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Los Angeles

Mechanisms of spinal learning related to a simple motor task in a  
mammalian system

A dissertation submitted in partial satisfaction

of the requirements for the degree

Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

by

Michael Selvan Joseph

2016



# ABSTRACT OF THE DISSERTATION

Mechanisms of spinal learning related to a simple  
motor task in a mammalian system

by

Michael Selvan Joseph

Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

University of California, Los Angeles, 2016

Professor Victor R. Edgerton, Committee Chair

Even in the absence of brain input, the spinal cord has the ability to learn dynamically complex motor tasks such as stepping and standing with repetitive training. (Hodgson, Roy et al. 1994, Harkema, Hurley et al. 1997, Edgerton, Tillakaratne et al. 2004, Fong, Cai et al. 2005). Although there is substantial behavioral evidence of the spinal plasticity following injury, molecular mechanisms of plasticity are largely unknown. To examine the mechanisms of plasticity or learning in spinal cord of locomotor trained animals proved difficult due to the complexity the circuitry involved.

In an attempt to understand the synaptic mechanisms involved in the performance of motor tasks in mammals, we adopted a simple model of learning in the rat paw withdrawal learning (PaWL) to the hindlimbs of mice (Jindrich, Joseph et al. 2009). Learning to avoid a shock by limb withdrawal was originally reported by Horridge (Horridge 1962, Chopin and Buerger 1976) in insects, later adapted for spinal rats (Chopin and Buerger 1976), (Grau, Crown et al. 2006). In PaWL paradigm, spinal mice dorsiflex the hindpaw in response to position-dependent mild electric

shocks to the tibialis anterior muscle and learn to hold the paw to minimize the net exposure to shocks. Although important molecular substrates of instrumental learning in the spinal rats have been validated by pharmacological manipulations (Joynes and Grau 2004, Gomez-Pinilla, Huie et al. 2007, Ferguson, Bolding et al. 2008), neither physiological characterization nor cellular mapping of learning-associated spinal neurons has been examined. This simple *in vivo* learning model provides an opportunity to identify molecules and neural pathways that mediate spinal learning in mammals, utilizing genetic and molecular tools of key learning-associated signaling molecules in the brain (Gomez-Pinilla, Huie et al. 2007, Joseph, Tillakaratne et al. 2012).

Through EMG analysis in tibialis anterior hindlimb muscle, the primary ankle flexor involved in the task, we showed that the proprioceptive learning during PaWL is mediated through specific sensory-motor spinal network (Chapter 3). Specifically, 1) We measured muscle activity using chronically and acutely implanted EMG electrodes during PaWL testing in several hind limb muscles of Master and Yoked groups, and showed that the sustained hindpaw dorsiflexion during learning is mediated through the activation of the TA motor pool in learned (Master) but not in the failed (Yoked) group (Chapter 3); 2) We compared EMG activity in the TA muscle under passive paw withdrawal learning condition, where electrical shock is removed and only the proprioceptive foot position is applied to complete spinal transected mice, we showed under these conditions, mice cannot learn to maintain the dorsiflexed hindpaw position (Chapter 3); 3) By selectively blocking the TA muscle or the cutaneous afferents, only blocking of muscle afferents led to failure of PaWL (Chapter 3).

Using the activity dependent marker c-fos in combination with the transsynaptic pseudorabies virus (PRV) or the learning marker, CaMKII, we identified activated neurons in specific laminae in mice that successfully learned the PaWL task (Chapter 4). We found that the

number of activated neurons (Fos+) were higher in the ipsilateral spinal cord of both groups of mice that received contingent (Master group) and non-contingent shocks (Yoked group) to the TA, but the number of activated neurons was inversely correlated with the degree of learning, only in the mice receiving the contingent shocks. CaMKII immunoreactivity in the ipsilateral spinal cord was highest in L4 segment in the lumbosacral spinal cord in Master mice. These mice also had more Fos+ CaMKII+ neurons in laminae IV-VI compared to the yoked mice. Pseudorabies virus (PRV) that labels functionally connected circuitry, when injected into tibialis anterior, the primary muscle involved in PaWL, showed that majority of activated neurons in L4 and activated CaMKII+ neurons in laminae IV-VI were also PRV+.

We then disrupted PaWL by interfering with cAMP response element binding protein (CREB) function in CaMKII expressing neurons using the conditional CREBIR transgenic mice, that express CREB repressor protein (CREBIR) with tamoxifen (Chapter 4). We found that disrupting CREB function in these transgenic mice while not affected the early learning, interfered PaWL when retested 24 hrs. later. Furthermore, the failure to learn the PaWL task was correlated with decreased levels of pCaMKII, and pCREB proteins in only in the CREBIR mice when induced by tamoxifen. Our data showed that compared to wildtype mice, the mutant mice showed reduced number of activated spinal neurons in laminae IV-VI of the spinal cord. For example, wild type spinal mice contained higher numbers of both Fos+ and CaMKII+ in lateral lamina II and laminae IV-VI at L4-L5 compared to CREBIR mice. The location of active spinal neurons in mice with normal CREB function, but not observed in transgenic mice with targeted CREB disruption, suggests that these neurons are important in Paw withdrawal learning. These results indicate that specific neurons in the L4 segment are part of the circuitry associated with hindlimb flexion during paw withdrawal learning. We showed that fewer neurons are activated in paw withdrawal

learning than stepping, allowing the mapping of circuitry in this simpler and feasible task. Identification of the spinal networks for this simple motor task will be a step toward understanding cellular and synaptic mechanisms of more complex learning such as standing and stepping after a complete spinal cord transection.

The dissertation of Michael Selvan Joseph is approved.

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## DEDICATION

In loving memory of my mother Elizabeth M. Joseph and father Nelson Joseph Sr.

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Jindrich DL, Joseph MS, Ootshi CK, Wei RY, Zhong H, Roy RR, Tillakaratne NJ, Edgerton VR. Spinal learning in the adult mouse using the Horridge paradigm. *J Neurosci Methods*. 2009 Sep 15; 182(2):250-4. Epub 2009 Jun 9. PubMed PMID: 19520117

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Joseph, M.S., Y Faynerman, S Sasthri-Rajaputrage, R Cruz, P Duong, D Jindrich, H Zhong, R R Roy, A J. Silva, V. Reggie Edgerton, N JK Tillakaratne, Paw withdrawal learning is impaired in spinal cord transected adult transgenic mice that are unable to phosphorylate cyclic AMP response element binding protein (CREB) *Soc Neurosci. Abstr*. 2009

Joseph, M.S., Rus Cruz, Lily Ying, Niranjala JK Tillakaratne, Roland R. Roy, Hui Zhong, Fernando Gomez-Pinilla V. Reggie Edgerton . The combination of omega-3 Fatty acid and curcumin dietary supplementation enhances learning of a sensorimotor task in the spinal cord. *Soc Neurosci. Abstr*. 2010

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Joseph, M.S., Griffis, K., Zhong, H., Roy, R.R., Tillakaratne, N.J.K., Edgerton, V.R. Tibialis anterior muscle activity during paw withdrawal learning paradigm. *Soc Neurosci. Abstr*. 2016

## CHAPTER OVERVIEW

The overall goal of my dissertation is to identify the neural circuitries and mechanisms associated with the paw withdrawal learning (PaWL) paradigm in the mouse spinal cord, in absence of the brain inputs. In PaWL paradigm, the spinal mice dorsiflex the hindpaw in response to position-dependent mild electric shocks and learn to flex the paw to minimize the net exposure to shocks. My overall hypothesis is that learning to hold the paw in a flexed position is mediated through a sensory-motor network that can control this specific movement of the tibialis anterior muscle. Figure 1-1 shows the specific aims of the dissertation.

**Chapter 1** contains brief literature review of background significance of PaWL including its the impact as a simple model of learning in the spinal network of a mammal under in vivo conditions. Spinal learning is reviewed in this chapter with emphasis on some early concepts of spinal conditioning, and instrumental learning and mechanisms of learning in brain and in spinal instrumental learning and learning deficit.

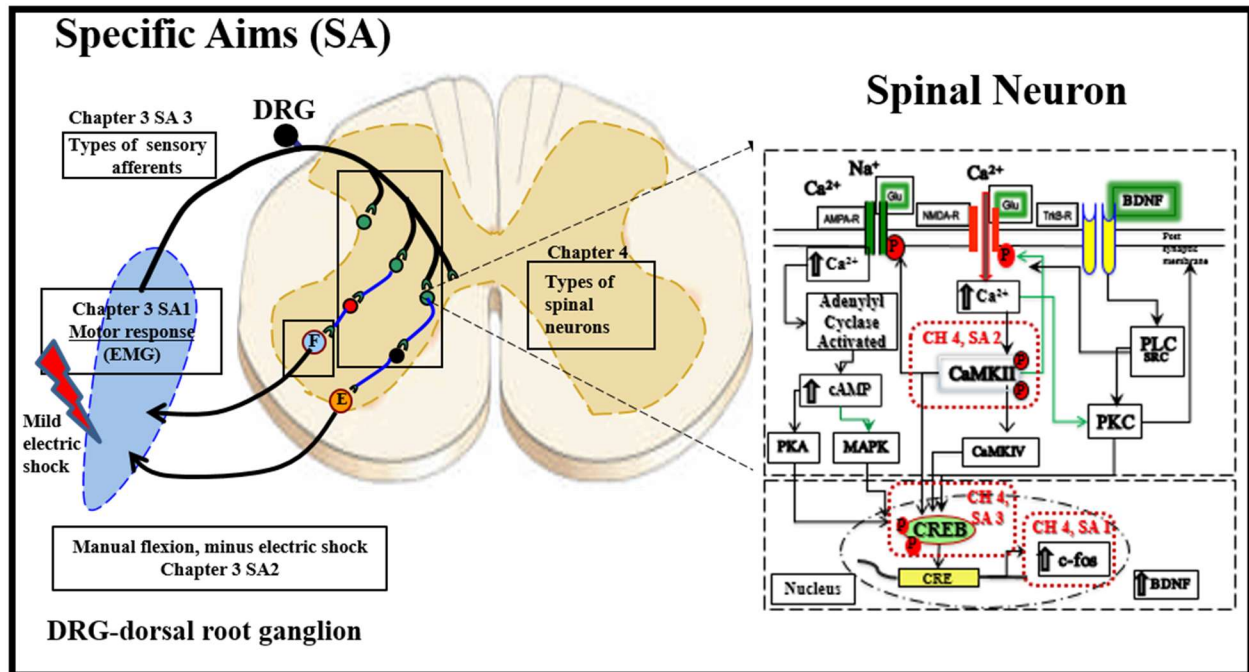
**Chapter 2** describes the Paw withdrawal paradigm (PaWL), with detailed methodology including testing and recording, and learning in both Swiss Webster and C57BL6 mouse strains.

**Chapter 3** sets out to demonstrate that during PaWL, the acquisition of the de novo flexed hindpaw is mediated through muscle-specific proprioceptive inputs and a time dependent modification of spinal interneuronal network associated with the tibialis anterior (TA), the primary muscle involved in the dorsiflexion of the paw. I show that, during PaWL, 1) the time-dependent modifications in spinal neuronal activity in the learned event is reflected in characteristic EMG activation patterns in the TA in the master but not the yoked (Hypothesis 1), 2) learning occurs when the sustained hindpaw dorsiflexion is mediated through changes in stimulus-driven paw position and not when it is passively imposed, without shocks (Hypothesis 2), 3) blocking

proprioceptive (muscle) afferents and not the cutaneous afferents will interfere with the paw withdrawal learning (hypothesis 3). To address hypothesis 1, EMG activities of the TA (ankle flexor) and its antagonist, medial gastrocnemius, and a synergist, vastus lateralis muscle, were measured during PaWL (experiments 1 and 2). Hypothesis 2 was addressed by recording the TA EMG activity during manual flexion of the hindpaw to mimic a sustained paw flexion, but without the application of the contingent shock (experiment 3). TA Muscle afferents and cutaneous afferents were blocked using lidocaine to address hypothesis 3 (experiment 4).

**Chapter 4** includes studies that examine the PaWL circuit by identifying neurons involved in PaWL using a combination of anatomical and genetic tools in spinal cord transected mice.

Specifically, certain populations of activated neurons expressing the learning associated-marker, Calcium/Calmodulin Kinase II (CaMKII) (hypothesis 1) in TA-associated circuitry (hypothesis 2) and CREB pathway (Hypothesis 3) are critical for PaWL. To address hypothesis 1, the activated neurons in the spinal circuitry involved in the PaWL, were immunohistochemically identified using the activity-dependent marker, c-fos, and CaMKII in lumbar spinal cord (Experiment 1). To address hypothesis 2, activated neurons were identified in functionally connected neurons in the hindlimb circuitry utilizing the transsynaptic pseudorabies virus (PRV-152) injected into the TA muscle (Experiment 2). To address hypothesis 3, CREB function in CaMKII expressing neurons were disrupted using the conditional CREBIR mice treated with tamoxifen to induce CREB-repressor fusion protein (CREBIR) and activated spinal interneurons critical for PaWL were identified (Experiment 3)(Kida, Josselyn et al. 2002). Each of these experiments have provided insight into the mechanisms of learning and memory in a spinal network controlling a simple motor task in a mammalian system.



**Figure 1-1: Specific aims of the dissertation.**

A schematic of the spinal cord shows muscle afferent inputs, few types spinal interneurons and the motor output. Green circles depict interneurons receiving afferent inputs. Flexor (F, blue circle) and extensor (orange circle, E) motoneurons receive inputs from secondary interneurons (Red and black circles). This schematic is a simplified version and does not include all types of interneurons or motoneurons. The larger version of the spinal neuron, shows CaMKII and CREB-mediated pathways of learning. BDNF, brain-derived neurotrophic factor; CaMKII, Calcium/calmodulin dependent Kinase II; CRE, cyclic AMP response element; CREB, cyclic AMP response element binding protein; DRG, dorsal root ganglion; MAPK, mitogen- activated protein kinase; PKA, Protein Kinase A; PKC, Protein Kinase C.

## CHAPTER 1: Background and Significance

### **Impact of Spinal Cord Injury**

The average prevalence of people living with SCI in the US population alone is approximately 276,000 persons and range between 240,000 and 337,000 persons(DeVivo and Chen 2011) with approximately 12,500 new cases per year(Selvarajah, Hammond et al. 2014). The average age of injury has increased from 29 yrs. during the 1970s to 42 yrs in 2010 (Molton, Terrill et al. 2014, Matsuda, Verrall et al. 2015). The etiology of SCI is largely due to vehicular crashes, falls, gun violence, and sports mediated injuries (Oteir, Smith et al. 2014). Worldwide incidence of SCI has also increased due to a greater use of automobiles, violence, and accidental falls(Jazayeri, Beygi et al. 2015).

The total economic cost, which includes medical, adaptation, material, administrative, and first-responder costs including the loss of productivity to the patient is approximately \$131-\$302 million (Garcia-Altes, Perez et al. 2012). The lifetime cost can vary based on severity and onset of injury. Individual costs vary, as more severe SCI (high cervical tetraplegia) is estimated to cost approximately \$1 million in the first year following the injury and \$184,000 each subsequent year. Recent developments in therapies and clinical care advancement have improved reduced the length of initial hospitalization and respiratory- mediated mortality (Ploumis, Kolli et al. 2011) and resulted in impairment in a wide range of sensory-motor tasks and autonomic functions. It is clear there is a global need to seek out and develop therapies that regain patient function and improve their quality of life, and decrease the burden of care on the immediate family as well as the society at large. Currently, finding cures for spinal cord injuries requires developing a better understanding of fundamental spinal cord physiology, plasticity, including learning and memory which promotes

these fundamental basis plasticity of motor tasks that can be learned with in the isolated spinal network.

### **The automaticity of the spinal cord**

The neurophysiology of locomotion involves the integration of sensory inputs locally at the spinal cord and in brain for complex tasks, with supraspinal inputs of descending and ascending tracts of reticulospinal, vestibulospinal, rubrospinal, and corticospinal tracts. Two levels of control of locomotion were proposed by Mark L. Shik and Grigori N. Orlovsky (1976). The first level embodies the local tonic output from the mesencephalic that activates spinal interneurons and eventually to motoneurons generating a cyclic activity of many motor pools in a manner that results in stepping. The second level involves the cortical integration of all afferent sensory inputs from the proprioceptive position of the head, torso, and limbs. In addition, the visual and auditory inputs direct and fine tune motor control from the cerebellum via the descending tracts (Shik and Orlovsky 1976).

Independent of supraspinal inputs, the spinal cord is capable of an extensive level of automaticity in controlling and coordinating the locomotor activity at the spinal cord level. The spinal neuronal network is capable of intricate timing and activation, resulting in locomotion. For example, Grillner and colleagues showed that the lumbar spinal neurons are capable of timing and activation of large and small hindlimb muscles during locomotion at variable treadmill speeds and loads on the hindlimbs of cats, whose spinal cords were completely transected at mid-thoracic vertebral levels (Andersson, Forssberg et al. 1981). Sensory feedback and modulation at the spinal level was clearly seen in the alternation of EMG activation at the gastrocnemius and semitendinosus muscles and changes in hip, knee and ankle angles during treadmill speed and load modulation in these chronic complete spinal cats.

The automaticity or rhythmogenic capacity of the spinal cord is thought to be mediated through a network of neurons named central pattern generators (CPG)(Grillner and Zangger 1979). In the invertebrate and vertebrate systems, the CPG represents a circuitry of neurons involved in generating an oscillatory and highly coordinated motor efferent patterns under conditions where there is no external drive oscillatory input (Zucker, Kennedy et al. 1971, Moore and Buchanan 1993). Using spinal cord isolation studies in cats, Grillner and Zangger identified the CPG network to be distributed from L6-S1. The cat spinal isolation consisted of one transection at the thoracic 12-13 level followed by a second transection performed variable between levels between L3-L7. Then the dorsal roots were dissected between L3-L7 while the ventral roots remained intact. Locomotion in the spinal isolated cats was achieved using L-DOPA and monoamine oxidase inhibitor Nialamide. They were able to show alternation of activity between flexors and extensors of foot, ankle, and knee. Furthermore, Grillner and Zangger identified the CPG network involved in hindlimb stepping to be distributed from L6-S1(Grillner and Zangger 1979). Subsequently it was shown that the interneurons within these spinal segments are rhythmically active and highly limited to the motor effect pattern during fictive locomotion(Edgerton, Grillner et al. 1976). Similar location of CPG was established in rodents (Bertrand and Cazalets 2002) and turtles (Berkowitz 2004). The administration of 5-hydroxytryptamine (5-HT) and N-methyl-D-aspartate (NMDA) have been shown to trigger induction of fictive locomotion in hindlimbs (Barbeau and Rossignol 1994).

## **Rehabilitation after Spinal cord injury**

Spinal cord injury often results from blunt force trauma, vertebral disk compression or disk displacement that cause paralysis and loss of function. The dysfunction can also be described as



the disruption of cortical and subcortical structures to drive spinal networks. The severity of spinal cord injury in patients can range from a complete functional loss to a partial paralysis.

Numerous animal studies show that even in the absence of any supraspinal inputs, with repetitive training, animals can acquire complex motor tasks such as standing, stepping and adapt to perturbation (Lovely, Gregor et al. 1986, Barbeau and Rossignol 1987, Lovely, Gregor et al. 1990, Barbeau and Rossignol 1994, de Leon, Hodgson et al. 1998, Fong, Cai et al. 2005, Liu 2007, Courtine, Song et al. 2008, Ichiyama, Broman et al. 2011, Zhong, Roy et al. 2012). For example, low thoracic spinal cord transected cats, trained daily to step executed greater number of consecutive plantar steps and also had the ability to support more weight-bearing, during treadmill stepping (Edgerton, de Leon et al. 1997, de Leon, Hodgson et al. 1998). Furthermore, the electromyography (EMG) recordings of soleus (ankle extensor) and tibialis anterior (ankle flexor) muscles at 2 week and 12 weeks with treadmill step training lead to increased muscle activities (Lovely, Gregor et al. 1990, Roy, Hutchison et al. 1991, de Leon, Hodgson et al. 1994). In complete adult spinal cats and rats that were trained to step on a treadmill when presented with an obstacle along the path of the treadmill as a trip perturbation, they were able to reprogram/adapt their step trajectory to avoid the object (Liu 2007, Zhong, Roy et al. 2012). Moreover, the EMG data suggested that the primary knee flexor semitendinosus contributes to this adaptive response to avoid the perturbation (Liu 2007, Zhong, Roy et al. 2012).

Intensive activity-based locomotor therapy can improve functional recovery in humans with chronic incomplete SCI. For example, patients with incomplete spinal cord injuries (American spinal injury association impairment scale, AIS grade C and D), improved balance and walking after 20 sessions of body weight support locomotor treadmill training (Dobkin 1995, Harkema, Hurley et al. 1997, Lunenburger, Bolliger et al. 2006). Advances in SCI research has

focused on combining of spinal cord epidural stimulation (ES) in regions below the injury site to facilitate locomotion. For example, ES and subdural lumbar stimulation in decerebrate cats can induce fictive hindlimb locomotion (Iwahara, Atsuta et al. 1992, Iwahara, Atsuta et al. 1992). More recently, ES in mid-thoracically spinal cord transected rats lead to facilitation of the lumbar spinal network when treadmill stepping with body-weight support (Ichiyama, Gerasimenko et al. 2005, Gerasimenko, Lavrov et al. 2006, Gerasimenko, Ichiyama et al. 2007, Edgerton, Courtine et al. 2008) In humans, the combination of ES and locomotor training resulted in improved standing with and without standing cage, improved bladder function and increased voluntary control of legs (Harkema, Gerasimenko et al. 2011, Roy, Harkema et al. 2012, Rejc, Angeli et al. 2015). Following an approach similar to ES facilitation of the spinal motor network, the use of non-invasive transcutaneous stimulation (TS) in healthy subjects suspended in a gravity neutral position generated alternating leg movements. The application of transcutaneous surface array in a SCI patient showed EMG facilitation at the rectus femoris during step cycling (Gerasimenko, Gorodnichev et al. 2015, Gerasimenko, Gorodnichev et al. 2015, Sayenko, Atkinson et al. 2015, Sayenko, Atkinson et al. 2015). Although ES and TS can improve motor ability in SCI patients, the mechanism of plasticity or the underlying circuitry is poorly understood. All of these imposed motor functions could be possible only if the spinal networks contained mechanisms for learning and memory.

## **Spinal learning or plasticity**

### *Early concepts of Spinal conditioning or plasticity*

Spinal plasticity related research began with focus on examining habituation of spinal reflexes as a spinally-mediated event. Notably, Sherrington (1906) described the fatigue of scratch and flexion reflexes in spinal cord transected dogs (Sherrington 1906). He defined fatigue as the

diminished spinal reflexes as a response to repeated cutaneous stimulation at the hind limb. Shurrager and Shurrager (Shurrager and Shurrager 1946) and later Patterson, Cegavske and Thompson (Patterson, Cegavske et al. 1973) showed that the spinal cord can undergo classical conditioning. Dykman and Shurrager showed that the spinal cord was capable of motor conditioning following complete spinal cord transection in cats (Dykman and Shurrager 1956). The motor conditioning was achieved when a weak mechanical (Sable hair brush on the fur) or electric stimulation to the tail (conditioned stimulus, CS) is combined with a strong electrical shock (unconditioned stimulus, US) to the ipsilateral medial semitendinosus (a hindpaw flexor muscle), resulting in the flexion of the hind leg (conditioned response, CR). With ~ 2 hours of training, some animals learned to associate CS to CR. The extinction of the CR was also evident in all the animals that learned. The existence of extinction supported that the behavior was a learned response. In another study, chronic spinal kittens given a tail pinch (CS) and toe pinch (US), showed EMG increases over training sessions in the paired group with only response habituation in the unpaired group (Buerger and Dawson 1968).

Patterson and Thompson studying Pavlovian conditioning in the spinal cord also showed modified response for the CS-US pairing was not an outcome of habituation or sensitization at the spinal cord (Groves, Glanzman et al. 1970, Cegavske, Patterson et al. 1979). In an intact or acutely spinal cat, habituation is a response to weak repeated cutaneous stimuli with a diminishing amplitude of hindlimb flexion reflex response, whereas sensitization or windup occurs when given the repeated stimuli increases flexion amplitude response (Shurrager and Shurrager 1946, Wall 1958). Fitzgerald and Thompson showed during classical conditioning of the hindlimb flexion reflex in acute decerebrate spinal cats, when the animals are given a shock (CS) to the skin of the thigh skin paired with a shock (US) to the toe pads of the same leg, only the paired trials resulted

in significant increases in the leg flexion (CR) response to the CS, but not to unpaired stimuli (Fitzgerald and Thompson 1967). Furthermore, during the extinction trials which was conducted with increasing CS intervals, the CRs decreased over time demonstrating that the learned CR is novel in the spinal cord and it is not a product of spinal sensitization. Patterson and Thompson developed an acute spinal cat preparation under anesthesia with Flaxedil to control body and leg movements during spinal conditioning (Patterson, Cegavske et al. 1973). Groves and Beggs utilized this preparation to study the neural mechanism underlying the spinal conditioning, and using microelectrodes placed in the afferent terminals area or near motoneurons they showed that the increased CR amplitude with CS-US pairing was not taking place at the afferent terminals (Groves, Glanzman et al. 1970) or in the motoneurons (Beggs 1986), but in the interneuron fields of the cord (Patterson, Steinmetz et al. 1982, Patterson 2001). In a later study, Durkovic showed that the spinal conditioning survives with greater interval of testing following CS-US training, suggesting that the spinal conditioning is due to modification of the neurons participating in a post-tetanic potentiation driven by a mechanism of long term potentiation (Durkovic 1985). Durkovic and Prokowich used an NMDA receptor antagonist, D-2-amino-5-phosphonovalerate (APV), to block the induction of associative long-term potentiation during the spinal conditioning that showed no signs of reflex potentiation in spinal cat (Durkovic and Prokowich 1998). These results demonstrated the similarities in motor adoptive response in spinal cord classical conditioning and long-term potentiation in the hippocampus.

### *Instrumental learning/conditioning in absence of supraspinal inputs*

Horridge demonstrated that headless insects can learn new leg positioning upon stimulation as described below (Horridge 1962). Horridge inserted one electrode into a hindlimb flexor muscle of a headless insect, and a second grounding electrode attached to the tarsus of the same leg,

positioned over a saline water bath kept at a critical point (threshold). When the electrode hanging from the insect's tarsus, made a contact with saline, the insect received shocks. During the training, one insect received shock contingent on the leg position (master), while a second insect that was paired electrically to the master, received the same stimulus, but non-contingently (yoked). Horridge concluded although the yoked received higher frequency of stimulation similar to the master, only the master insect was capable of flexing the leg to avoid the shock (Horridge 1962).

Instrumental conditioning in adult spinal rat was modeled after the Horridge learning paradigm by Buerger and Fennessy (Buerger and Fennessy 1970), Chopin and Buerger (Chopin and Buerger 1975) and Grau (Grau, Barstow et al. 1998) to show that spinal rats can also learn to acquire and maintain a new leg position in a Pavlovian conditioning protocol. In Horridge-like instrumental conditioning, the rats whose spinal cords were completely lesioned at low thoracic level by cauterization, began the conditioning 24-48 hrs later. One electrode inserted into the TA muscle and a second thick-electrode (ankle electrode) placed along the flat bottom of the ankle protruding ahead of the toes, were connected to a stimulator that is connected serially to a saline bath. The vertical threshold was imposed by placing the tip of the protruding ankle electrode 4mm below the surface of the saline solution. When the ankle electrode comes in contact with the saline solution, the completed circuit generates a shock at the TA muscle. As the ankle begins to flex, it withdraws the ankle electrode out of the saline solution breaking the circuit and halts the electrical shocks at the TA muscle.

Both Chopin and Grau used the electrically paired master and yoked animals as in the insect model as described earlier (Horridge 1962). Chopin and Buerger also used this paradigm to show that the master spinal rats acquired foot flexion from 4 mm to 8 mm vertical threshold, when the vertical threshold was raised by 1 mm increments imposed after 2 min of sustained foot holding

above the threshold. These results showed that the spinal cord is capable of learning a new task and there is a behavioral memory component during instrumental conditioning (Chopin and Buerger 1975). Grau and colleagues showed that the intensity of stimulation was also critical for the learned response-outcome relationship (Grau, Crown et al. 2006). The current required to generate a force between 0.4N-0.6N was optimal to induce learning. A stimulation current that is too low or too high, producing a force (0.2N or 0.8N respectively), resulted in habituation. Crown and Grau (Crown, Ferguson et al. 2002) proposed that the yoked animal's inability to learn is not a consequence of poor performance but an acquired learning deficit due to the non-contingent shock inducing a greater habituation (Joynes and Grau 1996). Furthermore, when the ipsilateral or contralateral hindlimb of previously trained master or yoked was retested under the contingent shock condition, only the master reacquired the learning task in either of the hindlimbs. Grau suggested that transference memory from prior exposure facilitated the learning in the master while the prior learning deficit inhibited the subsequent learning in the yoked group (Grau, Barstow et al. 1998, Crown, Ferguson et al. 2002).

## **Mechanisms of learning**

### *Learning and memory in brain*

#### **Protein Kinase A (PKA) pathway, and Ca<sup>++</sup>/calmodulin kinase II (CaMKII)**

A primary component of neuronal mechanism of learning and memory in the vertebrate mammals is the formation of long term potentiation (LTP) and long term depression (LTD) between presynaptic and postsynaptic neurons (Dunwiddie and Lynch 1978, Siegelbaum and Kandel 1991). The induction of early LTP requires an activity dependent activation (4 pulse at 100Hz stimulation) at the presynaptic neuron (Rose and Dunwiddie 1986, Roberts and Glanzman 2003). Arrival of action potential leads to an influx of calcium via calcium channels (Zucker 1999).

Serotonergic interneuronal input both at pre- and post-synaptic terminal initiate a Serotonin (5HT) mediated the adenylate cyclase catalyzed cascade that converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) and subsequent activation of protein kinase A (PKA)(Tanaka and Nishizuka 1994, Abel, Nguyen et al. 1997, Chitwood, Li et al. 2001, Glanzman 2010). Protein kinase A then increases presynaptic vesicle release of glutamate into the synapse and facilitates gene transcription in the nucleus (Abel, Nguyen et al. 1997, Roberts and Glanzman 2003). The glutamate binding to  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and NMDA receptors initiate movement of cations ( $\text{Ca}^{++}$  and  $\text{Na}^{+}$ ) into the post-synaptic bouton(Grover 1998, Liu, Wong et al. 2004). Serotonin from interneurons act at the postsynaptic terminal to initiate the phospholipase C and insostal-1,4,5-triphosphosphate cascade to release stored calcium (Taufiq, Fujii et al. 2005, Glanzman 2006). In the presence of  $\text{Ca}^{++}$ /calmodulin, the Thr286 becomes autophosphorylated that activates  $\alpha\text{CaMKII}$  which binds to the NMDA receptor subunit NR2B in the post synaptic density (Lisman, Yasuda et al. 2012). Calcium then binds to  $\text{Ca}^{++}$ /calmodulin kinase II (CaMKII), a mediator for NMDAR-dependent LTP, and PKA to increase trafficking and insertion of AMPAR into the post-synaptic membrane(Naisbitt, Kim et al. 1999, Lu, Man et al. 2001). The increased levels of cAMP also initiate the cAMP response element-binding protein 1(CREB1) pathway to increase gene transcription which is required for LTP stabilization between the pre- and post-synapse (Silva, Kogan et al. 1998). The transition from early to late LTP occurs with continued activity dependent pre- and post-synaptic induction that lead to CREB1-mediated gene transcription that ultimately stabilizes the plasticity.

### **BDNF and Tropomyosin-related Kinase B (TrkB) signaling**

In addition to Glutamate, neurotrophic factors such as BDNF and neuromodulatory transmitter dopamine are involved in maintaining, remodeling and stabilizing the synapse. Brain-derived neurotrophic factor (BDNF)/TrkB signaling pathway is involved in learning and memory mainly through the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway. Binding of BDNF protein to the TrkB receptor result in the dimerization and activation of the intracellular tyrosine kinase domain. Autophosphorylation of the TrkB dimer initiates three downstream cascades; activation of intracellular signaling cascades, including the Ras–MAPK, phosphatidyl inositol-3 kinase (PI3K)/serine threonine kinase (Akt) and phospholipase C (PLC)- $\gamma$  pathways. Common but not limited to these pathway, is the initiation of transcription factors such as CREB to induce LTP, Zif268 in formation of long term memory and for neuronal survival, and growth(Whitlock, Heynen et al. 2006). Behavioral evidence support the role of BDNF-TrkB in LTP. For example, deletion of BDNF in mice disrupted the induction of early-LTP (E-LTP) in the CA1 region of hippocampal slices(Korte, Carroll et al. 1995). Inhibition of TrkB signaling with TrkB IgG antiserum 1-2 hours before high frequency stimulation impaired the induction of L-LTP maintenance significantly in hippocampal slices, indicating importance of BDNF-TrkB signaling in the maintenance of LTP(Korte, Kang et al. 1998).

### **MAPK /ERK1/2 pathway**

The presynaptic release of glutamate binds to AMPA receptors and NMDA receptors will allow the influx of calcium into the post synaptic density as described earlier. High levels of calcium activate several kinases such as CamKII, PKC and PKA. An increase in PKC activity initiates the downstream cascade of the mitogen activated protein kinase (MAPK) pathway(Wiegert and Bading 2011). Additionally, PKC activation of small GTPase RaP-1 or Ras results in phosphorylation of Raf proteins with in turn phosphorylate MEK1/2 intermediate, that



subsequently phosphorylate the ERK1/2 protein (Derkach, Oh et al. 2007, Patterson, Szatmari et al. 2010). Phosphorylation of ERK1/2 modulate AMPA receptor trafficking that increases the postsynaptic density in stabilization of the synapse. Furthermore, ERK1/2 also phosphorylates specific transcription factor such as Elk-1 and mitogen- and stress activated kinase 1 (MSK1) which in turn phosphorylates CREB to initiate downstream protein synthesis(Davis, Vanhoutte et al. 2000, Wiegert and Bading 2011). Other transcription factor important for synaptic plasticity regulated by the ERK1/2 are Zif268, BDNF, Arc, c-fos and c-Jun(Guzowski, Miyashita et al. 2006, Reul, Arthur et al. 2007). In contextual fear memory conditioning, the intrahippocampal injection of the MEK inhibitor U0126 before contextual and shock conditioning phase blocked the activation of both ERK1/2 and CREB when only the context was presented alone and correlated to behavioral memory impairment(Trifilieff, Herry et al. 2006).

### **c-Fos protein role in learning and memory**

The Fos protein is a 62kDa protein that is a member of the family of immediate early gene transcribed in response to increased cellular activity (Hu, Mueller et al. 1994). C-fos has been widely used as an activity dependent marker of neuronal activation (Hoffman, Smith et al. 1993). A minimum of 15min of exercise or treadmill running can lead to increased c-Fos protein levels in the cortex and spinal cord(Brudzynski and Wang 1996, Ahn, Guu et al. 2006). The activation of the proto-oncogene c-fos was first observed in the brain following seizure activity and noxious stimulation. Since then, a many stimuli have been identified to initiate c-fos expression; cutaneous input (Alberini, Ghirardi et al. 1995, Chiasson, Hong et al. 1997), odor (Amir, Cain et al. 1999, Jahng, Choi et al. 2003, Kippin, Cain et al. 2003), neurotransmitters(Hu, Liu et al. 2002, Chen, Dong et al. 2003), vision(Craner, Hoffman et al. 1992, Kaczmarek and Chaudhuri 1997, Hannibal, Vrang et al. 2001), hormones (Rachman, Unnerstall et al. 1998), stress (Serova, Saez et al. 1998),

and locomotion (Brudzynski and Wang 1996, Iwamoto, Wappel et al. 1996, Ichiyama, Gilbert et al. 2002, Ahn, Guu et al. 2006, Tillakaratne, Duru et al. 2014, Duru, Tillakaratne et al. 2015).

Activity dependent activation leads to the increased expression of the immediate-early-genes, c-fos and c-Jun. Both c-fos and c-Jun combine to form a heterodimer AP-1. One identified function of the AP-1 transcription factor is to regulate target genes involved in cell growth, inflammatory responses, and repair processes (Yu, Chen et al. 1997, Sinitsyna, Reznikova et al. 2010). In a recent study by Ivashkina and colleagues, a single episode of contextual fear conditioning was shown to increase c-fos and Arc proteins in the dentate gyrus and hippocampal CA1 and CA3 fields (Ivashkina, Toropova et al. 2016). The direct learning gene targets of c-fos or AP-1 have not been identified but the majority of cognitive studies have established the upregulation of c-fos is strongly associated with the process of acquisition of a behavioral task and demonstrates learning, memory, and consolidation.

### **CREB mediated plasticity**

Many studies have attempted to unravel the molecular mechanisms of learning in the brain by focusing on the various learning associated proteins; NMDAR, AMPAR subunit GluR1, CaMKII, BDNF, and CREB and their role in the learning pathways. In Aplysia and hippocampal learning and memory, NMDA mediated plasticity is critical for developing long term plasticity (Dudek and Bear 1992). Along with NMDAR,  $\alpha$ CaMKII is a key protein implicated in LTP and spatial learning and memory (Achterberg, Buitendijk et al. 2014, Arruda-Carvalho, Restivo et al. 2014). Mice with  $\alpha$ CaMKII mutation exhibited spatial learning deficits when compared to their wildtype controls. For example, Silva and colleagues showed that although the mutant mice could learn to find the visible platform initially, but once the platform was hidden, needed more time compared to the wildtype controls (Silva, Paylor et al. 1992). In another study, Frankland and

colleagues showed the importance of  $\alpha$ CaMKII in contextual fear conditioning using mice with  $\alpha$ CaMKII T286 mutation (Frankland, Josselyn et al. 2004). Percent of “freezing” in the mutant mice was significantly less than the wildtype mice. They also showed the importance of NMDA in fear conditioning, by demonstrating decreased percent of freezing in wildtype mice injected with  $[\pm]$ -3-[2-Carboxypiperazin-4-yl]propanephosphonic acid (CPP), a selective NMDA receptor antagonist, compared to saline injected mice (Frankland, Josselyn et al. 2004).

Much of the NMDAR-dependent LTP has focused on the mechanisms responsible for the initial increase in synaptic strength lasting 30–60 min (Blitzer, Wong et al. 1995). In the invertebrates, the mechanisms that allow LTP to last hours involve activating the Aplysia CCAAT enhancer-binding protein (ApC/EBP) mediated gene transcription (Alberini, Ghirardl et al. 1994). In vertebrates, the cyclic AMP response element binding (CREB) protein family of transcription factors are critical for formation and retention of memory (Taubenfeld, Wiig et al. 2001). In mice, the disruption via null mutation of the CREB  $\alpha$  and  $\delta$  isoform mutants showed normal short-term but abnormal long-term memory (Kogan, Frankland et al. 1997). Kida and colleagues showed that conditionally blocking the phosphorylation of CREB did not affect the acquisition of fear conditioning, but the mice had difficulty in consolidation of long term conditioned fear memories (Kida, Josselyn et al. 2002). Using a Pavlovian contextual fear-conditioning task, Franklin and colleagues using CREB  $\alpha,\delta^{-/-}$  showed that the formation of lasting memory of context and shock in contextual fear conditioning required CREB dependent transcription and protein synthesis (Frankland, Josselyn et al. 2004).

There are other transcription factors beyond CREB1 involved in memory formation. CCAAT-enhancer binding protein (C/EBP) and early growth response protein 1 (EGR1) are two transcription factors with a role in learning and memory and are transcriptionally regulated by

CREB1. Both C/EBP $\delta$  and Zif268 (aka EGR1) are transcription factors that can in turn regulate the expression of other genes. While CREB is required for memory consolidation and reconsolidation, the role of C/EBPs in memory consolidation does not parallel that of CREB. The injection of C/EBP $\beta$  antisense oligomers into the hippocampus blocks memory consolidation, but the same injection does not affect memory reconsolidation (Taubenfeld, Milekic et al. 2001). The data suggest that reconsolidation is independent of hippocampal expression of C/EBP $\beta$ . In another study, mice without Zif268 exhibit reduced protein synthesis and deficits in late-LTP and hippocampal dependent memory, but maintained normal short-term memory function (Jones, Errington et al. 2001). These data support the hypothesis that Zif268 acts as part of a cascade of transcription factors required for protein-synthesis dependent L-LTP and long-term memory formation (Jones, Errington et al. 2001). In addition to CREB1, both C/EBP and Zif268 transcription factors can regulate downstream genes related to LTP, learning, and memory (Matynia, Kushner et al. 2002).

Learning and memory as a function of synaptic plasticity has been shown to be dependent on many signaling pathways that lead to the activation of many intermediate proteins that regulate protein synthesis. Although we have focused on the glutamate induced AMPA/NMDA-mediated plasticity, there are other parallel signaling pathways such as the voltage-gated calcium channels involved in downstream activation of many similar synaptic plasticity-related transcription factors. We focused on CaMKII and CREB dependent transcription synthesis as key markers of early and long term learning to demonstrate spinal cord learning using the PaWL paradigm.

Increased levels of CaMKII and the phosphorylation of CaMKII and CREB transcription factor are key indicators of learning and memory. The key step of LTP induction is CaMKII auto-phosphorylation that increases AMPA receptor mobilization and CaMKII facilitation of NMDA

receptors. In long-term learning and memory requires the phosphorylation of CREB by multiple kinases including PKA, RSK-2, CaMKII, and CaMKIV intermediates which suggests the convergence of multiple signaling cascades that can result in phosphorylate CREB as a key step in establishing long term memory.

### *Mechanisms of in spinal cord instrumental learning*

Grau and colleagues demonstrated through pharmacological studies that induction and maintenance of spinal learning under the master or controllable shock conditioning required facilitation through the NMDA and AMPA receptors pathway (Joynes, Janjua et al. 2004, Hoy, Huie et al. 2013) (Table 1-1). For example, intrathecal delivery of the competitive NMDAR antagonist AP5 or competitive AMPAR antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), into the spinal cord before or towards the end of the 30 min contingent shock learning, dose dependently blocked the induction and maintenance of hindlimb flexion, respectively.

Gómez-Pinilla in a collaboration with Grau's group (Gomez-Pinilla, Huie et al. 2007) showed that instrumental learning in spinal master rats was impaired by AIP (a myristoylated form of Autocamtide-2-Related Inhibitory Peptide (AIP), a highly potent and specific substrate competitive inhibitor of CaMKII), demonstrating that CaMKII is also critical in spinal learning (Gomez-Pinilla, Huie et al. 2007). Furthermore, in the lumbar spinal cord segments of rats that were given contingent shocks (master condition), the levels of BDNF, CaMKII, CREB, and Synapsin I mRNA were significantly increased when compared to the rats that received uncontrollable shocks (Yoked) and in spinal rats that did not receive shocks (unshocked control). Blocking the BDNF pathway with TrkB IgG also inhibited the learning, further showing the importance of BDNF in this instrumental learning (Gomez-Pinilla, Huie et al. 2007, Huie, Garraway et al. 2012, Joseph, Ying et al. 2012). In a subsequent study, Huie et al demonstrated

the rescue of spinal learning following intrathecally delivered BDNF protein in rats that were preconditioned with the uncontrollable shock (Huie, Garraway et al. 2012). In a maladaptive conditioning of the injured spinal cord, using the intermittent noxious tail stimulation in SCI rats showed reduction of BDNF, TrkB, CaMKII, ERK1/2 protein at 1hr, 24 hr and 7 day after stimulation in the dorsal horn (Garraway, Turtle et al. 2011). The brain derived neurotrophic factor (BDNF) has been implicated in various roles such as glutamatergic signaling, NMDA receptor activity, GABAergic signaling, and in learning related synaptic transmission. In hippocampus and spinal cord, BDNF is upregulated due to activity and promotes learning and memory (Shieh and Ghosh 1999, Soya, Nakamura et al. 2007, Joseph, Tillakaratne et al. 2012).

### *Molecular mechanism of instrumental learning deficit*

The meta-plasticity of spinal learning includes both the positive acquisition of leg flexion to avoid shock under contingent shock training and the acquisition of learning deficit from uncontrollable shock (yoked) or intermittent noxious tail conditioning of the spinal cord (Crown, Ferguson et al. 2002, Joynes, Ferguson et al. 2003, Grau, Washburn et al. 2004, Ferguson, Crown et al. 2006, Ferguson, Bolding et al. 2008, Ferguson, Huie et al. 2012, Ferguson, Huie et al. 2012). Grau and colleagues showed the deficit learning is also mediated through similar molecular learning markers. Pharmacological studies by Grau and colleagues showed that the learned deficit in the uncontrollable shock (yoked) trained condition is dependent on NMDA, AMPA receptor, CaMKII and GABAergic mediated plasticity. The intrathecal delivery of pharmacological agents such as MK-801 and AP5 (Joynes, Janjua et al. 2004, Ferguson, Crown et al. 2006), or CPCCOEt (mGluR1 antagonist) (Ferguson, Crown et al. 2006, Ferguson, Bolding et al. 2008), or CaMKII inhibitor (C1360) (Baumbauer, Young et al. 2007) or a PKC inhibitor, staurosporin-derived BIM (Ferguson, Bolding et al. 2008) or Anisomycin, a protein synthesis inhibitor (Baumbauer, Young

et al. 2006), all effectively blocked the learning deficit by the uncontrollable Yoked condition or using preconditioning with intermittent noxious stimuli (Table 1-1). Even though retaining of yoked rats as master with controllable shock condition was not successful for 48 hours (Crown, Ferguson et al. 2002, Crown, Ferguson et al. 2002), the yoked animals overcame their learning deficit, when treated with these pharmacological agents.

**Table 1-1: Learning-associated markers in instrumental learning in adult spinal rats.**

<b>Learning associated maker</b>	<b>Action</b>	<b>Pharmacological agent</b>	<b>Master</b>	<b>Yoked</b>	<b>Intermittent noxious shock</b>	<b>Reference</b>
Protein Synthesis	inhibitor	Cycloheximide, Anisomycin	blocked learning	blocked yoked inhibition	blocked learning deficit	Patton et al., 2004, Baumbauer et al, 2006
NMDA	inhibitor	AP5, MK-801	blocked learning	N/A	blocked learning deficit	Ferguson et al, 2006; Joynes et al, 2004
PKC	inhibitor	PKC inhibitor	N/A	N/A	blocked learning deficit	Ferguson et al, 2008
CaMKII	inhibitor	AIP	blocked learning	N/A	blocked learning deficit	Baumbauer et al, 2006, 2004; Gomez-Pinilla et al 2007
BDNF	Inhibitor	TrkB IgG	blocked learning	N/A	blocked learning deficit	Gomez-Pinilla et al 2007, Huie et al, 2005, 2013
AMPA, mGluR1 blocker	inhibitor	Naspm trihydrochloride	blocked learning	blocked yoked inhibition	blocked learning deficit	Ferguson et al, 2008; Huie et al, 2015
AMPA	inhibitor	CNQX	blocked learning	N/A	blocked learning deficit	Hoy et al, 2013,
CaMKII level	Measured mRNA	RT-PCR	Increased	Decreased	N/A	Gomez-Pinilla 2007
BDNF	Protein	Western,	Increased	Decreased	Decreased	Huie, 2005, 2013, Gomez-Pinilla et al 2007; Garraway et al 2011
BDNF level	mRNA	RT-PCR	Increased	N/A	N/A	Gomez-Pinilla et al 2007, 2002



## CHAPTER 2: Paw Withdrawal Learning Paradigm (PaWL)

### INTRODUCTION

A simple instrumental learning paradigm in headless insects was first developed by Horridge (Horridge 1962). He inserted electrodes into the hindlimb of a headless insect to elicit a flexion response, when the foot dipped below a preset vertical threshold, into a saline bath, thus completing the circuit. The outcome of the experiment was that the headless insect acquired a new foot position to avoid the shock. Horridge further developed this model to include an experimental (master) and control (yoked) groups, where only the master headless insect received the shock contingent on the foot extended below the threshold, while the electrically coupled yoked insect to the master, received shocks irrespective of its foot position. The master insect acquired a new flexed elevated foot position to avoid the shocks, while the yoked did not. This Horridge paradigm was subsequently studied in other insects, frogs and rats (Buerger and Fennessy 1970, Chopin and Buerger 1975, Joynes and Grau 1996).

Instrumental conditioning of the Horridge paradigm was adapted in rats and further studied at great length by Grau and colleagues (Grau, Barstow et al. 1998, Crown, Ferguson et al. 2002, Crown, Ferguson et al. 2002, Joynes and Grau 2004, Liu, Ferguson et al. 2005, Grau, Crown et al. 2006, Bigbee, Crown et al. 2007). Grau and Joynes showed under the master contingent condition, proper stimulating current was critical to successfully acquire ankle flexion and not habituation (Joynes and Grau 1996). The uncontrollable (yoked) shocks resulted in failed learning in the contralateral leg, even when it was given controllable shocks in subsequent trial (Grau, Barstow et al. 1998). This learning deficit was persistent for the next 22-hrs. In a second bout (10 min after the first bout) of instrumental conditioning at the ipsilateral or contralateral limb, only the master group, showed facilitation of acquisition of the foot flexion at a higher (~2x) criterion (Crown,

Ferguson et al. 2002). When sciatic nerve was cut or intrathecal lidocaine delivered to block the sensory input into spinal cord, both master and yoked failed to learn the flexion task (Joynes, Ferguson et al.). However, 10 min later, instrumental conditioning of the contralateral leg of the yoked rat, now under controllable stimulus (master) condition, resulted in a learned flexion response, suggesting the previous negative conditioning did not engage the spinal cord due to application of lidocaine on to the spinal cord (Crown, Ferguson et al. 2002). Lesion of the spinal segments from L1-S3 progressively, showed that the L4-L5 was critical for the activation of TA muscle and acquisition of the foot dorsiflexion (Liu, Ferguson et al. 2005). Together, these studies showed that the spinal cord was the central mediator of the behavioral plasticity in instrumental learning of foot flexion. Furthermore, pharmacological studies (Table 1-1, Chapter 1) showed that learning-associated markers in the spinal cord such as NMDAR, AMPAR, CaMKII, PKC, and BDNF were involved in the instrumental spinal learning in rats (Joynes, Janjua et al. 2004, Garraway, Anderson et al. 2005, Gomez-Pinilla, Huie et al. 2007, Ferguson, Bolding et al. 2008, Baumbauer, Huie et al. 2009, Huie, Garraway et al. 2012, Hoy, Huie et al. 2013, Huie, Stuck et al. 2015). Levels of mRNA for CREB, CaMKII, and BDNF in the spinal cord were increased in master rats while decreased in yoked rats (Gomez-Pinilla, Huie et al. 2007).

In an attempt to understand the synaptic mechanisms involved in more complex motor tasks, we developed a simpler model of learning (Jindrich, Joseph et al. 2009) (Fig. 2-1), i.e., the paw withdrawal learning (PaWL) in mice, originally developed for headless insects by Horridge and then for rats by Grau and colleagues as described above and in Chapter 1. This simple learning model in mice allows us to examine the role of specific signaling proteins in paw withdrawal learning using transgenic mouse lines. We used CREBIR transgenic mice whose spinal cords were

completely transected to examine the role of CREB in learning to withdraw the paw in response to leg shock (Chapter 4).

## **MATERIALS AND METHODS**

### *Animal Groups*

All surgical and experimental procedures were conducted under strict guidance provided by UCLA Chancellor's Animal Research Committee. Adult female C57Bl6 mice (n=52; Table 3-1, Chapter 3), or Swiss Webster mice (n= 16;) and CREBIR mice and their littermates (n= 65; Table 4-1 Chapter 4), of ~30-45 body weight were used for PaWL experiments.

The development to of the PaWL paradigm was originally conducted in Swiss Webster (SW) strain (Jindrich, Joseph et al. 2009). The majority of the background strain used in developing transgenic mice are C57BL6 mice. Given this, we set out characterize PaWL in both the SW and C57BL6 strains.

### *Spinal cord transection Surgery*

All surgical procedures were conducted under aseptic conditions. In preparation for the surgery, 45 minutes prior to surgery the mice were given an analgesic (Buprenex, 0.05-0.1 mg/kg) subcutaneously to alleviate pain associated with the surgical procedure. The back and hindleg were carefully shaved with clippers (#50 clipper blade) in a wide margin around the incision sites. The surgical site was scrubbed three times with betadine scrub solution, and an ethyl alcohol (70-90%) wipe was conducted after each scrub application. Ophthalmic ointment was applied to both eyes immediately before surgery to prevent drying of eyes. A sterile drape was used to cover the majority of the mouse while exposing the surgical site. The animals were anesthetized using isoflurane gas mixed with oxygen at 1.0%-2.5% and delivered via facemask to maintain a surgical level of anesthesia. The animal's breathing was visually monitored (chest movement 1 breath/1sec) and anesthesia was modified accordingly.

All surgeries were conducted on a water-circulating heating pad maintained at 37° degrees Celsius to help maintain body temperature. Under anesthesia, the spinal cord of adult mice was transected at a mid to low thoracic level (T8-T9). A dorsal midline skin incision was made from T5 to L2 and the musculature covering the dorsal vertebral column from T6 to T13 was separated to reveal the spinal lamina. A laminectomy was performed by removing the spinous processes and the dorsal and lateral aspects of the vertebral column to expose the spinal cord between T7 and T10. Lidocaine (1%) was used as a local anesthetic on the dura and spinal cord. The dura was then opened along the midline of the spinal cord. The spinal cord was then lifted and transected completely at the T8-T9 vertebral level, using micro-scissors. Two surgeons verified the completeness of the transection by lifting the cut ends of the spinal cord and passing a glass probe through the lesion site. Gel foam was inserted into the gap created by the transection as a coagulant and to separate the cut ends of the spinal cord. All incision areas were irrigated liberally with warm, sterile saline solution. The overlying muscles were sutured with DEXON 6.0 sutures and the wound was closed using Ethilon 6.0 sutures and 2% Lidocaine ointment was applied on the sutures for additional analgesia. The oral analgesics acetaminophen (300mg/kg body weight, orally) was provided following the completion of the surgery and continued for a minimum of 3 days (Jenkins 1987). The mice were allowed to recover in an incubator maintained at 37°C until they are fully awake and mobile with the use of their forelimbs. The mice were given 2 ml of lactated Ringer's solution subcutaneously following surgery to minimize dehydration. The hindlimbs were gently taped to prevent leg extension during recovery (Jindrich, Joseph et al. 2009). The recovered animals were then returned to their home cages until the PaWL test. The mice were housed individually in cages that had ample CareFresh bedding, and the bladders were expressed manually 3 times daily. All of these surgical procedures and postsurgical animal care are performed routinely

in our laboratory and have been described previously (Courtine et al., 2009; Jindrich et al 2009; Gad et al., 2013).

### *PaWL Procedure*

#### **The experimental (master) and control (yoked) pairing in PaWL**

Preparation of mice for PaWL began ~20-22 hrs after the after spinal cord transection, while the PaWL procedure was conducted ~24 hrs as described before (Jindrich et al 2009). One hindpaw of the Experimental mouse (master) is exposed to a contingent stimulus protocol, where the mouse receives a stimulus whenever the ankle drops below the set threshold vertical position (Fig. 2-1A, B). When the paw falls below the determined vertical threshold, a shock is delivered at ~20 Hz to the tibialis anterior muscle until the paw is flexed above threshold. A second mouse is electrically coupled (“yoked”) to the “master” mouse, and receives the shock irrespective of its paw position. The leg stimulus of the master and yoked mouse are described as contingent (controllable stimuli), and non-contingent (uncontrollable stimuli), respectively.

#### **Electrode implantation**

The hind legs were shaved using a small electric clipper (#50 clipper blade) and painted black using a non-toxic marker to optimize camera tracking (Fig. 1). A small white mark was placed on the fifth metatarsal-phalangeal (MTP) joint using non-toxic paint to track using the digital camera (Fig. 2-1). Next the optimal location for the implantation of the electrode in TA was visually determined by the most sensitive insertion point (optimal twitch response). Fine-wire electrodes were constructed by removing the insulation from the tips (~1mm) of 1000 gauge (50 $\mu$ m) nylon-insulated single strand stainless steel wires (California Fine Wire Co., Grover City, CA). The fine wire was threaded through a 32-gauge hypodermic needle. The needle end of the wire was de-shielded to expose the contact surface and barbed to hook and remain in the TA

muscle once it is inserted. Then, the electrode (anode) was inserted into the belly of muscle, medial to the tibial bone, which gave the optimal ankle flexion. The second (cathode) electrode was inserted following the application of a small amount of 2% lidocaine spread locally over the lateral malleolus. A second fine wire electrode de-shielded and barbed, was inserted below the skin over the lateral malleolus. This was an improvement over the previous procedure, in which the ground electrode secured to the ankle using a small bolus of Spectra 360 electrode gel (Parker Laboratories, Inc., Fairfield, NJ) on the skin externally then secured with rubber cement. In this configuration the ground electrode at times would dislodge and stimulation would be lost. The distal ends of the fine wires were then attached to a stimulator (S88, Grass Product Group; W. Warwick, RI), through a stimulus isolation unit (SIU5) and a constant current isolation unit (CCU1) which converts the alternating current (AC) to direct current (DC) output to the muscle (Fig. 1). The stimulation parameters used for these experiments were a 50ms square wave direct current followed by a 10ms delay between repeated stimulations. Subsequent to the electrode placement, the force to current relationship was established and stimulus intensity was determined as described below (Fig.2).

### *Determination of stimulus intensity*

For each animal, the force generated at the ankle dorsiflexion following electrical stimulation was measured through a series of increasing currents (0.10mA), until the maximum and supramaximal force was realized. From each force curve the optimum current producing a submaximal contraction was determined by taking the 2/3 the maximal force and its correlated current. To do so, a #4 surgical Silk thread was tied across the paw on the hindlimb with implanted stimulating electrodes. The #4 silk thread was then attached to the force transducer (Dual Mode Muscle Lever 300BLR, Aurora Scientific Inc., Aurora, Ontario, Canada). Force resulting from a

series of single 50ms square-pulse stimuli from 0 to 3.0mA, in increments of 0.10mA, with a 30 sec delay between each stimulus, were recorded and displayed by a custom written LabVIEW program. When the stimulus series was completed, the thread was removed from the foot. Force was recorded for each increment step of the current. The force curve was generated via delivery of a single 50 ms stimulus every 30 sec to the TA muscle (Fig. 2-2). In majority of the experiments, a current of 0.25-0.30 mA generated 0.6N of force at the ankle during paw dorsiflexion.

### **Digitizing the foot position**

Acquisition of the two-dimensional foot position was accomplished by digitizing the white mark placed on the MTP with a video-based point tracking system (CMUCam2; Carnegie Mellon University). The CMUCam2 performs automatic, hardware-based point tracking of region fitting user-specified color and size criteria and reports the centroid of the region to the computer. The LabVIEW (National Instruments, Inc., Austin, TX) software was used to control the CMUCam2 and collect tracking data streaming from the camera. The software then calculated the range of colors associated with the marker and because of the high-contrast (white on a black background), resulted in accurate tracking points. Once the desired parameters were established, they were used to configure the point tracking by the CMUCam2 and the LabVIEW software. The camera has a field of view of 15mm×15mm, or a resolution of 8 pixels/mm, positioned 40mm away from the foot. Following the stimulation parameters (Force curve) was set the acquisition of foot position and, a resting ankle position was determined after 3 priming stimulations. The vertical threshold was assigned based on the post-primed foot position. A threshold of 1mm above the resting position, was set for both the master and yoked mice with a threshold parameter in the LabVIEW program. The paw withdrawal test began with the initiation of the stimulator for 30 min, set to the stimulus parameters consisting of a 50 ms square wave pulse followed by a 10 ms delay between consecutive impulses.



### **Response Duration**

The spinal cord learning over the 30 min of the PaWL testing was measured as response duration (RD), calculated according to the equation below. The response duration takes into account the number shocks (measured as a foot flexion response) during the binned time.

$$\text{Response Duration (min)} = \frac{(60\text{sec} - \text{time the paw was below the threshold})}{\text{Number of responses} + 1}$$

The numerator represents the time the paw is above the imposed threshold. The number of paw flexion or responses are the result of shocks received when the paw was extended below the threshold. Given the length of stimulus pulse duration (50ms) followed by a 10ms built-in delay, the maximum possible stimulation frequency is 16.7Hz and minimum is zero during period of paw hold greater than 60sec. To eliminate the possibility of zero in the denominator, the total number of response for each minute binned is increase by 1. For example, during a one minute period where 1 response will yield RD= 29.996 min, for 2 responses RD= 19.967 min and for 5 response RD = 9.967 min. To obtain a response duration curve, the calculated response duration for each binned min (y-axis) was plotted as a function of time (x-axis), over the length the 30 min experiment (Fig. 2-3A and C).

### *Statistics*

Statistical comparisons of learning between groups across time (binned min) during PaWL test and interaction between group affect over time were determined using a Repeated Measure two-way ANOVA. Sidik multiple group post-test was conducted to show statistically significant differences between master and yoked group over time (Fig. 2-3A, C). Total response duration between master and yoked were compared using the paired Wilcoxon Test (Fig. 3B and D). To quantify the rate of learning in the master group, Boltzmann Curve fit analysis was performed. The

confidence bands (dotted lines) indicate the likely location of the true fit curve (Fig. 2-3A and B). The maximum response duration of the curve fit is reported and the rate of learning was determined by taking the time at half-the-maximum ( $T_{50RD}$ ) of the response duration curve. All analyses were performed using GraphPad 7.0 (GraphPad Software Inc. San Diego, CA). The level of significance was selected as  $P < 0.05$  for all comparisons. Data are reported as the mean values  $\pm$  standard error of the mean (SEM).

## RESULTS

### *Successful instrumental learning in PaWL Paradigm*

In Swiss Webster mice, the master group learned to hold the paw above the 1mm threshold while the paired yoked group did not (Fig. 2-3A, 2-3B). A Repeated Measure two-way ANOVA analysis shows master significantly different compared to the yoked group ( $F(1, 6) = 19.45$ ,  $P = 0.0045$ ), with significance differences over time ( $F(29, 174) = 4.213$ ,  $P < 0.0001$ ) and interaction between master vs. yoked group over time ( $F(29, 174) = 3.69$ ,  $P < 0.0001$ ). The Sidak post-hoc test showed that the first significant difference between master vs yoked began at the 15<sup>th</sup> min and then continued to the 30<sup>th</sup> min (Fig. 2-3A). The total response duration between matched pairs of master and yoked groups was also significantly different (Fig. 2-2B, Wilcoxon Test  $P < 0.05$ ), (Fig. 2-3B).

In C57BL6 mice, the master group was able to acquire the new paw position while the yoked paw remained extended. The repeated measure two-way ANOVA comparison showed significant difference in response duration between master and yoked group ( $F(1, 6) = 21.19$ ,  $P < 0.01$ ), with significance temporal differences over 30min test period ( $F(29, 174) = 2.689$ ,  $P < 0.0001$ ) and interaction of master vs. yoked group difference over time of the test ( $F(29, 174) = 3.672$ ,  $P < 0.0001$ ). The Sidak post-hoc test between group and over time showed, that the first significant difference of the response duration between master vs yoked began at the 8-9 min and then continued from 11 to the 30 min (Fig. 2-3C). The comparison between mean total response duration between matched pairs of master and yoked animals showed the master group with significantly greater than all the yoked animals (Fig. 2-3D, Wilcoxon Test  $P < 0.05$ ), (Fig. 2-3D).

To compare the rate of spinal learning, the Boltzmann's curve fit analysis was performed to identify the time at which half-the-max ( $T_{50RD}$ ) response duration for SW and C57BL6 mouse

strains. With the curve fit, the Top RD (maximum curve fit response duration value) for SW mice and C57BL6, were 51.89 sec (Fig. 2-3A) and 53.61 sec (Fig. 2-3C), while  $T_{50RD}$  was 15.67 min and 4.98 min, respectively. These results showed that the C57BL6 mice learned paw holding task faster than the SW mouse strain.

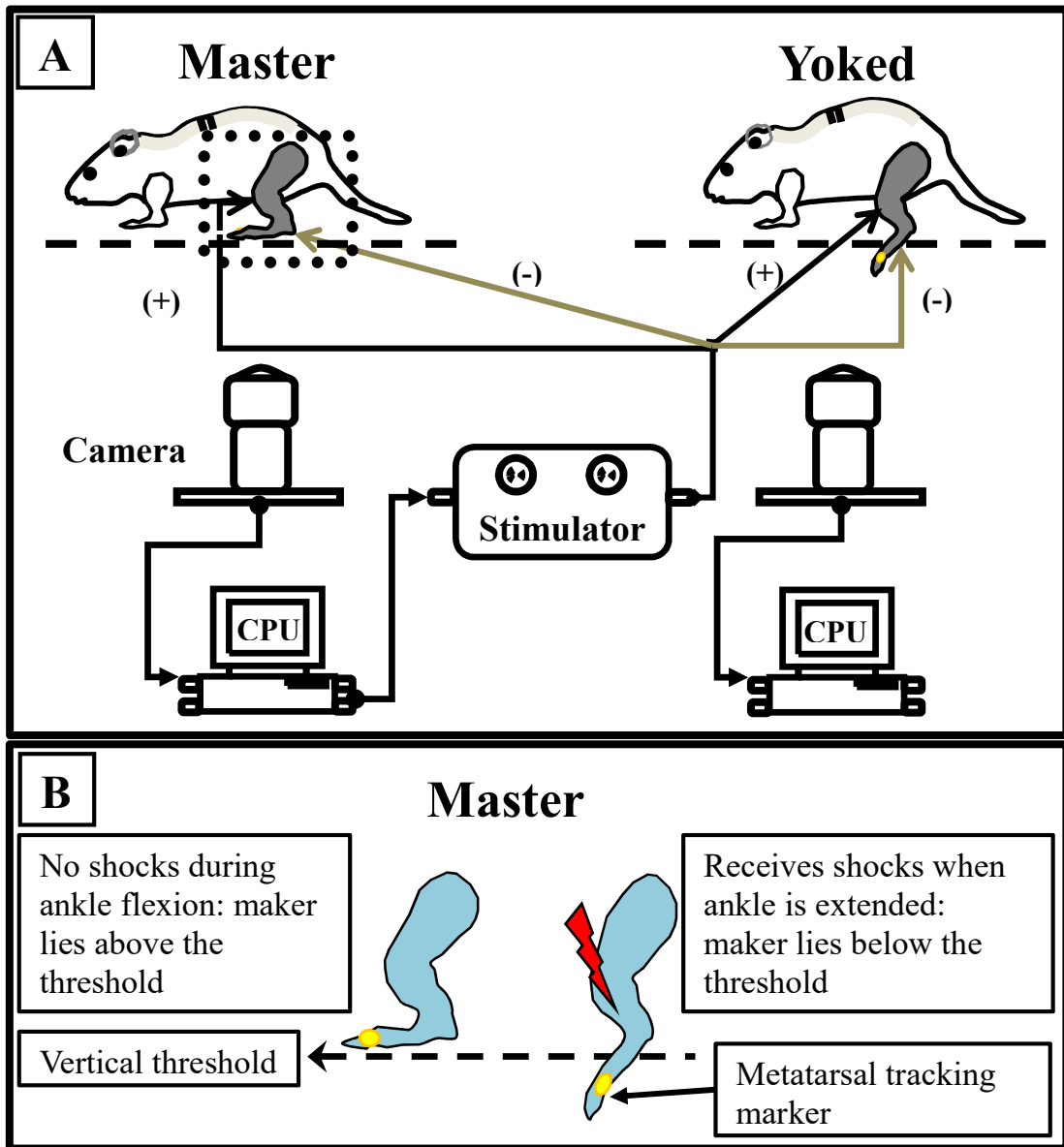
## DISCUSSION

Instrumental spinal learning of sustained paw flexion was successfully demonstrated in two strains of mice using the PaWL paradigm. Adaptation of similar instrumental learning model from rats to mice required modifications to address the smaller size of mice. The rat instrumental model uses thick gauge wire as the electrodes glued to the bottom of the hindpaw. The paw electrode is positioned over or submerged in a well containing saline solution. The vertical threshold is imposed by lowering the rat into the saline solution (Grau, Barstow et al. 1998). The small physical size of the mouse creates challenges in attaching a large gauge stiff wire to the hindpaw and detecting changes in the paw position. Use of stiff wire electrode attached to the mice hindpaw will generate additional weight and torque to the limb. This additional load may adversely affect the outcome of learning. Furthermore, the sole of the hindpaw is populated with sensory receptors critical for generation locomotion (Lovely, Gregor et al. 1986, de Leon, Hodgson et al. 1998). In addition to unloading the hindpaw of the mice, by not attaching the electrode to the sole, also prevents the unintentional stimulation of hindpaw sensory afferents. The real-time video camera identifies the paw position in space using a paw marker at the metatarsals. The contrast mark on the paw allows the researcher to generate a center value (centroid) which is used to impose a precise threshold that can be modified with ease without moving the mice or use of a well filled with saline. With these modifications, we were able to show successful learning in the master compared to the yoked group of mice in the PaWL paradigm. The master group receiving the paw position based stimuli learned to hold the paw above the 1mm threshold. The yoked group having received the identical electrical stimuli at the TA muscle did not acquire the flexion response.

Strain-specific comparisons showed that the C57BL6 mice were faster at acquiring the PaWL spinal learning task than the SW mice. Strain specific differences was also shown in

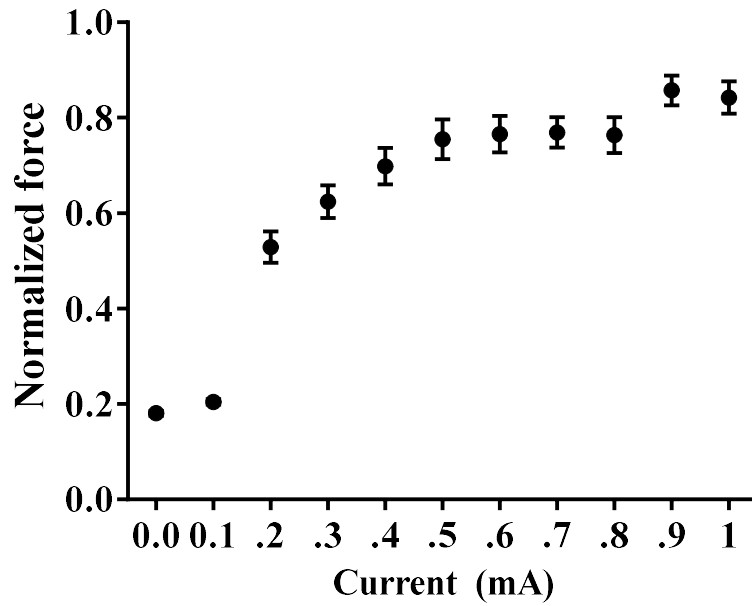
cognitive spatial learning. The C57BL6 strain were better able acquire and retain the hidden platform Morris water maze (MWM) task when compared to the SW strain. Comparison of the SW strain to Dilute Brown Agouti (DBA) and C57BL6 strains the ability to locate the hidden platform in Morris water maze (MWM) test, the SW consistently failed to locate the platform at 4 different positions and also traveled the greatest distance, when compared to the other two strains (Petrie 1995). In a more recent study, C57BL6 mice found the platform within significantly shorter time and distance traveled when compared to the SW strain that performed similar to the p75 NGF knockout mice. The study suggests the SW mice performance is similar to that of p75 knockout mice that is known to demonstrate spatial learning deficits (Wright, Alt et al. 2004). However, the underlying basis for the difference in the learning abilities between these two strains are unknown.

In conclusion, the successful adaptation of the rat instrumental learning model to mice (PaWL paradigm) presents a unique opportunity to study spinal plasticity in a simplified manner. The advantage of the developing a mouse model of spinal learning is that it provides the opportunity to use transgenic and knock out mice using molecular techniques to dissect the learning mechanism in the spinal cord.



**Figure 2-1: Paw withdrawal learning paradigm.**

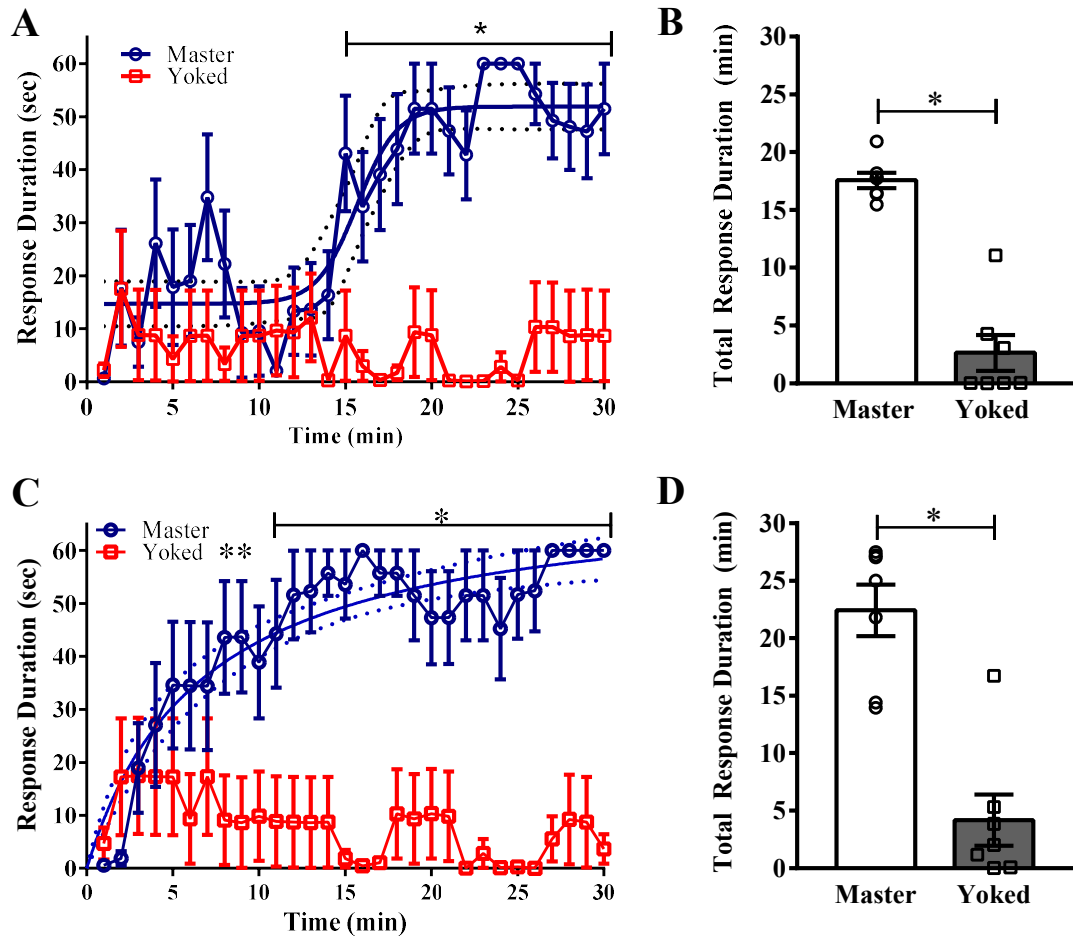
A) Schematic diagram illustrating the PaWL setup. The image (paw position) from the camera allows for the LabVIEW software to track the metatarsal marker and generate a signal to the stimulator when the paw position is below the threshold (B). The stimulator is connected to both master and yoked animals in parallel. Both the master and yoked computers record the paw position in real time for subsequent analysis (Jindrich, Joseph et al. 2009).



**Figure 2-2 Force curve relationship to current.**

A force curve was generated before the start of PaWL to determine the current needed to obtain a submaximal force. The submaximal force was normalized across all currents for each animal (n=33). For PaWL tests, the submaximal current applied ranged between 0.25-0.30mA generating approximately 0.6N of force.





**Figure 2-3: PaWL in Swiss Webster and C57BL6 strain of mice.**

A) The master group of Swiss Webster mice, (SW, n= 6) showed significant increase in response duration compared to the yoked group from 15<sup>th</sup> -30<sup>th</sup> min. B) Total response duration in the SW master group was also significantly greater than the yoked. C) Compared to the yoked, the master group of C57BL6 (n=7) mice showed greater response duration at the 8-9, and 11-30 min during learning. D) The total response duration in C57BL6 master group was also significantly higher compared to the yoked. From the curve fit analysis, the master C57BL6 mice reached half-the-maximum response duration faster ( $T_{50RD} = 4.982$  min) than SW master mice ( $T_{50RD} = 15.92$  min). Confidence bands show the range or likely location of the true curve.

## CHAPTER 3: EMG characterization during PaWL

### INTRODUCTION

Complex tasks like locomotion cannot be explained with simple neuronal interactions. Mendell and Henneman revealed a single muscle input to the spinal cord can have more than sixty Ia afferents in various combination projecting to approximately 300 homonymous alpha motor neurons(Henneman and Mendell 2011). Each sensory input can have collaterals that engage interneurons which in turn can synapse to multiple combinations of motoneurons(Scheibel and Schiebel 1969). Given the complexity of network modeling has primarily been accomplished using cultured cortical neurons, in hippocampal slices and computer based modeling. The Horridge paradigm in insects and instrumental spinal learning in rats, represent a simpler model that can be tested for its consistency with a corroborating behavior.

Spinally transected rats given shock to a hind leg when the leg was extended (contingent shock) learned to maintain the shocked leg in a flexed position that minimized net shock exposure (Joynes and Grau 1996, Grau, Barstow et al. 1998). This instrumental learning was not observed in yoked rats that were electrically linked to the master rats, but received noncontingent shock irrespective its paw position. We adopted the paw withdrawal learning (PaWL) paradigm to mice using real-time video to track paw position in the X-Y plane to allows for a precise shock delivery(Jindrich, Joseph et al. 2009).

A series of experiments have been performed to understand the mechanism of the spinal learning phenomena. Both a systematic isolation of the spinal network via severed sciatic nerve and intrathecal delivery of lidocaine to the lumbar enlargement in spinal cord transected rats blocked the instrumental spinal learning, suggesting the mechanism of learning is mediated

through the L4-S2 spinal segments(Liu, Ferguson et al. 2005). It remains unclear as to whether the physiological mechanism demonstrating paw flexion is a mechanical response to shock or whether it is an active learning event that involves the sensorimotor network in the spinal cord.

A series of experiments were conducted to determine whether the acquisition of the de novo flexed hindpaw is mediated through muscle-specific proprioceptive inputs and if so, are the mechanism of activation of the proprioceptive motor pools reflected in the EMG activation patterns of the primary muscle of interest, for example the tibialis anterior (TA) muscle. Specifically, during PaWL, 1) are the time-dependent modifications in spinal neuronal activity in the learned event unique to the characteristics of EMG activation in the tibialis anterior (TA) motor pools in the master compared to the yoked (Hypothesis 1), 2) does learning occurs when the sustained hindpaw dorsiflexion is mediated through changes in stimulus-driven paw position and not when it is manually imposed, without shocks (Hypothesis 2), and 3) will blocking proprioceptive (muscle) afferents and not the cutaneous afferents interfere with the paw withdrawal learning (hypothesis 3).

To address the hypothesis 1, EMG activities of the TA (ankle flexor) and its antagonist, medial gastrocnemius, and a synergist, vastus lateralis muscle, were measured during PaWL (experiments 1 and 2). The hypothesis 2 was addressed by recording the TA EMG activity during manual flexion of the hindpaw to mimic a sustained paw flexion, but without the application of the contingent shock (experiment 3). To further define the neural mechanism, the TA Muscle afferents and cutaneous afferents were blocked using lidocaine to address hypothesis 3 (experiment 4). EMG was used to address a majority of the hypotheses, since it provides an individual motor unit activity or overall recruitment of multiple motor units during contraction.

## **MATERIALS AND METHODS**

### *Animal Groups*

All surgical and experimental procedures were conducted under strict guidance provided by UCLA Chancellor's Animal Research Committee. Adult female C57Bl6 mice of ~30-45 body weight (n=52), were used in the four experiments shown in the Table 3-1 (Total=52).

### *PaWL Paradigm*

PaWL paradigm was conducted as previously described in Jindrich et al 2009. Spinal cords of adult mice were completely transected at mid-thoracic level (T7-T9), 24 hours prior to the PaWL procedure. The hindlimb of the mouse was shaved and disinfected with isopropanol and iodine, a very fine wire electrode was inserted into the belly of the tibialis anterior (TA) muscle, and a surface ground electrode was placed on the dorsal surface of the ankle adjacent to the lateral malleolus (Fig. 3-1). Two additional electrodes were implanted into the belly of TA in parallel, when EMG is recorded acutely. The other ends of the stimulating electrodes were attached to a lead from medical-grade constant current (60 Hz, AC) stimulator (S88 Grass Product Group, Warwick, Rhode Island). The ends of the EMG electrodes were connected to the A-M system Model 1700 Differential AC amplifier (Carlsberg, WA) to amplify the signal. All electrodes described above were made from 0.5mm nylon-insulated single strand stainless steel wires (California Fine Wire Co., Grover City, CA).

The video-acquired paw position was used to determine the starting paw position and using the LabVIEW software a vertical threshold at 1mm was imposed, such that shocks are delivered in the experimental animal (master) only when the paw is extended and below 1mm threshold in a real-time manner. In Experiment 1, a control (yoked) was coupled the master animal, to such

that it received the same electric shock as the master, independent of its paw position. Experiment 2-4, only consisted of PaWL conducted with the master condition only (Table 3-1). The EMG activity was recorded during PaWL while the animal was secured with a body weight support device using a cloth harness attached to a stand and positioned for the real time video acquisition of the paw position (Jindrich et al 2009; Fig. 2-1, Chapter 2).

The PaWL paradigm consisted of 30 min where the master mice received contingent electrical shock (master) or non-contingent shock (yoked) with a defined vertical 1mm threshold above the initial resting position. The spinal learning ability is measured as the response duration (RD) of paw held above the threshold and calculated by the following equation  $RD = (\text{time of paw held above threshold}) / (\text{number of stimulations} + 1)$  per each minute binned over the 30 min trial (Grau, Barstow et al. 1998, Jindrich, Joseph et al. 2009).

### **Experiment 1. EMG activity in TA muscle during PaWL in the master and yoked pairs**

EMG activity in TA muscle in the Master and Yoked pairs (n=6) were recorded during 30 min of PaWL using acutely implanted EMG electrodes (Table 3-1). The amplified EMG signal was filtered at (1Hz-1kHz band-pass) and sampled at a higher rate 10kHz using a custom data acquisition program in LabVIEW (National Instruments, Austin, TX) as previously described (Courtine, Gerasimenko et al. 2009, Gad, Lavrov et al. 2013). Data analysis consisted of burst amplitude and frequency/power domain analysis over time to show differences in EMG activity temporally throughout the PaWL.

### **Experiment 2. EMG activities in multiple hindlimb muscles during paw flexion**

In Experiment 2, EMG activities of three hindlimb muscles, TA (ankle flexor), medial gastrocnemius (MG, ankle extensor) and vastus lateralis (VL, knee flexor) were recorded using

chronically implanted bipolar EMG electrodes during PaWL in master mice that received the contingent shock (n=4, Table 3-1) (Roy, Hutchison et al. 1991, Courtine, Song et al. 2008). EMG The electrodes made of Teflon-coated wires (AS631-2, 40 gauge; Cooner Wire, Chatsworth, CA) were passed through the mid-belly of each muscle. Afterwards, a small notch was made in the Teflon coating (~0.5 mm) of each wire and positioned in the mid-belly. EMG and stimulation wires were subcutaneously routed through the back with slack for movement. The wires were then secured to the fascia with a suture at their entry and exit points from the muscle. All EMG, stimulating and ground electrode wires were connected to a 12 pin head plug that was secured to the skull of the animal using dental cement. EMG was amplified and filtered at (1Hz-1kHz band-pass) and acquired at a sampling rate of 2000Hz using a custom data acquisition program in LabVIEW (National Instruments, Austin, TX) as previously described (Courtine, Gerasimenko et al. 2009, Gad, Lavrov et al. 2013). After two weeks of recovery, ground walking was used to verify EMG function prior to spinal cord transection and initiation of the PaWL testing. In this experiment, the EMG electrodes were chronically implanted in multiple muscles, instead of acute electrodes, since the electrodes made from thicker wires reduced the noise improving the signal and also subcutaneous arrangement of multiple EMG wires reduces movement artifacts.

### **Experiment 3. EMG activities during manual dorsiflexion of the hindpaw without electric shocks**

The PaWL paradigm was modified by removing the contingent shock that is paired with paw position and the normally associated proprioceptive position by manually imposing a flexion with EMG electrodes inserted acutely in the TA muscle (n=6, Table 3-1). All procedures for PaWL paradigm was followed as before which including the implantation of electrodes, placing the mice in the harness, and video tracking of the hindpaw. We monitored the EMG activity in the TA

muscle with acute electrodes. The initial 5 minutes after the video-acquisition of the baseline paw position, the ankle was manually held at a flexed position for 15 min above the 1mm threshold, using a spatula attached to a micromanipulator. The hold was then released gently and left free of any manipulation for the next 10 min. Throughout the 30 min of the experiment, the paw position was video-tracked and the EMG activity in the TA muscle was recorded during paw position at rest, at 1mm with manual flexion and after releasing the paw from the hold.

#### **Experiment 4. Application of lidocaine to block skin and muscle afferents**

The cutaneous and proprioceptive inputs to the spinal cord was minimized via Lidocaine that blocks voltage gated sodium channels, preferentially (Sheets and Hanck 2003). Blocking the  $Na_v1.7$ , 1.6 or 1.3 by lidocaine leads to the inhibition of action potential propagation (Sheets, Heers et al. 2008). Lidocaine gel (2%) was applied topically to the entire hindlimb, 15 min prior to conducting the PaWL (n=6). In four additional groups of mice, consisting of n=6 per group (Table 3-1) received; 6 $\mu$ l or 30 $\mu$ l of lidocaine solution (20mg/mL) or 6 $\mu$ l or 30 $\mu$ l of saline was injected intramuscularly into the TA muscle, 5 min prior to conducting the PaWL test.

#### *EMG analysis*

EMG during shocks were not analyzed due to the electrical and movement artifact. Therefore, for all EMG analysis, only the signals during the periods of flexion above the threshold (between shocks) were analyzed. The Butterworth band pass filter was applied to the raw EMG signal, to reduce noise and smooth the signal (20Hz-500Hz). Amplitudes of the EMG bursts during each period of flexion during PaWL were determined as the integral EMG (iEMG). The mean iEMG (Fig. 3-2C, 3-5B) and percent cumulative iEMG of EMG bursts (Fig. 3-2D) was compared between master and yoked groups to show the relative EMG activation over time. The iEMG data was normalized to the baseline for each mouse in both groups. To demonstrate differences between

the master and yoked groups, the cumulative total of the master group for each pair was used to compute percent cumulative values (Fig. 3-2D).

### *Spectral analysis*

The use of Fourier Transform (FT) allows to us examine the time frequency features of the EMG wave form. FT takes the complex EMG signal within a given time constraint and decomposes the waveform to multiple subcomponent sine waves or to its frequency and power spectrum. These sine waves are then grouped to frequency and the repeating subcomponent sine waves are then grouped to represent as power. The FT presented in graphical form of change in frequency (x-axis) and power (y-axis) for a given length of EMG signal. The FT of large time segments of EMG (min) does not allow for accurate temporal resolution in change in frequency and power. As a remedy, a short time (200ms) window Fourier transform (STFT) was developed to improve temporal resolution in the frequency and power spectra. In addition, to create a smoother transition between adjacent small time windows (300ms) we adopted a window smoothing function (Hanning) with 100ms overlap, to ensure smooth 200ms windowed FT analysis (Hu, Luk et al. 2002, Hosokawa, Hasebe et al. 2004). The short Time Fourier Transform (STFT) was applied to the EMG signal in master and yoked animals to characterize frequency power relationship in a time dependent manner. Multivariate regression analysis showed that the iEMG frequency range of 10-100Hz and not the 100-250Hz indicated a positive linear relationship with the dependent variable paw dorsiflexed position. Therefore, for all spectral characterization 10Hz to 100Hz frequency range was considered. To compare the EMG characteristics during PaWL, we selected paw flexion holds lasting *30, 60 and 180* sec. During these three different duration (30, 60, and 300sec) the first 5 sec, the middle 5 sec and the final 5 sec periods were



analyzed and compared to its corresponding changes in displacement of paw at or above the 1mm threshold (Fig. 3-4A-C).

### *Statistical analysis*

Statistical comparisons of learning between groups across time (binned minute) during PaWL test and interaction between group affect and over time were conducted using a Repeated Measure two-way ANOVA and Sidik multiple group post test was conducted to show difference between master and yoked group and over time (Fig. 3-2A). Total response duration between master and yoked compared using the paired Wilcoxon Test (Fig. 3-2B). Statistical comparison for between groups was determined by ordinary One-Way ANOVA, and Bonferroni multiple group post-hoc test (Fig. 3-2C and F, Fig. 3-4A-C). To show how the iEMG was change over the length of PaWL, we plot the Gaussian percent cumulative relationship between the master and yoked group (Fig. 3-2D). All of the analyses were performed using GraphPad 6.0 (GraphPad Software Inc. San Diego, CA) or JMP Pro 12.0.1 software (SAS Inc., Cary, NC, 1989-2015). The level of Significance was chosen as  $P < 0.05$  for all comparisons. Data are reported as the mean values  $\pm$  standard error of the mean (SEM).

## RESULTS

### *Increased response duration during PaWL*

Spinal cord learning under instrumental/operant conditioning shows that the master group learned to acquire and maintain a dorsiflexed paw position at or above the 1mm threshold (Fig. 3-2A and B). A Repeated Measure two-way ANOVA analysis shows significantly difference in response duration between master and yoked group ( $F(1, 6) = 44.77, P < 0.0001$ ), with significance differences over time (1<sup>ST</sup> -30<sup>TH</sup> min) ( $F(29,174) = 4.404, P < 0.001$ ) and interaction between master vs. yoked over time ( $F(29,174) = 2.26, P < 0.001$ ). The Sidak post-hoc test reveals, first significance difference between master vs yoked began at the 5<sup>th</sup> minute and then continued from 8<sup>th</sup> to the 30<sup>th</sup> minute. The effect size between master and yoked group (n=6 pairs) was large and distinct. Furthermore, the total response duration contrast between matched pairs of master and yoked groups was significantly different (Fig. 3-2B, Wilcoxon Test  $P < 0.05$ ). The inter pulse interval (IPI) during the first 5 min of PaWL in master animals ranged from 81.9ms to  $3.6 \times 10^4$ ms (Fig. 3-7).

### *Increased EMG activity in TA during PaWL*

To further understand the mechanism of learning, we compared the physiological changes in the TA muscle by recording EMG activity during PaWL. Only the TA muscle showed significantly higher EMG activity across the 30 min period of PaWL compared to medial gastrocnemius (MG) and vastus lateralis (VL) (Fig. 3-2F). For example, the EMG activity as a percent at rest (baseline) was ~78.45, 7.88 and 5.72, respectively in TA, MG and VL (Fig. 3-2F).

EMG activation during periods of paw dorsiflexion was analyzed as integrated EMG (iEMG, Fig. 3-2D-F, 3-4B) or STFT (Fig. 3-3E-F, and 3-4C). The integral EMG (iEMG, mV-sec)

data was further normalized by time (sec) and compared between master and yoked group (Fig. 3-2C). The mean iEMG during rest (baseline) and dorsiflexion between master and yoked groups showed significant differences between the groups (one-way ANOVA,  $p < 0.0001$ ,  $n=6$ ). The mean iEMG during dorsiflexion of the master group was significantly greater than the yoked ( $p= 0.0019$ , Bonferroni's multiple group comparison). The mean iEMG during dorsiflexion was also significantly higher compared to the baseline, in both master ( $p= 0.0004$ ) and yoked ( $<0.05$ ) groups. The percent cumulative iEMG during bouts of paw dorsiflexion plotted over time showed two distinct populations and regression lines representing the master (blue circles and line) and yoked (red squares and line) (Fig. 3-2D) with quantified goodness of curve fit adjusted  $R^2$  squared values of 0.9444 and 0.780 for master and yoked groups, respectively.

### *Time course of paw displacement and corresponding EMG activity in TA during PaWL*

Fourier Transform (FT) and Short time Fourier transform (STFT) of the EMG was used to illustrate the changes in frequency and power domain during bouts of paw dorsiflexion. An example of FT and STFT of raw EMG during a 29.4 sec of paw dorsiflexion in one master and yoked pair is shown (Fig. 3-3A-F). Figures 3-3A and 3-3B show the raw EMG signal of the TA in master and yoked, respectively. FFT of the EMG activity shows differences in frequency-power range (10-500Hz) in master (Fig. 3-3C) and the yoked (Fig. 3-3D). Frequency features in the time domain show the master animal with greater range and power during the initial 3 sec of EMG burst (Fig. 3-3E), whereas Yoked did not show similar frequency and power relationship (Fig. 3-3F). Decreased paw position corresponded to the decrease in EMG activity and a decrease in frequency range and its respective power.

In order to examine the characteristics of paw flexion, the chronological first occurrence of paw holding for 30 sec (30 sec hold), 60 sec (60 sec hold) and 180 sec (180 sec hold) in the master animals and corresponding bouts for the yoked animals were selected (Fig. 3-4). The temporal occurrence of these bouts vary between the master animals, but generally falls within 2, 2-4 and 8-15 min for short, moderate and long bouts, respectively. We observed changes in the paw position and the EMG activity within each type of bouts of dorsiflexion or hold. Between the different bouts of dorsiflexion, we selected 5 seconds period from the beginning (B), middle (M) and end (E) of each type of bout (double arrows in Fig. 3-3E, F) to compare with the paw position (Fig. 3-4A), iEMG (Fig. 3-4B), STFT frequency (Fig. 3-4C) and power (Fig. 3-4D).

#### **Paw displacement**

In all three types of bouts, the master group showed significantly greater paw displacement above the threshold when compared to the yoked group (Fig. 3-4A). Furthermore, the paw displacement in master animals was greatest during the beginning of a bout in all bout types. For example, the paw displacement at the beginning of the 30 sec bout ( $0.83 \pm 0.28$  mm) was significantly higher than at its middle ( $0.36 \pm 0.007$  mm) or the end ( $0.28 \pm 0.03$  mm) ( $p < 0.0001$ ) of the hold. The paw displacement throughout the 180 sec bout in master animals was also significantly higher compared to 30 sec and 60 sec bouts. At all corresponding time points, the yoked animals failed to dorsiflex above the threshold.

#### **EMG activity**

All bout types of master animals showed significantly higher EMG activation during the beginning of a paw dorsiflexion than the middle or the end (Fig. 3-4B). Furthermore, the mean iEMG during beginning of a bout decreased as the mouse learned to hold for longer periods. For example, the 30 sec bout ( $1.6 \times 10^{-3} \pm 2.9 \times 10^{-4}$  mV-sec) showed significantly higher iEMG during

the beginning of the bout compared to the 180 sec bout ( $9.0 \times 10^{-4} \pm 1.9 \times 10^{-4}$  mV-sec;  $p \leq 0.05$ ). Although the yoked animals did not dorsiflex the paw above the 1mm threshold, considerable EMG activations were observed during the 60 and 180 sec bouts, with minimal activity during the 30 sec bout. These EMG activities were probably due to hyperextension of the hind limb indicated by being at a well below the initial starting position.

The average median frequency was used to identify the differences in spectral features of EMG of TA motor pool activity during paw dorsiflexion. STFT frequency range was broader during the beginning of the bout compared to the middle and the end (Fig. 3-3E, F). Since only the 10-100 Hz frequency range showed a positive linear relationship with the position of the flexed paw, EMG during all three bout types were analyzed to examine the temporal changes in EMG in this STFT frequency spectrum (Fig. 3-4C). The STFT analysis at the 10-100Hz range showed that the STFT median frequency of the master group was significantly less than the yoked group in all three types of holding bouts (Fig. 3-4C). The STFT median frequency in master animals decreases as the bout progresses in each bout type (Fig. 3-4C). The middle of a bout that represents the maintenance phase of paw dorsiflexion, showed significantly higher STFT frequency in the 60 sec bout than the other two bouts, with the lowest in 180 sec (Fig. 3-4E, F). While the iEMG and STFT frequency during the beginning, middle and the end of 180 sec bout were lower than those in the 30 sec and 60 sec bouts, the paw displacements were higher (Fig. 3-4D). STFT frequencies and paw displacements of yoked animals did not show any significant changes during or across all three types of durations.

*Manual dorsiflexion does not lead to successful paw withdrawal learning*

When the paw was manually dorsiflexed, instead of through pairing with a contingent electric shock, the animals did not learn to hold the paw position when the manual paw dorsiflexion was released (Fig 3-5A). The mean iEMG levels during manual dorsi-flexion ( $1.02 \times 10^{-3}$  mV·sec) and during the release period ( $1.09 \times 10^{-3}$  mV·sec) showed no significant difference compared to the baseline ( $1.52 \times 10^{-3}$  mV·sec) (Fig. 3-5B). Comparing the iEMG activity in the master group ( $1.82 \times 10^{-2}$  mV·sec) was significantly greater than its baseline ( $7.93 \times 10^{-4}$  mV·sec) and greater than the 5min baseline, during the 15min. manual dorsiflexion, and release period ( $P < 0.0001$ ) (Fig. 3-5B).

#### *Intramuscular administration of 2% Lidocaine into TA interferes with PaWL*

Intramuscular injection of 30  $\mu$ l of 2% lidocaine (0.6 mg) into TA resulted in complete failure to learn PaWL task, while group that received 6  $\mu$ l of lidocaine (0.12 mg) showed moderate level of learning (Fig. 3-6A and B). The PaWL was not blocked by the saline injections. For example, the total response durations of paw dorsiflexion over 1 mm threshold during 30 min in master animals that received intramuscular injections of low and high dose lidocaine were 12.6 and 0.34 min compared to the  $\sim 20$  min in saline group. Cutaneous sensory block of the TA muscle with 2% Lidocaine gel did not show any significant effect on the PaWL.

## DISCUSSION

In the PaWL paradigm, a submaximal force stimulation paired to a de novo proprioceptive position results in a learned response only in the positively paired condition (master group), but not in the randomly paired yoked group. Although it is well established that the plasticity event is neurally mediated in the spinal cord (Crown, Ferguson et al. 2002), this is the first study designed to understand how learning to hold the paw to minimize shock exposure can be mediated through muscle proprioceptive input that reflects a specific joint position. The principle focus was on the activation features of the tibialis anterior motor pool to show that the engagement of specific spinal networks produces an efferent output leading reflecting a learned behavior.

### *EMG characterization during PaWL*

The significant increase in the EMG amplitude in the TA muscle and time-frequency and power relationships in the master group, but not the yoked, suggests that the successful learning requires temporally mediated engagement of the spinal sensorimotor network. Furthermore, higher activation at the TA compared to other muscle antagonists or synergists in the master group suggest that the learning mechanism is mediated through a limited, and specific network. During all dorsiflexion bouts, the master group exhibits higher amplitude burst pattern and higher STFT median frequency and greater power in the time-frequency spectrum during the beginning of the bouts, which was associated with the highest paw displacement. Although the STFT frequency spectrum is not a direct measure of specific motor unit activity, changes in the median frequency spectrum reflects a relative change in a population of motor units activity in the TA motor pool and muscle (Beck, Housh et al. 2007). Komi and Tesch (1979) and Solomonow et. al., (1990) showed, the frequency-power spectrum is a good indicator of motor unit modulation during fatigue and recruitment (Komi and Tesch 1979, Solomonow, Baratta et al. 1990, Soderberg and Knutson

2000). The decrease of median STFT frequencies and EMG amplitudes during the middle and the end, compared to the beginning, suggests that fewer motor units are involved during the maintenance and the ending periods of a paw flexion.

Similar types of TA EMG modulation during foot flexion have been reported in normal human subjects when the foot was flexed by vibrational stimuli applied at the tendon. Their analysis of the EMG motor unit recruitment changed during the beginning and the maintenance of the foot flexion response. One population of motor units became active during the beginning of foot flexion followed by a second and third population of motor unit types, characterized by decreasing in amplitude in a time dependent manner (Grimby and Hannerz 1968). How these populations differ remains unknown. Another example of similar EMG recruitment patterns have been observed during lifting of a loaded limb in normal human subjects from a resting to holding position going through isotonic contraction to isometric hold, respectively (Loscher, Cresswell et al. 1996, Thomas and del Valle 2001, Adam and De Luca 2003). During the isotonic phase of the muscle flexion, fast acting, fatigable fiber type II type were activated, but once the isometric maintenance phase was initiated, the Type II fibers dropped out and slower acting Type I were recruited to maintain the voluntary hold. Given these observations, our data suggest a higher motor unit recruitment during the beginning of a paw dorsiflexion bout and then narrowing to more selective recruitment during the maintenance of paw above the threshold. The strong EMG activation during the beginning of paw dorsiflexion increase the probability of the recruitment of more Type II fibers, since the TA muscle in C57BL6 mouse shown to contain primarily of fast twitch, fast fatiguing fiber types; Type IIB, IID and IIBD hybrid fibers (Augusto, Padovani et al. 2004). Future experiments, however, will be required to identify specific motor unit recruitment



type during PaWL. We also suggest that the paw position is maintained through the activation of slow twitch and slow fatiguing muscle fiber consistent with a steady maintained paw position.

### *Effect of removal of the stimulus on learning*

Removal of the shocks, while maintaining the paw position manually alone was not sufficient to establish PaWL, Grau and colleagues showed that low and high intensity shocks also disrupted the instrumental learning in rats (Grau, Barstow et al. 1998). Presentation of limb proprioceptive position alone in absence of electric shocks did not result in modulation of TA EMG activity. Removal of the electrical shock may uncouple the time dependent modification at the sensorimotor network resulting in the observed lack of efferent output. Classical conditioning and instrumental learning model defines the behavioral learning as a response-outcome (R-O) relationship to a stimulus (Grau, Barstow et al. 1998). In PaWL or instrumental learning in rats, the electric shocks (stimuli) are delivered when the paw is below a given threshold, resulting in paw flexion (response). The outcome is the maintaining the paw flexion to minimize the shock exposure. When the outcome (maintenance of paw flexion) was manually imposed without the stimulus- mediated flexion response, the R-O relationship failed, demonstrating the inability to maintain paw position and having minimal TA EMG activity.

### *Interference of muscle afferents*

The interference of axonal conduction at the sensory afferents via sodium channel blockers diminished or blocked spinal learning. Dose dependent block by intramuscular, but not topical lidocaine shows the critical role of the muscle afferents and not the skin afferents in engaging sensory motor network for PaWL spinal learning. Lidocaine blocks the voltage gated Na<sup>+</sup> channels critical for propagation of action potential in muscle spindle afferents to the dorsal horn and

potentially the end plate potential at the neuromuscular junction (Kaji, Rothwell et al. 1995, Gingrich and Wagner 2016). Lidocaine preferentially block large diameter Ia sensory afferent in rat (Buffenoir, Decq et al. 2005).

Importance of the muscle afferents in Operant conditioning of the H-reflex (the electric analog of the spinal stretch reflex) was shown by Wolpaw and colleagues in intact rats. They showed that the up-conditioning of the soleus H-reflex response was affected following sciatic nerve transection and repair. After up-conditioning of the soleus H-reflex for 4 months following injury, the background soleus EMG activity recovered faster and significantly larger in the conditioned compared to non-conditioned rats. Furthermore, anatomical studies showed that SOL H-reflex up-conditioning strengthened primary afferent re-innervation of SOL motoneurons. In another example, the trajectory of the wiping reflex of the frog is affected by eliminating specific afferents. Cutting the specific dorsal roots that carry a majority of hind limb peripheral afferent information to the lumbar enlargement altered the trajectories of the wiping reflex of frogs (Giszter and Kargo 2000). The wiping reflex in frogs with intact dorsal roots involves the activity of knee extensors followed by hip extensors. Following de-afferentation, the EMG activities in ipsilateral knee extensors were reduced, and produced synchronous activation with the hip extensors, resulting in the modified trajectory of the wiping reflex.

### *Does Catch-like facilitation occur in TA muscle during PaWL?*

A potential explanation of the significantly lower TA activation during maintenance than the beginning of the paw flexion could be due to some catch-like muscle property in TA. Catch-like muscle property is described as an increase in the generation of force due to an initial short high frequency pulse followed by low frequency stimuli which results an increased force

generation (Burke, Rudomin et al. 1970, Burke, Rudomin et al. 1976, Binder-Macleod and Barrish 1992). This force augmentation response is a characteristic property of the skeletal muscle tissues where an increase in sarcoplasmic release of calcium sensitivity to prolonged power stroke resulting in increased force. Modulation of calcium level was shown to directly modulate the number of cross-bridges. Thus muscle tension (or the number of active actin myosin cross bridges) is a function of calcium concentration. In PaWL, the repeated electric shocks could result in prolonged contraction and with greater force. However, the observed inter pulse intervals (IPI) during PaWL suggests that the sustained flexion is not the result of catch like property. In order to have a catch like facilitation, the IPI must be ~10-15ms (Burke, Rudomin et al. 1976, Binder-Macleod and Kesar 2005, Nielsen 2009). Given that the shortest IPI (81.9ms) observed during the initial learning was larger than the required IPI for catch-like facilitation in slow (40 ms) and fast (10 ms) twitch muscles, it is unlikely that sustained paw flexion is mediated through catch-like property of the TA(Fig. 3-7). However, to definitively rule out the role of catch-like facilitation during PaWL, the calcium levels should be measured in TA, in a time dependent manner in both master and yoked groups.

### *Is PaWL, a network learning event?*

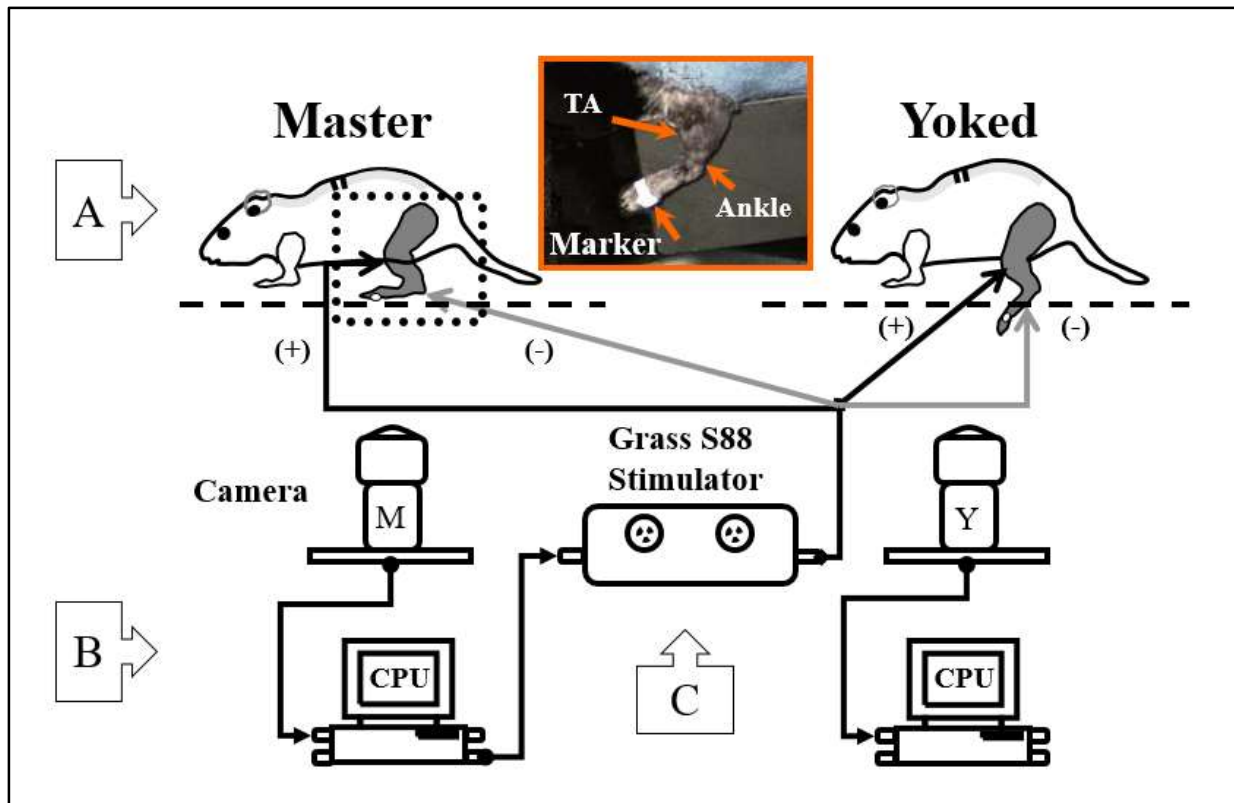
During PaWL, hindpaw position-dependent shock, delivers proprioceptive sensory inputs (Ia, Ib and II afferents) and project to interneurons in the spinal cord, and then these interneurons in turn can project to order interneurons and/or to both alpha and gamma motoneurons. We suggest that in PaWL, the repeated stimulation events are coupled to proprioceptive paw position that initiates/engages a naïve spinal network in Hebbian type network association (Hebb 1949, Kepler, Marder et al. 1990, Soto-Trevino, Thoroughman et al. 2001). The Hebbian learning is described as naïve group of neurons that become linked to form a network due to activity-dependent

modifications in a spatial and temporal dependent manner (Turrigiano, Leslie et al. 1998, Xie and Seung 2003). The strengthening or weakening of the pre to post synaptic plasticity is mediated through the spike time- dependent long term potentiation (LTP) or long term depression (LTD), respectively(Soto-Trevino, Thoroughman et al. 2001). The role of intracellular calcium, expression of NMDA, and AMPA receptors, and downstream CREB-mediated events, determine the time requirement of synaptic plasticity, ranging from seconds, minutes to hours (Bliss and Collingridge 1993, Hunt and Castillo 2012).

We suggest in PaWL, it is the repeated stimulation event coupled to proprioceptive paw position that initiates/engages a naive spinal network in Hebbian type network association. The network that is formed during the initial paw flexion becomes efficient with time. For example, the longer bouts of paw hold, may be mediated through increasingly more efficient spinal network, because when the limb drops due to gravity or fatigue, very few shocks (sometimes, only one shock) are needed to correct and reinstate the paw position. In contrast, the lack of temporal reinforcement of the spinal network seen in the yoked disrupts the Hebbian type network solidification resulting in failed paw flexion. The presentation of the shock at the proper muscle joint position is critical for the development of the Hebbian style learning. In conclusion, our data support that the PaWL is a spinal learning event in which the proprioceptive input coupled to the presentation of the electrical stimulus that can engage the sensorimotor neurons to create a more efficient learned network.

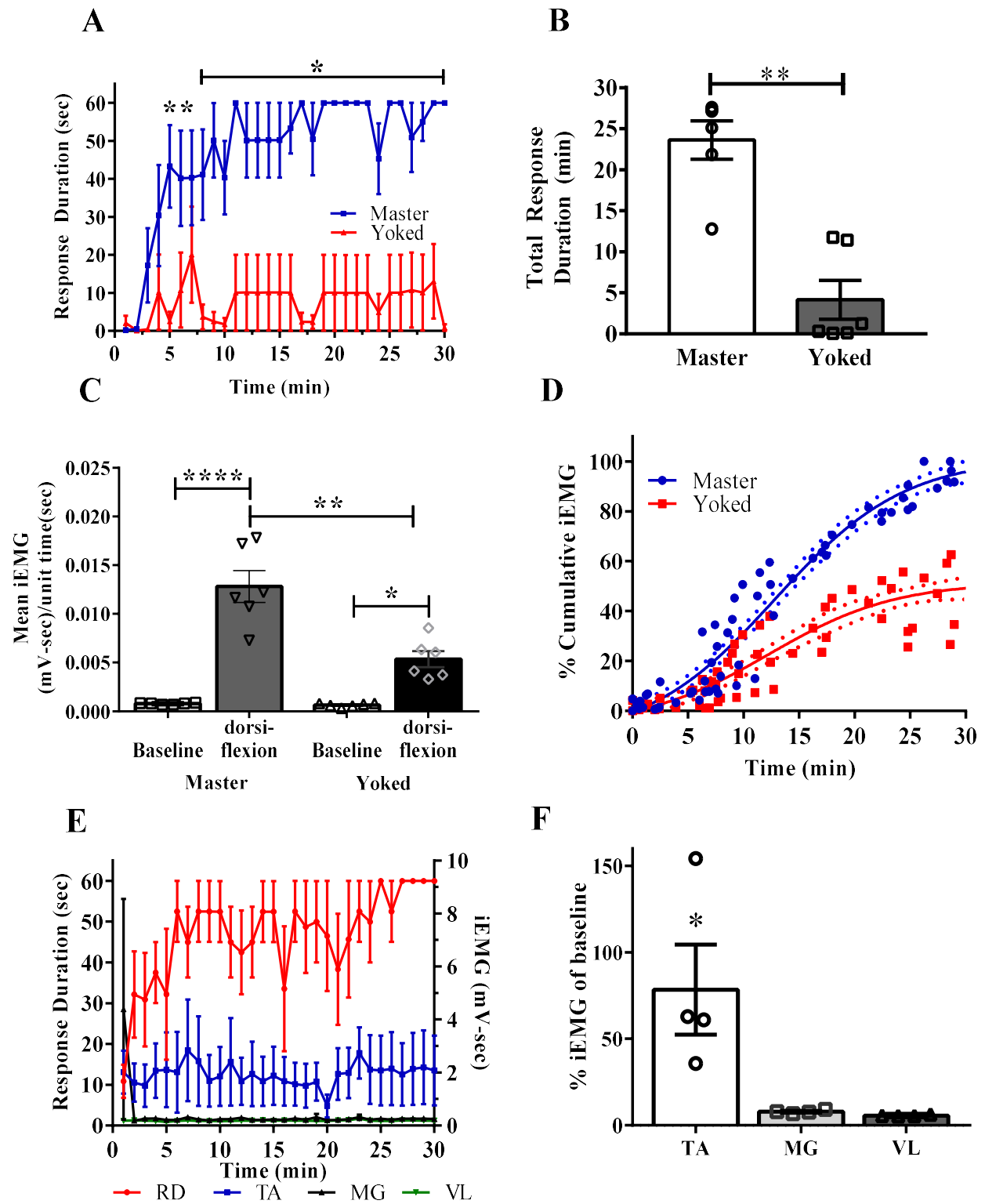
**Table 3-1: Experimental design**

<b>Experiment</b>	<b>Experimental procedure</b>	<b>Animal groups</b>
1. EMG activity in TA muscle during PaWL	Recording of EMG in TA muscle during PaWL	Master=6
		Yoked=6
2. EMG activities in multiple hindlimb muscles during paw flexion	Recording of EMG in TA, VL, and MG muscles during PaWL	Master=4
3. EMG activities during manual dorsiflexion of the hindpaw without electric shocks	Recording of EMG in TA muscle during manual dorsiflexion	Master=6
4. Application of lidocaine to block skin and muscle afferents	Application of 2% lidocaine on the skin of hind leg	Master=6
A) Intramuscular injection of lidocaine (20mg/ml) into TA	6 $\mu$ l	Master=6
	30 $\mu$ l	Master=6
B) Intramuscular injection of saline into TA	6 $\mu$ l	Master=6
	30 $\mu$ l	Master=6
TA, tibialis anterior, VL, vastus lateralis, MG, medial gastrocnemius		Total mice=52



**Figure 3-1: PaWL paradigm in spinal mice.**

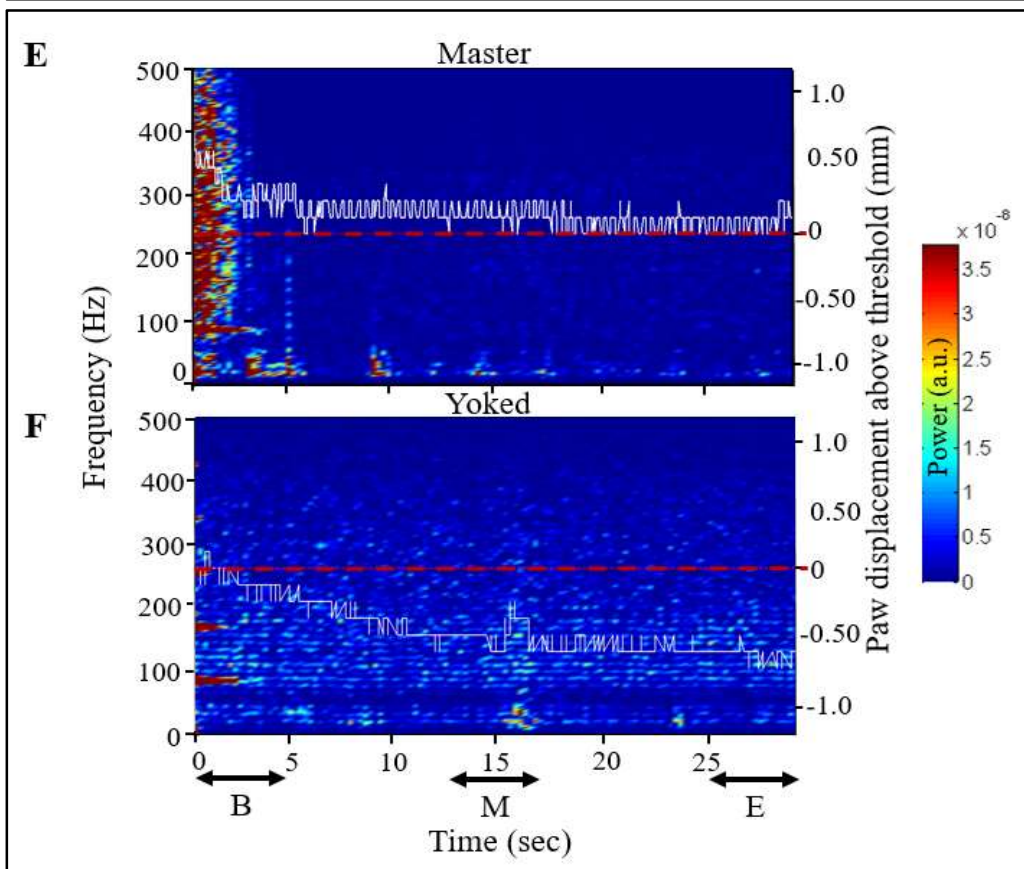
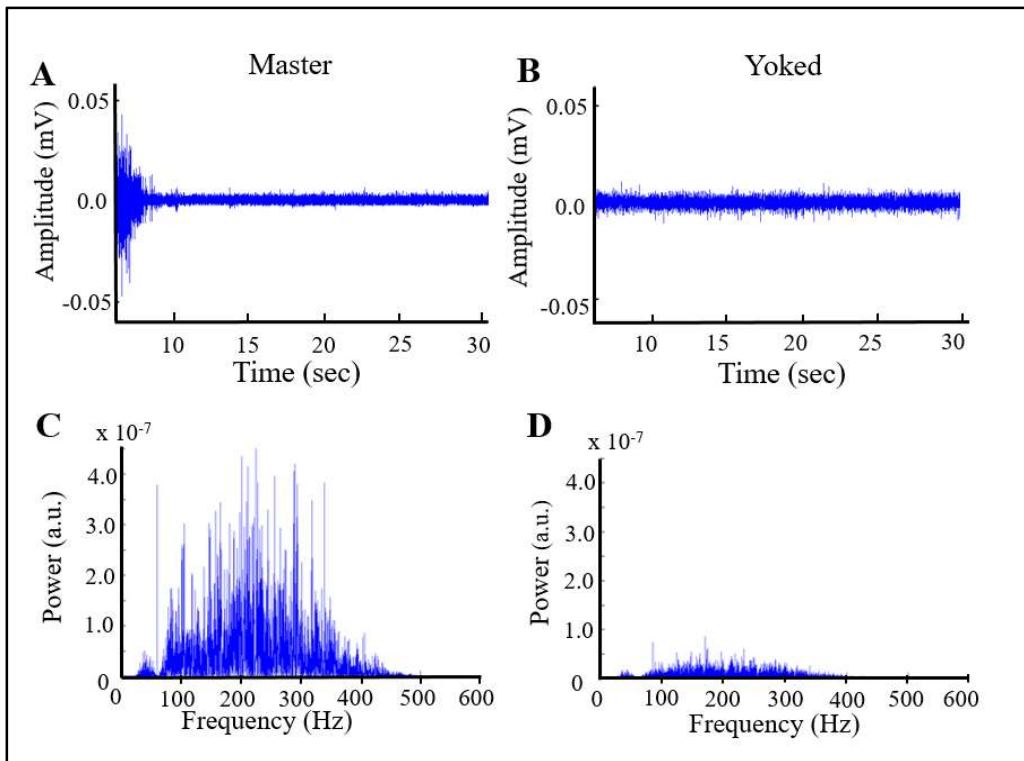
A) The experimental mouse (master, M) is given an electric shock to the tibialis anterior (TA) muscle via fine wire electrodes, when the paw is extended below the imposed 1mm threshold (dotted line). The control (yoked, Y) mice is electrically coupled to the master and receive the same pattern of stimulation, but independent of the limb position. B) A real-time video tracking system acquires the paw position in space. The LabVIEW software collects vertical and horizontal paw position from the mice impose the 1mm threshold level, and (C) the Grass S88 stimulator delivers a direct current (DC) electric shock to the TA. Information of the paw position and paw response to shock in master and the yoked mice are recorded by computers (CPU) in real-time (Jindrich, Joseph et al. 2009).



**Figure 3-2: The tibialis anterior (TA) is differentially activated between master and yoked mice during PaWL.**

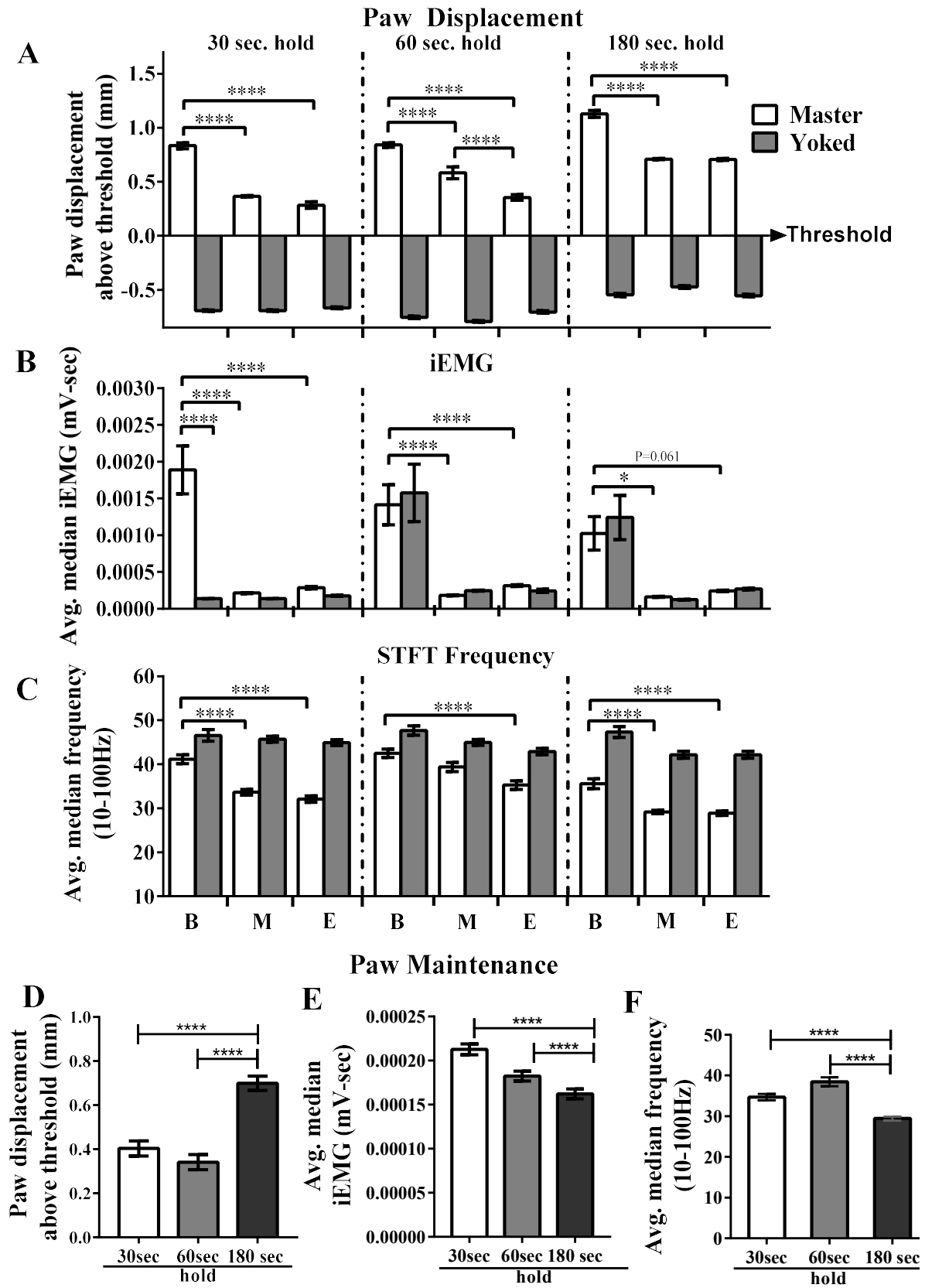
Both master and yoked mice were implanted with EMG electrodes acutely in the TA (A-C, n=6 pairs) while only master mice had chronic EMG implants in TA, medial gastrocnemius and vastus lateralis muscles (D & E, n=4). Response durations, the total integrated EMG (iEMG) and the percent cumulative iEMG in the TA of master and yoked animals during a 30 min PaWL session are shown in A, B, and C, respectively. Mean response durations per min (A) and corresponding cumulative iEMG and IEMG are plotted over a 30 min period in D and E, respectively. iEMG and percent iEMG of the baseline in three hindlimb muscles (TA, MG and VL) during PaWL in master animals are shown in D and E, respectively. The mean iEMG shown was normalized to the total paw holding period above the threshold for both master and yoked groups. Values are mean  $\pm$  SEM. \*  $P < 0.05$  (A, C), \*\* is  $P < 0.01$ , \*\*\*  $P < 0.001$  (A-C, F). Statistical analysis: repeated measure two-way ANOVA with Sidik post hoc (A), Wilcoxon test (B), one-way ANOVA with Bonferroni Post hoc (C, F). Gaussian % cumulative curve analysis with confidence bands showing the likely location of the true curve (dashed lines), adjusted  $R^2 = 0.94$  and  $0.78$ , for master and yoked groups, respectively (D).





**Figure 3-3: Time frequency spectral analysis.**

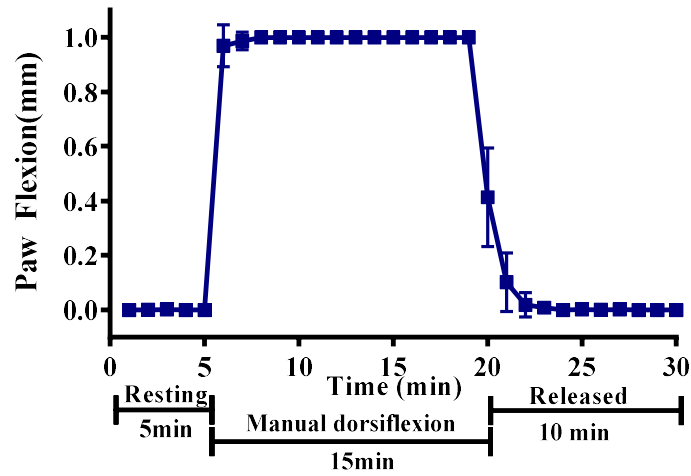
The raw EMG signal in the TA in a master (A) and yoked (B) pair during a 29.4 sec of paw dorsiflexion. Fast Fourier transform (FFT) of the EMG activity is shown in master (C) and yoked (D). Spectral characteristics of EMG using short time Fourier transform (STFT) is shown in the master (E) and yoked (F). Red dashed line indicates the threshold set position which initiates the stimulation. White line indicates the paw displacement above or below the threshold. B, M and E, indicates the beginning 5 sec, the middle 5 sec and the end 5 sec of the paw hold, respectively. The iEMG, STFT frequency and Power values in Fig. 3-3 were determined for B, M and E during various paw holding periods.



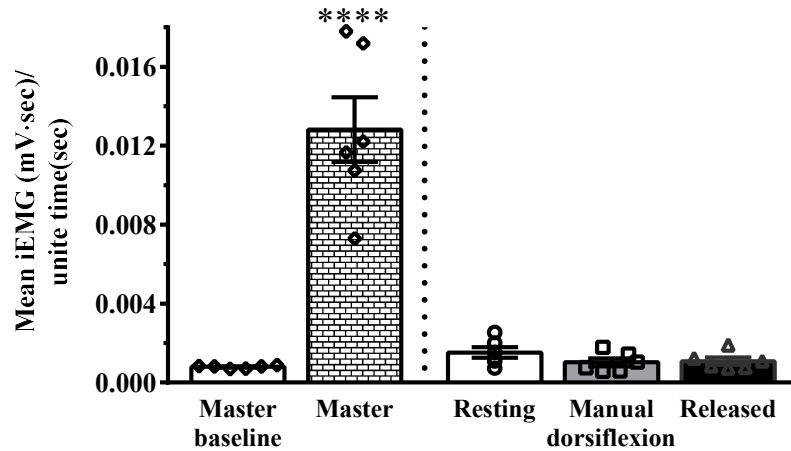
**Figure 3-4: Analysis of frequency spectrum analysis over time domain.**

Panel (A) shows the paw displacements of the master animal during three 5 sec time points, the beginning (B), middle, (M) and end (E) of the first 30 sec hold, , or 60 sec hold, or the 180sec hold. Panel (A) also shows the corresponding paw positions of the yoked animal. (B), and (C), shows the corresponding iEMG (integral EMG), Median STFT Frequency spectrum of the TA, respectively, for master and yoked animals (n=6 pairs). In master group only, changes in paw displacement (D), iEMG activity (E) and median frequency during the maintenance phase of the 30sec, 60sec, and 180sec hold. Values are mean  $\pm$  SEM. \* P< 0.05, \*\* is P< 0.01, \*\*\* P<0.001, \*\*\*\*P<0.0001. Zero value in (A and D) represents the threshold at 1 mm above the resting paw position.

A

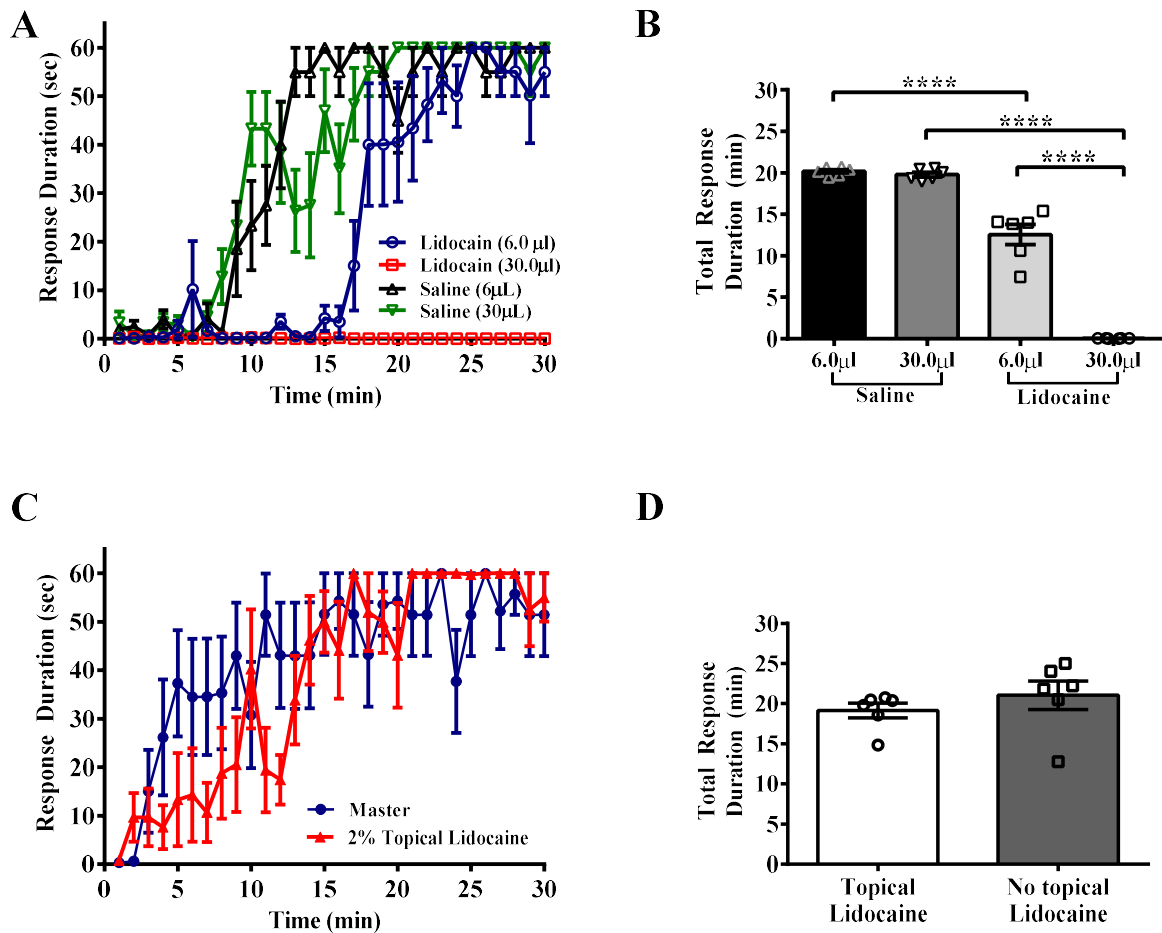


B



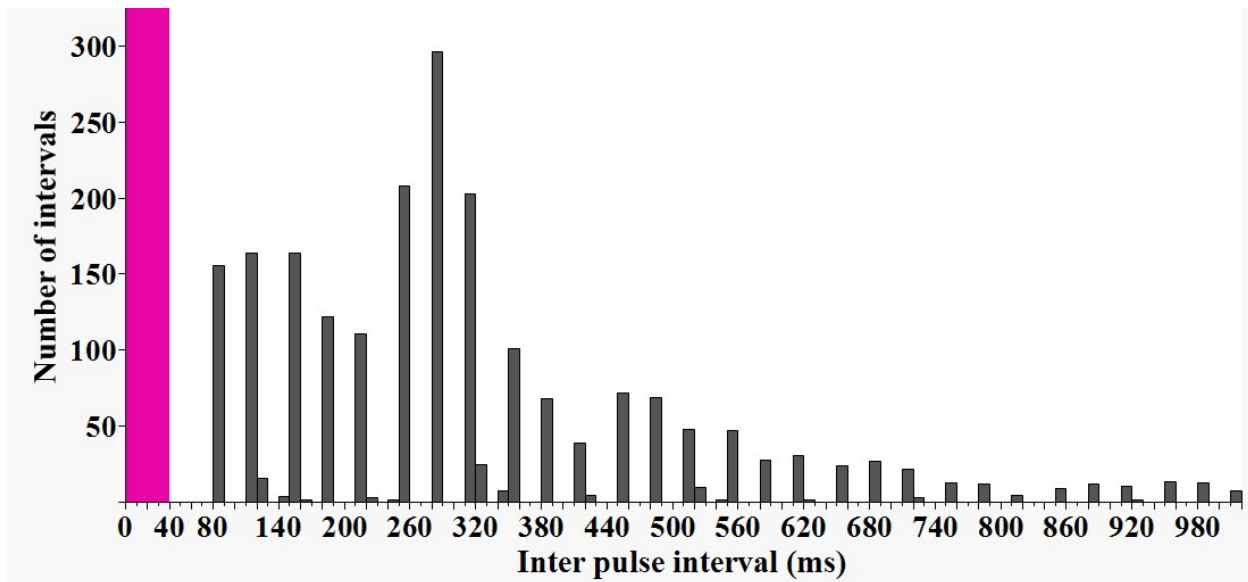
**Figure 3-5: EMG activity of the TA during manually dorsiflexed paw.**

The paw was at rest for 5 min, and then manually dorsiflexed at 1 mm for 15 min (A). The hold on the paw was then released for the next 10 min. The paw position was recorded by the video tracking and the EMG of the TA was recorded during the 30 min of the experiment. No shocks were delivered to the TA muscle at any time. (B) Corresponding mean EMG activity normalized to time during PaWL master baseline, master and the above three periods during manual dorsiflexion. Master EMG is significantly greater all other periods, with no significance between the three period during manual dorsiflexion trials (n=6).



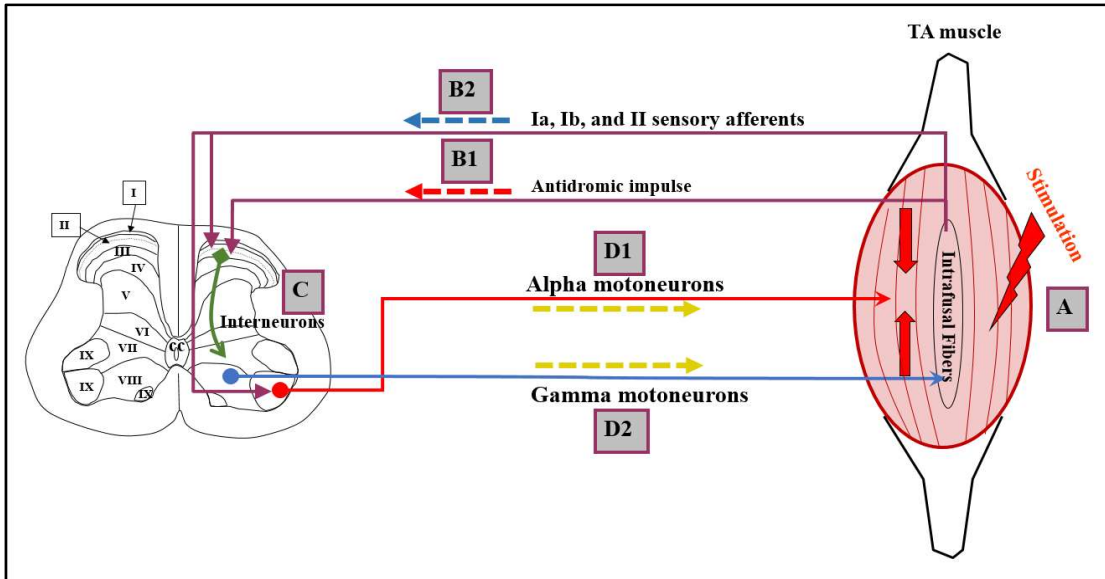
**Figure 3-6: Anesthesia of muscle afferents, but not skin afferents, affects the PaWL.**

Response duration during PaWL is shown when lidocaine or saline was injected into the TA, 5 min before (A, n=6) or when lidocaine cream was topically applied to the leg, 15 min before the experiment (C, n=6). B and D, show the corresponding total response durations during PaWL, respectively. \*\*\*\* p<0.0001, One-way ANOVA with Bonferroni Post hoc (B). No statistical significance between with and without topical lidocaine (D, t-test, p>0.05)



**Figure 3-7: The distribution of Inter pulse interval (IPI) between stimuli during the first 5 minutes of PaWL paradigm.**

The Y-axis represents the number of intervals occurring at the specific binned duration of the IPI represented by X-axis. Only up to 1000ms IPI is plotted here. The purple band represents the minimum 40ms IPI required for a positive Catch-like-response. From a total of 2320 stimuli taken from 6 master animals during the first 5 min of the PaWL, the minimum and maximum IPI recorded was 81.9ms and  $3.64 \times 10^4$ ms, respectively.



**Figure 3- 8: Proposed mechanism of PaWL.**

The sensorimotor network in the spinal cord consists of proprioceptive sensory inputs from Ia, Ib and II afferents (Purple lines) projecting to interneurons (Green) where signal integration occurs and projects output to motoneurons (blue and red) which generates efferent output. At the beginning of PaWL, when the hindlimb is extended, the tibialis anterior (TA) muscle (A) receives an electrical shock. The electrical shock at the TA muscle sends an antidromic impulse (B1) that travels back to dorsal horn of the spinal cord. The spread of the electrical current at the TA muscle results in a muscle contraction that initiates the signal (B2) to the spinal cord. Presumably, there are sensory afferents that interpret the paw position associated with the conditioning stimulus. At interneurons (C), the paired arrival of the sensory afferent signal and the antidromic pulse modifies the spinal network that results in an efferent motor output (D1-2) (McLeod and Wray 1966, Mayer and Feldman 1967, Thomas, Johansson et al. 2002). The spike timing dependent plasticity of the interneurons network over repeated shocks builds the Hebbian type association resulting in the spinal network that in the master animals (Kepler, Marder et al. 1990, Soto-Trevino, Thoroughman et al. 2001).



## CHAPTER 4: Identification of activated spinal neurons in the Paw Withdrawal Learning paradigm

### INTRODUCTION

It is clear that learning-related events occur within the spinal circuitry when animals whose spinal cords are completely transected at a low thoracic level are trained to stand or step (Barbeau and Rossignol 1994, Edgerton, Leon et al. 2001, Edgerton, Tillakaratne et al. 2004, Edgerton, Courtine et al. 2008). There also is convincing evidence that the spinal neural circuitries can learn to modulate the magnitude of the H-reflex response (Wolpaw and Lee 1989, Carp and Wolpaw 1995, Chen, Chen et al. 2006, Grau, Crown et al. 2006, Bigbee, Crown et al. 2007, Gomez-Pinilla, Huie et al. 2007, Chen, Wang et al. 2016 and to avoid a shock based on the position of the ankle {Horridge, 1962 #1481). Thus, both very complex and very simple motor tasks can be learned by the spinal circuitries. However, simple models of learning will be necessary to understand the synaptic mechanisms involved in the learning of more complex motor tasks. Unlike in the *Aplysia* (Byrne, Castellucci et al. 1978, Murphy and Glanzman 1997), there has been no established mammalian experimental model of learning developed to identify a complete circuit of sensory neurons and interneurons that can directly interpret and project to an effector neuron.

While it is not as simple as the *Aplysia* model, the paw withdrawal learning paradigm in mice (Fig. 1, Chapter 2) has some components of locomotion, but with reduced complexity (Jindrich, Joseph et al. 2009). For example, collaboration with Grau and colleagues, we demonstrated that spinal neural circuits that are modified in PaWL are associated with changes that also occur in stand training, a much more complex motor task in which learning has occurred over a period of weeks (Bigbee, Crown et al. 2007). For example, we showed that spinal rats that had been trained to maintain a standing posture (potentiation of extensor neurons) for weeks had

a reduced capacity to learn the paw withdrawal task (potentiation of flexor neurons). Both the ipsilateral and contralateral hindlimbs were impaired significantly in their acute learning potential. It seems more likely that the PaWL paradigm in genetically modified mice can be used as a model to identify circuitry anatomically and understand spinal learning mechanisms.

Although important molecular substrates in PaWL in spinal rats have been shown through pharmacological manipulations (Joynes and Grau 2004, Gomez-Pinilla, Huie et al. 2007, Ferguson, Bolding et al. 2008), anatomical locations of spinal neurons associated with this learning have not been examined.

Our overall hypothesis is that PaWL activates specific neurons in the spinal cord (Specific Aim 1) in TA-associated sensory-motor circuitry (Specific Aim 2). We propose that PaWL is mediated through learning mechanisms similar to the brain, specifically CREB pathway (Specific Aim 3).

We used an activity dependent marker, c-fos, to identify activated neurons in the spinal circuitry involved in the PaWL. Expression of the c-fos protein (Fos) has been used effectively to map neuronal activity in the nervous system at the cellular level (Huang, Noga et al. 2000, Dai, Noga et al. 2005, Ahn, Guu et al. 2006, Tillakaratne, Duru et al. 2014, Duru, Tillakaratne et al. 2015). Furthermore, we identified populations of activated neurons that expressed  $\alpha$ CaMKII neurons in specific spinal laminae. We injected pseudorabies virus (PRV152) into the tibialis anterior (TA) muscle, the primary hindlimb muscle involved in the PaWL paradigm, to label functionally connected neurons associated with TA, and then identified activated neurons during PaWL.

We also used CREBIR transgenic mouse line developed by Dr. Alcino Silva and colleagues, for cell-specific manipulation of CREB function (Silva, Kogan et al. 1998, Kida,

Josselyn et al. 2002, Matynia, Kushner et al. 2002, Frankland, Josselyn et al. 2004) in cells involved in the processes of learning and memory. A central constituent of the pathways involved in memory consolidation is the transcription factor CREB that is activated by phosphorylation by specific kinases during the process of learning (Silva, Kogan et al. 1998). Multiple signaling pathways converge to phosphorylate CREB that in turn mediates CRE-dependent transcription of genes required for long-term memory (Alberini, Ghirardi et al. 1995, Alberini 1999, Kida, Josselyn et al. 2002, Balschun, Wolfer et al. 2003, Chen, Ohno et al. 2006). The CREBIR transgenic line conditionally can express the CREB-repressor fusion protein (CREBIR) that is unable to phosphorylate CREB, thus allowing it to compete with endogenous CREB and disrupt CRE-mediated transcription. We used CREBIR mice to identify spinal neurons in PaWL by disrupting the role of CREB signaling in the PaWL task.

The roles of these genes in the spinal cord may be important not only in understanding the basic form of learning with PaWL, but also may allow for increased understanding of other types of spinal learning. Better understanding of spinal learning will ultimately facilitate efforts to restore neuromotor function after a spinal cord injury or other debilitating conditions.

## **MATERIALS AND METHODS**

### *Animals*

Swiss Webster mice (Experiment 1, n=11, master/yoke pairs), (Experiment 2, n=5, master), and CREBIR and wildtype littermates (Experiment 3, Table 3-1, n= 52, master) were used. All animals were euthanized at the end of the experiments following the American Physiological Society Animal Care Guidelines and protocols approved by the Animal Use Committee at UCLA. Pseudorabies virus (PRV) usage was approved by UCLA Institutional Biosafety Committee (IBC). PRV handling, injections, animal care and terminations were carried out under Bio Safety Level

II in a negative air flow lab space inside fume hood according to the IBC approved standard operating procedure. PRV was kindly provided by Dr. Lynn Enquist at Princeton University, Center for neuroanatomy with Neurotropic Viruses (CNNV grant no. P40RR018604).

### *PaWL paradigm*

PaWL paradigm was conducted ~ 24 hrs after spinal transection as described before ((Jindrich, Joseph et al. 2009); Chapter 2, & 3). Briefly, while suspended in a harness, adult mice whose hindlimbs were completely paralyzed were given a mild electric shock to the tibialis anterior (TA) muscle to one leg when the hindpaw was in extended position (master mouse; contingent shock). A yoked mouse, that is experimentally coupled to the master mouse also get shocked, but the shock delivery is not dependent on its paw position yoked mouse; (non-contingent shock). Force calibration was done using a force transducer to optimize current to elicit a submaximal contraction. The position of the foot, the height of the vertical threshold, and the number of stimulations were recorded for over the course 30 min during the PaWL trial. The threshold for all experiments was 1mm, except second day of testing in Experiment 3 set at 2mm. To assess paw withdrawal learning, we compared the response duration, during which the animal holds it paw above the threshold. The measure of response duration was defined by the equation,  $(\text{time above threshold}) / (\text{number of stimulations} + 1)$ , was calculated for each of the 30 minutes of the trial. This quantitative method was developed by Grau and colleagues (Grau, Barstow et al. 1998). Sixty min after the PaWL testing the mice were terminated and the spinal cord was saved for tissue analysis.

### **Experiment 1 (Specific Aim 1). Identification of neurons activated in the spinal cord during PaWL**

PaWL was carried out in Swiss Webster mice as Master and Yoked pairs (n=11, 6 for initial Fos quantification, and 5 for CaMKII+ Fos+ quantification) at 1mm threshold. In some mice (n=3), 10  $\mu$ l of 2% fluorogold was injected into multiple sites in the TA muscle to identify, spinal cord sections containing TA motoneurons, 5 days before spinal cord transection. Mice were returned to the cages after completion of PaWL testing and terminated 60 min later via transcardial perfusion with 4% paraformaldehyde, and processed for Fos protein, CaMKII analyses, described in detail under tissue processing.

### **Experiment 2 (Specific Aim 2). Activated neurons in functionally connected TA hindlimb circuitry**

The attenuated pseudorabies virus strain Bartha (PRV-Bartha) that expresses the enhanced green fluorescent protein (PRV 152) was unilaterally injected into the TA muscle to identify the functionally connected TA neurons in the lumbar spinal cord, by transynaptic and retrograde labeling. PaWL was carried out in Swiss Webster mice as Master and Yoked pairs (n=5) at 1mm threshold.

### **Experiment 3 (Specific Aim 3). Effect of perturbation of CREB mediated learning pathway on PaWL.**

To begin to delineate the neural circuitry of PaWL paradigm using a conditional transgenic mouse strain that expresses an inducible CREB repressor fusion protein (CREBIR) that disrupts the CREB function. The induction of CREBIR is regulated by tamoxifen (TAM), allowing it to compete with endogenous CREB and disrupting the CRE-mediated transcription.

*CREBIR mice*

The CREBIR transgenic line was developed in Dr. Alcino Silva's laboratory at UCLA by fusing a CREB repressor ( $\alpha$ CREB isoform with a S133A mutation  $\alpha$ CREBS133A) to a ligand-binding domain (LBD) of a human estrogen receptor with a G521R mutation (LBDG521R), whose activity is regulated not by estrogen but by the synthetic ligand Tamoxifen (TAM)(Kida, Josselyn et al. 2002). In the absence of TAM, the LBDG521R–CREBS133A fusion protein is inactive. However, administration of TAM activates this inducible CREB-repressor fusion protein (CREBIR), allowing it to compete with endogenous CREB and disrupt cAMP-responsive element (CRE)–mediated Transcription.

### *Experimental groups*

Four groups of adult mice (CREBIR and wild type mice, with DMSO or with tamoxifen) underwent a complete mid-thoracic spinal cord transection (Table 4-1). Six hours before conducting PaWL, one group of WT (group 1) and CREBIR (group 3) were intraperitoneally (i.p) injected with vehicle Dimethyl sulfoxide, while the second group of WT mice (group 2) and CREBIR mice (group 4) were injected with Tamoxifen TAM (16 mg/kg, i.p.). Twenty-four hrs later, the mice were retested for PaWL on the same leg, at 2 mm vertical threshold. Both days animals received contingent shocks (master set up). In previous PaWL paradigm for mice or instrumental learning in rats, in data indicates rodents pre-trained to avoid 1mm for mice and 4mm for rats of threshold can learn to acquire a more challenging task of 2mm for mice and 8mm in rats respectively (Crown, Ferguson et al. 2002, Gomez-Pinilla, Huie et al. 2007).

### *Surgical Procedures*

Mice were handled to minimize stress for two weeks before the surgery. Spinal cords of mice were completely transected at the mid-thoracic level (T7-T9), and were allowed to recover

overnight with pain management, hydration and temperature control (for details, see Chapter 2, methods). Both hindlimbs were taped overnight to immobilize to prevent hyperextension of the limbs (Grau et al, Jindrich et al 2009). All procedures described for the care and maintenance of mice are described in Chapter 2.

### *PRV injection*

PRV-152 virus was injected into multiple sites of the TA muscle of same leg to be tested for PaWL in intact female Swiss Webster (~30-45g, n= 6), 52 hours prior to the spinal transection surgery. Mice were anesthetized using isoflurane gas at 1.0%-2.5% via facemask to maintain a surgical level of anesthesia, and PRV injections were performed on a water-circulating heating pad maintained at 37<sup>0</sup> C. A small skin incision (~2 cm) was made and fascia below the skin was cleared to expose the belly of the TA muscle. PRV 152 (~15 µl of 1.24x10<sup>9</sup> pfu/ml; total virus 1.9x 10<sup>7</sup>) was injected into the TA using a 10 µl Hamilton syringe equipped with a 30-gauge needle (Hamilton, Reno, NV). In order to maximize the viral spread within the TA muscle, 3 injections were made proximal to and 2 injections were made lateral to the tibial bone in the TA. The virus was injected slowly to minimize leakage and the area was flushed with warm sterile saline to clear the area of any virus that may have leaked out. The skin was closed with 5.0 Vicryl sutures as described before using the running subcuticular technique and 2% Lidocaine ointment applied on the sutures for pain relief (Chapter 2). The mice were allowed to recover from the anesthesia in a warm incubation chamber and once fully awake and mobile, the mice were returned to disposable cages. Fifty two hrs post-PRV injection, complete spinal cord transection surgery was performed as described before and the mice were allowed to recover overnight. The PaWL was conducted in master and yoked pairs at 1mm vertical threshold, ~24 hrs after the spinal transection surgery, and

60 min later, the animals were transcardially perfused with 4% paraformaldehyde under deep anesthesia.

### *Tissue Preparation*

To ensure time for maximal Fos levels in the spinal cord, the mice were returned to their cages and perfused 60 min after the PaWL test (Morgan, Cohen et al. 1987). Animals were deeply anaesthetized with Eutha-6 (100mg/kg i.p.) approximately 5 min before perfusion would take place. All animals were perfused transcardially with a fixative solution of cold 4% paraformaldehyde (PF) in phosphate buffered saline (PBS; 130 mM NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>; Sigma, St. Louis, MO) under deep anesthesia with approximately 150 to 200ml of 4% PF for 15-20 min. After the perfusion, the spinal cord was dissected, rinsed with 1XPBS buffer and place in a 30% sucrose for cryoprotection for 4-5 days. With the dorsal root ganglion of L5 as a landmark, the spinal cords were divided into T13-L2, L3-L5, and L6-S2 blocks, then embedded in Richad-Allan Neg50 frozen section medium frozen with Neg 50 (Thermo Scientific, Hudson, NH), and stored at -80<sup>0</sup> C until ready to cut. The spinal cord blocks were cryosectioned serially at 30µM thickness and collected and stored in sequence as free floating sections in 1XPBS with 0.02% sodium azide (Sigma). -filled wells, 4 sections/well in 96-tissue culture well plates and stored at 4<sup>0</sup>C. The lesion site and adjoining rostral and caudal spinal cord segments were cryosectioned (20 µm thick sections) and alternate sections were mounted on slides in a sequential fashion for histological staining. Luxol blue (myelin) and cresyl violet (neurons and glia) stains were used to verify the completeness of the spinal cord transection (Kluver and Barrera 1953).

### *Immunohistochemistry (IHC)*



Immunofluorescent histochemistry with anti-c-Fos (Sc7202, Santa Cruz Inc., CA) and CaMKII (6G9 clone, Millipore, CA) was used to identify activated CaMKII neurons (CaMKII +/Fos+) in the spinal cord (Table 4-2). For the analysis of segmental distribution of activated spinal neurons, we used 5-8 sections each from L1-S2 lumbar segments. We counted Fos+, CaMKII+/Fos+ neurons in 8-12 sections in all laminae of L4 segments to provide an adequate sampling of activated spinal neurons. To detect PRV+, Fos+ and CaMKII+ cells, 8-12 spinal cord sections from PRV-injected mice gone through PaWL were processed by IHC with GFP antibody (Aves Labs, Tigard, OR) and c-fos antibody and  $\alpha$ CaMKII antibody (Table 4-2).

For immunohistochemistry, sections were processed as free-floating sections. Briefly, sections were transferred into Costar netwells (15 mm membrane diameter, 74  $\mu$ m mesh) and rinsed for 30 min in 1xPBS. The sections then were transferred to 24-well plates containing 200  $\mu$ l/well of a mixture of Fos antibody (1:400) with CamKII (1:500) in 1xPBS containing 0.3% Triton X100 (PBST) and incubated overnight at 4°C on an orbital shaker. Sections were washed in 1xPBS as follows: 2 quick rinses, followed 2x5 min rinses, and 2x10 min rinses. Sections then were incubated in a mixture (200  $\mu$ l/well, 24-well plate) of secondary antibodies (anti-rabbit Dylight 594 1:500 and anti-goat FITC 1:500, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hr at room temperature. Sections were washed extensively as above, mounted on Fisher Superfrost slides (Fisher Scientific, Pittsburg, PA) and cover slipped with VectaShield mounting media containing DAPI (Vector Labs, Burlingame, CA). See Table 4-2 for a list of antibodies and dilutions used for IHC.

### *IHC image analysis*

Spinal cord sections processed for fluorescent immunohistochemistry were examined under Zeiss (Axiophot) microscope under appropriate fluorescent filter sets. Digital images of

neurons labeled with Fos, DAPI, GFP and CaMKII were acquired using a Spot RT CCD Slider color camera (Diagnostics Instruments, Inc., Sterling Heights, MI) and the Image Pro Plus 7 image analysis program (Media Cybernetics, Rockville, MD). Composite images of Fos, DAPI, and CaMKII or Fos, GFP, CaMKII were created using color composite feature of Image Pro. Neurons of the following types were tagged using the manual tag analytical feature of Image Pro Plus: 1) activated (Fos<sup>+</sup>) spinal neurons (Fos<sup>+</sup> nuclei), 2) activated CaMKII<sup>+</sup> interneurons (Fos<sup>+</sup> nuclei and cell bodies labeled by CaMKII, (Fos<sup>+</sup>/CaMKII<sup>+</sup>)) 3) activated, GFP<sup>+</sup> interneurons (Fos<sup>+</sup>/GFP<sup>+</sup> IN), 4) activated GFP<sup>+</sup> motoneurons (Fos<sup>+</sup>/GFP<sup>+</sup> MN), and 5) non-activated GFP<sup>+</sup> motoneurons (Fos<sup>-</sup>/GFP<sup>+</sup>); 6) activated GFP<sup>+</sup> CaMKII<sup>+</sup> interneurons (Fos<sup>+</sup>/GFP<sup>+</sup>/CaMKII<sup>+</sup>). Laminae borders were drawn onto spinal cord sections with tagged neurons according to the spinal cord atlas of the adult mice (Watson et al., 2009) and the numbers of activated interneurons in individual spinal cord lamina were counted. The number of activated CaMKII<sup>+</sup> neurons in laminae IV-VI (i.e., CaMKII<sup>+</sup> Fos<sup>+</sup>) was calculated as a percent of total activated (Fos<sup>+</sup>) interneurons (i.e., CaMKII<sup>+</sup> Fos<sup>+</sup> divided by the total number of Fos<sup>+</sup> interneurons).

### *CaMKII immunoreactivity in the dorsal horn in L3-L6 lumbar segments*

Five spinal cord sections/segment from L3-L6 segments, processed for IHC with CaMKII antibody were selected for optical density measurements. Regions of interest (ROI; dorsal horn) were outlined manually on the saved images, and objects identified above background threshold were measured. Pixels outside the ROIs were removed using the qualify feature. We added the edit option on the work file to erase, redraw, or individually remove obvious background spots before the final data acquisition. The zoom and roam feature was used on the image on display for detailed examination. The customized work files were saved and loaded to collect data from the saved images and the data were copied to Excel spreadsheets. The optical density of CaMKII

immunoreactivity measured as the total object area, was divided by corresponding area of the ROI and expressed as a percentage.

### *Western blot analysis*

The fresh spinal cord segments at the L4 level was collected from CREBIR and wild type littermate controls (Table1). Spinal cord tissue were homogenized in a freshly prepared lysis buffer (137 mM NaCl, 20 mM Tris-HCl pH 8.0, 1% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 1 µg/mL leupeptin, 0.5 mM sodium vanadate). Homogenates were centrifuged at 4°C 12,000 g for 10 min to remove insoluble material. The supernatants were collected into clean 1.5 ml tubes and stored at -80°C. The total protein concentration of homogenates was determined with a MicroBCA kit (Pierce, Rockford, IL, USA), using BSA as a standard. A total of 50 µg of protein from each sample was separated on 10% SDS-polyacrylamide gels, and then transferred onto PVDF membranes. Nonspecific bindings in the membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST). The membranes were incubated with a primary antibody overnight at 4°C, followed by a secondary antibody for 1 hr at room temperature. The following primary antibodies were used (Table 4-2): HA-antibody (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, California), phospho-CREB (1:1000, Millipore, CA), phospho-CaMKII (1:1000, Santa Cruz Biotechnology, CA), total-CaMKII (1:1000, Millipore, CA) and total-CREB (1:1000 Millipore, CA). The secondary antibodies used were anti-goat or anti-rabbit IgG- HRP (1:10,000, Santa Cruz Biotechnology, CA). After rinsing with buffer (0.1% Tween-20 in TBS), the immunocomplexes were visualized by chemiluminescence using the Amersham ECL Plus Western Blotting Detection kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) according to the manufacturer's instructions. The film signals were digitally scanned using a HP Scanner (HP Scanjet 3970) and quantified with c-Imaging

Image software. Actin was utilized as an internal control for sample loading, and each blot was normalized to its corresponding actin value.

### *Generation of heat maps*

Fos+ nuclei were manually tagged using manual tag feature of the Image Pro program. The tagged coordinate points were imported to Microsoft Excel that translated the coordinate points into a spreadsheet grid that represented the distribution of Fos+ nuclei in the spinal cord section when divided into a  $80 \times 50$  frame grid. Grid values indicate the number of nuclei counted in that particular frame. A color gradient was applied based on the value in each grid frame. A heat map color gradient was adjusted for according to its highest value. A diagram of the laminae distribution in each segment was overlaid on each heat map to quantify the number of Fos+ found in each lamina. A macro for heat map was generated by Kevin Truong in Reggie Edgerton's laboratory. To calculate the % of total Fos+ in each lamina on ipsi and contralateral sides, the number of quantified Fos+ per in each laminae was expressed as percentage of the total number of Fos+ in ipsi and contralateral sides, respectively.

### *Statistical Analysis*

Western blot data were shown by mean and standard deviation and the results were analyzed using JMP Pro 11.2.0 and GraphPad 11. Ordinary One-Way ANOVA with Bonferroni's multiple comparison between group test to show significance and compare between groups (Fig. 4-1B, Fig. 4-5B, and 4-5C, Fig. 4-6C-D ). Two-Way ANOVA followed by Bonferroni's multiple comparison was used to show over all significance and differences between groups (Fig. 4-2A). Mann-Whitney test of used to show differences between master vs yoked groups (Fig. 4-3E, Fig. 4-4C). To quantify the rate of learning in the master group, Boltzmann Curve fit analysis was

performed. The confidence bands (dotted lines) indicate the likely location of the true fit curve (Fig. 4-3A and B). The maximum response duration of the curve fit is reported and the rate of learning was determined by taking the time at half-the-maximum ( $T_{50RD}$ ) of the response duration curve. Significant statistical difference was determined to be when  $P < 0.05$ . All of analyses were performed using GraphPad 7.0 (GraphPad Software Inc. San Diego, CA) or JMP Pro 12.0.1 software (SAS Inc., Cary, NC, 1989-2015). The level of Significance was chosen as  $P < 0.05$  for all comparisons. Data are reported as the mean values  $\pm$  standard error of the mean (SEM).

## RESULTS

### *Activated neurons is highest in the lamina V, and are directly correlated with the degree of PaWL in the Master mice*

We examined paw withdrawal learning in Swiss Webster spinal mice during one training trial, and only the mice receiving the contingent shock (master mice) successfully learned to maintain the shocked leg in a flexed position that minimized the net shock exposure (Chapter 2, Fig. 2-1). The mice terminated 60 min after PaWL and were processed by immunohistochemistry to identify activated neurons using c-fos as an activity marker (Fig. 4-1A). The number of activated neurons (Fos+) were significantly higher in the ipsilateral compared to the contralateral spinal cord, in both the master and yoke mice (Fig. 4-1B, C). L4 segment, where TA motoneurons are located, had the highest Fos+ neurons when compared to rest of the lumbar spinal cord (data not shown). Taken together, the total Fos+ in all laminae in the ipsilateral half was similar in both master and yoke mice, the master mice showed a strong negative correlation between total Fos+ and response duration of PaWL (Fig. 4-1D). Although the Fos+ in the ipsilateral individual laminae showed similar distribution between respective spinal laminae in both master and mice (Fig. 4-2A), the Fos+ in laminae II (Fig. 4-2B) and IX (Fig. 4-2C), were negatively correlated while in laminae V (Fig. 4-2D), showed a strong positive correlation with degree of learning in master mice only. Lamina V contained the highest Fos+ in all spinal laminae in the stimulated side of the spinal cord both master and yoked animals (Fig. 4-2A). Combination of laminae IV-VI also showed a strong positive correlation ( $R=0.87$ ) with the response duration of learning.

### *CaMKII neurons are activated in ipsilateral IV-VI in the master mice*

CaMKII immunoreactive terminals were highest in the ipsilateral laminae I-III (Fig. 4-3A), with many strongly labeled CaMKII+ cell bodies in medial IV-VI (Fig. 4-3B, C). Comparison of

CaMKII<sup>+</sup> staining in L3-L6 segments showed that in both master and yoked mice, CaMKII<sup>+</sup> staining in the ipsilateral DH is stronger than the contralateral side (Fig.4-3D). Of all the lumbar segments, the master animals contained significantly higher levels of CaMKII immunoreactivity in L4 dorsal horn than the yoked mice (Fig. 4-3D). Fos<sup>+</sup> CaMKII<sup>+</sup> cell bodies (Fig. 4-4A) were tagged individually with Image Pro and heat maps were constructed for master and Yoke (Fig. 4-4B) mice. Master mice contained significantly higher activation of Fos<sup>+</sup> CaMKII<sup>+</sup> neurons in ipsilateral IV-VI compared to the yoked mice (Fig. 4-4C) and also positively correlated with the PaWL ability (Fig. 4-4D).

*In functionally connected TA circuitry, the laminae IV-VI contain most of the activated CaMKII<sup>+</sup> neurons*

To identify the functionally connected TA circuitry associated with PaWL in the master mice, pseudorabies Virus (PRV) Bartha-152, a recombinant PRV that expresses green fluorescent protein (GFP), was injected into the TA muscle, the primary hindlimb muscle involved in the learning paradigm, 76 hrs prior to PaWL testing. PRV<sup>+</sup> motoneurons were located primarily in the L4 segment. A time course of post-viral injection times showed that at 72 hrs most of the labeling was in the motoneurons, but at 76 hrs interneurons (presumably pre-motoneurons) also get labeled. (data not shown). ~77% PRV<sup>+</sup> interneurons were located in the ipsilateral side and most of these PRV<sup>+</sup> interneurons (66%) were activated (Fig. 5B). About 78% of the ipsilateral PRV<sup>+</sup> CaMKII<sup>+</sup> were activated and distributed mainly in laminae IV-VI (Fig. 4-5C, E). The total ipsilateral PRV<sup>+</sup> Fos<sup>+</sup> strongly correlated negatively with PaWL in this group of master mice similar to the Fos<sup>+</sup> in the previous group (Fig. 4-1D).

*CREBIR mice treated with Tamoxifen, interferes with PaWL*

Since the transgene is under the control of the CaMKII promoter, we used CREBIR transgenic mice that are unable to phosphorylate cyclic AMP binding protein (CREB) at S133A site to examine the role of phosphorylation of CREB in long-term memory formation during PaWL. Since the CREBIR is conditionally expressed upon treatment with Tamoxifen (TAM), comparisons were made in CREBIR and wildtype littermates injected with TAM and control (DMSO), 6 hrs before testing PaWL on the first day (Table 4-1). The ability to form long-term memory for PaWL was measured by comparing the paw withdrawal responses on the first and the second day in all four groups. All mice learned PaWL on the first day (Fig. 4-6A-D). On the second day, all but CREBIR mice injected with TAM were able to learn, demonstrating the importance of CREB phosphorylation during spinal learning.

Ordinary one-way ANOVA analysis shows significantly difference in total response duration between CREBIR+TAM day 1, CREBIR+TAM day 2, WT+TAM Day 1 and WT+TAM Day 2 groups ( $F(3, 32) = 18.08, P < 0.0001$ ). The Bonferroni post-hoc test reveals, CREBIR+TAM day 2 significance is significantly less total response duration than CREBIR+TAM day 1, WT+TAM Day 1 and WT+TAM Day 2 groups. (Fig. 4-6C, Bonferroni-post hoc test  $P < 0.05$ ). In all the groups given +DMSO as vehicle control demonstrated no significant differences between the groups (Fig. 4-6D). The curve fit analysis, the CREBIR+TAM day 1 group reached half-the-maximum response duration faster ( $T_{50RD} = 5.936$  min with maximum = 57.76sec), compared to the CREBIR+TAM day 2 group reached a maximum response duration(RD) of only 14.5sec with a  $T_{50RD} = 4.246$  min, suggesting it failed to acquire the 2mm threshold set (Fig. 4-6 B). For the WT+TAM day 1 the maximum RD = 50.12 sec reaching the  $T_{50RD} = 4.51$  min demonstrating normal rate of learning and for WT+TAM Day 2 with maximum RD= 59.97 sec with  $T_{50RD} = 9.334$  min showing learning occurring at a slower than Day1 control (Fig. 4-6 B).



### *Phosphorylated CaMKII protein significantly decreased in CREBIR TAM+*

On the second day, level of phosphorylated CaMKII protein was significantly decreased in CREBIR TAM+ compared to WT TAM+ and CREBIR DMSO+ mice (Fig. 4-7B, 4-7C, 4-7D). Endogenous pCREB levels were also low, 6hrs and 24 hrs after TAM+ injection in TAM+ CREBIR mice. On day 2, TAM+ CREBIR mice showed significantly reduced levels of pCaMKII protein (Fig. 4-7B; D), but this decrease is not due to uneven loading or of proteins as seen from the actin control. Endogenous pCREB levels are also low, 6hrs and 24 hrs after TAM+ injection. There was no similar reduction in PCREB in wildtype mice (Fig.4-7C, 4-7D). The % PCaMKII/total CaMKII and % PCREB/total CREB, significantly correlated to response duration in only the CREBIR mice (Fig. 4-7E, G).

### *Reduced Fos+ CaMKII+ in laminae IV-VI in CREBIR TAM+*

Fos+ CaMKII+ in L4 spinal cord sections of all four groups were tagged under the microscope at high magnification, using the manual tag feature of the Image-Pro image analysis software. CREBIR TAM+ tested on second day, had reduced number of Fos+ CaMKII+ neurons in spinal laminae IV-VI, compared to TAM and DMSO treated groups of wildtype mice on both days and CREBIR TAM+ mice treated with TAM and DMSO on first day (Fig. 4-8).

## DISCUSSION

### *Using a simple model to examine learning related events*

Learning related events occur within the spinal circuitry even after there has been a complete spina transection at mid-thoracic level and are trained to stand or step. The spinal circuitry can learn to modulate the magnitude of the H-reflex response (Wolpaw and Lee 1989, Chen and Wolpaw 1995, Chen, Chen et al. 2006) and it can learn to avoid a shock based on the position of the ankle. Thus, very complex and very simple motor tasks can be learned by the spinal circuitry. Given the extensive involvement of millions of neurons and many circuits that generate coordinated posture and locomotion in mammals, the combination of inhibitory and excitatory synapses in the alternating flexion and extension combined with abduction and adduction, all components of normal posture and locomotion. It is quite evident that a simpler model of learning was necessary to understand the synaptic mechanisms involved in the more complex motor tasks, hence, we used the paw withdrawal learning paradigm.

Chen and colleagues showed that rats that received up conditioned H-reflex training that received a thoracic, lateral column lesion, had greater improvement in the symmetry of locomotion following injury when compared to the negative conditioning (Chen and Wolpaw 1995, Chen, Chen et al. 2006, Chen, Chen et al. 2006). This outcome implies a simple monosynaptic reflex has functional consequences performing a complex task of stepping. These authors suggested that precise methods for reeducating the spinal cord neurons and synapses are needed for restoring effective function. Second, Bigbee et al demonstrated that spinal rats that had been trained to step had similar acute learning potential as non-trained, while the Stand-trained rats had a reduced capacity to learn the acute task (Bigbee, Crown et al. 2007). Further investigation of the Stand-trained group showed that, while both the ipsilateral and contralateral hindlimbs were significantly

impaired in their acute learning potential, the contralateral, untrained hindlimbs exhibited significantly greater learning deficits. These results suggest that different types of chronic peripheral input may have a significant impact on the ability to learn a novel motor task. Both of these simple models of learning in the spinal cord involve neural circuits that are modified by one simple paradigm and this modification impacts a much more complex motor task and vice versa.

Changes in levels of expression of neurotransmitter and learning related molecules have been observed in response to the spinal cord injury as well as due to training induced recovery of function (Tillakaratne, de Leon et al. 2002, Fong, Cai et al. 2005, Edgerton, Courtine et al. 2008, Khristy, Ali et al. 2009, Noga, Johnson et al. 2009, Noga, Johnson et al. 2011). There is considerable lack of knowledge about neurons that are involved in spinal learning. c-Fos has been used reliably to identify locations of spinal neurons that are activated during stepping in intact and spinal cord transected animals (Huang, Noga et al. 2000, Ahn, Guu et al. 2006, Noga, Johnson et al. 2009, Tillakaratne, Duru et al. 2014, Duru, Tillakaratne et al. 2015). In this chapter we used c-fos to identify the neural circuitries and mechanisms associated with paw withdrawal learning (PaWL) in spinal mice.

### *Number of activated neurons in the Master mice correlates with the degree of learning.*

While the total number of Fos<sup>+</sup> neurons were similar, differences in activation patterns in specific spinal laminae and correlation with degree of learning were observed between Master and Yoked mice. The negative correlation of total number of Fos<sup>+</sup> neurons with the PaWL ability found in master mice suggests that generally when animals don't learn well, more neurons are activated. Of the individual lamina, lamina II and IX, were negatively correlated. The number of shocks received by mice that hold the paw above the threshold for longer periods are lower than

moderate learners or failed mice. The increased number of shocks may contribute to the activation of lamina II neurons responding to nociceptive stimuli through pain-related afferents. We did not however identify these activated neurons with respect to pain related markers, or in association with muscle-specific afferents. Intrathecal delivery of NMDA in artificial CSF was shown to induce massive expression of Fos protein in spinal dorsal horn neurons in intact anesthetized rats, but failed to produce long-term changes of spinal nociception (Sandkühler, Treier et al. 1996). Given that both the master and the yoked groups show similar distribution of Fos+ neurons in Lamina I-II, suggests the potential activation of lamina I-II nociceptive related afferents may not be sufficient to interfere with PaWL in the Master animals.

In a previous study, we showed that compared to non-stepped rats, intact rats stepped on treadmill at a 0° incline showing significantly higher activation of interneurons associated with muscle-specific TA afferents in laminae IV, V and VII (Tillakaratne, Duru et al. 2014). Unlike activated neurons pattern in PaWL, the stepping at 0° incline of the treadmill (no nociceptive stimuli) did not activate significantly higher Fos+ in the laminae I, II or III. Unlike laminae II and IX, number of Fos+ interneurons in lamina V was positively correlated with degree of PaWL, only in Master mice. These interneurons may be responding to proprioceptive cues of the paw position. Then we examined if these neurons are a subpopulation of CaMKII+ neurons in the spinal cord, since CaMKII has been shown to be important in PaWL in rats (Gomez-Pinilla, Huie et al. 2007).

#### *Activated CaMKII interneurons in laminae IV-VI.*

The Ipsilateral CaMKII immunoreactivity was highest in the L4 segment where majority of the TA motoneurons and interneurons are located, when compared to rest of the lumbosacral spinal segments, suggesting learning-associated changes in CaMKII expression occurring in L4. We found cell bodies with strong CaMKII immunostaining in laminae IV-VI in L4, and the master

group showed significantly higher number of activated CaMKII in laminae IV-VI compared to yoked group. A high percent of PRV<sup>+</sup> interneurons in laminae IV-VI in L4 spinal sections were also positive for Fos and CaMKII. These results indicate that CaMKII<sup>+</sup> neurons in the L4 segment are part of the circuitry associated with hindlimb flexion induced during paw withdrawal learning. Although we can't definitely identify if these activated CaMKII<sup>+</sup> interneurons are premotor neurons in the TA neural circuitry, we used PRV post-viral injection survival times that indicated labeling of premotor interneurons. Although the use of monosynaptic rabies virus would be more suitable for definitive identifying premotor neurons of the TA muscle, due to failure of the rabies viral contracts to infect muscles of mice older than P15 mice, combined with the fact that PaWL cannot be done in these young mice due to their small paw size, we resorted to transynaptic labeling through PRV (Wickersham, Finke et al. 2007, Callaway 2008)).

Levine and Colleagues identified a spatially and molecularly defined population of neurons in the deep dorsal horn of the mouse spinal cord that they suggest as candidates to encode the programs for motoneuron activation patterns motor synergies encoders (Levine, Hinckley et al. 2014). They showed a population comprising of a network of interneurons, sufficient to elicit reliable and coordinated motoneuron activity (motor synergy encoder (MSE) neurons). Labeling of first-order spinal neurons targeting gastrocnemius motoneurons (an ankle flexor) by monosynaptic rabies viral tracing in P0-P15 mice, they showed 49% of the labeled neurons in the medial deep dorsal horn (medial laminae V-VI), spanning from L4-L5, and direct activation of these neurons by optical stimulation was sufficient evoke detectable motoneuron responses. Of three candidate genes that are expressed in the medial deep dorsal horn interneurons, more than 93% received proprioceptive synaptic contact from corticospinal neurons. Similarly, these MSE neurons may be receiving proprioceptive contacts from muscles since primary proprioceptive

afferents have dense terminations in the medial deep dorsal horn (Scheibel and Schiebel 1969, Ishizuka, Mannen et al. 1979).

### *Is PaWL mediated through CaMKII/CREB?*

To examine if the learning to hold the paw dorsiflexion is mediated through the CaMKII and CREB mediated pathway, we used conditional CREBIR transgenic mice, that is under the CaMKII promoter, and can be induced to make a CREB repressor protein that is unable to phosphorylate upon tamoxifen administration, thus interfering with CREB function by competing with endogenous CREB (Kida, Josselyn et al. 2002). Learning in the PaWL paradigm was not affected by the induced CREB repressor on the first trial, but significantly affected on the subsequent trial, 24 hrs later. This was similar to Silva and colleagues who showed that the interfering with CREB function was not required for the short-term memory, but required for consolidation of long-term conditioned fear memories (Silva, Kogan et al. 1998, Kida, Josselyn et al. 2002).

We also showed that phosphorylated CaMKII and CREB protein levels are decreased in CREBIR mice treated with tamoxifen that induces the CREB repressor. This agrees with CREBIR mice with induced CREB repressor disrupting the binding of pCREB to CRE site, and reducing the CRE-mediated gene transcription of genes (eg. CaMKII and CREB) required for long-term memory. In addition, compared to control groups of wildtype and CREBIR mice, L4 spinal cord section of CREBIR with the induced repressor demonstrated reduced number of activated CaMKII<sup>+</sup> neurons in laminae IV-VI. More activated CaMKII neurons were detected in mice with normal CREB function, but not observed in transgenic mice with targeted CREB disruption, suggests that these neurons are important in Paw withdrawal learning. These results indicate that

these specific neurons in the L4 segment are part of the circuitry associated with hindlimb flexion during paw withdrawal learning.

CREB is within the common path that many other learning related/signaling pathways converge upon and express genes required for the process of learning and memory in the brain. pharmacological studies (Chapter 1, Fig. 1-1) in rat model of PaWL has shown that learning related events similar to the hippocampus occurring in spinal rats during PaWL. For example, Fernando Gómez-Pinilla in a collaboration with Grau's group (Gomez-Pinilla, Huie et al. 2007), measured mRNA levels of learning related markers in rats that have completed instrumental training. In the lumbar spinal cord segments of rats that were given controllable shock (Master), the levels of BDNF, CaMKII, CREB, and Synapsin I mRNA were significantly increased than that of the uncontrollable shocked (Yoke) and unshocked control groups suggesting instrumental training modulated expression of these genes. Furthermore, the increases in BDNF, CREB, and CaMKII were proportional to the learning performance. Pretreatment with CaMKII inhibitor also blocked the facilitated learning when rats were tested with a higher response criterion. In addition, blocking the BDNF pathway with TrkB IgG inhibited instrumental learning further adding to the importance of BDNF as a potential factor in instrumental learning. We have also observed that mice fed a diet containing DHA/Cur performed better in PaWL paradigm than mice fed a diet deficient in DHA/Cur and this enhanced performance was accompanied by increased mRNA levels of molecular markers of learning, i.e., BDNF, CREB, CaMKII, and syntaxin 3 (Joseph, Ying et al. 2012). Here we show that PaWL is a robust model for understanding spinal learning mechanisms at synapses that can be identified anatomically and through genetic manipulation and behavior.

**Table 4-1: Groups of CREBIR and wildtype mice**

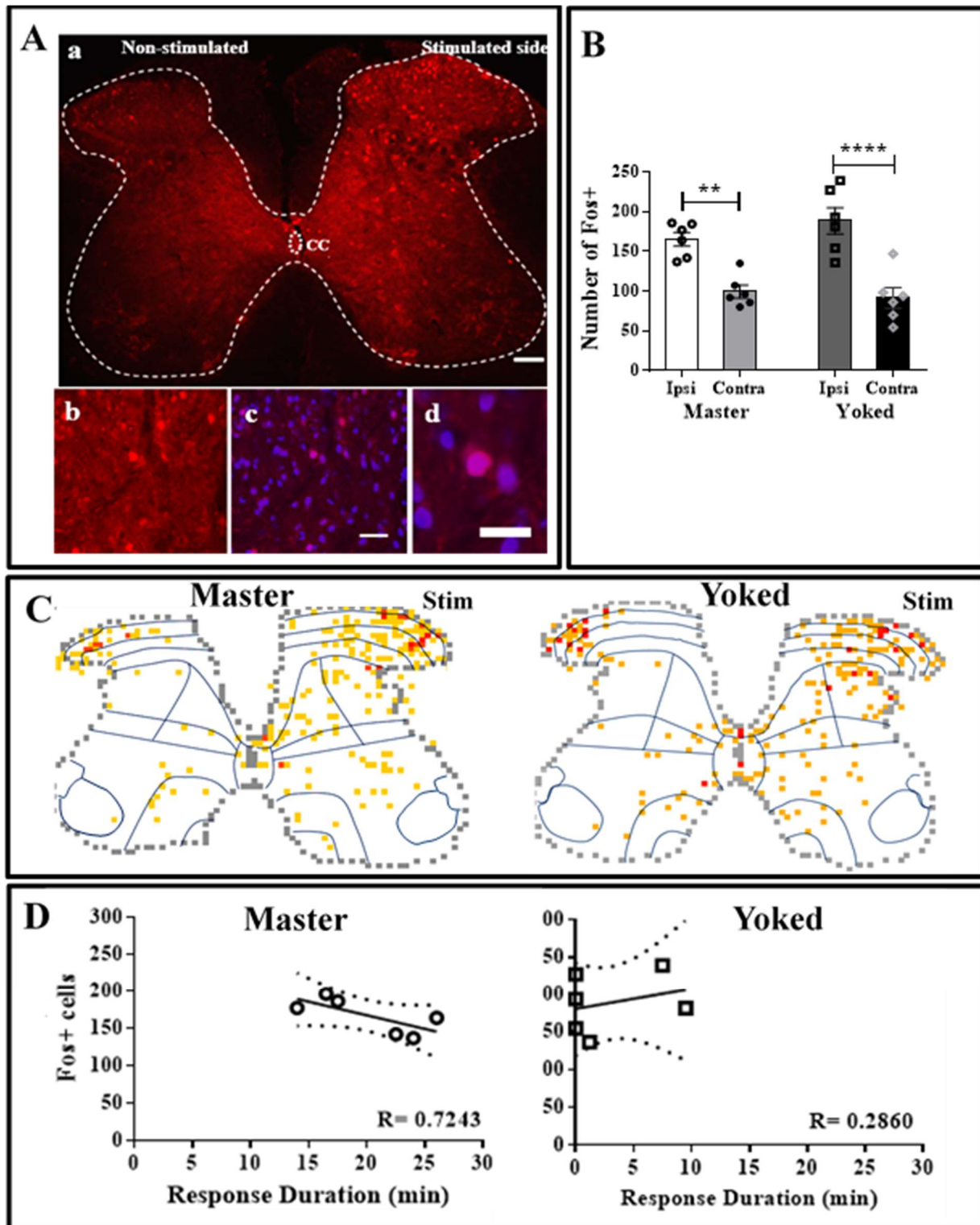
	<b>Animal Type</b>	<b>Injection</b>	<b>Number</b>	<b>Day 1</b>	<b>Day2</b>
<b>1</b>	ST- Wildtype	DMSO+	5	X	X
<b>2</b>	ST- Wildtype	TAM+	8	X	X
<b>3</b>	ST- CREBIR	DMSO+	6	X	X
<b>4</b>	ST- CREBIR	TAM+	10	X	X
<b>5</b>	ST- Wildtype	DMSO+	6	X	NA
<b>6</b>	ST- Wildtype	TAM+	6	X	NA
<b>7</b>	ST- CREBIR	DMSO+	6	X	NA
<b>8</b>	ST- CREBIR	TAM+	6	X	NA
<b>9</b>	ST- Wildtype	DMSO+	3	NA	NA
<b>10</b>	ST- Wildtype	TAM+	3	NA	NA
<b>11</b>	ST- CREBIR	DMSO+	3	NA	NA
<b>12</b>	ST- CREBIR	TAM+	3	NA	NA

X-Conducted PaWL as receiving contingent shocks; NA, PaWL not conducted



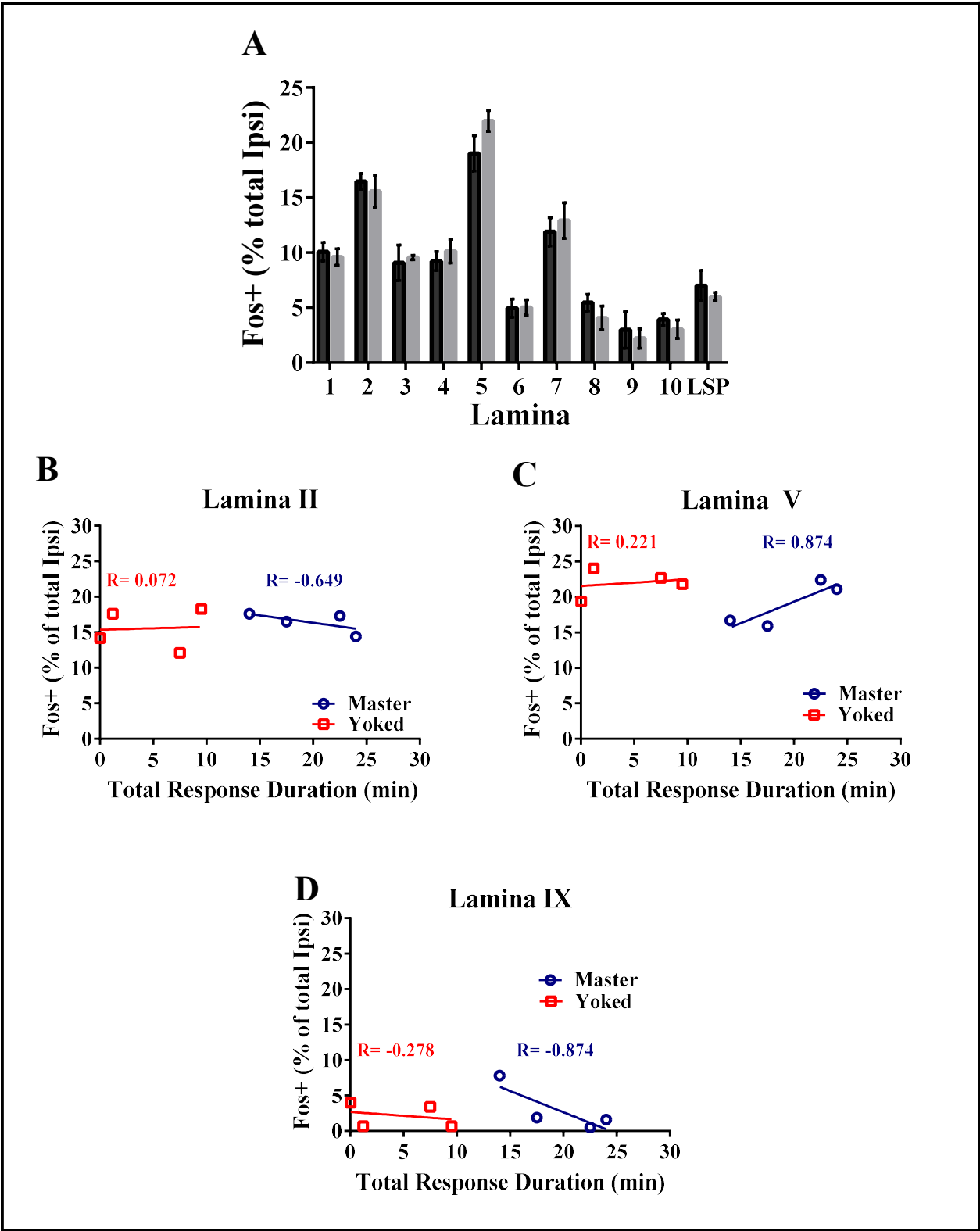
**Table 4-2: Antibodies used in immunohistochemistry and Western blots.**

<b>Primary Antibody</b>	<b>Dilution</b>	<b>Secondary Antibody</b>	<b>Dilution</b>
c-fos SC7202 (rabbit), Santa Cruz Biotechnology Inc., CA; (SCBT)	1:400 (IHC)	Anti-rabbit DyLight 594 Jackson ImmunoResearch Laboratories, Inc., PA	1:500
CaM Kinase II, $\alpha$ subunit Clone 6G9 (mouse), 05-532- Upstate, Millipore, CA). (no reactivity for phosphorylated or non-phosphorylated $\beta$ subunit	1:500 (IHC)	FITC anti-mouse Jackson ImmunoRes	1:500
GFP, 1020 (chicken) from Aves Labs, Tigard, OR	1:1000 (IHC)	Anti-chicken Alexa fluor 488, Jackson ImmunoRes	1:500
HA-probe (Y-11): sc-805 (rabbit); SCBT, CA. CREBIR fusion protein ~79.5 KDa	1:200 WB	goat anti-rabbit IgG-HRP: sc-2054 (SCBT)	1:10,000
p-CaMKII $\alpha$ (Thr 286), sc-12886-R (rabbit); SCBT, CA. (detects protein band ~50KDa)	1:1000 WB	goat anti-rabbit IgG-HRP: sc-2054 (SCBT))	1:10,000
CaMKII, (M-176), sc-9035 (rabbit), SCBT, CA. (detects protein band ~50KDa)	1:1000 WB	goat anti-rabbit IgG-HRP: sc-2054 (SCBT)	1:10,000
Anti-phospho-CREB (Ser133), (rabbit) Upstate 06-519. Millipore, CA. (detects protein band ~43KDa)	1:1000 WB	goat anti-rabbit IgG-HRP: sc-2054 (SCBT)	1:10,000
Anti-CREB CREB, 06-863 (rabbit);   Millipore, CA. (detects protein band ~43KDa)	1:1000 WB	goat anti-rabbit IgG-HRP: sc-2054	1:10,000
actin (I-19), Cat #: sc-1616 (goat) SCBT, CA. ((detects protein band ~43KDa)	1:1000 WB	donkey anti-goat IgG-HRP, SCBT (sc-2020)	1:10,000



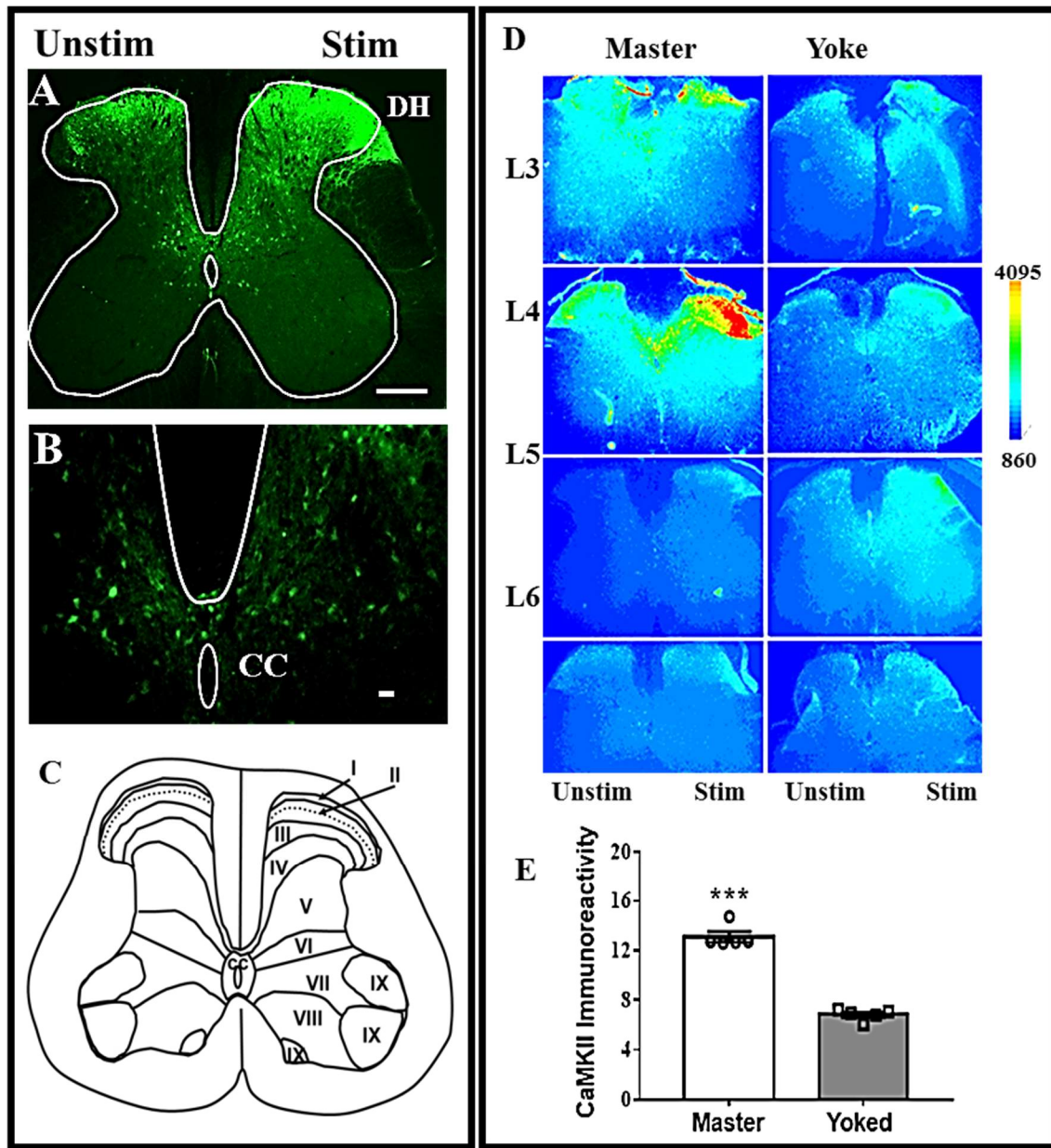
**Figure 4-1: Number of activated neurons in the master mice correlates with the degree of learning.**

c-Fos antibody was used in immunohistochemistry to identify activated neurons during PaWL. Panel A shows Fos<sup>+</sup> nuclei in a lumbar 4 (L4) spinal cord section of a master mouse, Aa, Fos<sup>+</sup> immunostaining at low magnification; Ab and Ac, shows overlap of Fos and DAPI (nuclear marker); Ac, double-labeled Fos<sup>+</sup> and DAPI<sup>+</sup> nuclei at high magnification. Scale bars in a=100  $\mu$ M and b-d=20  $\mu$ M. Total Fos<sup>+</sup> in ipsilateral and contralateral spinal cord shown in B. Fos<sup>+</sup> nuclei in acquired images were tagged individually using manual tag feature of Image Pro program, and representative sections for master and yoke mice are shown in C. Total Ipsi Fos<sup>+</sup> in master (D) and yoke (E) mice were correlated with the response duration (measure of PaWL). N=6, master/yoked pairs, 10 sections/animal). Although there are significantly more Fos<sup>+</sup> in stimulated side compared to the non-stimulated side, in both master and yoked mice, the total Fos<sup>+</sup> was similar between the two groups of mice (B). However, there was a significant negative correlation between total Fos<sup>+</sup> and the degree of learning in master mice, only.



**Figure 4-2: Fos+ in lamina V shows positive correlation with PaWL.**

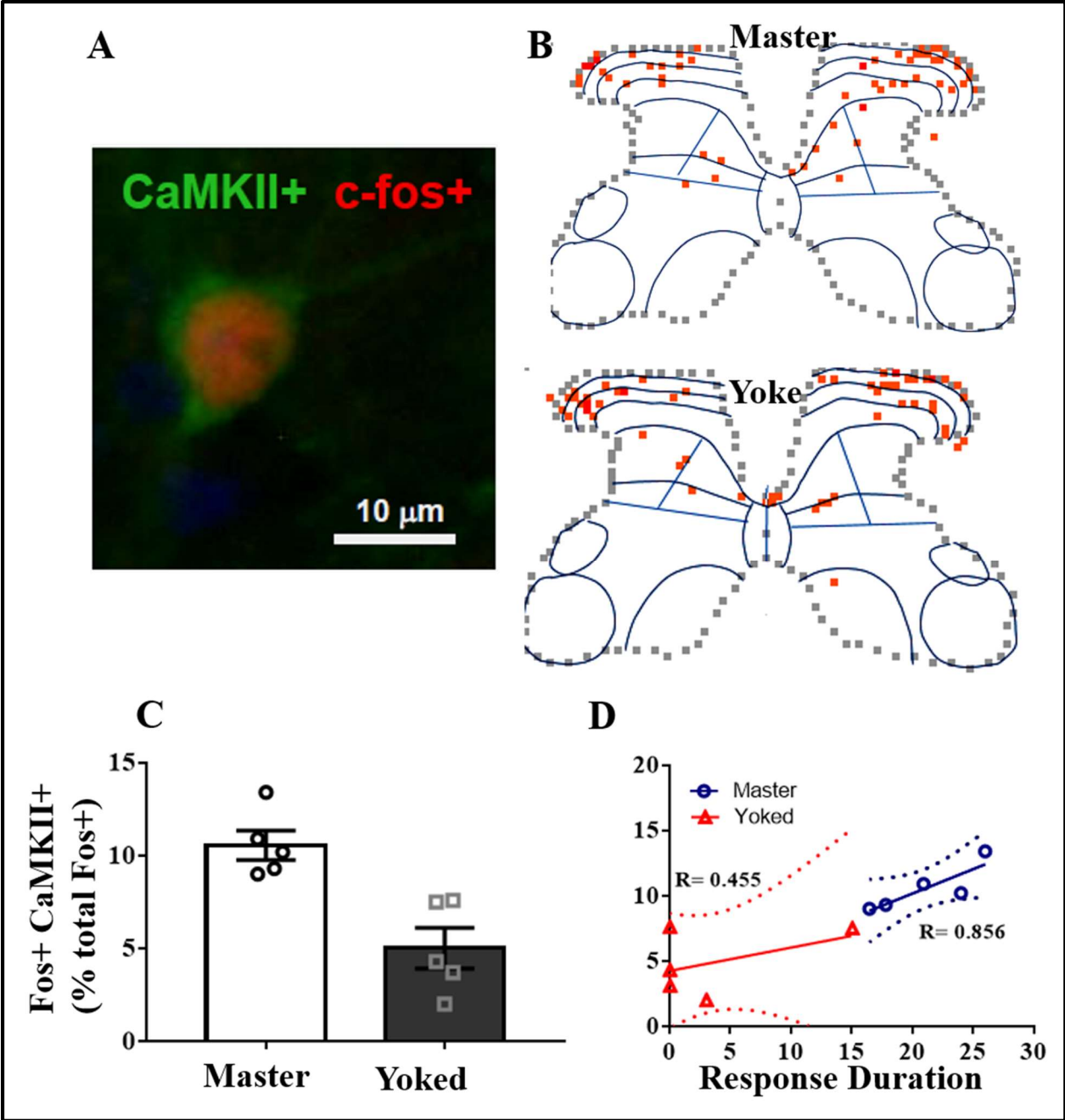
Laminae distribution of Fos+ in ipsilateral spinal cord in master and yoke mice shown in A. Correlation of response duration with Fos+ in lamina II (B), laminae IX (C) and V (D) are shown with the Pearson correlation coefficient R. LSP, lateral spinal nucleus. N=4, master/yoked pairs, 10 sections/animal). Fos+ nuclei in individual spinal laminae showed highest activation in laminae II and V. Only the laminae II, IX and V, correlated strongly with the response duration, with laminae II and IX showing a negative and lamina V, a positive correlation.



**Figure 4-3: CaMKII immunoreactivity is increased in the dorsal horn (DH) in master, but not in yoked mice during PaWL.**

Spinal cord sections from L3-L6 from master and yoked mice were processed for CaMKII immunoreactivity. L4 section of a master mouse showing strong CaMKII<sup>+</sup> staining (cell body and terminals) in laminae I-III (A) and IV-V (B) on the ipsilateral side. Schematic of a Lumbar 4 section showing lamina borders (C). Comparison of CaMKII<sup>+</sup> staining in L3-L6 segments of master/yoked pairs show strong CaMKII<sup>+</sup> staining in the ipsilateral DH than the contralateral side

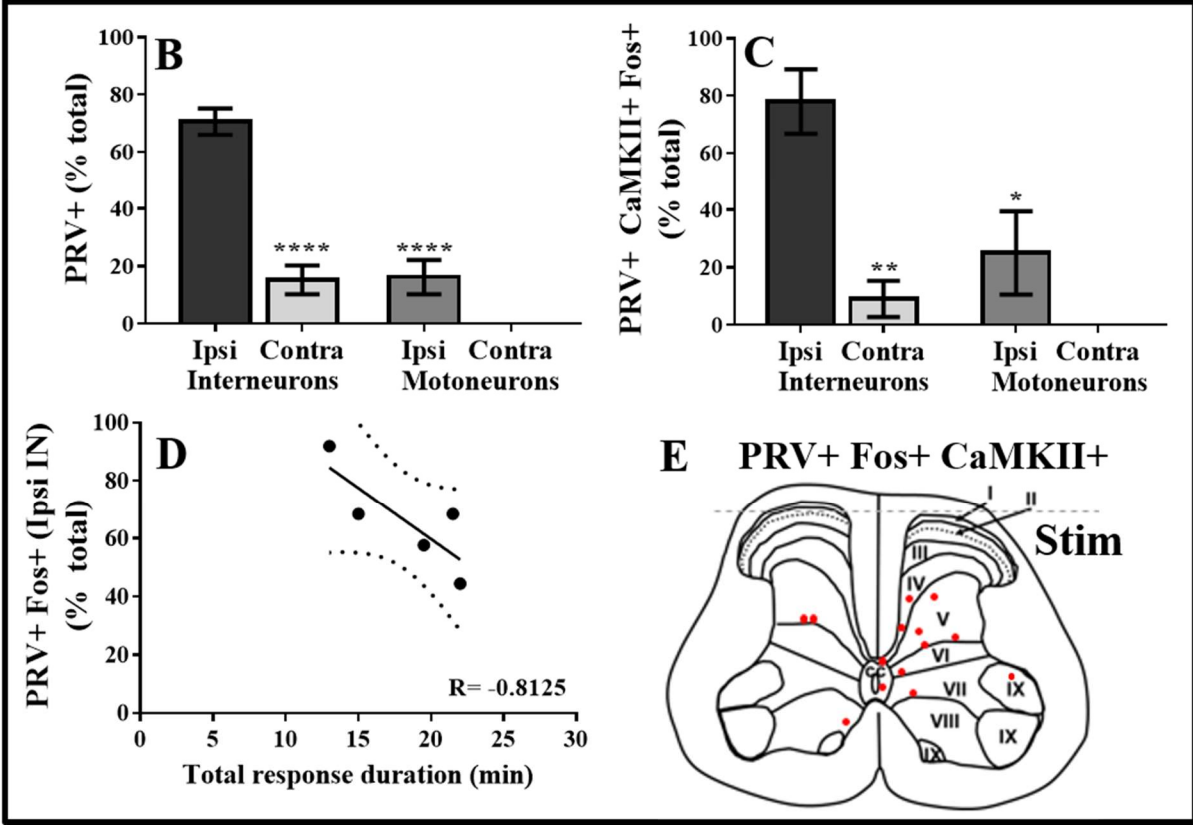
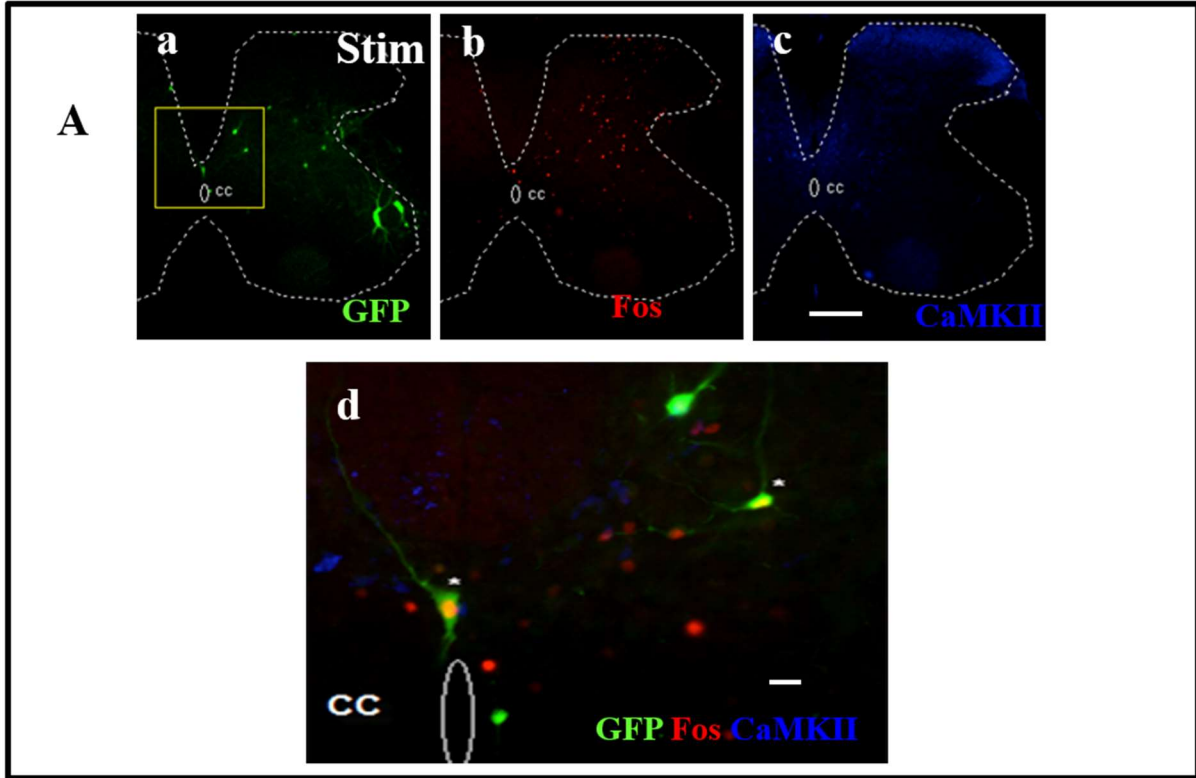
(D). Pseudo color images of staining were generated using the Image-Pro Plus software for better visualization, where red is the most intense in the color gradient (D). Staining intensities in concurrently processed sections were quantified at a selected threshold above background. Master animals contained significantly higher levels of CaMKII in L4 dorsal horn than the yoked mice ( $p < 0.05$ ,  $N=5$ , master/yoked pairs, 5 sections/ animal) (E). Scale bars in (A) & (B) =100  $\mu\text{m}$ , (C)=20  $\mu\text{m}$ , respectively. CC, central canal.





**Figure 4-4: Master mice have more activated CaMKII neurons in laminae IV-VI, which correlate positively with learning.**

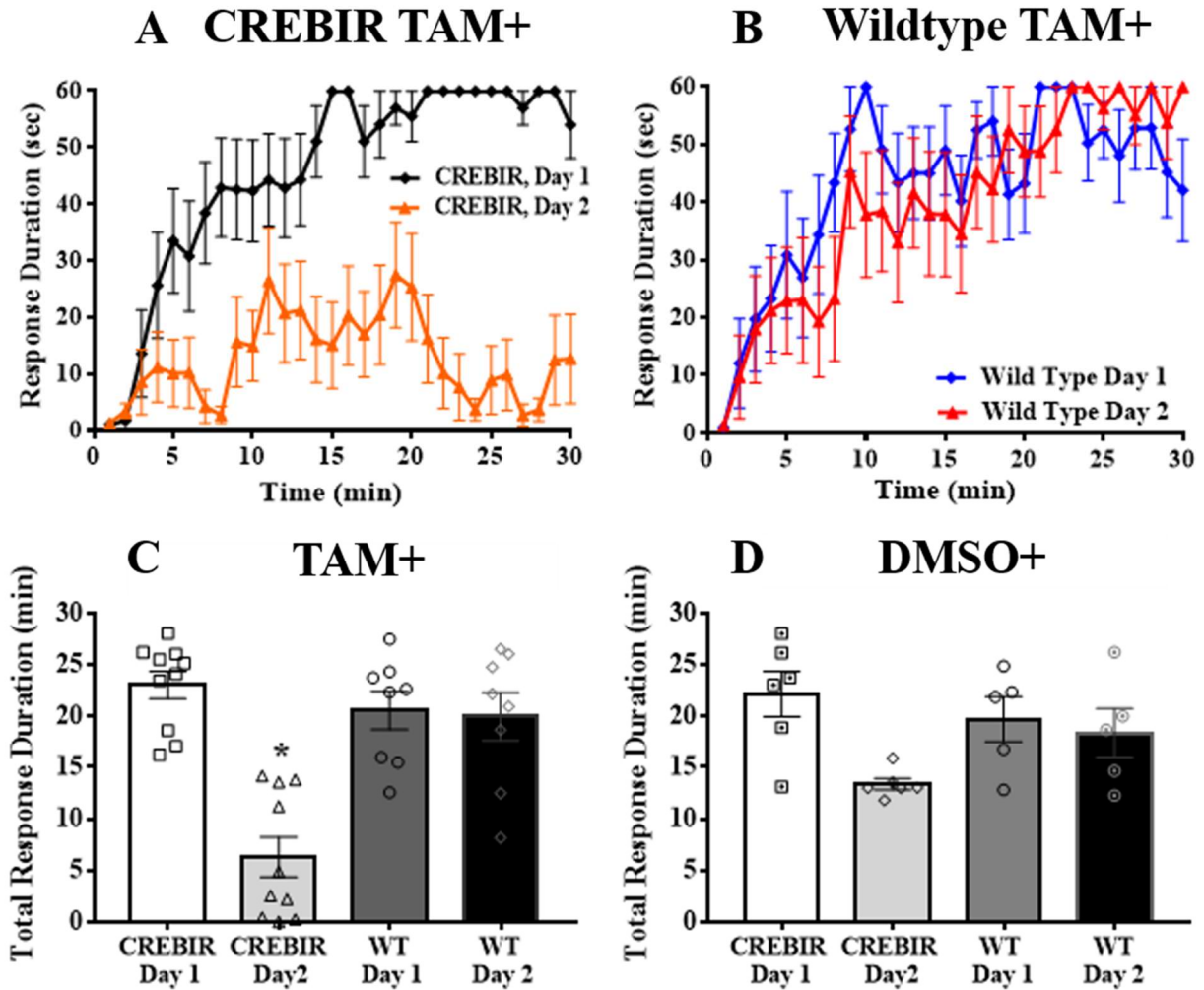
Activated CaMKII neuron is shown in A. Nuclei double-labeled for Fos and CaMKII (Fos+ CaMKII+) were identified under the microscope and marked on acquired images using manual tag feature of Image Pro program, and representative sections for master and yoke mice are shown in B. Percent of activated Fos+ CaMKII+ in the ipsilateral spinal cord was significantly higher in master than the yoked (C) and also significantly correlated positively with the response duration.



(Abarbanel, Lask et al. 1994)

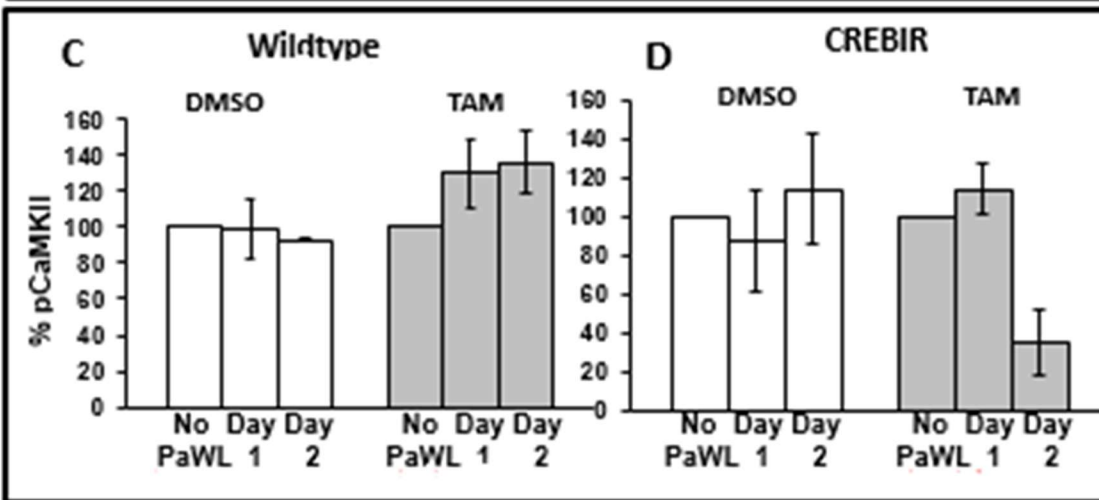
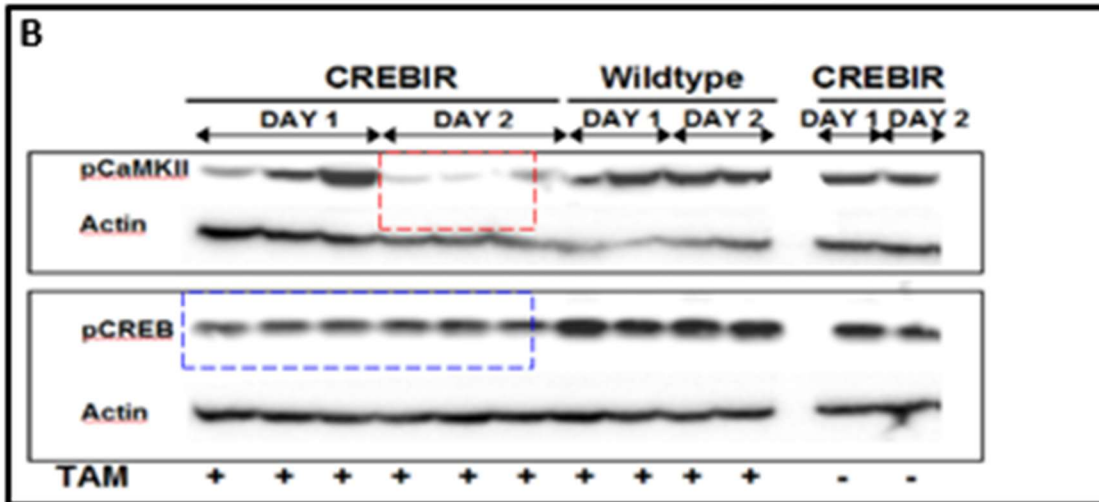
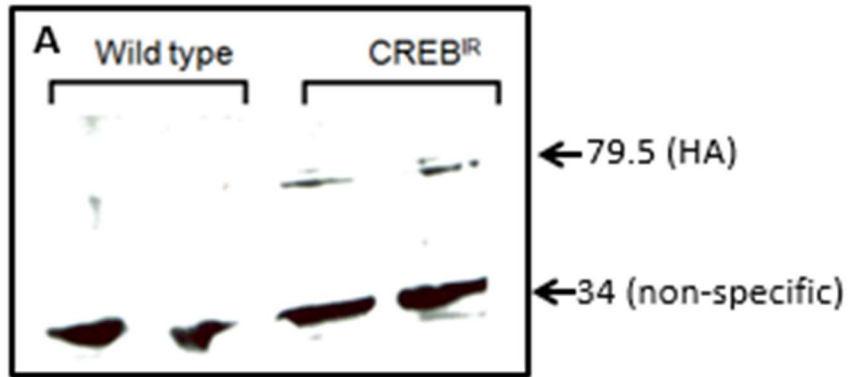
**Figure 4-5: Identification of TA network specific activated neurons showing PaWL.**

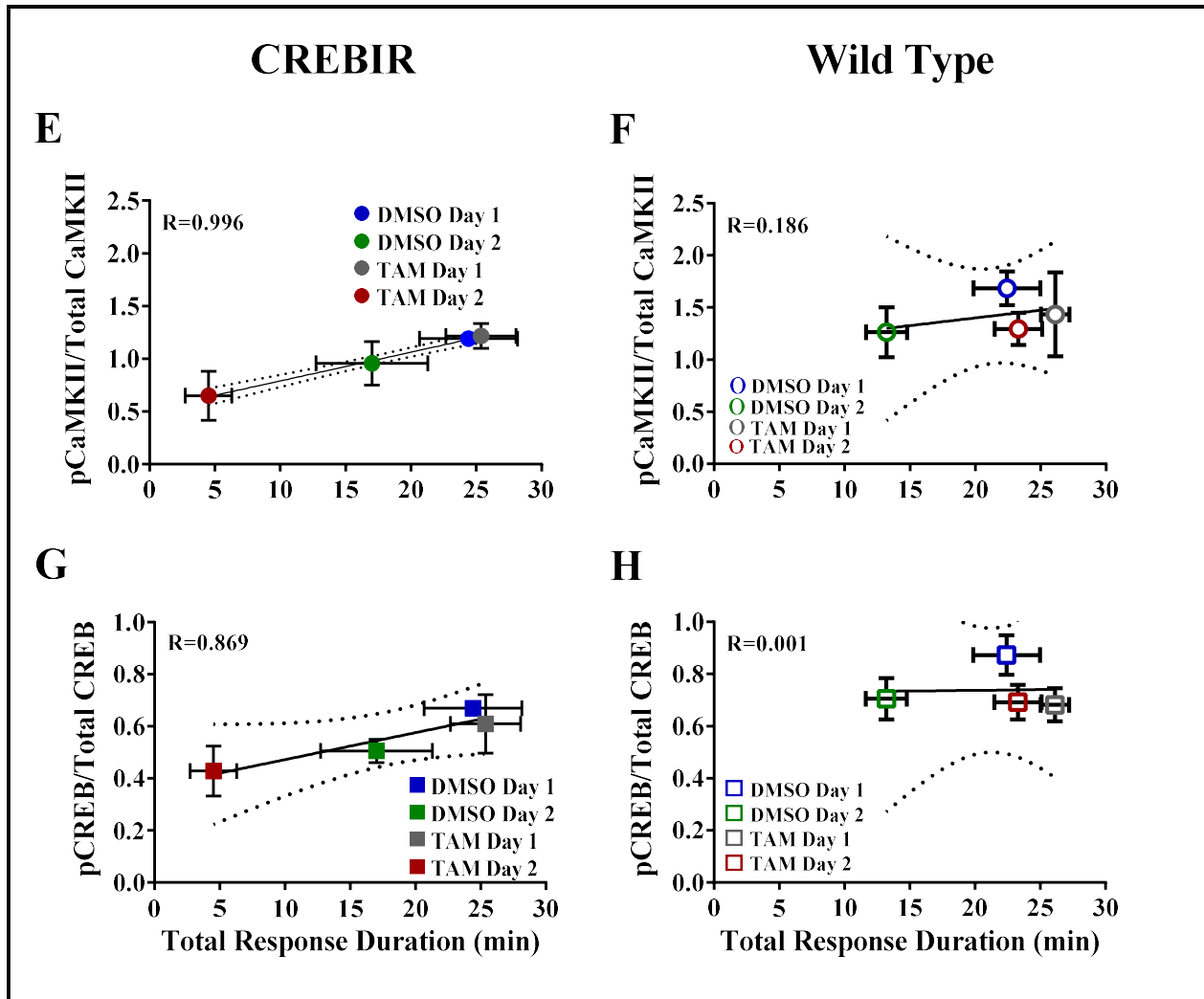
Transverse lumbar 4 spinal cord sections from master mice with pseudorabies virus (PRV-152) injected into the TA muscle, were processed for triple labeled Immunohistochemistry (A). PRV+ (a), Fos+ (b), and CaMKII+ (c), and magnified image of a merged composite of the 3 immunostaining (d) are shown. \*Asterisks in d marks PRV+ CaMKII+ Fos+ cells. cc, central canal, Stim, Stimulated side. Scale bar in c and d are 200 and 20  $\mu\text{m}$ , respectively. Quantification of PRV+ and PRV+ Fos+ CaMKII+ cells in the spinal cord are shown in B and C, respectively. D shows correlation of the response duration with % PRV+ CaMKII+ in ipsilateral neurons. Representative lamina distribution of PRV+ Fos+ CaMKII+ is shown in E. N=5, master/yoked pairs, 10 sections/animal). Bonferroni's multiple comparisons showed % of PRV+ and % of PRV+ CaMKII+ Fos+ interneurons in ipsilateral spinal cord is significantly higher than both ipsilateral motoneurons and contralateral interneurons, in both master (B) and yoke (C) mice. The PaWL performance is negatively correlated with activated ipsilateral PRV-labeled interneurons (D). R, Pearson correlation coefficient.



**Figure 4-6: PaWL is disrupted in CREBIR mice treated with tamoxifen.**

Both CREBIR and wildtype mice received tamoxifen (TAM) or DMSO 6 hrs before the first PaWL testing. Mice underwent one PaWL session on the first day or received an additional bout of PaWL, 24 hrs later. No TAM was given on the following day. All mice except CREBIR mice that received TAM learned to hold the paw above the threshold successfully (A, B). There was no significant difference in total response duration in DMSO-injected CREBIR and WT mice on both days (C). However, the total response duration during PaWL on second day was significantly reduced in TAM+ CREBIR mice, and not DMSO+ CREBIR mice or TAM+ or DMSO+ wildtype mice (D).





**Figure 4-7: pCaMKII protein levels are decreased in TAM+ CREBIR.**

Western blot analysis showed expression of CREBIR protein in the spinal cords of CREBIR, but not in WT littermates. The CREBIR protein containing the HA tag at 79.5 kDa, and a HA-labeled nonspecific band at 34 kDa, are detected (A). The first 10 lanes from immunoblots 1 and 2, are proteins from lumbar spinal cord of CREBIR and wildtype mice that received tamoxifen (TAM) 6 hrs. before the PaWL training (B). The last two lanes are from CREBIR mice that received the vehicle control (DMSO). Mice either underwent one PaWL session (Day 1) or received an additional training 24 hrs. later (Day 2). Immunoblots showing pCaMKII, PCREB and actin, and

quantified pCaMKII protein levels are shown in B and C, respectively. pCaMKII/total CaMKII and pCREB/total CREB were calculated for all four groups (E-H) and correlated with response duration. On day 2, TAM+ CREBIR mice showed significantly reduced levels of pCaMKII protein (red box in B; D), but this decrease is not due to uneven loading or of proteins as seen from the actin control. Endogenous pCREB levels are also low, 6hrs and 24 hrs after TAM+ injection (blue box). There was no similar reduction in pCREB in wildtype mice (C, D). The % pCaMKII/total CaMKII and % pCREB/total CREB, correlated to response duration in only the CREBIR mice (E, G).

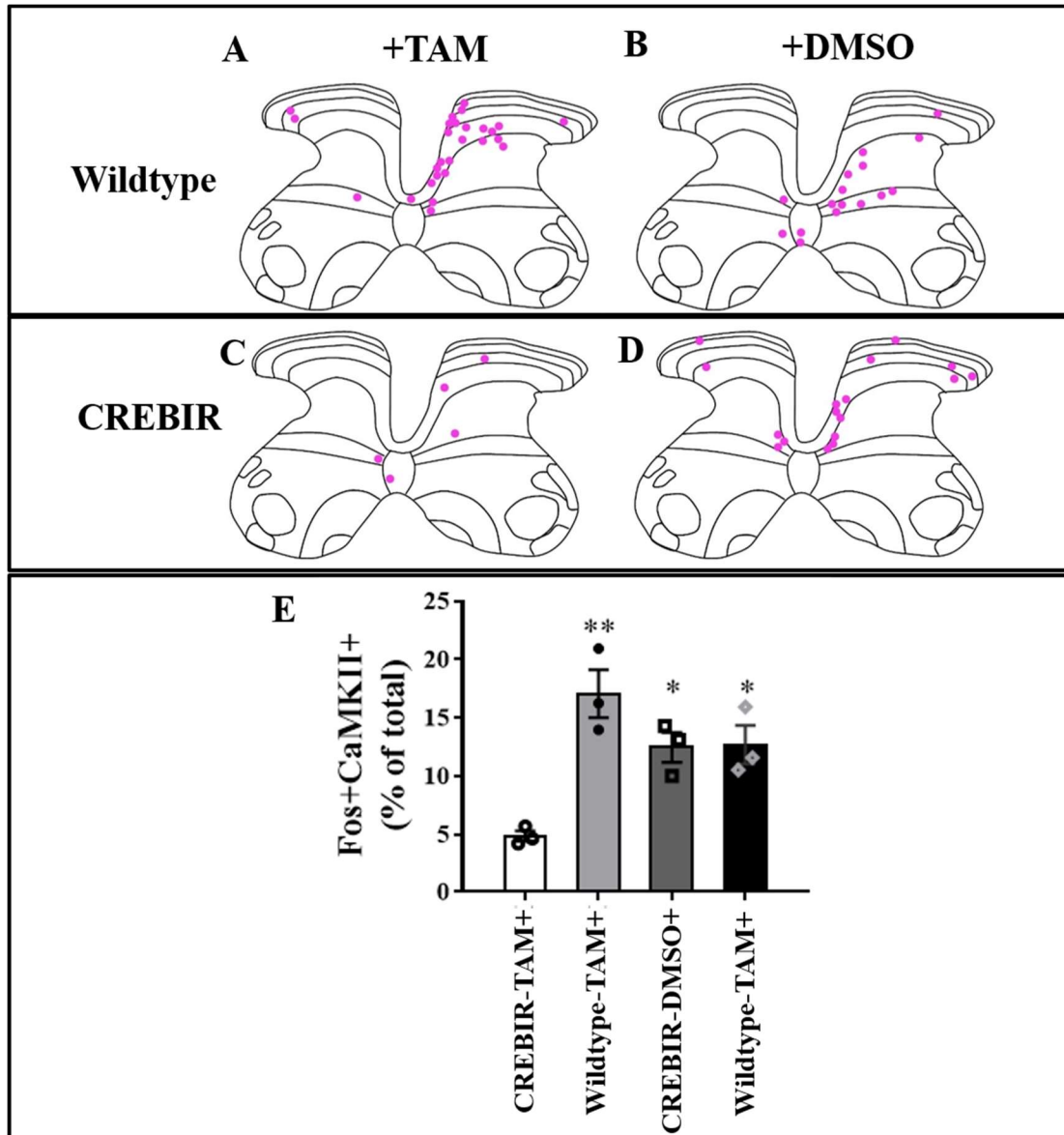


Figure 4-8: LBD-CREB transgenic mice induced to express CREB repressor have reduced number of Fos+ CaMKII+ cells in laminae IV-VI.

Representative L4 spinal cord sections containing Fos+ CaMKII+ cells of all four groups are shown (A-D). Bonferroni's multiple comparison showed that CREBIR +TAM (C) has significantly decreased number of Fos+ CaMKII+ cells in laminae IV-VI, compared to wildtype TAM+ (A), wildtype DMSO+ (B) and CREBIR DMSO+ (D). n=3/group, 5 sections/animal. \*, P<0.05, \*\*, P<0.01.



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