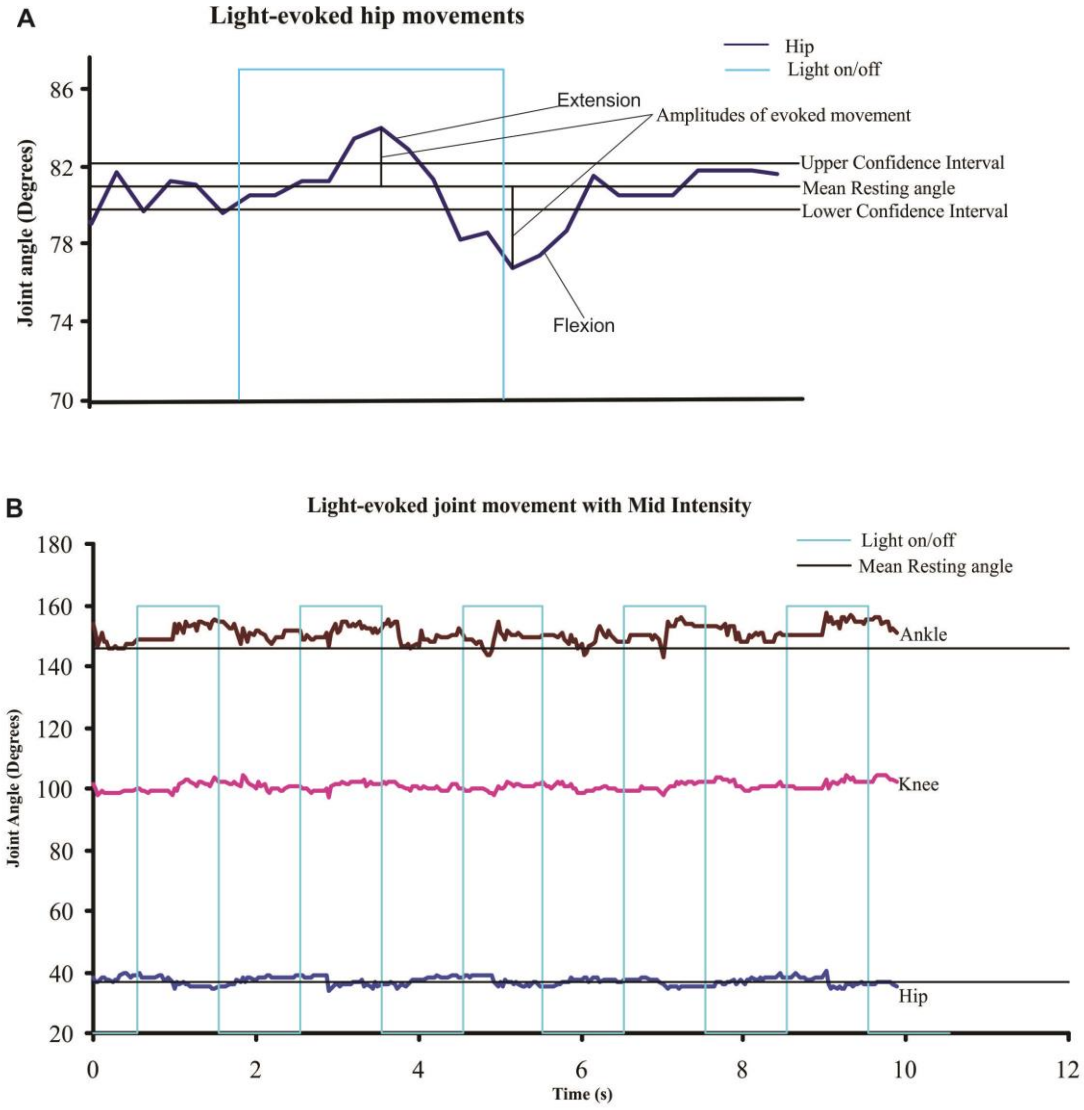

Figure 4. Light-evoked variable joint movement for a 2s period protocol. A. Evoked hip movements with one pulse of high intensity light showing extension and flexion (Data were taken from Animal E). B. Evoked joint movements with mid intensity light (Data were taken from Animal B). Hip extensions were evoked on 1st-3rd and 5th pulses while hip flexions were evoked from 1st to 4th pulse. Extensions occurred before flexions.

Effect of 1st light pulse on the amplitudes of evoked movement

The first pulse of light was very critical to this study since the animal has no recent exposure to light. Subsequent pulses could cause different responses due to changes in neuronal circuitry or muscle physiology. The following paragraphs will describe the results of each of the pulses of light intensities on movements of the hip, knee and ankle of the animals.

Effect of 1st pulse on evoked hip movements

Figure 5 shows an example of evoked movements in response to light of different intensities using the 2 s period protocols (animal C). Notice that the first pulse of low intensity light evoked only extension, while mid and high intensities evoked only flexion. Also, there was an increase in movement amplitude with light intensity. The maximum amplitude (8°) was evoked with high intensity light. Changing from flexion to extension as a function of light intensity was present in 3/5 of the animals. In one of the animals, extensions were evoked by only low and mid intensity lights. The mid light intensity also evoked flexion while the high light intensity only evoked flexion. In this animal, the amplitudes of evoked flexions increased with intensity from mid to high light intensity and there were also increased extension amplitudes with intensities from low to mid. The maximum amplitude (about 7˚) was evoked by the high intensity light. In the other two animals, the first pulse of all intensities evoked only flexions. For one of these animals, there was no evoked movement with the low intensity light, but there was an increase in amplitude from the mid (6˚) to the high (7˚) intensity light.

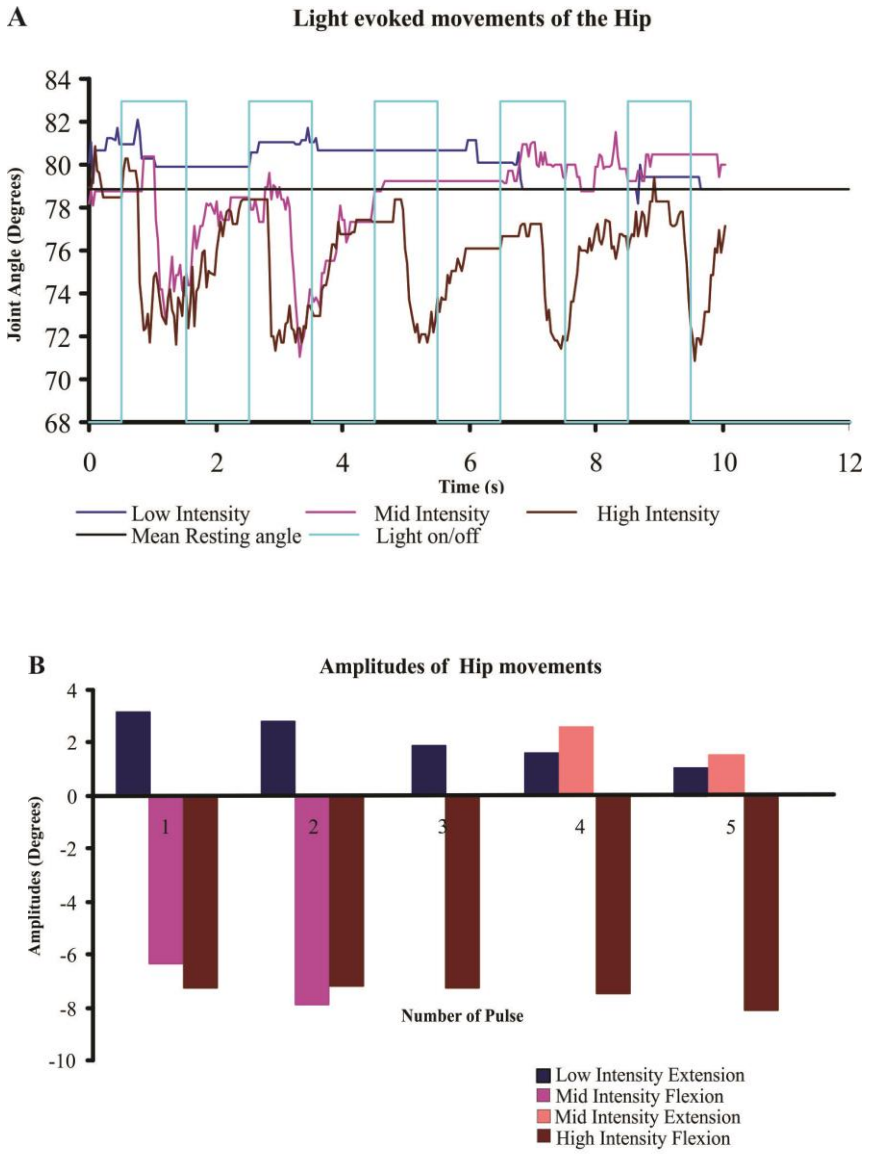

Figure 5. Hip movements evoked by light for animal C. A. Joint angle vs. time plots and B. Amplitudes of evoked movement of the Hip by the three Light Intensities for 2s period protocol. Consistent maximum amplitudes were evoked by High light Intensity. Mean Resting Angle = 79˚, (SD = 0.321).

Effect of 1st pulse on evoked knee movements

In the knee, there were evoked knee flexions in response to light of different intensities using the 2s protocol (animal C) as shown in Figure 6. Observe that there was an increase in amplitudes from the low to the mid intensity light and a slight reduction with high intensity light. The mid light intensity evoked the maximum amplitude. This was closely followed by amplitude of the high intensity. This pattern of evoked movement was present in 1/5 of the animals. The amplitude of the low intensity evoked was the smallest. In 2/5 of the animals, knee extensions were evoked with all intensities. In one of these animals, there was a decrease in amplitudes with increasing intensity while the other one had an increase in amplitudes from the low to the mid and a slight reduction with high light intensity. In the other 2/5 of the animals, there were variations of evoked flexion and extension by all intensities. One of them had a reduction in amplitudes from low to mid and increase with high intensity. The maximum amplitude of 13˚ was recorded with the high intensity.

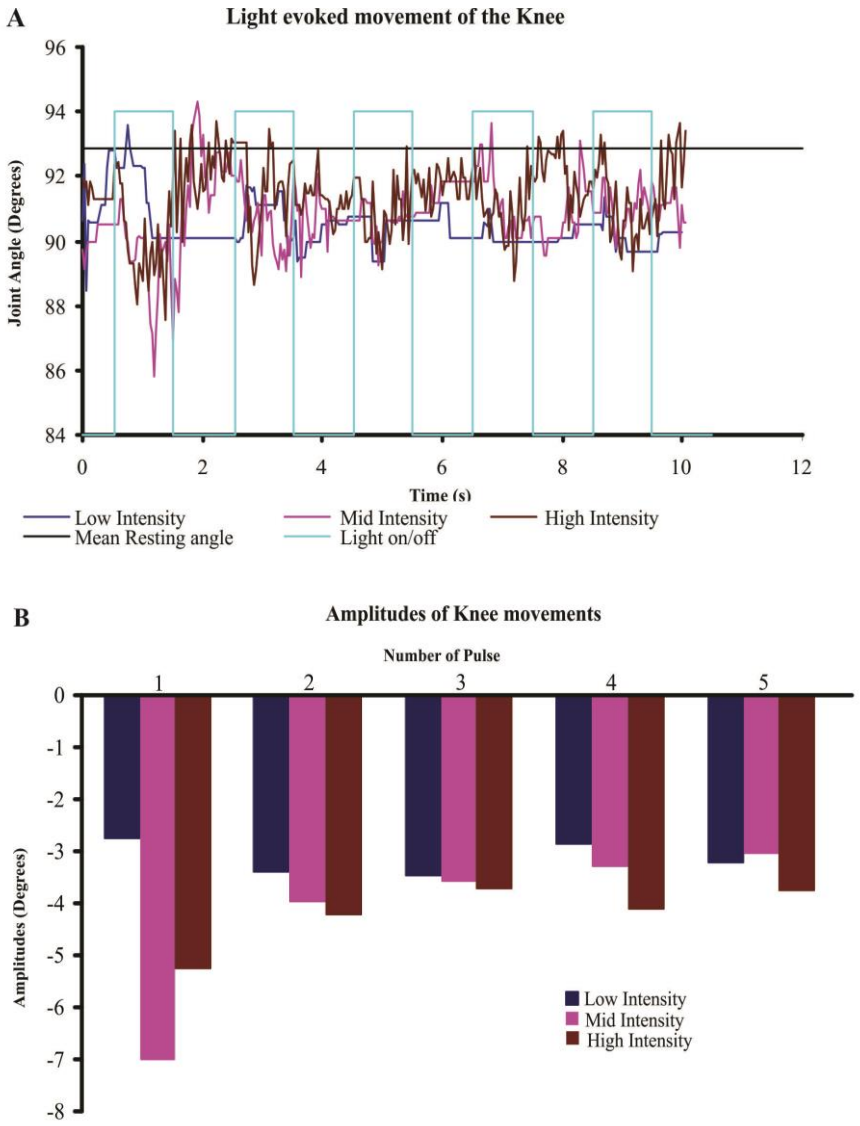

Figure 6. Knee movements evoked by light for animal C. A. Joint angle vs. time plot and B. Amplitudes of evoked movement of the Knee by the three Light Intensities for a 2s- periodic protocol. Consistent bigger amplitudes were evoked by High and mid light Intensity. Mean Resting Angle = 93˚ (SD = 0.314).

Effect of 1st pulse on evoked ankle movements

Figure 7 shows an example of evoked ankle flexions in response to the three light intensities using the 2s protocol. There was increasing flexion amplitudes with intensity, with the maximum amplitudes (22˚) evoked by the high intensity. Evoked flexions by all intensities were present in 2/5 of the animals. One showed an increase in amplitude from low to the mid intensity and a minor reduction with the high intensity. Maximum amplitude (23˚) in that animal was evoked by the mid intensity. The other two animals showed differential results with all intensities for the first pulse. In one animal, the high intensity light evoked extension before flexion and showed a minor reduction in extension amplitude from mid to high. There was no evoked movement with the low intensity. For the other animal, the high intensity evoked ankle flexions before extension. In this animal, there was a minor reduction in flexion amplitudes from the low to the mid light intensity and the high light intensity. Also, the low intensity did not evoke any movement while the high intensity light evoked the only extension. In 1/5 of the animal, there was a decrease in joint amplitudes from the low to the high intensity light, but increased extension amplitudes from the low to the high intensity light.

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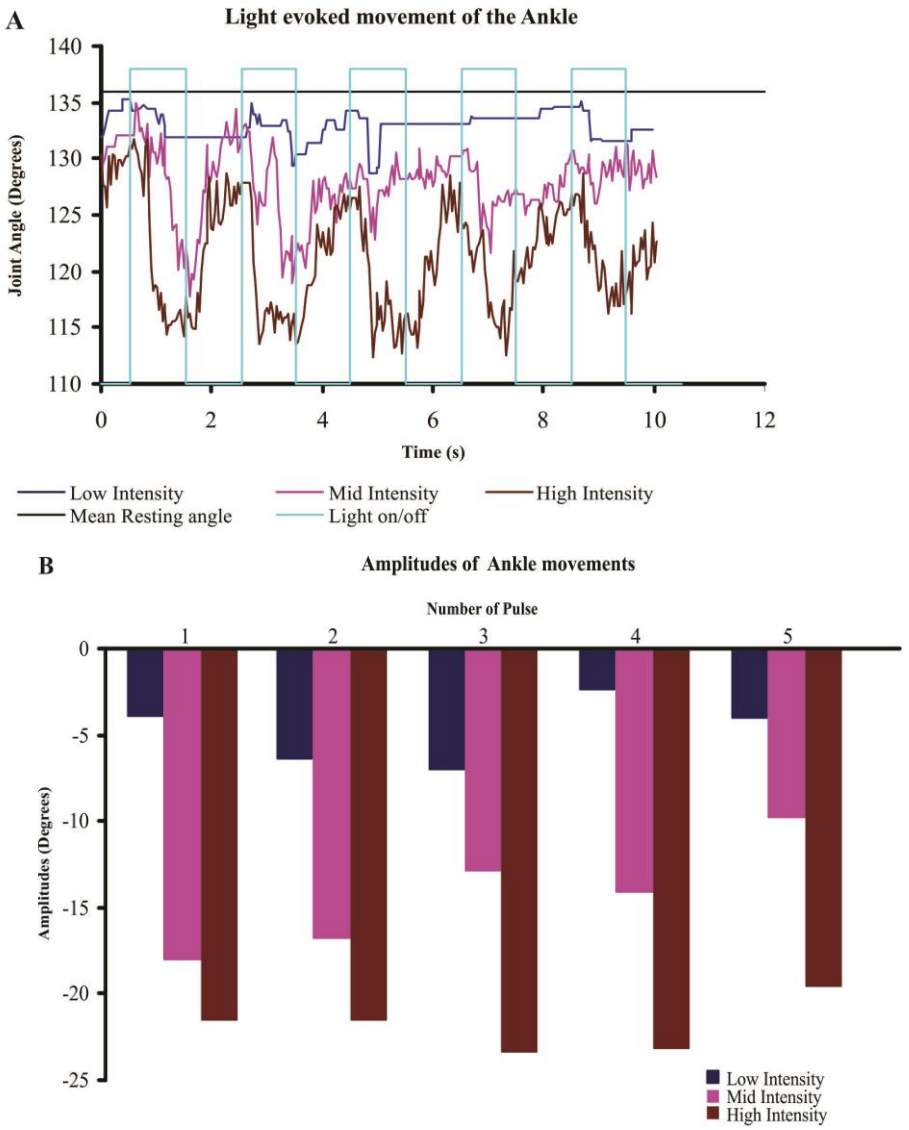

Figure 7. Ankle movements evoked by light for animal C. A. Joint angle vs. time plot and B. Amplitudes of evoked movement of the Ankle by the three Light Intensities for a 2s period protocol. Consistent bigger amplitudes were evoked by high and mid light intensity. Mean Resting Angle = 136˚ (SD = 0.712).

Effect of 2nd light pulse on amplitudes of evoked movements

With prior experience of the first light, it was expected that that would increase in amplitudes of evoked movements with increasing intensity with respect to amplitudes of evoked movements by the 1st pulse. This is because with higher intensities, there will be increased probability that ChIEF molecules will be activated. This will lead to increased rate of firing of action potentials by motor neurons. Thus, there would be increased muscle tension and contraction, resulting in increased movements. The results are as follows;

Effect of 2nd pulse on evoked hip movements

The low light intensity evoked extensions while both the mid and high light intensities evoked only flexions which decreased slightly with high light intensity as shown in Figure 5. The maximum amplitude (8˚) was, therefore, produced by the mid light intensity, closely followed by that for the high light intensity (7°). This pattern of extension occurring before flexion with light intensity was present in 3/5 of the animals. The other 2/5 of the animals exhibited flexion amplitudes in the hip. In one animal, there was a general increase in amplitude with increasing intensity. Amplitudes in this animal for the low and mid intensities were almost the same as those observed in the first pulse, the amplitude of the high intensity increased by about 1˚. In the other animal, which had the maximum amplitude of 10˚, there was a major reduction in amplitudes from the low to the mid light intensity and a sharp increase with the high light intensity.

Effect of 2nd pulse on evoked knee movements

As shown in Figure 6, there were evoked flexions, which showed general increased amplitudes with intensity. This was present in 1/5 of the animals with the maximum amplitude of 4˚ evoked with high intensity light. In 2/5 of the animals there were evoked extensions with all intensities. The first animal had bigger amplitude with the low intensity light, then decreased with mid intensity light and increased moderately with the high intensity light. In the other animal, there was an increase in amplitudes from the low to the mid and minor decrease with high light intensity. In 1/5 of animals, there were different evoked responses with the intensities. There were evoked flexions before extensions with the high intensity light, only flexion with the mid intensity light and extension with the low intensity light. Both knee flexions and extensions with this animal were decreasing with intensity. The last 1/5 also had extension with low intensity light and flexions with mid and high light intensities.

Effect of 2nd pulse on evoked ankle movements

The ankle showed increased flexion amplitudes with increasing intensity as depicted in Figure 7 (animal C). The high intensity light evoked maximum amplitude of 22˚. Evoked ankle flexions were present in 2/5 of the animals. In the other one of the animals, the ankle produced wider and bigger amplitudes with the mid and high light intensities across all pulses. The low light intensity evoked movements with increased amplitudes with increasing pulses until the 4th pulse and decreased on 5th pulse. However the amplitudes evoked with low light intensity were still smaller than the mid and high light intensities. Apart from the first evoked flexion that returned to its rest position, the

2^{nd} to 5^{th} peaks did not return to rest before the next light pulse. The ankle in this animal showed increased amplitudes from the low to the mid light intensity and a minor reduction with the high light intensity. The other 3/5 of the animals gave variable results with intensities; one of them showed flexion amplitudes only with high intensity and also extension with the mid and high light intensities, low light intensity did not evoke any extension. In the other animal, there was evoked flexion with the low and the mid light intensities and extension with the high light intensity. In the last one, there were evoked flexions with all intensities and extensions with the high light intensity. In all animals studied, the maximum amplitude of 23˚ was evoked by the mid light intensity, but joint amplitude by the high light intensity was not far from that by the high light intensity (21˚).

Effect of last 3 light pulses on amplitudes of evoked movement

With previous experience of the two light pulses, it was still expected that there would be increased joint amplitudes of evoked movements (in relation to the other two pulses) with increasing intensity across pulses due to increased tension in muscles. The rationale for this is that, the probability that more ChIEF molecules will be activated is increased, thereby leading to increased rate of firing of action potentials by more motor neurons. The increased rate of firing action potentials would lead to increased muscle tension due to increased muscle contraction. This will therefore result in increased joint movement. However, there were variable observable results in the joints of the animals.

In the hip, there were evoked extension with the low and the mid light intensities and flexions with the high light intensity for a 2s period protocol as shown in Figure 5.

Flexion amplitudes evoked by the high light intensity increased across pulses. Extensions with the low and the mid light intensities decreased respectively from 3^{rd} – 5^{th} and 4^{th} -5^{th}, which is shown in Figure 5. The high intensity light had maximum amplitudes (about 9˚) in the hip on the 5^{th} pulse in one of the animals. This pattern of variation in evoked movements with intensity was present in 3/5 of the animals. One animal showed differential results by the evoking both flexions and extensions with low and mid light intensities, but the high intensity evoked flexions across pulses and extensions on the 3^{rd} and 4^{th} pulses. In the last animal, the high intensity light evoked extensions before flexions, with extension amplitudes decreasing from the 3^{rd} to the 5^{th}pulse. The low and mid light intensities evoked only flexions with the low intensity evoked the maximum hip flexion on the 5^{th} pulse in that animal and the high intensity light evoking the minimum on the 4^{th} pulse. There were flexions for the 2/5 of animals. In 1 of these 2 animals, the low and mid light intensities did not evoke flexions from the 3^{rd} - 5^{th} pulse and from 4^{th} – 5^{th} respectively. The high intensity light evoked increasing flexion amplitudes from 3^{rd} to 5^{th} pulses. In the other 1 of the animals, the low intensity light evoked amplitudes bigger than that of the 2^{nd} pulse, reduced slightly on 4^{th} and reduced further on 5^{th} pulse.

In the knee, there was an observable increasing amplitudes with increasing intensity as shown in Figure 6. This pattern was present in 1/5 of the animals (animal C). In 2/5 of the animals there were only extensions with all intensities while in the other 2/5, one produced both flexion and extension with high light intensity, flexions with the mid light intensity and only one evoked movement with low intensity on the 2^{nd} pulse. The other one had flexions from the 3^{rd} to the 5^{th} pulse with all intensities. In the animals that showed extensions, one of them showed a reduction in amplitudes with intensity on the

3rd and 4th pulses and an increase on the 5th pulse. In the other animal there was increasing amplitudes with pulse from 3rd – 5th with the mid intensity light. It did not depict an increase in amplitude with increasing intensity. The other 2/5 animals, one had differential results that did not follow any pattern from the 3rd to the 5th pulse. The other one showed an increasing trend of amplitudes with intensity, which had the maximum amplitude of about 17˚.

In the ankle, there were bigger amplitudes, which were increasing with increased intensity from the 3rd to the 5th pulse as shown in Figure 7. Flexions were evoked with all intensities in 2/5 of the animals. The other one of the animals, flexions on the 3rd pulse showed increasing amplitude with increasing intensity. The other 3/5 produced variable movements with each of the intensities. Both evoked extensions and flexions with all intensities were increasing with intensity. Extensions were evoked before flexions. The amplitudes of evoked movements on the 4th and on the 5th pulse showed an increase from the low to mid and moderate reduction with high light intensities in one animal and an increasing trend in amplitudes with intensity in the other animal.

Effect of varying period of stimulation on amplitudes of evoked movements

Since stimulation with high light intensity for a period of 2s, produced consistent and larger joint amplitudes of evoked movements, there was the need to determine the effect of varying the period of stimulation with high light intensity on amplitudes of evoked movements. Also, there was the need to determine the temporal effects of increasing the period of stimulation. The period of stimulation was then changed to 3s and 4s with the high intensity and the results were as follows. Figures 8 and 9 show joint

angle plot and amplitudes of evoked movements for all the three joints for the 3s/4s protocol for 1 animal (animal D).

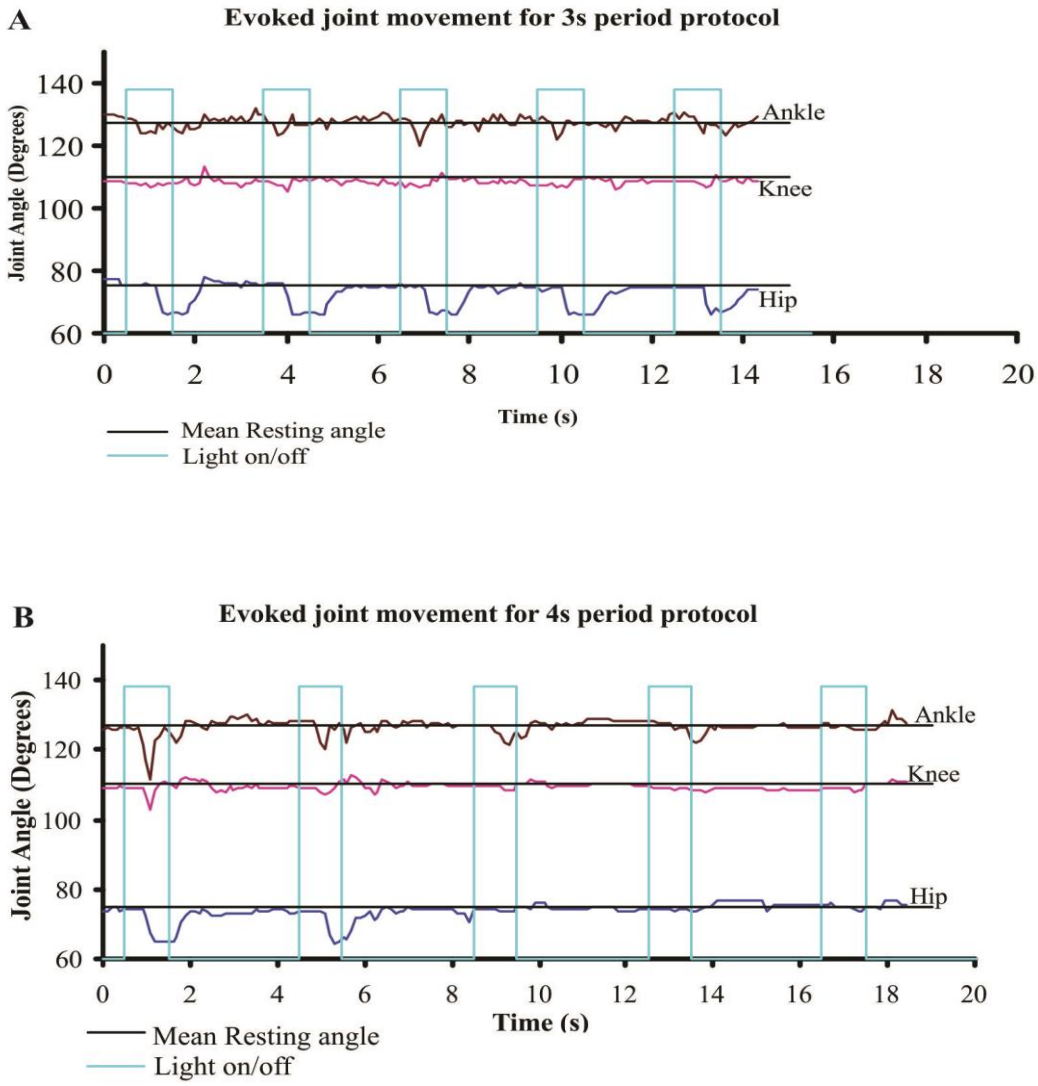

Figure 8. Evoked movements by light for the 3s/4s period protocol (animal D). Joint angle vs. time plots Hip, Knee and Ankle for A. 3s period protocol B. 4s period protocol and amplitudes of evoked movement of the by Light Intensities for the 3 and 4s period protocol by. Mean Resting Angle: Hip =75˚ (SD = 0.627); Knee = 110 (SD = 0.322); Ankle = 127 (SD = 0.842)

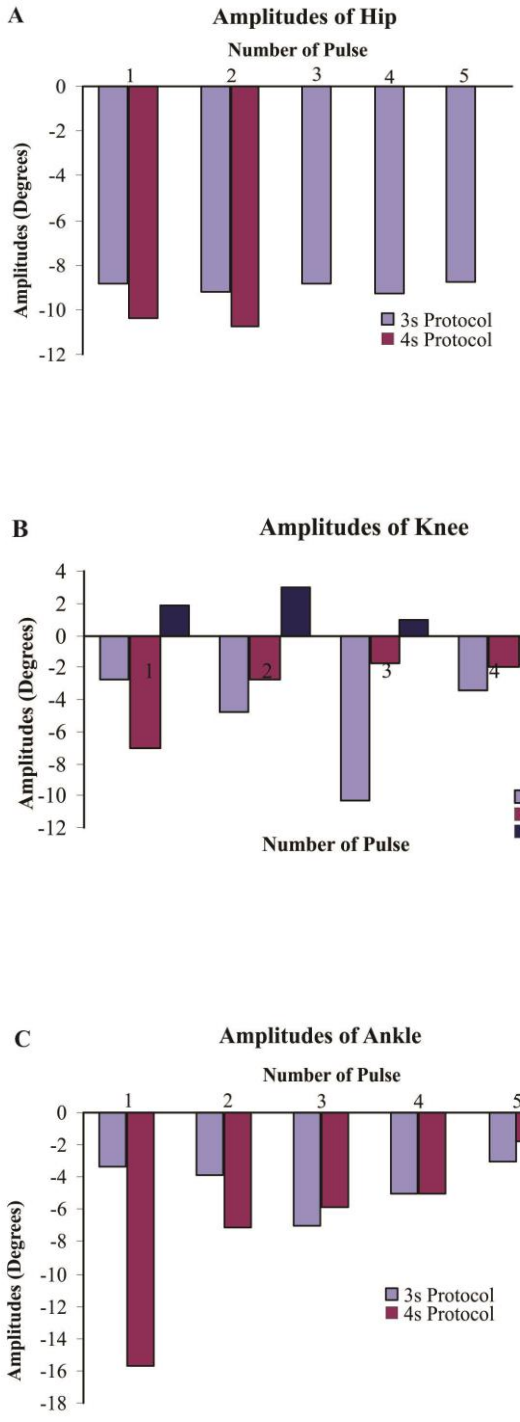

Figure 9. Amplitudes of evoked movements of joints for 3s/4s period protocols. A. Hip B. Knee and C. Ankle. Data were collected from animal D.

For the 3s protocol as shown in Figure 9, there was an increase in hip, knee and ankle flexions with the maximum amplitude of 10.33˚ recorded in the knee on the 3rd pulse. The hip showed an increase in joint amplitude from the 1st to the 2nd, a reduction on the 3rd pulse, increase on the 4th and further reduction on the 5th pulse. Maximum hip flexion was evoked on the 4th pulse. In the knee and ankle, there was sharp increase in joint amplitudes from the 1st to 3rd pulse and then decreased from 3rd to 5th pulse.

For the period of 4s as shown in Figure 9, the hip only produced flexions with the 1st and 2nd pulses and no movement from rest position with other pulses. The knee produced flexions before extensions. Knee flexions decreased from 1st to 5th pulse, knee extension no follow any regular pattern. In the ankle, flexions were evoked with amplitudes that decreased from 1st to the 5th pulse. Maximum joint angle was evoked in the ankle on the 1st pulse.

The results showed that stimulation with inter-pulse duration of ≤2s produced consistent joint amplitudes. An increase of inter-pulse duration of ≥ 2s did not produce regular pattern especially in the hip of one animal (animal D).

Temporal effects of light

Effect of light intensity on onset latency of evoked movement

With increasing light intensity, it was expected that there would be decrease in onset latency of evoked movements as a result of increased muscle tension. The increased tension in muscles may stem from increased activation of ChIEF molecules. This would result increased rate stimulation of more motor neurons to fire action potential and lead to

increased muscle contraction and tension as well as faster movement. The results are shown in Table 1 and Figure 10 and description is as follow:

Table 1

Onset latencies (s) of evoked movements by light for 2s period protocol. For Animal C. NR =No response

Joints	**Pulse #**	**Low Intensity**		**Mid Intensity**		**High Intensity**	
		Extension	**Flexion**	**Extension**	**Flexion**	**Extension**	**Flexion**
Hip							
	1	0.17			0.52		0.25
	2	0.89			0.63		0.26
	3	1.16		NR			0.43
	4	0.22		0.24			0.6
	5	0.22		0.24			0.61
Knee							
	1		0.4		0.49		0.05
	2		0.8		0.09		0.22
	3		0.28		0.2		0.1
	4		0.29		0.24		0.1
	5		0.19		0.28		0.18
Ankle							
	1	0.13	1.14		0.98	0.03	0.94
	2	0.34	0.64		0.67	0.14	0.64
	3	0.34	0.84		0.67	0.3	0.64
	4	0.54	1.14		0.78	0.24	0.84
	5	0.24	0.74		1.18	0.43	0.34

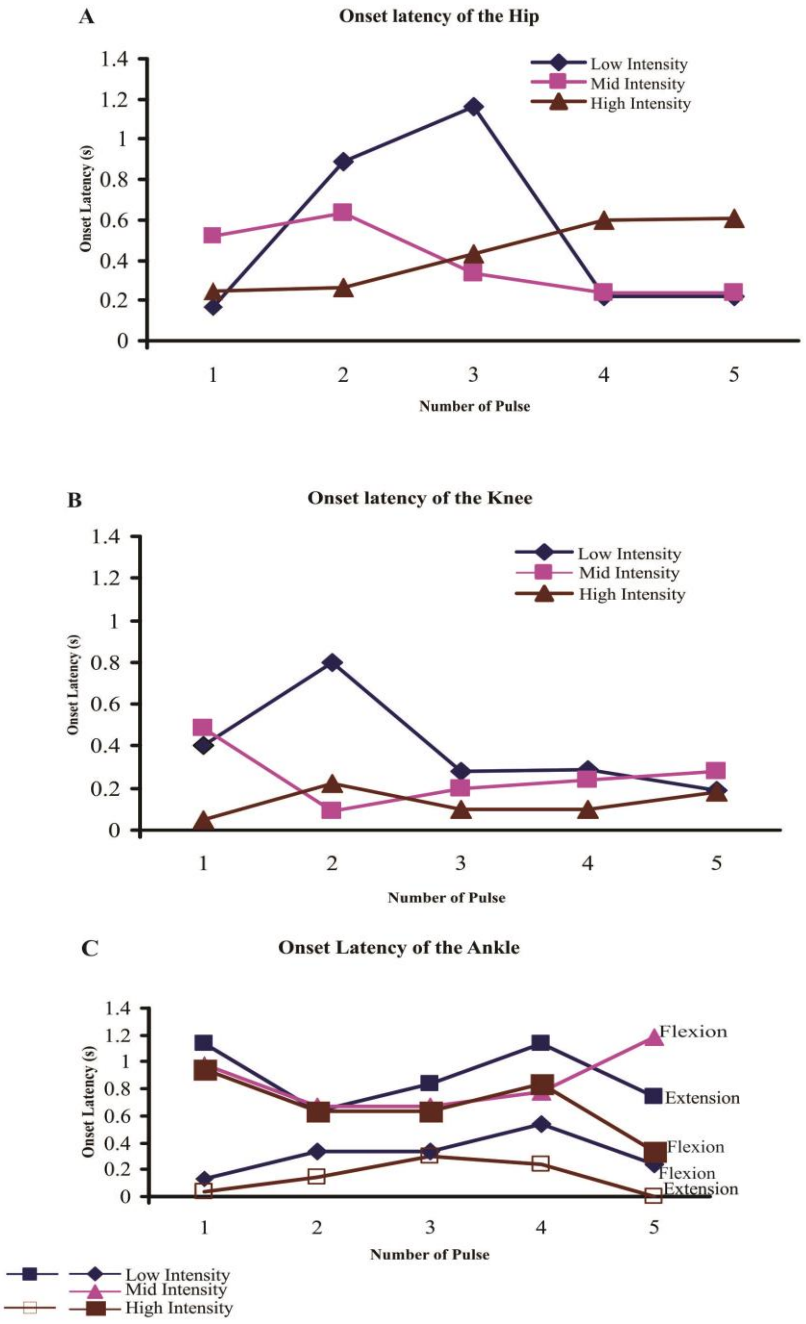

Figure 10. Onset latencies of evoked movement for 2s period protocol. A. Hip – data were collected from animal C, B. Knee – data were collected from animal C and C. Ankle – data were collected from animal E with light intensities. Onset of evoked movement with the high intensity had mostly the lowest onset. The onset latency of evoked movement with high intensity was increasing from the 1st to the 5th pulse in the hip and from the1st to the 2nd pulse in the ankle as expected.

Onset latency of 1st pulse of light intensities

In the hip, the low intensity light had the smaller onset latency of evoked movement, but the difference between that of the high intensity light and low intensity light was very minute as shown in Figure 10A (animal C). This trend was present in 2/5 of the animals. In 1/5 of the animals, smaller onset latency of movements was evoked with the high intensity light. For evoked movement in the other 2/5 of the animals, the mid intensity light had the lowest onset latency.

Figure 10B shows the onset latency of evoked movements in the knee with different intensities (animal C). It can be observed that, the onset latency of evoked movement of the 1st pulse was shortest with the high intensity light. This was evident in 2/5 of the animals. The mid intensity light had the smallest onset latency in 3/5 animals. In one of these, extension amplitudes by mid intensity light had the shortest onset latency of all movements, but the high intensity light had the lowest onset latency with flexion amplitudes.

In the ankle, the high intensity light had the smallest onset latency with both flexion and extension as depicted in Figure 10C (animal C). The high intensity light had the smallest onset latency in 4/5 of the animals while the low intensity had the longest onset latency of evoked movement. In 1/5 of the animal, the high intensity light had the longest onset latency of evoked movements compared to that of the mid intensity light, since the low intensity did not evoke any movement.

Effect of intensity on onset latency across pulses

In the hip, there was a general increase in onset latency of evoked movements from the 1st to the 5th pulse with high intensity light. This was evident in 2/5 of the animals as shown in Figure 10A. The onset latency of evoked movements for low intensity light was decreasing from the 1st to the 5th pulse in 1/5 of the animals. The onset latency of evoked movements for mid intensity light also showed an increasing trend in 2/5 of the animals. However, the onset latencies of the evoked movements for the high intensity light were always longer than that by the mid intensity especially from the 3rd to the 5th pulse as shown in Figure 10A.

In the knee, the onset latency of evoked movements for the high intensity light increased from the 1st to the 2nd pulse and then decreased on the 3rd pulse before showing a rising trend from the 4th to the 5th pulse as shown in Figure 10B (animal C). This was present in 2/5 of the animals. The onset latency of evoked movements for the high intensity light was increasing in the last two pulses for 3/5 of the animals. The onset latency of evoked movements for the other intensities did not follow any regular pattern. But the onset latency for the movements evoked by mid intensity light only increased from the 3rd to the 5th pulse in 3/5 of the animals while the onset latency of movements evoked by of low intensity light showed a decreasing trend from the 4th to the 5th pulse in 2/5 of the animals.

Evoked moments in the ankle did not show any regular pattern at any intensity. Figure 10C, shows an example of onset latency of evoked movements in the ankle by all three intensities using the 2s period protocol. The onset latency of evoked movements for

high intensity light was increasing from the 1st to the 3rd pulse. This was evident 1/5 of the animals. The onset latency of movements evoked by the high intensity light then decreased on the 4th and on the 5th pulses in 2/5 of the animals. In 1/5 of the animals, the onset latency of evoked movements by high intensity light increased from the 1st to the 2nd pulse. The onset latency of evoked movements by the high intensity light then decreased on the 3rd pulse and then increased on the 4th and a minor decline on 5th pulse. In the same animal, the onset latency of movements evoked by mid intensity light increased from the 1st to the 4th pulse and then decreased moderately on 5th pulse. The onset latency of evoked movements for low intensity light in another animal sharply decreased from the 1st to the 3rd and then increased sharply from the 3rd to the 5th pulse.

Effect of light intensity on peak latency of evoked movement

As more of the ChIEF molecules are activated with increased intensity, it was expected that there would be a decrease in peak latency of evoked movements with increased light intensity. This is because with increased light intensity, the will be increased activation of ChIEF molecules. This will lead to increased rate of firing of action potential by motor neurons and increased muscle contraction and increased tension. It would take less time for a joint to reach its peak joint angle. Table 2 and Figure 11 summarize the results of the peak latencies of evoked movements for the 2s period protocol.

Table 2

Peak latencies (s) of evoked movements of by light for 2s period protocol. Hip (Animal C), knee (Animal C) and ankle (Animal B). NR= No response

Joints	Pulse #	Low Intensity		Mid Intensity		High Intensity	
		Extension	**Flexion**	**Extension**	**Flexion**	**Extension**	**Flexion**
Hip							
	1	0.44			0.65		0.42
	2	0.71			0.79		0.39
	3	0.68		NR			0.67
	4	0.42		0.5			0.97
	5	0.63		0.41			1.05
Knee							
	1		0.74		0.65		0.35
	2		1.13		0.86		0.33
	3		0.45		0.4		0.61
	4		0.45		0.54		0.67
	5		0.36		0.45		0.65
Ankle							
	1	NR		1.02	NR	0.58	1.12
	2	NR		1.16	NR	0.27	0.9
	3	NR		0.43	NR	0.58	NR
	4	0.42		0.7	0.47	1.25	NR
	5	NR		0.48	NR	1.06	1.33

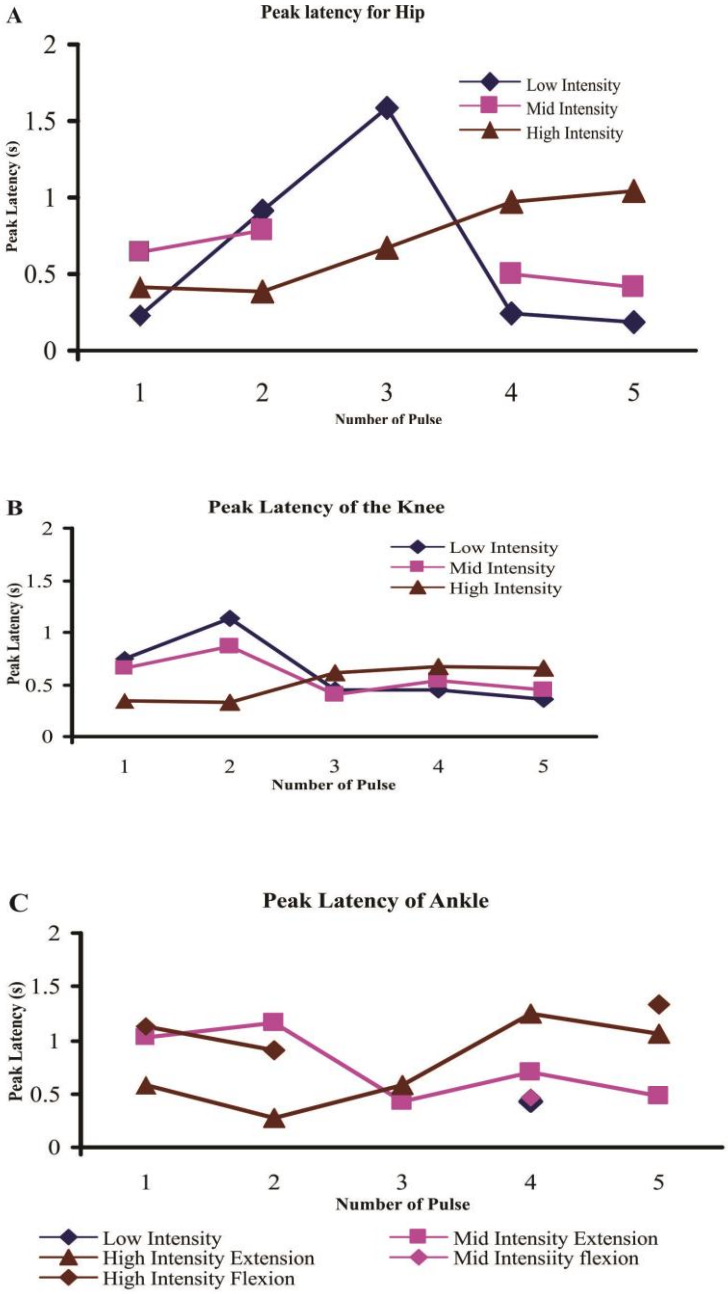

Figure 11. Peak latencies of evoked movement for 2s period protocol. A. Hip – data taken from animal C B. Knee – data taken from animal C and C. Ankle – data taken from animal C. Peak latency was increasing from the 1st to the 5th pulse in the hip with high intensity light, increasing in the knee and increasing only from the 2nd to the 4th pulse with ankle extensions and decreasing from the 1st to the 3rd with ankle flexions. In the hip, the peak of evoked movement by the mid intensity was increasing from the 2nd to the 4th pulse after an initial decrease.

Effect of the 1st light pulse on peak latency

Figure 11A shows an example of peak latency of evoked movements in the hip with different intensities for the 2s period protocol (animal C). In this animal, the peak latency of the evoked movement for the low intensity light was the shortest, but this was closely followed by the peak latency of movements evoked by the high intensity light. The shortest peak latency with low intensity light was observed in 3/5 of the animals. In the other 2/5 of the animals, the shortest peak latency of evoked movements was recorded with the mid intensity light.

Figure 11B depicts an example of peak latency of evoked movements in the knee with different intensities for the 2s period protocol (animal C). This animal produced the shortest peak latency of evoked movement with high intensity light. There was also a reduction in peak latency of evoked movements with increased intensity. This was evident in only 2/5 of the animals. The peak latency of evoked movements for the mid intensity light was the shortest in 3/5 of the animals.

Figure 11C shows an example of peak latency of evoked movements in the ankle with different intensities for the 2s period protocol (animal B). In this animal extension were evoked before flexion with high intensity light and the evoked extension had the smallest peak latency. The peak latency of evoked ankle flexion with mid intensity light was almost the same as that of evoked extension with the high intensity as shown in Figure 11C. The pattern of high intensity light with smallest peak latency of evoked movement was present in 2/5 of the animals. In the ankle of the other 3/5 of the animals, the mid intensity had the minimum peak latency of evoked movements. In one of the

three especially, peak latency of evoked movement for the high intensity light was the longest.

Effect of intensity on peak latency across pulses

In Figure 11A, there was an increase in peak latency across pulses in the hip with high intensity light as expected. This was partly present in all of the animals as other animals had the peak latencies with high intensity increasing on certain pulses. For example, one animal (animal D) had its peak latency increasing from the 2nd to the 4th pulse and a reduction on the 5th pulse. The peak latencies with the other intensities did not show any regular pattern. For instance, in Figure 11A the peak latency of the low intensity increased sharply from the 1st to the 3rd pulse and a sharp reduction from 3rd to 4th and reduced further on the 5th pulse.

Figure 11B depicts an example of peak latency of evoked movements in the knee with different intensities for the 2s period protocol (animal C). There was gradual increase in peak latency across all pulses with high intensity light. It showed a decrease in peak latency with intensity on the 2nd pulse and the reverse on the subsequent pulses. The increase in peak latency with intensity was present especially, from the 4th and on 5th pulses in 4/5 of the animals

Figure 11C shows an example of peak latency of evoked movements in the ankle with different intensities for the 2s period protocol (animal B). Notice that the peak latency was decreasing with intensity on the 2nd pulse, but increasing with intensity from the 3rd to the 5th pulse. The peak latency of evoked movements with high intensity light showed an increasing trend from the 2nd to the 4th pulse. This was evident in 2/5 of the

animals. In the other 3/5 of the animals, there was no regular trend in peak latency. For instance, in one of the animals, the peak latency for the low intensity light showed an increasing trend from the 2nd to the 4th, while in another the peak latency of the low intensity showed a decreasing trend.

Effect of light intensity on Relaxation/fall time of evoked movement

It was expected that there would be increase in relaxation time with increasing intensity. The rational for this is that, with increasing intensity, there would be increased muscle tension. The increased muscle tension would be as a result of increased activation of ChIEF molecules leading to firing of action potentials by motor neurons and increased muscle contraction. With increased muscle tone, the joint would spend more time in motion and therefore it would take longer time for the leg return to its resting angle. Table 3 and Figure 12 represent a summary of the results.

Table 3

Relaxation/ fall times of evoked movement for 2s period protocol. Hip (Animal D), Knee (Animal B) and Ankle (Animal A). NR= No response

Joints	Pulse #	Low Intensity		Mid Intensity		High Intensity	
		Extension	Flexion	Extension	Flexion	Extension	Flexion
Hip							
	1		NR		0.03		0.83
	2		1.00		0.17		1.26
	3		NR		NR		1.30
	4		NR		NR		1.03
	5		NR		NR		0.67
Knee							
	1	NR		0.34		0.67	
	2	0.33		0.54		0.53	
	3	NR		0.29		1.1	
	4	0.033		0.4		0.43	
	5	0.07		0.23		0.17	
Ankle							
	1		0.2		1.03		1.03
	2		0.5		0.33		0.67
	3		0.39		0.8		0.6
	4		0.93		0.27		0.5
	5		0.2		0.33		0.4

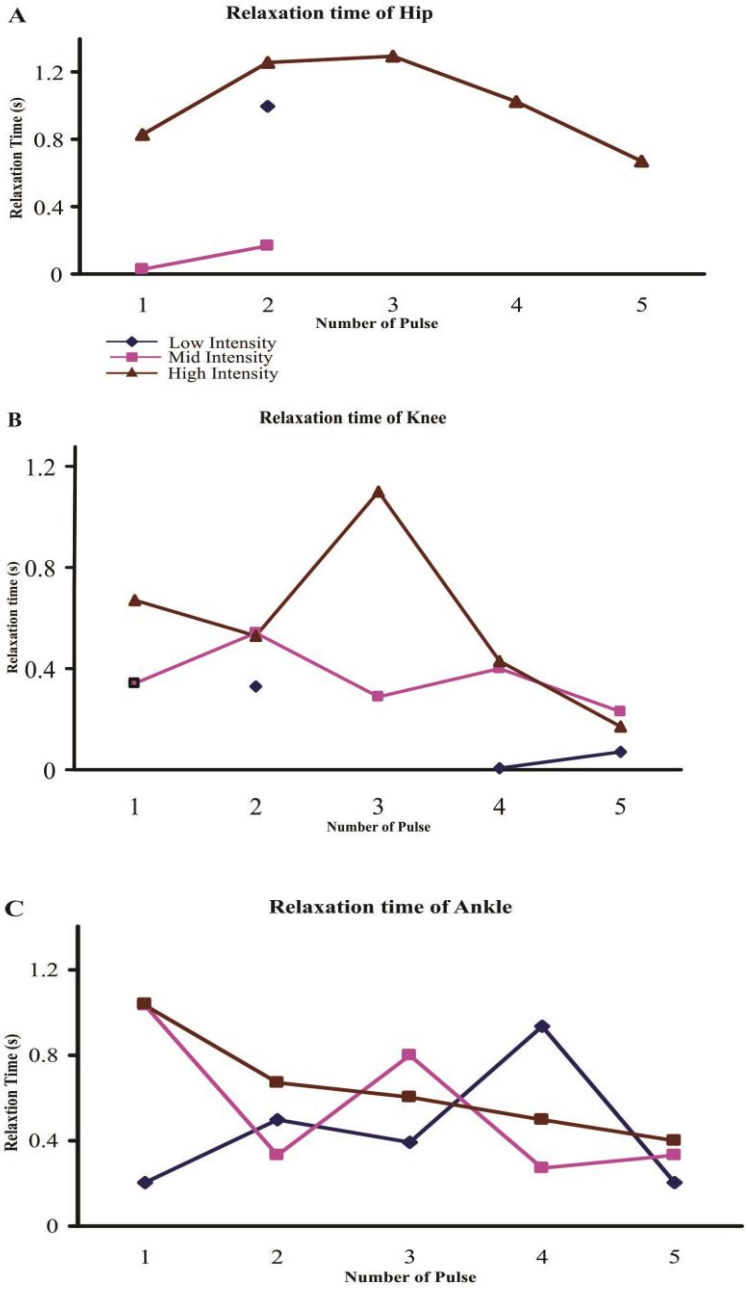

Figure 12. Relaxation/fall times of evoked movements for 2s period protocol. A. Hip – data were taken from Animal A; fall time of high intensity increased from 1st to 3rd and decreased then on the 5th pulse B. Knee- data were taken from Animal B; showed no regular pattern C. Ankle – data were taken from animal D; the fall time of evoked movement by the high intensity light was decreasing from 1st to 5th pulse as predicted.

Effect of 1st pulse on relaxation/fall time of evoked movement

Figure 12A shows an example of the fall time of evoked movement in the hip in response to light of different intensities for a 2s period protocol (animal D). Observe that in the 1st pulse, the fall time was increasing with intensity. This increasing trend was present 4/5 animals. In the other 1/5 of the animals, the high intensity light had the smallest fall time whereas the longest fall time was produced by the evoked movement by mid intensity light.

Figure 12B is an example of the fall time of evoked movement in the knee in response to light of different intensities for a 2s period protocol (animal B). Notice that in the 1st pulse, the fall time was increasing with increased intensity. This was evident in 1/5 of the animals. In the other 4/5 of the animals, the fall time decreased with intensity.

Figure 12C shows an example of the fall time of evoked movements in the ankle in response to different light intensities for a 2s period protocol (animal A). It can be observed that, in the 1st pulse the fall time increased with intensity. The fall time for the mid intensity was however the same as that of the high intensity. The increased pattern of the fall time was present in 2/5 of the animals. In other 2/5 of the animals, there was no regular pattern, but the fall time of evoked movements for the high intensity light was the shortest. In the other 1/5 of the animals, the low and the mid light intensities did not evoke any movement. In this animal, the high light intensity evoked ankle flexions before extensions.

Effect of intensity on Relaxation/fall time across pulses

In the hip, the relaxation times showed a gradual decrease from the 1st and 5th with the high intensity light as shown in Figure 12A. This trend was observed in 3/5 animals. In the other 2/5 of the animals, there was no regular trend. For example, one had an increasing trend with high intensity light from the 1st to the 3rd pulse and a decreasing trend from the 3rd to the 5th pulse. But the fall time on the 5th pulse in that animal was still bigger than that of the 1st pulse.

In the knee, the fall times for all intensities did not show any regular trend for the 2s protocol as depicted in Figure 12B. It decreased on the 2nd pulse, increased sharply on the 3rd pulse and then sharp decrease from the 3rd to the 5th pulse. It still showed an increasing trend with intensity on the 3 and 4th pulses. The irregular trend in fall times for all intensities was present in all the animals

In the ankle, there was a decreasing trend in fall time with high intensity light as shown in Figure 12C. This was present in 2/5 of the animals. The other animals showed variable trend across animals and with various intensities. For instance, one animal (D) showed an increasing and decreasing trend with both high mid light intensities form the 2nd to the 5th pulse.

Latencies of evoked movements for 3s/4s periods of stimulation

Stimulation was then performed with high intensity light for periods of 3s and 4s and pulse duration of 1s and their latencies are described in Tables 4-6 and Figures 13-15.

Table 4

Onset latencies of evoked movement for the 3s/4s period protocol. The 3rd to the 5th pulse of the 4s period protocol did not evoke any movement in the hip and no evoked extension on 4th pulse in the knee, so there was no recorded latency.

Joint	Pulse #	3s-Protocol	4s-Protocol	
		Flexion	Flexion	Extension
Hip				
	1	0.7	0.5	
	2	0.5	0.5	
	3	0.61	NR	
	4	0.61	NR	
	5	0.71	NR	
Knee				
	1	0.2	0.3	1.3
	2	0.31	0.3	0.91
	3	0.21	0.71	1.31
	4	0.31	0.71	NR
	5	0.41	0.32	1.72
Ankle				
	1	0.3	0.4	
	2	0.3	0.5	
	3	0.3	0.51	
	4	0.41	0.9	
	5	0.61	1.12	

Table 5

Peak Latencies(s) of evoked movement for 3s/4s period protocol. NR = No response.

Joint	Pulse #	3s-Protocol	4s-Protocol	
		Flexion	Flexion	Extension
Hip				
	1	0.9	1	
	2	0.6	0.81	
	3	0.8	NR	
	4	0.71	NR	
	5	0.81	NR	
Knee				
	1	0.6	0.6	1.4
	2	0.5	0.61	1.21
	3	0.41	0.81	1.41
	4	0.41	1.31	NR
	5	0.71	0.92	1.62
Ankle				
	1	0.4	0.6	
	2	0.3	0.61	
	3	0.41	0.81	
	4	0.41	1.11	
	5	0.5	1.22	

Table 6

Relaxation/ fall times of evoked movement for 3s/4s period protocol.

Joint		3s-Protocol	4s-Protocol	
	Pulse #	Flexion	Flexion	Extension
Hip				
	1	0.8	0.9	
	2	1.2	1.1	
	3	0.8	NR	
	4	1.2	NR	
	5	1	NR	
Knee				
	1	0.7	0.2	0.5
	2	0.1	0.3	0.2
	3	0.3	0.2	0.3
	4	0.5	0.4	NR
	5	0.2	0.1	0.3
Ankle				
	1	0.3	0.4	
	2	0.2	0.2	
	3	0.2	0.5	
	4	0.2	0.4	
	5	0.1	NR	

Figure 13A shows the onset latencies of evoked movements of the hip for both 3s and 4s period protocols (animal D). The onset latency of evoked movements decreased from the1st to the 2nd pulse while that of the 4s protocol remained the same. The onset latency of evoked movements of the 3s protocol then increased slightly on the 3rd pulse. The onset latency remained the same on the 4th and then increased on the 5th pulse. The onset latency of evoked movements for the 4s period protocol did not evoke any movement in the hip from the 3rd to the 5th pulse.

Figure 13B shows the onset latencies of evoked movements of the knee for both 3s and 4s period protocols (animal D). The onset latency of evoked knee movements for the 3s protocol decreased from the1st to the 2nd pulse. The onset latency then increased from the 2nd to the 5th pulse. With the 4s protocol, the onset latency of evoked knee movements remained the same from the 1st to the 2nd pulse, it increased on the 3rd pulse and had a decreasing trend from the 2nd to the 5th pulse.

Figure 13C shows the onset latencies of evoked movements of the ankle for both 3s and 4s period protocols (animal D). For the ankle, there was an observable increasing trend for onset latency of evoked movements for both 3s and 4s protocol from 1st - 5th pulse by a magnitude of between 0.1-0.3s and 0.1-0.7s respectively. The onset latencies across pulses were longer with the 4s period protocol in relation to the 3s period protocol.

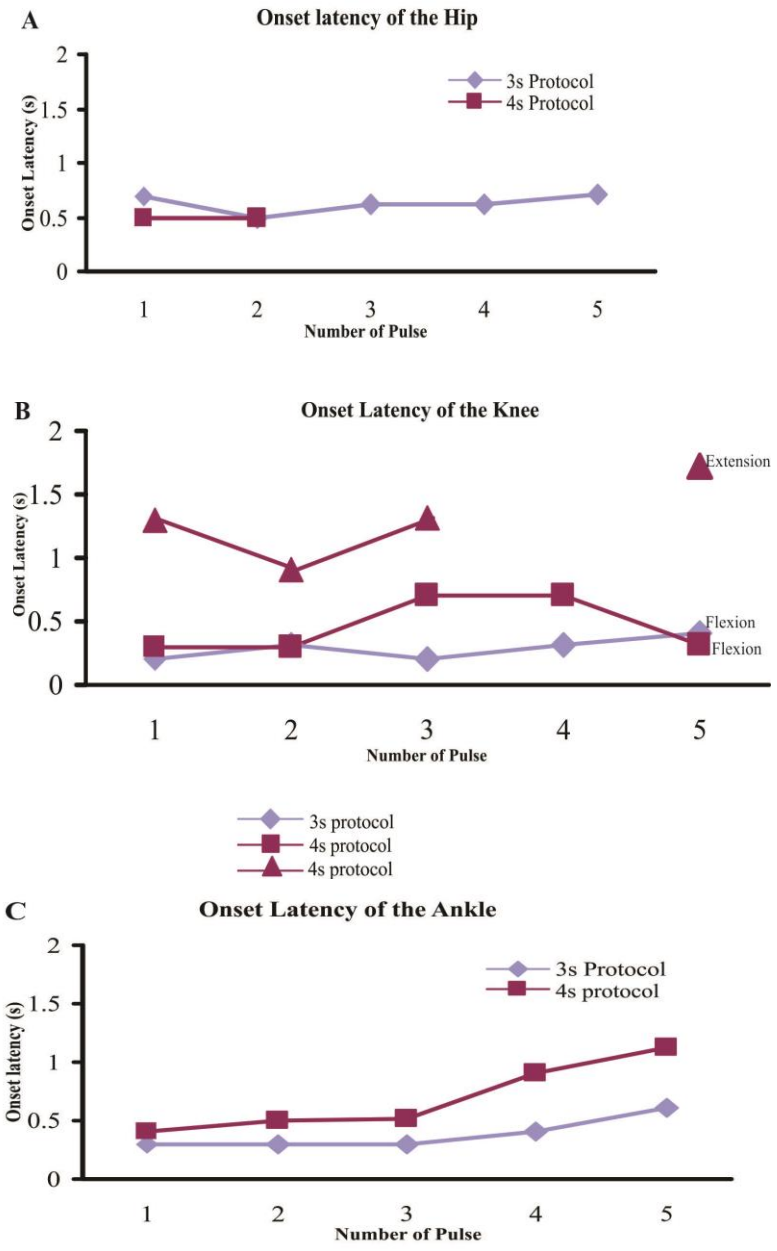

Figure 13. Onset latencies of evoked movement for 3s/4s period protocol (animal D). A. hip, B. knee and C. ankle with 3s/4s period protocol: the onset of evoked movement was increasing from the 1st to the 5th pulse in the ankle for both protocols.

Figure 14 shows the peak latencies of evoked movements of the hip, knee and ankle for both 3s and 4s period protocols (animal D). In the hip, the peak latency decreased from 1^{st} – 2^{nd} pulse for both protocols as shown in Figure 14A. The peak latency for 3s period protocol then increased on the 3^{rd}, decreased on the 4^{th} and then increased on the 5^{th} pulse. With the 4s period protocol, there was no evoked hip movements from the 3^{rd} to the 5^{th} pulse.

With knee flexion, both protocols decreased from 1^{st} - 4^{th} pulse. It then increased on the 5^{th} pulse with 3s period protocol and decreased with the 4s period protocol. Knee extension evoked with the 4s period protocol did not show any pattern (see Figure 14B).

In the ankle, the peak latency of 3s period protocol had a minor decrease from 1^{st} - 2^{nd} and minor increase from 2^{nd} - 5^{th} pulse. For 4s protocol, the ankle showed relatively moderate increase in peak latency from the 1^{st} to the 5^{th} pulse. The peak latencies across pulses for 4s period protocol were longer than that for the 3s period protocol (see Figure 14C).

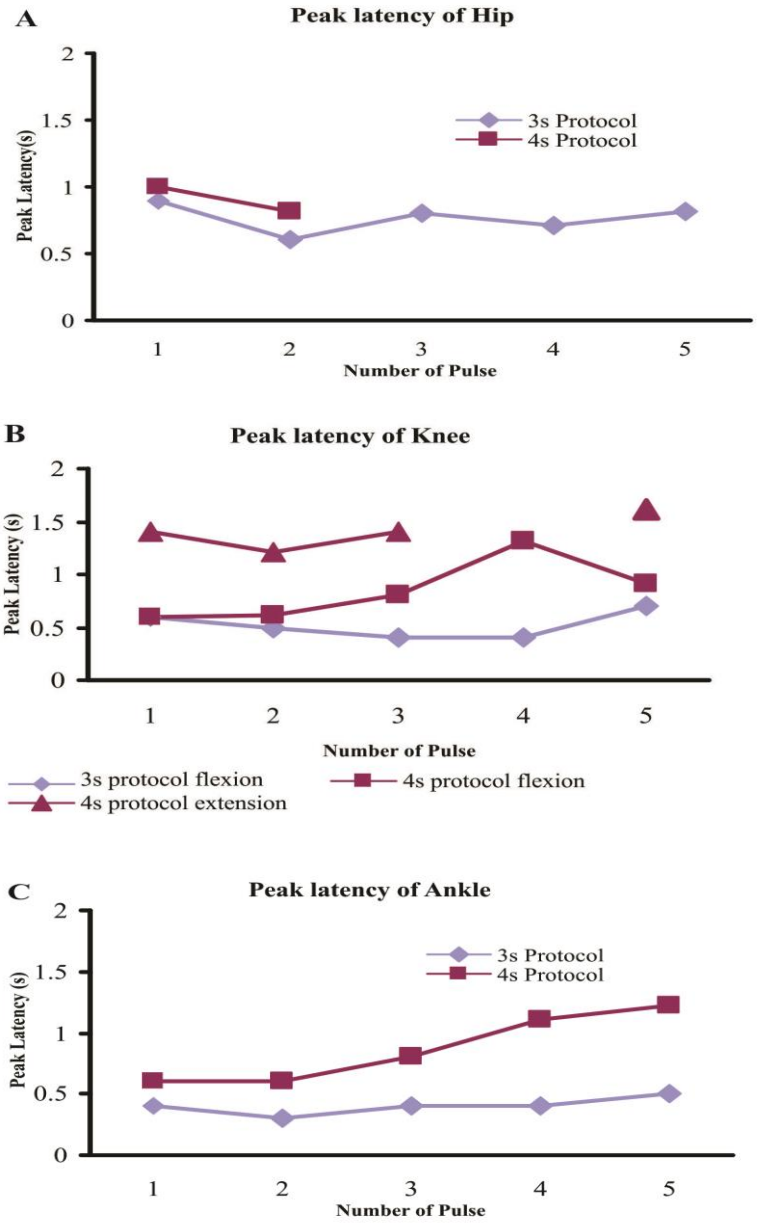

Figure 14. Peak latencies of evoked movements for 3s/4s period protocol (animal D). A. Hip B. Knee and C. Peak latency in the ankle for 4s period protocol was increasing in the ankle from the 1st to the 5th pulse. 3s period protocol only increased from the 2nd to the 5th pulse after an initial decrease from the 1st to the 2nd pulse.

Figure 15 shows the relaxation or fall times of evoked movements of the hip, knee and ankle for both 3s and 4s period protocols (animal D). Relaxation or fall times increased in the hip with both 3s and 4s-protocols from the 1st to the 2nd pulse. In the 3s protocol, it decreased on the 3rd pulse, increased on the 4th and then reduced on the 5th. There were no evoked hip movements with the 4s period protocol (see Figure 15A).

In the knee, the fall time for 3s protocol decreased from 1st to 2nd pulse and then increased from 2nd to 4th and a moderate reduction on the 5th pulse. With the 4s period protocol, the fall time increased and decreased alternately from the 1st to the 2nd, from the 2nd to the 3rd, from the 3rd to the 4th and from the 4th to the 5th (see Figure 15B).

In the ankle, the 3s protocol had a fall time that was decreasing across pulses as expected. For the 4s protocol, it only decreased from the 1st- 2nd and from the 3rd to the 5th pulse. The fall times for the 4s period protocol was slightly longer across pulses in relation to the 3s period protocol (see Figure 15C).

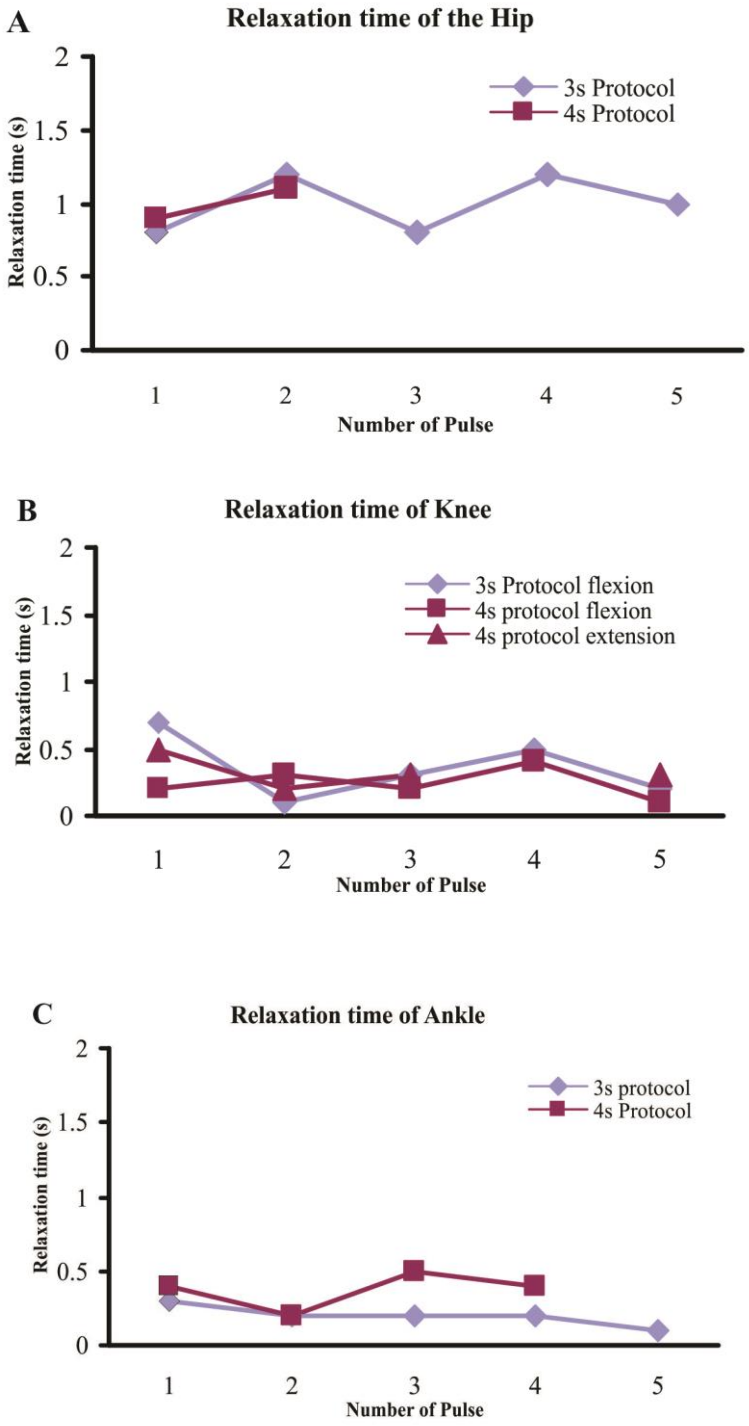

Figure 15. Relaxation/fall times of evoked movement for the 3s/4s period protocol (animal D). A. Hip, B. Knee and C. Ankle Fall time was decreasing from the 1st to the 5th pulse with the 3s period protocol

Effect of training on spontaneous movement

It was expected that after an hour of stimulation, there would be increase in the maximum and minimum joint angles as well as joint excursion as a result of strengthening of synapses and subsequent increase in muscle strength. It was also expected that after an hour of training, there would be increase in movement time or duration due to increased excitability of motor neurons and increased synaptic strength. This would lead to increased muscle strength and increased movement duration. There would be decreased inter-movement intervals (IMI) and an unchanged period of motility, due to increased spinal or motor excitability and therefore animal tends to move more than rest. Since the period is the sum of the movement time and IMI, it was expected to remain the same.

Effect of 1 hour of training on joint angles and excursion

Figures 16-19 show representative spontaneous movement, maximum and minimum joint angles as well as joint excursion before during and after training for one animal (animal B).

Generally, from pre- to post- training, there were increases in both maximum (in 3/5 of the animals) and minimum (in 4/5 of the animals)) joint angles in the hip; in the knee, there was decreased maximum joint angle (in 4/5 of the animals) and increased minimum joint angle (in all animals); while in the ankle there were decreases in both maximum (in 4/5 of the animals) and minimum (in 3/5 of the animals) joint angles. There were some moderate changes in joint angles from pre- to post-training and during the stimulation. The maximum change in joint was observed in the ankle.

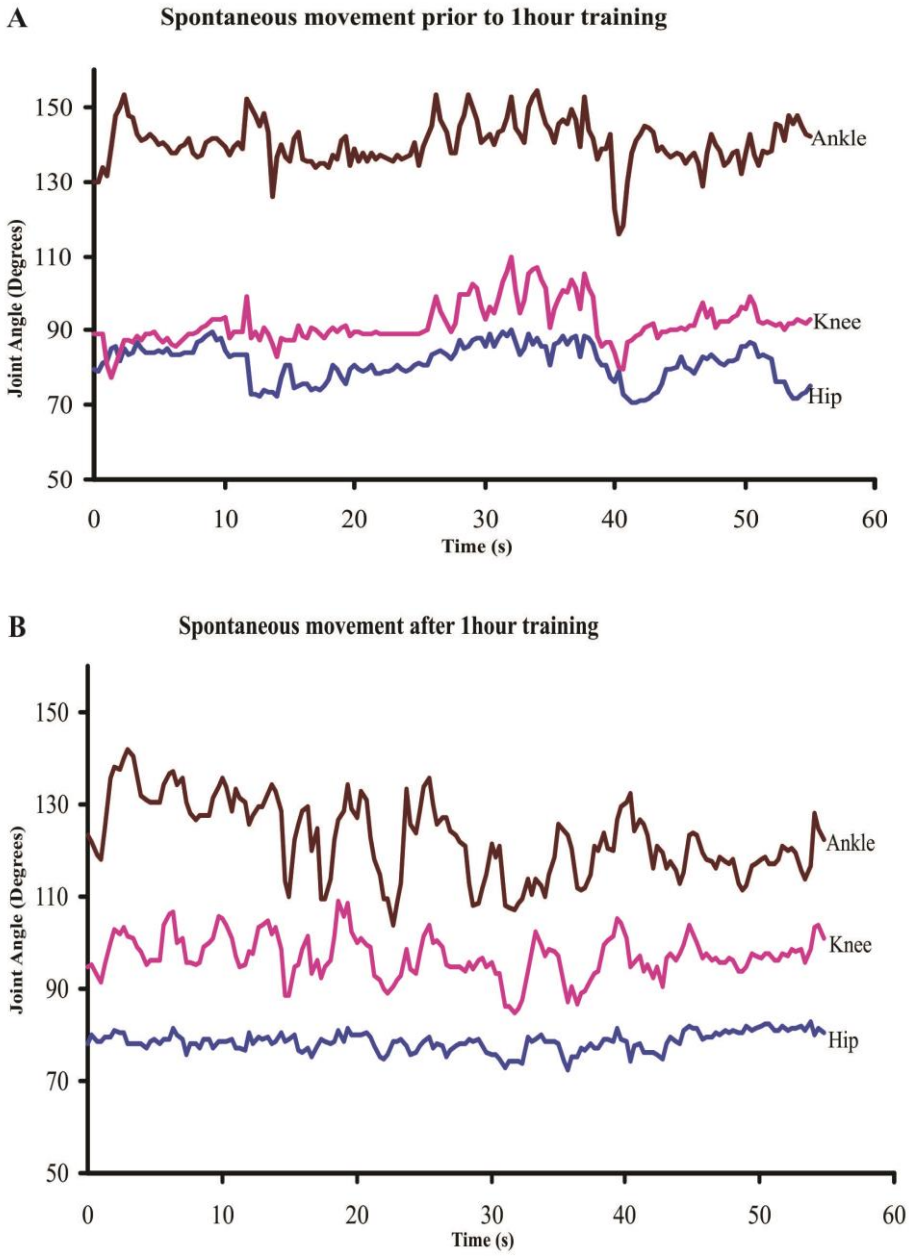

Figure 16. Representative Joint Angles vs. time plots for one animal (B). A. Movement episode prior to training (collected 8 mins and 9s prior to training protocol and B. Movement episode after training (collected 2 mins and 9s after training protocol

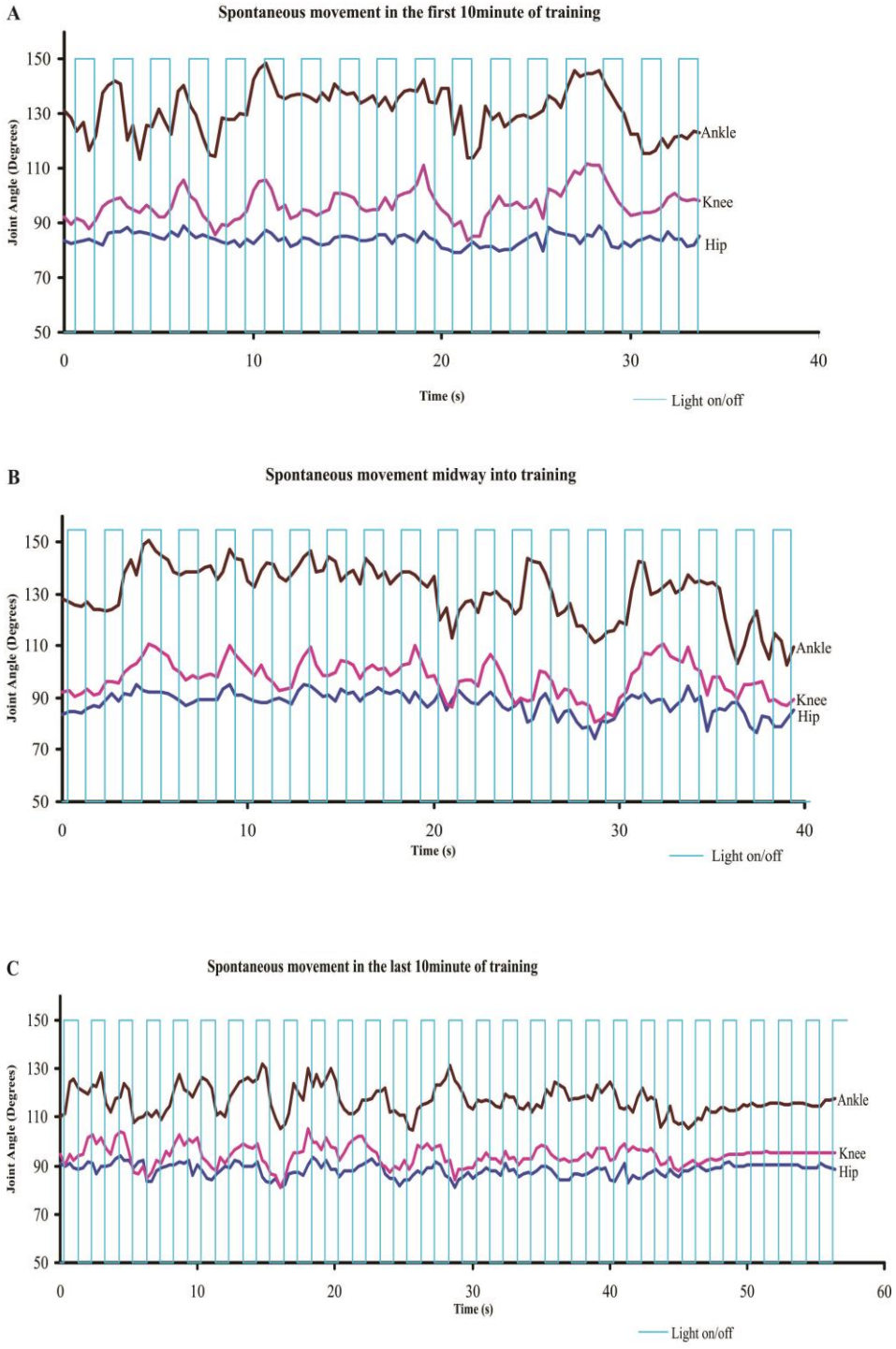

Figure 17. Representative Joint Angles vs. time plots during training (animal B). A. Movement episode in the 1st 10min (collected 2 mins and 19s into training) B. Movement episode in the mid 10min (collected 31mins and16s into training) C. Movement episode in the last 10min (collected 50mins:02s into training)

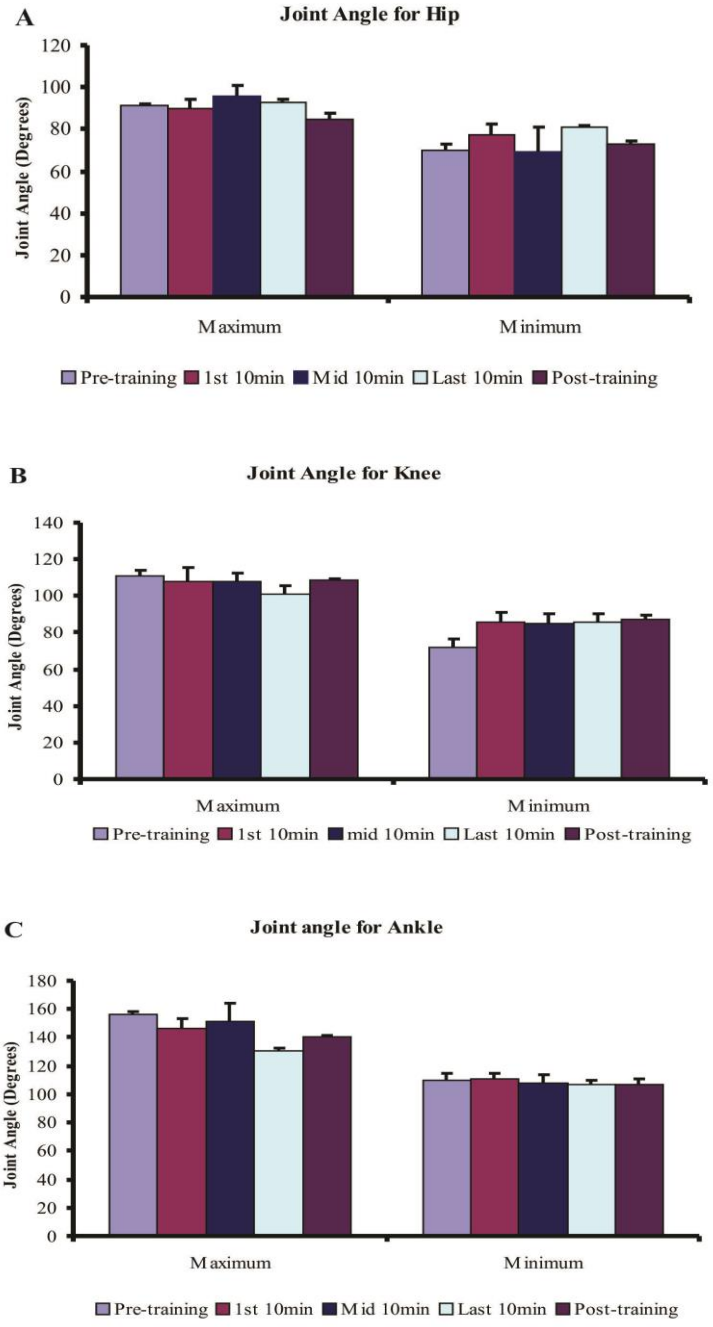

Figure 18. Average of Maximum and Minimum joint angles of hip, knee and ankle prior to, during and after training. Data collected for three (n =3) episodes of spontaneous movement collected for 1 animal (B). Errors bars represent standard deviations of joint angles.

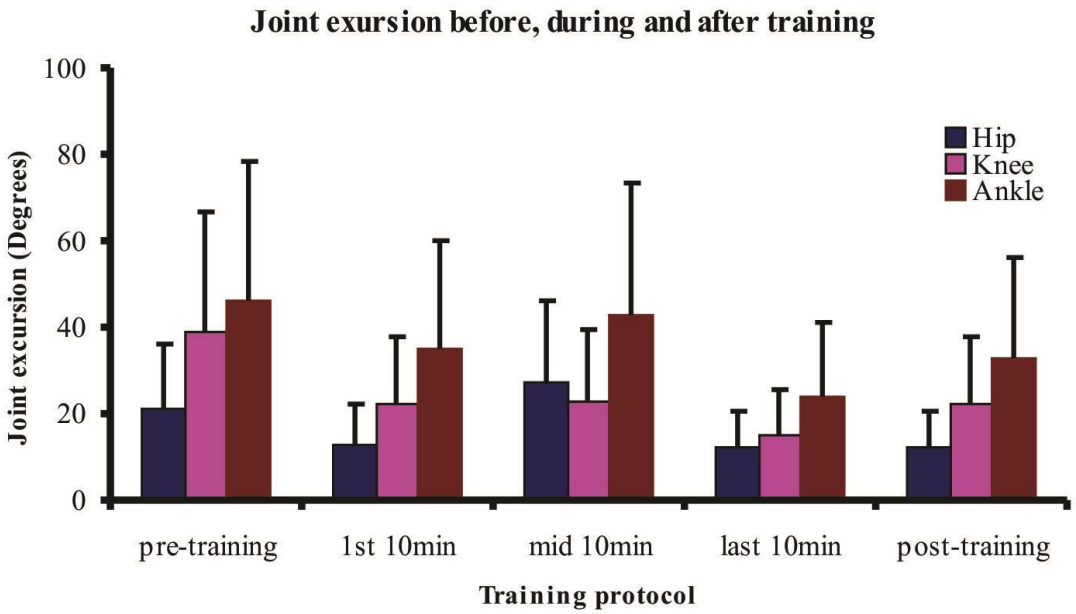

Figure 19. Plot of average joint excursion vs. training protocol. Average joint excursion of three (n =3) episodes of spontaneous movement collected for 1 animal (B). Errors bars represent standard deviations of joint excursions.

Effect of 1 hour of training on joint angles and excursion of the hip

Figures 18A and 19 show examples of maximum and minimum joint angles for the hip, as well as joint excursions for all training protocols (animal B). Notice that in the hip, there was minor decrease in maximum and an increase in minimum joint angles from pre to post training. This was present in 2/5 of the animals. The other 3/5 of the animals had a minor increase in maximum joint angle but a decrease in minimum joint angle in one of them and an increase in the other two.

During the training, there was increased maximum joint angle from the 1st 10min to the mid 10min and a decrease on the last 10min of training in all animals as shown in

Figure 18A. There was also decreased minimum joint angle from the 1st 10min to the mid and an increase on the last 10mins of stimulation. This was evident in 3/5 animals as shown in Figure 18A. The other 2/5 showed a minor increase in minimum joint angle from 1st 10min to mid 10min and slight decrease in the last 10min.

Figure 19 shows an example of joint excursion for the three joints of all training protocols (animal B). It can be observed that in the hip, there was a decrease in joint excursion in post-training in relation to pre-training. This pattern of reduction in joint excursion was present in 3/5 of the animals. In the other 2/5 of the animals, there was an increase in joint excursion from pre- to post-training. During the course of training, the joint excursion of the hip reduced in the 1st 10min from the pre-training, increased in the mid 10min, decreased in the last 10min and then increased in the post-training protocol. This pattern of joint excursion in the hip was replicated in 4/5 of the animals. In the other 1/5 of animals, there was reduction in joint excursion from the 1st 10min to the last 10min and an increase in the post-training. A maximum hip excursion of 27˚ was recorded in the mid 10mins of animal B, while the minimum excursion of 12˚ was recorded in the last 10min of training.

Effect of 1 hour of training on joint angles and excursion of the knee

Figures 18B and 19 show examples of maximum and minimum joint angles for the knee, as well as joint excursions for all training protocols (animal B). Notice that, in the knee, there was decreased maximum joint angle and increased minimum joint angle from the pre- to the post-training. This pattern of variation in joint angles was observed in

4/5 animals. In 1/5 of the animals, there were increased maximum and minimum joint angles from pre- to post-training.

In the course of training protocol, there was decrease in maximum joint angle from the 1st 10min to the last 10min of stimulation and an increase in post-training as shown in Figure 18B. This was present in 3/5 of the animals as while other 2/5 animals showed an increase

Figure 19 shows an example of joint excursion for the three joints of all training protocols (animal B). It is evident that the knee excursion decreased from pre-to post-training. This was observed in all animals. In the course of training, the knee excursion reduced in the 1st 10min from the pre-training, increased slightly in the mid 10min and then reduced in the last 10min before an increase in the post-training. This pattern of alternation of decreased and increased knee excursion was evident in 3/5 of the animals. In the other 2/5 of animals, one had an increase in excursion in the 1st 10min from pre-training and then decreased in the mid 10min and last 10min of stimulation before an increase in post-training. The other one had decreased excursion in the 1st 10min from that of pre-training. There was then an increase in the mid 10min and then decreased in the last 10min and post-training. The maximum joint excursion of 39˚ was recorded in pre-training while the minimum excursion of 5˚ was recorded in the last 10min of stimulation as shown in Figure 19.

Effect of 1 hour of training on joint angles and excursion of the ankle

Figures 18C and 19 show examples of maximum and minimum joint angles for the ankle, as well as joint excursions for all training protocols (animal B). Observe that in the ankle, there was a decrease in maximum joint angle from pre- to post-training while there was no change in the minimum joint angles. This was present in 4/5 of the animals. In the other 1/5 of the animals showed an increase in maximum joint angle and an increase in minimum joint angle from pre- to post-training.

In the course of training, there was decrease in maximum joint angle from pre-training to 1st 10min, increased in the mid 10min, reduced in the last 10min and then increased in post-training. This alternation in maximum joint angle across pulses was present in 4/5 of the animals as shown in Figure 18C. There were various variations among the animals with regard to the minimum joint angle. It increased in the 1st 10min and decreased in the mid and last 10min and remained the same at post-training. This was only present in 1/5 of the animals as shown in Figure 18C. In 2/5 of animals, there was decrease minimum joint angle in 1st 10min and increases in the mid and last 10min before a decrease in post-training. In the other 2/5 of animals, one showed decreases in minimum joint angles from 1st 10min to the last 10min and an increase in post-training. In the other one, there was an increase in the 1st 10min, reduced in the mid 10min, increased again in the 1st 10min and then decreased in post-training.

Generally, the ankle joint had the maximum excursion of all joints in all animals across all protocols. Figure 19 shows an example of joint excursion for the three joints of all training protocols (animal B). Observe that there was reduction in joint excursion in the ankle from pre- to post-training. This was present in 4/5 of the animals. The other 1/5

of the animals showed an increase in ankle excursion from pre- to post-training. This animal (animal C) had the maximum excursion of all joint of all animals (62˚) in the 1st 10min of the training. It can be observed in Figure 19, that during the training, the ankle excursion reduced in the 1st 10min, increased in the mid 10min, decreased in the last 10min before increasing moderately in post-training. This variation in ankle excursion was evident in 2/5 of the animals. In another 2/5 of the animals, there was increase in ankle excursion in the 1st 10min, decreases in the mid and last 10min and an increase in post-training. In the other 1/5 of animals, there was decrease in ankle excursion in the 1st 10min, an increase in the mid 10min and decreases in the last 10min and post-training. As shown in Figure 19, the maximum ankle excursion (46˚) was recorded in the pre-training with the minimum ankle excursion (24˚) recorded in the last 10min of training.

Evoked responses during training

It was expected that during the stimulation, there would be some evoked responses to light in all animals, at the beginning of training and a reduction of or no responses during the course of training protocol. This is due presumable to the inactivation of ChIEF molecules with prolonged exposure to light. This may lead to firing of action potential by few populations of motor neurons and reduced muscle strength. Figures 20 and 21 show examples of evoked responses during training in one animal (animal A).

However, there were some responses in only 2/5 of the animals during the course of stimulation. In both animals, as shown in Figure 22, the maximum number evoked responses were observed during the 1st 10min of stimulation as expected and the

minimum evoked responses during the mid 10min of training and the average were plotted. Responses in 2/5 of the animals during stimulation were stronger during the 1st 10min and decreased in the last 10min of stimulation. When there were evoked responses, the pattern of movement during stimulation was a period of evoked responses, followed by resting period, in which there was no response or no movement and a period of movement. In both animals, evoked responses occurred just before rest in the course of the training protocol. It should also be noted that, there seemed to be reduced strength of evoked responses at the latter part of training in both animals.

In 1 of the 2 animals (see Figure 22), which had evoked responses just before every episode of spontaneous motility for the selected times during stimulation, the maximum number of evoked responses were 26 during the 1st 10min of training and a minimum of about 7 evoked responses in the mid 10min of training. The last 10min of training recorded 9 evoked responses. In the other 1 of the animals (see Figure 22), which evoked responses just before every episode of motility, there were 5 bouts of response time during the 1st 10min, 1 bout of evoked responses during the mid 10min and 2 bouts of evoked responses in the last 10min of stimulation. The maximum number of responses was 14 during the 1st 10min of training and the minimum of number of evoked responses was about 1 in the mid 10mins of stimulation. The number of evoked responses during the last 10min of stimulation was 2.

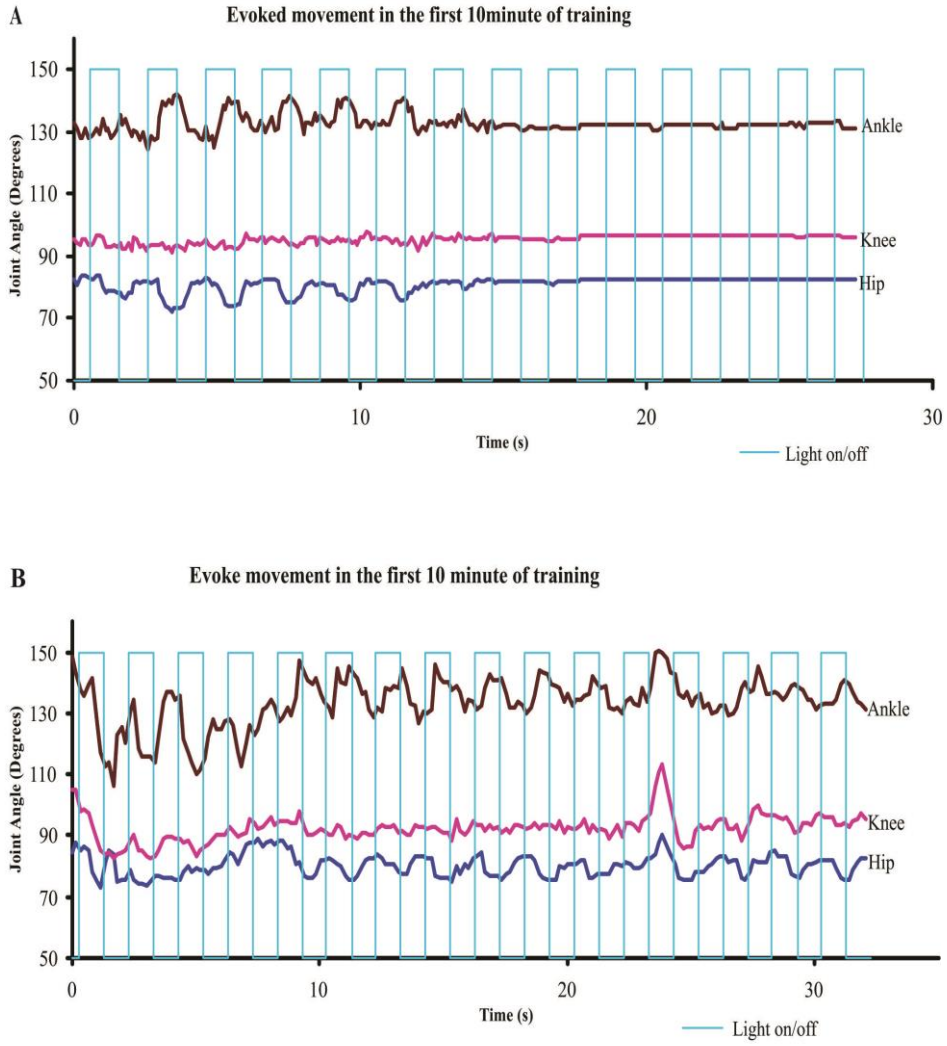

Figure 20. Representative Joint Angles vs. time plots of evoked movement for 1st 10min of training. A. Evoked movement, collected from 4 mins and 52s into training B. Evoked movement, collected from 7 mins and 14s into training for Animal A.

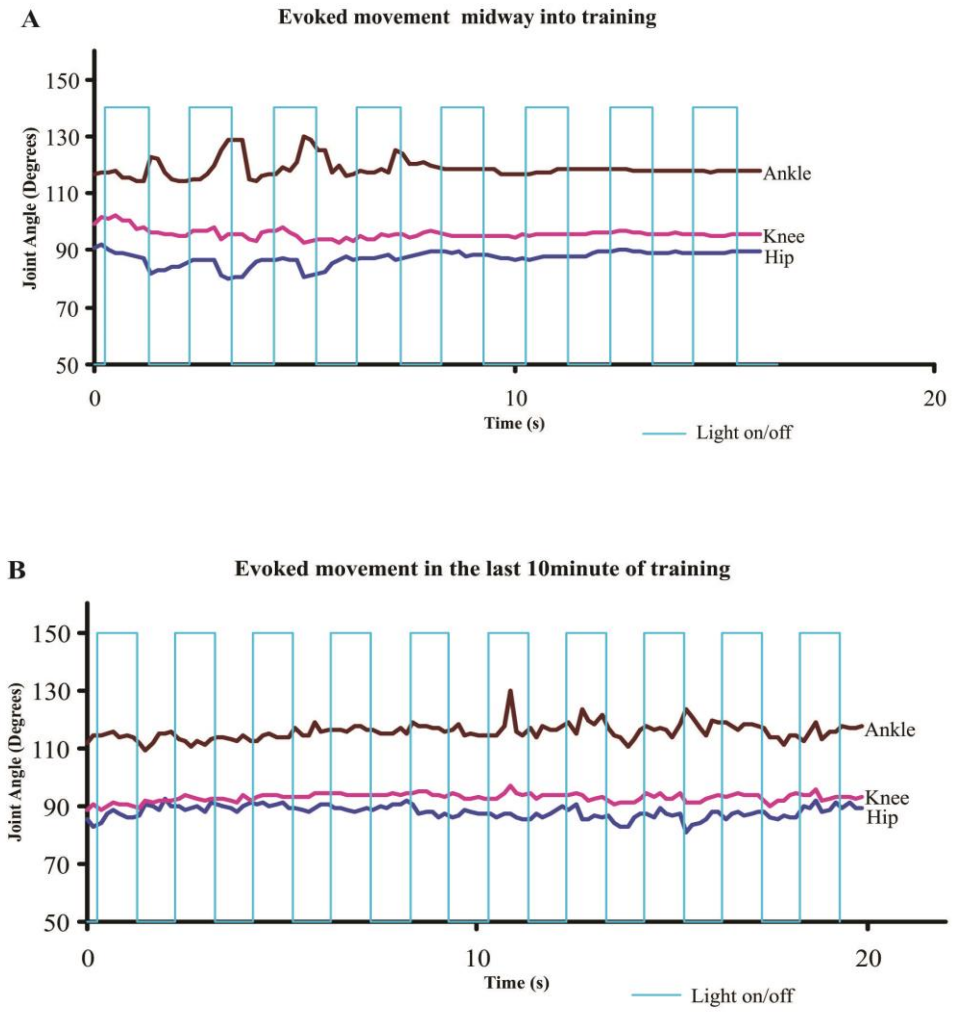

Figure 21. Representative Joint Angles vs. time plots of evoked movement during the mid 10min and last 10min of training for Animal A. A. mid 10min, collected from 35 mins and 15s into training and B. last 10min, collected from 54 mins and 22s into training.

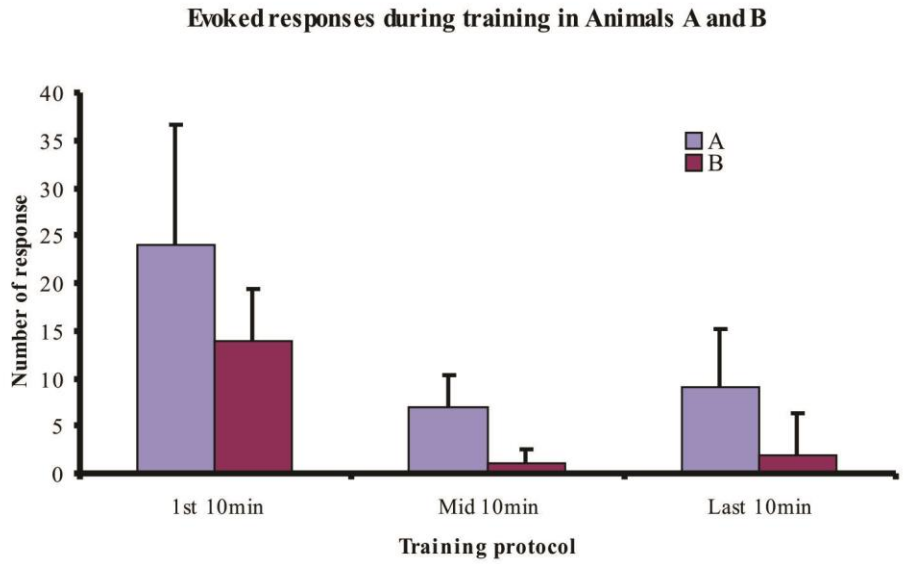

Figure 22. Average number of evoked responses during training in 2 of the animals (A and B). In Animal A, average number of responses was collected from 6 episodes for 1st 10min, 6 episodes for mid 10min and 7 episodes for the last 10min of training. In animal B, average number of responses was collected from 5 episodes in 1st 10min, 7 episodes for the mid 10min and 5 episodes for last 10min. Error bars represent standard deviations.

Effect of 1 hour training on episodes of spontaneous motility

Figure 23 shows an example of movement time, IMI and period of motility of spontaneous motility before, during and after training (Animal B). Observe that there was a linear trend with movement time, inter-movement interval and period of motility in all animals for pre- and post-training. This was present in all the animals. This linearity of the features of spontaneous activity during training was present in 4/5 of the animals. The other 1/5 of the animals showed a different trend during the last 10min of stimulation.

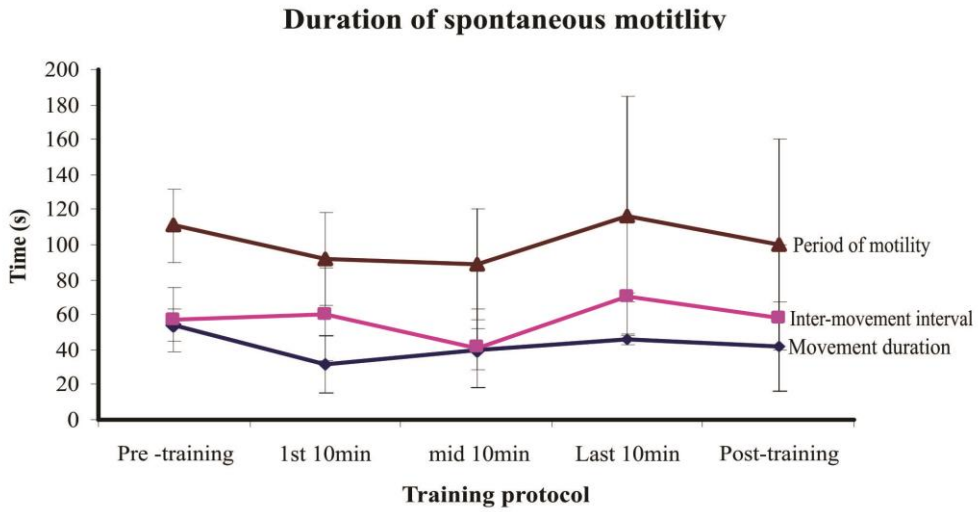

Figure 23. Representative episode of spontaneous movement for training protocol for an animal (B). Average of three (n =3) of spontaneous motility calculated for each training protocol. Error bars represent standard deviations.

Movement durations for Pre- and post-training

It was expected that there would be increase in strength of joint movement as a result of increased muscle strength from training. It was also expected that there would be some alteration in the spontaneous movement time, inter-movement interval and period of episodic motility, as a result of neuronal changes that may have taken place during training.

As it can be seen in Figure 23, there was decrease in movement duration from pre- to post training. This trend was present in all the animals. The maximum change in movement time from the pre- to the post-training was 21s in one animal and minimum change was about 1s in another 1/5 animal.

Movement durations during training

Movement time decreased in the 1st 10min from pre-training but showed an increasing trend through to the last 10min before reducing in post-training as shown Figure 23. This trend was evident in 3/5 of the animals. In 1/5 of the animals, movement time increased in the 1st 10min and then increased from the 1st 10min to the mid 10min and decreased in the last 10min. In the other 1/5 of the animals, movement time decreased from the 1st 10min to the mid 10min and a slight increase in the last 10min, although it was less than movement time in the 1st 10min.

Inter-movement interval for pre- and post training

As shown in Figure 23, there was an increase in inter-movement interval (IMI) from pre- to post-training. This was present in 3/5 of the animals. The maximum increment change was about 7s. In the other 2/5 of the animals there was decrease in the inter-movement interval from pre- to post-training. The maximum decreasing interval change was approximately 34s in one of the animals.

Inter-movement interval during training

As shown in Figure 23, IMI decreased from the 1st 10min to mid 10min and then increased during the last 10min of training. This was present in 2/5 of the animals. In 2/5 of animals, IMI increased from the 1st 10min of training to the mid 10min and then decreased during the last 10min. In 1/5 of the animals, there was a general increase in IMI from the 1st 10min to the last 10min of stimulation. The IMIs for the last 10min were

generally greater than that of the 1st 10min and mid 10mins of training (in 2/5 of the animals). The maximum IMI increase change from 1st 10min to mid 10min was about 25s.

Period of episodes of motility for pre- and post-training

Figure 23 shows an example of movement time, IMI and period of motility of spontaneous motility before, during and after training (Animal B). Notice that the period for an episode of spontaneous motility decreased in post training in relation to pre-training. This was present in 4/5 of the animals. The maximum decrement difference was approximately 55s. It only increased in 1/5 of the animals with an increase change of approximately 14 seconds.

Period of episodes of motility during training

Figure 23 shows an example of movement time, IMI and period of motility of spontaneous motility before, during and after training (animal B). In the course of training protocol, there was a decreased period of motility from the 1st 10min to the mid 10min and an increase in the last 10min of training. This was observed in 3/5 of the animals. In 2 of these animals the period or episode duration of the last 10min was greater than that of the 1st 10min, while the other 1 had the same durations for the 1st 10min and the last 10min of training. The other 2/5 animals, showed an increase in motility duration from the 1st 10min to mid 10min and a reduction in the last 10min of training. The episode of motility duration was greater in the last 10 than that of the 1st 10min in one animal and vice versa in the other.

CHAPTER 4

DISCUSSION

Motor behavior, and for that matter fetal motor behavior, is an example of implicit learning and could be affected by classical conditioning (Kandel et al, 2000). Therefore by using optogenetics as a training tool for an hour in an embryonic chick at E9, there may be disruption or changes in normal behavior as a result of experience. The changes in behavior may stem from mechanisms of plasticity that may be taking place in the neuronal circuitry of the embryo.

This study focused on two main goals; first, was to determine the optimal parameter of light that would be needed to cause plasticity and result in behavioral changes in terms of movement in an embryonic chick at E9. At E9, the sensorimotor system is sufficiently mature to perform some experimental manipulations. As described in the methods, the current intensities used were low (66.66%), mid (83.33%) and high (100%). These were arrived at upon testing various current intensity levels. It was determined that current intensities at or below 50% level evoked no observable movements. It was possible that current intensities below 50% were activating ion channels below the threshold for motor neuron and muscle to fire action potential and to result in movement.

It was observed that high current intensity produced the greatest and consistent responses in all animals studied. In the second aim for this study, high intensity light pulses were used as a training paradigm for an hour with a period of 2s, to determine if there were changes in the spontaneous movement that could have resulted from changes occurring in the neuronal circuitry that generates behavior. Stimulation was done for 2s

period due to the fact that continuous stimulation with light would lead to inactivation of ion channel. The frequency used also fell in line with the natural stimulating frequency (≤ 1Hz) of an embryonic chick (Sharp & Fromherz, 2011). It was also based on the fact that spaced training performed for long minutes or hours with rest was more efficient than massed training (with no rest) in influencing synaptic strength which form the basis for both short- and long-term memory (Kandel et al, 2000).

Effect of light intensity on evoked movement amplitudes

With no prior exposure to light, the effect of the first pulse of the 5-train of light pulses was very critical in determining the optimal light intensity to use for the training protocol as well as light intensity that may cause neuronal plasticity. In all the joints of most of the animals used, the maximum and consistent amplitudes of evoked joint movement were recorded with the high intensity of light. The mid intensity, however, also evoked some bigger flexions but was inconsistent in all animals and all joints. This fell in line with the fact that the higher stimulus intensity produces greater responses by increasing the probability of activating more molecules of ChIEF. This culminated in increased rate of firing of action potential by motor neurons and muscle and therefore bigger movement amplitudes. The low intensity generally produced small amplitudes by not activating all ChIEF.

In the hip (see Figure 5), the low intensity light evoked extensions with all pulses whereas; the high intensity light evoked only flexions through all pulses. The evoked hip extension with low intensity light may show that the hip extensors had a threshold for activation by low intensity light while hip flexors had the threshold for activation by the

high intensity light. The mid intensity evoked hip flexion with the first two pulses and extensions with the last two pulses. The variation in flexion and extension may mean that hip flexors were stimulated stronger than the extensors in the first 2 pulses and the extensors then took over in the last two pulses. It is probably that, extensors needed more stimulation time for them to be activated. Also the pattern of stimulation may have affected or shifted upward the resting joint angles of the animals. When the pattern of stimulation was started with high intensity and moved down to the low intensity, evoked movement by joints with the high intensity did not return to rest after the first evoked movement. Evoked movements by joints with the mid intensity did not return to rest after the first two pulses. By the time stimulation was performed with the low intensity, the rest position had shifted upward, presumably due to increased muscle tension build-up caused by the high intensity light. It could also be that stabilization by gluing animals to back paddle during the experiment was not effective and may have resulted in errors in digitizing in MATLAB.

In the knee (see Figure 6), all intensities evoked flexions, with the mid intensity light evoking the maximum amplitude of movement in the first pulse, but were closely followed by amplitude of movement evoked by high intensity light. The knee flexors seem to possess a threshold of activation below the low intensity light. The high intensity light then subsequently evoked maximum flexions from the 2^{nd} to 5^{th} pulses by increased activation of ChIEF molecules. The low intensity light produced smaller amplitudes across pulses. The pattern of stimulation also affected the knee especially with the evoked amplitudes by the low intensity light since they did not return to rest after the first evoked movement.

In the ankle (see Figure 7), flexions were evoked by all intensities with the high intensity light producing the maximum amplitudes of evoked movement across all pulses. This also signifies that ankle flexors had a threshold of activation by the low stimulus intensity. Since maximum amplitudes of movements were evoked with the high intensity light, it supports the hypothesis that high light intensity may have increased the probability of activating ChIEF molecules through all pulses while the low light intensity had decreased activation ChIEF molecules. As usual, the pattern of stimulation may also have affected resting angle after first pulse as explained above.

Effect of stimulus period on amplitude of evoked movement

By varying the period of stimulation to 3s and 4s with the high intensity light and keeping the pulse duration still at 1s, there were bigger amplitudes of evoked movements in the hip, and moderate amplitudes of evoked movement in the knee and ankle. Figures 8 and 9 give a summary of these results.

In the hip, 3s period protocol had the bigger and consistent uniform amplitudes of movements (flexions) through all 5 pulses while that of 4s period protocol only had 2 amplitudes of movement (though bigger than that for 3s-protocol) with the first 2 light pulses. In the 4s period protocol, there were no movement evoked with the last 3 light pulses. The longer inter-pulse interval may have resulted in variability of the response in 4s period protocol. The longer wait time for stimulus may have delayed the depolarization of motor neurons and therefore no actual muscle contraction and movement. It also showed that the inter-pulse interval of $\leq$2s (for 2s and 3s period protocol) may efficient in producing bigger and consistent joint angle as well as

activating sufficient ChIEF molecules in relation to the inter-pulse interval (3s) of the 4s period protocol.

In the knee, 3s period protocol produced a uniform pattern of flexion amplitudes while 4s period protocol produced flexions before extension on the 1st-3rd and on the 5th pulses. This could be due to increasing the inter-pulse interval to 3s in the 4s period protocol which by some means may have activated flexors more strongly than the extensors. The 3s period protocol produced the maximum amplitude at the 3rd pulse which may be attributable to lesser inter-pulse interval in relation to 4s period protocol.

In the ankle, the maximum amplitude of evoked movement was produced with the 4s period protocol on the 1st pulse and decreased from 2nd to 5th pulses. The 3s period protocol had a uniform distribution of its amplitudes of evoked movements with its maximum on the 3rd pulse which was smaller than that of 4s period protocol on the 1st pulse. Perhaps in the ankle, it takes up to the 3rd pulse to reach its peak flexion with the 3s period protocol as a result of gradual building of tension in muscles. But in the 4s period protocol, the amplitudes had a deceasing trend due to reduction of tension in muscles as a result of increased inter-pulse interval which may have led to decreased activation of ChIEF. It could also be as a result of habituation process taking place in the neuronal circuit, which gradually depresses the synaptic conductivity (Kandel et al, 2000)

Comparing the amplitudes of evoked movements for the 2s, 3s and 4s period protocols, it was established that that the 2s period protocol with high intensity light produced bigger amplitudes as a result of lower inter-pulse interval of 1s, which was in adherence to the normal natural frequency of stimulation of an embryonic chick of ≤1Hz (Sharp& Fromherz, 2011).

Temporal effects of light on evoked movements

Onset latencies of evoked movements

In the hip, the evoked movement by the low intensity light had the lowest onset latency, followed closely by that for the high intensity on the 1st light pulse. This may be due to the fact that the low intensity light evoked extensions and extensors had a low threshold for activation and therefore may have been activated faster than the flexors. The high intensity light may have activated flexors, which had the higher threshold for activation, and therefore the hip was slower in moving. The onset latency for hip movements with high intensity light was shorter for the first two pulses in relation to that of the mid intensity light. It also showed an increasing trend from the 1st to the 5th pulse as expected. This is probably as a result of decreased activation of ChIEF molecules and therefore decreased muscle tension resulting in a longer time for the embryo to move the leg. The onset latency of hip movements for the low and mid light intensities showed a decreasing trend from the 3rd to the 5th and from the 2nd to the 5th pulse respectively, potentially, as a result of an increase in latent muscle tension.

For the 3s/4s period protocol, the onset latency of evoked hip movement with 3s period protocol was longer in the 1st pulse than that of the 4s period protocol, but it decreased on the 2nd pulse to the same onset latency as the 4s period protocol. The onset latency for 3s period protocol showed an increasing trend from the 2nd to the 5th pulse while with the 4s period protocol there were no evoked hip movement from the 3rd to the 5th pulse and therefore no recorded latency of evoked movement. These results could attest to the fact that activation with 3s period protocol was probably done within the natural movement frequency of the embryonic chick. The onset latencies with 3s/4s

period protocols were however longer than that of the high intensity in the 2s period protocol. This may imply that the inter-pulse stimulus of ≤1s was optimal for proper and effective activation of motor neurons in the embryonic chick.

For the knee, the onset latency with high intensity light was lower in the 1st pulse increased on the 2nd pulse but showed a decreasing trend from the 2nd to the 4th pulse and a slight increase. In effect, the onset latency was increasing on 5th light pulse due decreased muscle tension. The onset latency for the mid intensity light decreased on the 2nd pulse and showed an increasing trend from the 2nd to the 5th pulse. This could be due to the gradual inactivation of ChIEF molecules, decreased firing rate of action potentials by motor neurons and decreased tension in muscles. For the low intensity, onset latency of evoked movements increased from 1st to 2nd pulse and then decreased from the 2nd to the 5th pulse likely as a result of initial decreased activation of ChIEF molecules and gradual increased activation and increased muscle tension in the later pulses.

For the 3s/4s period protocols, the onset latencies of evoked knee movements were generally smaller with 3s period protocol than with the 4s period protocol due its smaller inter-pulse interval which was within the framework of natural movement frequency of the embryonic chick. The onset latency of evoked movements for the 3s period protocol increased from the 1st to the 2nd pulse, decreased on the 3rd and then showed an increasing trend from the 3rd to the 5th presumably due to reduced activation of ChIEF molecules. For the 4s period protocol, flexors were activated before extensors. It showed an increasing trend of onset latency of evoked movements from the 1st to the 4th pulse with flexion and from the 2nd to the 3rd and on 5th pulse with extension. The longer

inter-pulse interval of 3s may have played a role in this trend of activation by inconsistently activating ChIEF molecules.

In the ankle of most animals, extensors were activated before flexors with low and high light intensities. Onset of evoked ankle extension and flexion, were generally faster with the high intensity light than with the low intensity light as expected. This is likely due to higher intensity stimulus increased activation of ChIEF molecules and an increase in residual muscle tension that contributed to early movement.

For the 3s/4s period protocol, the onset latencies for ankle movements depicted an increasing trend from the 1st to the 5th pulse in both protocols as expected, but the onset for the 3s period protocol were always faster than that of the 4s period protocol. This could be as a result of decreased activation of ChIEF molecules across pulses, leading to low firing rate of action potential by motor neurons and lower muscle tension to produce faster response. As usual, the lower inter-pulse interval in 3s period protocol may have played a role in producing lower latencies as activation was done within the natural movement frequency of the embryonic chick. Refer to Tables 1-6 as well as Figures 7-12 for a summary of these results.

Peak Latencies of evoked movements

In the hip, the peak latency of evoked movements on the 1st pulse for the low intensity light was slightly lower than that of the high intensity light. For the low intensity light, the peak latency of evoked movement increased from the 1st to the 3rd pulse and decreased sharply from the 3rd to the 5th pulse. This could be attributed to the generalized active state of the embryonic motor system and the initial increased activation of ChIEF

molecules. From the 1st to the 3rd pulse, there was reduced activation of ChIEF molecules and that may have led to increased peak latencies of evoked movements. Thereafter, there may have been increased activation of ChIEF molecules in latter light pulses leading to reduced peak latencies. The peak latencies of evoked movements for the high intensity light showed a gradual increasing trend from the 1st to the 5th as expected due to gradual reduction in the number of activated ChIEF molecules across pulses. For the mid intensity light, flexions evoked for first two pulses showed an increasing trend while extension for the last two pulses, had no movement on the 3rd pulse, decreased on the 4th and further decrease on the 5th pulse. This may be due to the fact that hip flexors had an activation threshold that was greater that of the low intensity light. In the process the threshold for activation of the extensors may have been raised slightly in order to be stimulated by the mid intensity in the later pulses. The extensors also need more stimulation time to be activated. Also, contraction of flexors may have overridden that of the extensors in initial pulses. The extensors then took over in the last two pulses.

For 3s/4s period protocol, the peak latency of movements decreased from the 1st to the 2nd pulse with both protocols, which may be due to increased activation of ChIEF, and therefore the hip reached its peak of movement faster. There was then alternation of an increase and a decrease of peak latency of hip movements from the 3rd to the 5th pulse with 3s period protocol. This may be due to alternation of decreased and increased activation of ChIEF with these light pulses. There were no evoked responses from the 3rd to the 5th pulse with the 4s period protocol, which may be attributable to the longer inter-pulse interval which may have resulted in increased activation of ChIEF to overcome the hyperpolarized state of the motor neurons.

For the knee, the peak latency of evoked movements showed a decreasing trend with the high intensity light from the 1st to the 2nd pulse and an increasing trend from the 3rd to the 5th pulse. This is because the higher stimulus initially activated almost all of the ChIEF molecules, for firing of action potential by motor neurons and an increased muscle tension leading to strong movement. The knee therefore reached its peak amplitude of movement faster. The low intensity light may have activated fewer amounts of ChIEF molecules in the initial two pulses but increased its activation of ChIEF in the latter three pulses. This resulted in lower peak latency of movements in the latter stages of stimulation.

For the 3s/4s period protocol, the peak latency of the knee had a decreasing trend from the 1st to the 4th pulse and a slight increase on the 5th pulse with the 3s period protocol. This may be due to increased ChIEF activation and decrement on the 5th pulse. With the 4s period protocol, it showed an increasing trend with flexion from the 1st to the 4th pulse and a decrease on the 5th pulse. This may be due to initial reduction in activation of ChIEF and an increased activation on the 5th pulse. It could be that the increase inter-pulse interval had a positive effect on activating knee flexors. Knee extension with 4s period protocol did not depict any regular trend.

For the ankle, the peak latency of evoked extension was smaller with the high intensity light. The peak latency of evoked movement decreased and increased respectively from the 1st to the 2nd pulse with high and mid light intensities. The decrease with high intensity light may be due to increase in activation of ChIEF. The increase with mid intensity light may be due to reduction in activation of ChIEF. The peak latency of evoked movements with the high intensity light showed an increased pattern from the 2nd

to the 4th pulse and decreased on the 5th pulse. This may be due to reduced activation of ChIEF from the 2nd to the 4th light pulse and increased activation on the 5th pulse. For the mid intensity light, the peak latency of evoked movements decreased on the 3rd pulse, increased on the 4th and then increased on the 5th pulse. There might have been alternation of increased and decreased activation of ChIEF from the 3rd to the 5th pulse. The only recorded peak latency of evoked movement for the low intensity which was recorded on the 5th pulse was smaller than the other intensities. There were no evoked movements with the low intensity light and no flexion with mid intensity light from the 1st to the 3rd and on the 5th because these two intensities may not possess enough energy to stimulate motor neurons and/or hip muscles to cause any movement.

With the 3s/4s period protocol, the ankle peak latency of evoked movements for the 3s period protocol decreased from the 1st to the 2nd pulse and depicted an increasing trend from the 2nd to the 5th pulse. The peak latencies of evoked movements for the 3s period protocol were lower than that of 4s period protocol across pulses. There may have been initial reduction in activation of ChIEF and a latter increased stimulation on subsequent pulses. The peak latencies of evoked movements for the 4s period protocol showed an increasing trend the across the pulses likely due to reduced activation of ChIEF molecules.

Relaxation/ fall times of evoked movements

The 1st pulse of light caused the biggest fall time for all joints with the high intensity light. This was very critical to show the effectiveness of the high light intensity in activating all of the ChIEF molecules. The activation of ChIEF resulted in increased

rate of firing of action potential and increased muscle tone. Therefore, it took a longer time for the muscles of the joints to relax and in turn cause the leg to return to its resting position more slowly.

The hip showed a decrease in fall time from the 1st to the 5th pulse in 60% (3/5) of the animals with low intensity light while the high intensity showed a decreasing trend in 40% (2/5) of the animals. This was because with the low intensity, fewer ChIEF molecules were activated across pulses and it therefore took lesser time for the hip to return to resting position. For the 3s/4s period protocol, there was decreasing trend from the 1st to the 2nd pulse for both protocols, which may be due may be due to reduced activation ChIEF molecules.

In the knee, the decreasing pattern of fall time with mid (from the 2nd to the 5th pulse) and high (from the 1st to the 2nd pulse) intensities light were as a result of decreased activation and conductivity of ChIEF as well as reduced muscle tension across pulses. It is possible that there may be short-term habituation occurring in the neuronal circuitry as a result of synaptic depression (Kandel et al, 2000).

The decreasing trend from the 1st to the 2nd pulse and increasing pattern from the 2nd to the 4th pulse with the 3s period protocol may be due to decreased and increased ChIEF activation with the initial light pulses and latter pulses respectively. The alternate pattern of increase and decrease in fall times with 4s period protocol may also be due to alternation of short-term synaptic habituation and sensitization.

In the ankle, the decreasing trend in the fall time across pulses was likely due to decreased activation of ChIEF, reduced muscle tension and also short-term habituation of synapses. The decreasing trend showed with the 3s period protocol may be due to

increased inactivation of ChIEF across pulses. The alternation of decreasing and increasing trend depicted by the 4s period protocol may also be due to alternation of inactivation and activation of ChIEF.

Effect of 1hour training on motility of embryonic chick

Joint angles and excursions

The maximum and minimum joint angles were determined to ascertain if there were any changes after training. For the hip, the maximum joint decreased moderately from the pre-training to the post-training. The biggest maximum joint angle was recorded during the mid 10mins of training, after an initial increase from the 1st 10min. It then decreased slightly during the last 10min. The decreased maximum joint angle in the post-training may due to short-term habituation of synapses that occurred during the last 10min after an initial sensitization of neuronal circuitry during the mid 10mins. The joint excursion (difference between the mean maximum and minimum joint angles) reduced from the pre- to the post-training, which could be attributable to fatigue after training or decreased muscle strength from prolonged exposure to light as a result of inactivation of ChIEF (Sharp & Fromherz, 2011). It could still be as a result of habituation from reduced synaptic conductivity or strength. As a result the muscle might not be adequately activated to produce increased joint angles (Kandel et al, 2000).

In the knee, there was decreased maximum joint angle from the pre- to the post-training. The joint angles remained the same during the 1st 10min of stimulation and midway through stimulation but decreased during the last 10min of stimulation and then increased slightly in post-training. This could be due to short-term habituation process

occurring during the last 10min and a long or short-term sensitization or facilitation of the synaptic circuitry in the post-training. This resulted in an increase of joint angle in post-training from the last 10min but just a mild decrease in relation to the pre-training. But joint excursion for pre-training (39˚) was greater than that for post-training (22˚), presumably due to reduced muscle strength as a result of habituation during and after the training.

In the ankle, there was a reduction in maximum joint angle from the pre- to the post-training while the minimum joint angles remained almost the same. During training, the maximum joint angle increased from the 1st 10min to the mid 10min and a decrease in the last 10min. This is consistent with the idea that sensitization of neuronal circuitry may be happening during the mid 10min and habituation in the last 10min. Maximum joint angle however, increased after the training but was still lower than that of pre-training. There may have been a mild sensitization occurring after stimulation. But the decrease in joint excursion in post-training (33˚) with relation to pre-training (46˚) may be attributable to reduced muscle strength or mild fatigue after training as a result of decreased synaptic strength from habituation.

Evoked movements during training

There were some evoked movements during stimulation in 2/5 of the animals studied. They both showed increases in the number of evoked responses in the 1st 10min, a reduction midway through training and an increase in the last 10min of stimulation. This may depict that with no prior exposure the neuronal system was in an elevated active or sensitized state to be readily stimulated by light, but habituation set in midway

through stimulation due to prolonged exposure. The neuronal system may have then undergone facilitation to respond better after recovering from its synaptic depression. There may also have been decreased muscle strength as a result of reduced synaptic strength. Also, it could mean that there was a gradual inactivation of ChIEF as a result of prolonged exposure during the training (Kandel et al, 2000; Sharp & Fromherz, 2011). The other 3/5 (of the animals) that did not respond to light may have had their neuronal system in a generalized hyperpolarized state at the time of training because these animals when tested later with brief pulses reacted to light. Refer to Figures 16-22 for a summary of effect of training on joint angle.

Effect of training on spontaneous activity

The study showed that after one hour of training, the movement duration decreased. This could be due to decreased excitation of spinal circuitry and decreased muscle strength as a result of the habituation process that may have occurred during the stimulation. Also, it could mean that there had been decreased synaptic strength, which served as a compensatory or energy conservation mechanism, such that there was efficient regulation of movement by central pattern generator. This is probably the result of plastic changes taking place in the neuronal circuitry. During the stimulation, the movement time dropped in the 1st 10min, increased during mid 10min and last 10min. By the end of training, the movement time had decreased. The spinal circuitry may have adjusted its activity in order to accommodate the external stimulus and therefore regulated its movement accordingly.

The inter-movement interval increased slightly after the training. This showed that movement duration and inter-movement interval were not directly related. It could be attributed to some plastic changes happening in the neuronal circuitry and, therefore, the animals rested a bit more, even though they moved less. It could also be an energy compensatory mechanism by the embryonic system. During the training, movement time increased in relation to pre-training during the 1st 10min, reduced midway through stimulation and then increased in the last 10min of stimulation before a decrease in relation to post-training. The reduction in inter-movement interval in the mid 10min could be during to plastic changes occurring as a result of habituation.

After training, the period of motility was directly related to movement time. The decrease in the period of motility could be attributed to same factors that affected movement time as describe in previous paragraphs. During the training, the period of motility decreased from the 1st 10min to the mid 10min and increased in the last 10min of stimulation. The increase in the last 10min was due to increased movement duration and inter-movement interval as they were directly related. It therefore could be the result of increased sensitization of the neuronal circuitry leading to increased movement time.

Concluding remarks

In summary, this study was able to show a direct relationship between light intensity and evoked response or movement in an embryonic chick. This was consistent with the fact that high electrical stimulus intensity produce major responses by the neuromuscular system of an organism. High electrical stimuli on nerves increase the rate of firing of action potentials and an increase in contractile force produced by muscle

(Kandel et al, 2000). The high light intensity evoked maximum joint amplitudes and lowest latencies of evoked movements across pulses while the low light intensity performed the direct opposite. The mid intensity light also evoked amplitudes very similar to the high intensity light, but its latencies of evoked movements were a bit longer. Electroporation proved to be a successful method of introducing DNA into an organism in this study. This is because all animals reacted adequately to even least low intensity light (i.e. 66.66% current capacity). It was also determined that stimulation with high light intensity of 1s duration for a period of 2s, was the optimal light parameters necessary to produce maximal embryonic response. This was consistent with the fact that the normal frequency of movement for an embryonic chick was ≤1Hz (e.g. Sharp & Fromherz, 2011). Light intensities lower than the low intensity used in this study did not evoke any response. Further investigations (electrophysiology/ electromyography) may need to be performed to determine if they actually caused any response in the neurons or spinal circuitry or even in the muscles.

The study seems to show some changes in joint excursions and episode of motility after prolonged stimulation. There may be some plasticity occurring in the nervous system of embryos as a result of learning or experience through training. Learning through mechanisms such as habituation and facilitation or sensitization may be happening in the course of training. It is also possible that short-term plastic changes may be happening during the 5-pulse train. Further investigations to ascertain the mechanisms of plasticity are required. For example, studies of the isolated spinal cord could be done where the spinal cord and nerves would be assayed to determine any changes in the spinal circuitry in terms of increases in number of neurons and number of synaptic

connections. Electrophysiological or electromyographical studies could also be done to determine any changes in synaptic strength and any changes in contractile forces of muscle tissues.

This study has demonstrated a direct input and output relationship between light stimulation and evoked movement of embryos. It has also provided a general knowledge on embryonic neuroplasticity and also how activity component of neuromuscular activity is built. It is expected that this preliminary study will provide general knowledge on treatment and rehabilitation of neurodevelopmental conditions. For example, the knowledge provided could be useful in the making of prostheses for people with paraplegia or Erb's palsy, who require stimulation of affected nerves.

Time constraints were a major limiting factor for this study. Incubated eggs did not always produce viable embryos. For example, sometimes only 2 out of 6 animals could survive after incubation for three days. Electroporation was not always successful and some animals died even before light stimulation could be performed. Also, some animals did not produce sufficient number of transformed neuronal cells for adequate responses to light. Additionally, it takes a long time to perform one experiment on one animal. Therefore it prevented further manipulations of light pulse duration and period of light stimulus for each animal and the number of parameter changes was limited. Some animals were also lost during preparation for light stimulation.

For future extensive studies, it is recommended that there be additional variations of times for all intensities in order to determine best temporal factors of stimulation. For example stimulation with all intensities could be performed for lower pulse durations of 0.25s and 0.5s for periods of 0.75s, 1s, 2s and 4s. Stimulation should be repeated with

each stimulus within an animal. It would also be necessary to determine the relationship between movement time, inter-movement interval and the period of motility of the embryonic chick. It will be necessary to use electrophysiological techniques to ascertain the effect of light on the motor or spinal circuitry, especially with low intensities, which did not evoke any movement. Simultaneously, electromyography should also be used to determine effect of the various light intensities on the muscle tissue of the leg of the embryo.

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