A non-invasive intervention (BreEStim) for management of phantom limb pain (PLP) after limb amputation

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Protocol (CPHS HSC-MS-20-1032)

1. **Protocol Title: A non-invasive intervention (BreEStim) for management of phantom limb pain (PLP) after limb amputation**
   PI: Sheng Li, MD, PhD
   Co-Investigators: Daniel Melton, MD; Shengai Li, MS
   Study Coordinator: not identified
   Population: amputee subjects
   Number of Sites: single site
   Study Duration: planned for 3 years
   Subject Duration: estimated 64 subjects

2. **Specific aims and research questions:**

   The overall goal of this project is to compare the effectiveness of innovative intervention of breathing-controlled electrical stimulation (BreEStim) and conventional electrical stimulation (EStim) in management of neuropathic phantom limb pain in patients after limb amputation.

3. **Background/literature review, Justification and Significance:**

a) Importance of the problem

1) <u>The need and target population</u>

In the United States, there are approximately 1.7 million people living with limb loss(Ziegler-Graham et al. 2008). It is estimated that one out of every 200 people in the U.S. has had an amputation(Adams et al. 1999). Limb amputation could lead to three non-exclusive phenomena, including phantom limb pain (painful sensation from the absent limb, i.e., phantom limb), phantom limb sensation (any sensation in the absent limb, except pain), and stump pain (pain localized in the residual limb) (Nikolajsen and Jensen 2001). Phantom limb pain (PLP) is a complex condition that is caused by peripheral nerve damage from amputation. Similar to neuropathic pain from other etiologies (e.g., spinal cord injury), PLP is characterized by spontaneous and ongoing pain, described as burning, shooting, prickling or electrical, and/or pain in response to innocuous stimuli (allodynia) and exaggerated pain in response to noxious stimuli (hyperalgesia) (Bennett 2010). PLP has increasingly been recognized as an important contributor to suffering, poor rehabilitation outcomes, reduced quality of life, and employment status of the persons with PLP after amputation. This is because of the following facts.

- PLP is very common(Werhagen et al. 2004). About 60-80% amputees have PLP at 2 years post-amputation (Jensen et al. 1985; Ephraim et al. 2005).
- PLP does not resolve over time. Phantom pain does not change after 6 months post-amputation (Jensen et al. 1985).
- PLP is difficult to manage (Woolf and Mannion 1999). Currently, pharmacological management is the standard of care for PLP. According to a Cochrane review(Alviar et al. 2011), there are 6 groups of medications with different pharmacological mechanisms have been used. Some medications demonstrated trends in short-term analgesic efficacy, such as gabapentin and

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morphine. But the studies lacked long-term efficacy and safety outcomes. Furthermore, more studies were not specifically for PLP(Hall and Eldabe 2018). Nevertheless, pharmacological interventions are often associated with side effects, such as addiction, withdrawal, and constipation, etc.

- PLP is related to poorer physical, psychological and social functioning(Stormer et al. 1997; Norrbrink Budh et al. 2005; Jensen et al. 2007); More importantly, it was found that employment status was related to the intensity of PLP. Unemployed amputees reported higher levels of PLP and lower levels of prosthesis use(Whyte and Carroll 2002).
- These challenges in treating neuropathic PLP likely contribute to opioid overuse and the opioid epidemics(Manchikanti et al. 2012). The U.S. Senate passed the Comprehensive addition and Recovery Act (CARA), which takes incremental steps to combat the epidemic. It was signed into law in July 2016(CARA 2016). To combat this epidemic and to help manage PLP in particular, it is critically important to develop non-pharmacological interventions.

Alternative, non-pharmacological treatments have been tried for management of neuropathic pain in the target population. Acupuncture and various electrical stimulation techniques have been used clinically and have demonstrated some success. Based on our pioneering discovery that human voluntary breathing could have systemic effects, including pain reduction(Li and Rymer 2011a), we propose an innovative treatment – Breathing-controlled electrical stimulation (BreEStim) for neuropathic pain management. Briefly, in the BreEStim treatment, a single-pulse electrical stimulation is delivered to peripheral nerve(s) when patients are taking a fast, strong, and deep inhalation, similar to a deep breath but faster and stronger. Patients control the intensity of electrical stimulation, to increase the intensity as tolerated gradually. As compared to conventional electrical stimulation (EStim), this patient-centered treatment of BreEStim increases its analgesic effectiveness by integrating various internal coping mechanisms, including voluntary and active involvement of patient in their own care, electroacupuncture effect, electrical stimulation effect, neurobehavioral modulations (habituation to aversive stimuli, and activation of the reward system), resulting in increased pain threshold and pain tolerance (Li et al. 2013). Our pilot data have demonstrated that BreEStim has better effectiveness in pain reduction in patients with above-the-knee amputation in our previous case report (Li et al. 2012a).

The proposed research will further compare the effectiveness of BreEStim and EStim in management of neuropathic pain in patients with PLP. This well-coordinated clinical study by an established scientist-physician team will provide direct evidence of clinical effectiveness of BreEStim in phantom limb pain management. As such, an alternative and effective non-pharmacological treatment of PLP could be developed.

2) *The proposed activities further the purposes of the Act*

The intention of the Rehabilitation Act of 1973 is to promote the right of individuals with disabilities. They constitute one of the most disadvantaged groups in society, to live independently, contribute to society, pursue meaningful careers, and enjoy full inclusion and integration into all aspects of mainstream American society. It ensures that the U.S. government plays a leadership role in promoting the employment of individuals with disabilities.

The proposed project seeks to develop an alternative, noninvasive, and non-pharmacological treatment (BreEStim) for phantom limb pain management. Comparisons of analgesic effects between BreEStim and conventional electrical stimulation technique (EStim) will demonstrate superior analgesic effects by BreEStim. This has been shown in our recent preliminary study using BreEStim for neuropathic pain after spinal cord injury(Li et al. 2016; Karri et al. 2018a; Li et al. 2018). With the evidence and results to be obtained from this project, we will be able to provide strong clinical and laboratory evidence that this treatment will provide more effective and longer lasting analgesic effects for these patients, and improve general well-being of these patients. As mentioned earlier, quality of life and unemployment status is related to the severity of PLP(Whyte and Carroll 2002). Therefore, the proposed activities will obviously benefit a large population of patients, who may experience different levels of impairment and disability, by reducing their pain, emotional tension, thereby maintaining and enhancing quality of life and employment for these patients and meanwhile reducing the burden on caregivers. In this manner, the proposed activity will clearly increase these patients'

ability to achieve the stated mandates of the Rehabilitation Act. The proposed activities, therefore, clearly further the goals of the Rehabilitation Act.

3) The proposed project will have beneficial impact on the target population.

As mentioned above, neuropathic phantom pain is very common, difficult to manage, and has increasingly been recognized as an important contributor to suffering, poor rehabilitation outcomes, reduced quality of life and employment status of the persons who are suffering chronic neuropathic pain (Stormer et al. 1997; Whyte and Carroll 2002; Norrbrink Budh et al. 2005; Jensen et al. 2007). BreEStim integrates multiple pain-coping mechanisms and provides better analgesic effects in neuropathic treatment. Our research will provide direct evidence of BreEStim as an effective alternative treatment for chronic neuropathic pain in patients suffering from amputation. As such, BreEStim could be added as a routine care for these patients. More specifically, the beneficial impact of the proposed research on BreEStim for neuropathic pain management can be summarized as follows:

- BreEStim is a non-invasive, non-pharmacological treatment. This is critical because most pain medications have side effects, sometimes very severe. These side effects could include addiction, overdose, withdrawal symptoms, and constipation, etc. These potential side effects could be avoided in the BreEStim treatment.
- BreEStim provides better analgesic effects. As compared to conventional electrical stimulation (EStim), this patient-centered treatment of BreEStim increases its analgesic effectiveness by integrating additional coping mechanisms. Patients are able to tolerate high levels of electrical stimulation, leading to enhanced analgesic effects. Such a positive feedback loop (activation of the reward system) results in a greater clinical efficacy.
- BreEStim is an alternative choice for PLP management. This alternative non-pharmacological treatment with better analgesic effects is important, particularly when PLP is difficult to manage. For example, only 7% of responders reported pharmacological treatment is effective for neuropathic pain in a postal survey (Finnerup et al. 2001a)
- BreEStim is patient-centered. As in our pilot study (Li et al. 2012b; Li 2013), patients will feel they actively participate in managing their pain, rather than “a passive participant in their own care”. This may enhance their treatment compliance on one hand, and psychological function on the other hand. Furthermore, BreEStim is specifically tested for this target population. In contrast, more studies with pharmacological agents on neuropathic pain are not specifically for PLP (Hall and Eldabe 2018).

b) Design of research activities

A. Comprehensive and informed review of the current literature

- Scope of the project and general introduction

Overall, mechanisms of phantom limb pain are not well understood. Definition of neuropathic pain is defined as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system” (Treede et al. 2008). Neuropathic pain can occur secondary to injury to peripheral nerves (peripheral neuropathic pain, e.g., amputation) or to spinal cord and brain (central neuropathic pain, e.g., post-traumatic headache (PTH) after traumatic brain injury (TBI)). PLP has been viewed as a maladaptive response after amputation. Possible mechanisms include ectopic impulse generation and transmission after nerve damage, amplification of impulses via peripheral and central sensitization, maladaptive changes in descending mechanisms (Costigan et al. 2009). Neuroimaging studies have demonstrated that both topographic shifts (Flor et al. 1995) and preserved representations (Makin et al. 2013) of the former hand area in patients with phantom limb pain. It has been proposed that PLP emerges as memory of phantom limb sensation in dynamic overlapping brain networks that is triggered and reinforced in the presence of associated distress (De Ridder et al. 2011).

Management of PLP in general has been a challenge for both physicians and patients. PLP is usually chronic and severe, and requires continuous analgesic treatment. The standard of care of PLP management is to prescribe pharmacological agents. Six groups of medications with different mechanisms of action have been used for PLP management. It is still difficult to draw definitive conclusions on efficacy of pharmacological management of PLP with available evidence(Hall and Eldabe 2018). Other than pharmacological management, there are numerous modalities have been used for management of neuropathic pain with some degrees of success, such as acupuncture (Hui et al. 2000;

Han 2004; Yoo et al. 2004; Cho et al. 2006; Dhond et al. 2008) and neuromodulation. Various neurostimulation techniques include transcutaneous electrical nerve stimulation (TENS) (Norrbrink Budh and Lundeberg 2004), spinal cord stimulation (Finnerup et al. 2001b), deep brain stimulation (Murphy and Reid 2001), and transcranial direct current stimulation (tDCS) (Fregni et al. 2006).

Taken together, it is very difficult to treat PLP. It is likely related to complex underlying mechanisms. New conceptualization is needed not only to understand the mechanisms, but also to provide a theoretical basis for development of new interventions. In this proposed project, we would like to focus on an innovative intervention that has been developed from our pioneering studies on the systemic effect of human voluntary breathing and its relation to chronic neuropathic pain. **In essence, we propose a new theoretic approach, as well as a breakthrough innovative non-pharmacological treatment – voluntary breathing controlled electrical stimulation (BreEStim)** (Li and Rymer 2011b; Li 2013; Li et al. 2013) for management of neuropathic PLP after amputation. The primary goal is to examine the effectiveness of BreEStim for PLP management.

We would like to start our application with a common observation: A 3-year-old girl cries after a fall which results in a minor skin abrasion on her right knee. She calms down when being rubbed on her right knee by her mom, and stops crying and continues to play when being re-directed to her favorite toy (cf.(Von Baeyer 1998)). This scenario exemplifies some features of pain. Pain is a subjective feeling that is multi-dimensional. Pain is not only sensory, but also emotional and could trigger behavioral responses (affective), e.g., crying. Distraction by re-directing attention does not make the sensation of painful stimuli (from knee) go away. However, the child does not feel bothered by the pain (affective aspect) when playing her favorite toy. It suggests that sensation (painful stimuli) and affective component of pain are two different, but parallel processes. Furthermore, crying may help pain relief. A shrewd observer may notice that the inspiratory phase is usually deeper and longer during the pain-induced cry. It suggests that effortful inhalation may help ease pain, rather than just emotional expression.

We will now review the relevant literature

- **Different aspects of multi-dimensional pain**

Sensory processing of neuropathic pain Pathways for sensory processing of painful stimuli are well documented. From the periphery to the spinal cord, noxious stimuli are primarily conveyed by Aδ and C-fibers. These primary afferents enter the CNS via the dorsal roots and terminate mainly in the superficial laminae (I and II) of the dorsal horn although they also send collateral projections to deep laminae (V, VI, and adjacent VII, and X) of the dorsal horn. Furthermore, the tactile (Aβ) fibers that terminate the intermediate (III, and IV) laminae, also send collateral projections to deep laminae (Willis and Coggeshall 1991). Noxious stimuli are transmitted in parallel to different subcortical and cortical areas, following different pain pathways (Gauriau and Bernard 2002; Bennett 2010), including Spino-thalamic projections, Spino-reticular projections, and Spino-parabrachial projections. Following transection of peripheral axons after amputation, sprouting occurs at the ventral terminals of the large myelinate axons. This sprouting allows the sensory axons to terminate in Lamina II instead of laminae III or IV, in other words, “wrong connections”. This may explain why light touch can trigger discomfort or even pain.

Affective processing of neuropathic pain (beyond sensation) Neuroimaging studies have consistently demonstrated increased activation in multiple cortical and subcortical areas in response to pain. Significant regions of activation during nociceptive stimulation include primary and secondary somatosensory cortex, the anterior cingulate cortex, insula, thalamus and prefrontal cortex (Peyron et al. 2000; Apkarian et al. 2005; Tracey 2005). Recent data from evoked potentials in humans with implanted electrodes in several brain structures indicate that painful stimuli are processed in parallel in the somatosensory cortex and anterior cingulate cortex (ACC). This suggests that the sensory and affective aspects are processed in parallel simultaneously and not serially (Frot et al. 2008).

Awareness of the noxious stimulus arises when this activity is connected to a larger coactivated awareness or perceptual network that involves anterior cingulate cortex (ACC), precuneus, parietal cortex, and frontal cortex. In other words, the stimulus becomes conscious only when its appropriate neural discriminatory representation in the somatosensory cortices is functionally connected to the awareness network. In persistent vegetative state, patients are awake but without awareness and without conscious percepts (Laureys 2007). Pain stimuli only activate the thalamus and the primary sensory cortex, since the primary sensory cortex is functionally disconnected from the secondary somatosensory cortex as well as from the above-mentioned awareness areas (Boly et al. 2008).

The awareness network, together with the posterior insula, is relevant for integration of sensory experience in bodily self-consciousness. Subjective experience of pain emerges when the posterior insula triggers the pain network, including the parietal operculum and the midcingulate gyrus (Isnard et al. 2011). The leading role of the insula on the affective dimension of pain is further supported by another study (Von Leupoldt et al. 2009). When the sensory intensity of experimentally induced pain was rated similarly by asthmatic patients with dyspnea and healthy subjects with comparable induced-dyspnea, ratings of the affective unpleasantness of pain were reduced in asthmatic patients. This perceptual difference was mirrored by reduced insular cortex activity, but increased PAG activity in patients (Von Leupoldt et al. 2009). This study suggests that a down-regulation of insular cortex activity secondary to a neuronal habituation mechanism after dyspnea in patients with chronic asthma is generalized to reduced affective unpleasantness of pain.

Descending modulation Different descending pathways are involved in modulation of pain. Descending pathways originated in the ACC, amygdala, and hypothalamus are relayed to the spinal cord through brain stem nuclei in the PAG and rostroventral medulla (RVM) (Fields and Basbaum 1999). The ACC is thought to encode unpleasantness, i.e., the emotional component of pain and anxiety. The ACC exerts top-down influences on the brainstem to gate pain modulation (Jones and Gebhart 1988; Calejesan et al. 2000), such as distraction (Valet et al. 2004). The PAG projects to the RVM which in turn sends projections to the spinal dorsal horn via the PAG-RVM pathway. Stimulation of either the PAG or the RVM produces inhibition of dorsal horn neurons including spinothalamic tract cells. Other common descending pathways originate in the reticular system in the RVM and pontine neurons. The reticular system uses serotonin as a neurotransmitter. The pontine noradrenergic cell groups use the neurotransmitter noradrenaline and produce inhibition of dorsal horn neurons.

The PAG is widely connected with other cortical and subcortical areas. Diffusion tensor imaging (DTI) is an MRI-based technique that can map white matter connections in the living human brain. Connections have been identified between the PAG and the prefrontal cortex, amygdala, thalamus, hypothalamus and rostroventral medial medulla bilaterally (Lorenz et al. 2003; Valet et al. 2004; Hadjipavlou et al. 2006).

With its wide connections with prefrontal and limbic areas and other subcortical areas, the PAG is viewed as the area housing “command neurons” (Iigaya et al. 2010). A recent human study supports the important role of the PAG in regulation of both respiration and pain(Von Leupoldt et al. 2009). In this study, dyspnea (induced by resistive loaded breathing) and heat pain were induced in both asthmatic patients and healthy controls. When the sensory intensity of both dyspnea and pain sensations was rated similarly by patients and controls, patients reported less unpleasantness of dyspnea and less pain. This perceptual difference was mirrored by reduced insular cortex activity, but increased PAG activity in patients during both increased dyspnea and pain. Connectivity analyses showed that asthma-specific down-regulation of the insular cortex during dyspnea and pain was moderated by increased PAG activity. This study suggests that a neuronal habituation mechanism reducing the affective unpleasantness of dyspnea in asthma is able to generalize to other unpleasant physiological sensations such as pain (Von Leupoldt et al. 2009).

The PAG could be maladaptive in response to chronic pain, however. It has been reported that the PAG and cingulate cortex were activated significantly less during suppression of pain in patients with complex regional pain syndrome I (CRPS I), as compared to healthy controls (Freund et al. 2011). On the other hand, increase in neuronal activity in the PAG was found to be the greatest in patients with neuropathic pain during anticipation of exercise or actual exercise. These changes were accompanied by changes in respiration, blood pressure and heart rate (Green et al. 2007). If activity of the PAG is increased by way of increased respiratory effort(Banzett et al. 2000; Von Leupoldt et al. 2009), it is hypothesized that the general function of the PAG is enhanced, including pain suppression. The effect of increased volitional breathing on systemic motor output has been recently studied (Li and Laskin 2006; Li and Yasuda 2007; Ikeda et al. 2009; Li and Rymer 2011a). Interestingly, along with reduction in muscle tone, reduction of central pain in chronic stroke patients has also been observed during voluntary-breathing controlled electrical stimulation (Li and Rymer 2011b; Li 2013) . This systemic effect of voluntary breathing on muscle tone and pain is possibly mediated via modulation of the PAG activity by voluntary breathing. We further infer that the enhanced PAG activity induced by voluntary breathing could lead to pain reduction.

- **Memory plays an important role in the awareness of persistent phantom limb pain** Memory plays an important role in the awareness of persistent PLP as well as in the reinforcement of the associated distress, particularly important if chronic PLP is secondary to a traumatic event. Traumatic injury is usually a single event. The memory of the event could last for the rest of life. When associated with a negative emotional context, PLP after amputation could be perceived as aversive, and re-triggered by a stressful life event (Jensen et al. 1985). Functional connectivity studies demonstrate a prolonged, enhanced functional coupling in the resting state between amygadala, ACC, anterior insula and the sympathetic locus coeruleus after psychological stress (van Marle et al. 2010). Localized micro-stimulation to the insular cortex, when delivered during peripheral aversive stimulation, leads to item-specific impairment of aversive memory reconsolidation, i.e., anterograde amnesia (Stehberg et al. 2009). In other words, peripheral aversive stimulation is not remembered. In our pilot study (Li et al. 2012a; Li 2013), shooting phantom pain in a patient who had an above-the-knee amputation disappeared after treatment with breathing-controlled electrical stimulation (BreEStim) to the ipsilateral forearm, but re-appeared 28 days later after receiving a sustained electrical stimulation accidentally. This observation suggests that the affective component of noxious stimuli for shooting phantom limb pain has been modified by the BreEStim treatment, but then re-triggered by the accidental stimulation. This pilot observation supports the notion that the affective processing of neuropathic pain could be modified, possibly via BreEStim.

New hypothesis on phantom limb pain after amputation

A novel hypermnesia-hyperarousal model is proposed specifically to account for pain persistence and pain sensitization following a traumatizing event(Egloff et al. 2013). According to this model, a traumatizing event, such as limb amputation, usually results in activation of two self-protective mechanisms: hyper-sensitization (i.e., to detect a potential hazard as early as possible) and hypermnesia (i.e., not to forget the event in order to recognize it again and to avoid it). In case of severe traumatization like amputation, intense hypermnesia and hyperarousal are likely imprinted on the victim's memories, and result in pain chronification and lower pain detection and tolerance thresholds (i.e., sensitization). We are aware that chronic PLP is multi-factorial. This model, however, offers a plausible explanation for chronic pain that is "unexplained" by structural damages after amputation. For example, when associated with a negative emotional context, phantom limb pain could be perceived as aversive, and re-triggered by a stressful life event(Jensen et al. 1985). Furthermore, the concept of trauma-induced pain sensitization and chronification has also provided a theoretical framework for a potential novel intervention, such as the one proposed in this application.

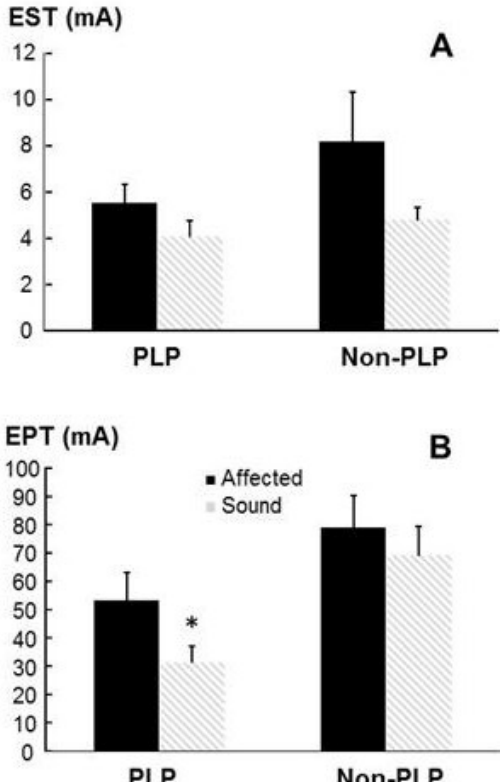

Figure 1 Electrical sensation threshold (EST) (panel A) and electrical pain threshold (EPT) (panel B) in the PLP and non-PLP group. Mean and standard errors are shown.

In our recent study (Li et al. 2015), we examined sensory thresholds in patients after traumatic amputation. We found hypersensitization in amputees with chronic phantom limb pain, but not in amputees without phantom limb pain (Figure 1). Thus, the findings support the concept of pain sensitization and chronification as a plausible underlying mechanism for neuropathic phantom limb pain. Specifically, it was the first time that tactile, thermal and electrical thresholds were comprehensively compared between the residual limb and the contralateral sound limb in patients with and without phantom limb pain. As shown in Fig 1, electrical pain threshold (EPT) was only decreased in the sound limb in the phantom limb pain (PLP) group and no difference among other three

limbs (Fig 1B), suggesting central sensitization in patients with PLP. In contrast, electrical sensation threshold (EST) was increased on the affected limb as compared to the sound limb within the PLP group, but there were no significant differences in EST between the PLP and non-PLP group. There was essentially no significant difference in other thresholds (thermal and tactile).

- **Non-pharmaceutical modalities (acupuncture, TENS, aversive electrical stimulation)for PLP management**

*Electrical stimulation (TENS) for pain*Various neurostimulation techniques, including transcutaneous electrical nerve stimulation (TENS) (Norrbrink Budh and Lundeberg 2004), spinal cord stimulation (Finnerup et al. 2001b), deep brain stimulation (Murphy and Reid 2001), and transcranial direct current stimulation (Fregni et al. 2006) have been used for management of neuropathic pain. We are particularly interested in non-invasive stimulation that is relevant to our study – TENS.

TENS is noninvasive, inexpensive, safe and easy to use. TENS for pain relief was fully accepted by the medical field after the publication by Wall and Sweet in 1967 (Wall and Sweet 1967) which provided experimental evidence supporting the gate theory of pain (Melzack and Wall 1965). TENS is usually applied at varying frequencies, intensities and pulse duration of stimulation for a prescribed treatment time. Frequency of stimulation is broadly classified as high frequency (>50Hz) or low frequency (<10Hz) or bursts of high frequency stimulation at a much lower frequency. Intensity is determined by patient's response as either at the sensory level or motor level TENS. With the sensory level TENS, the stimulation intensity (voltage, or amplitude) is increased until the patient feels a comfortable tingling/tapping sensation without muscle contraction (twitching). With the motor level TENS, the intensity is increased to produce a motor contraction, *but electrical stimulation is not noxious or painful*. The usual TENS settings are high-frequency and low-intensity, or low-frequency and high-intensity. TENS has been applied to a variety of pain conditions, including phantom limb pain. Overall, the clinical effectiveness of TENS is controversial (see Review(Sluka and Walsh 2003; Mulvey et al. 2010; Johnson and Bjordal 2011)).

Several mechanisms are used to support the use of TENS for pain relief. The most common one is the gate control theory (Melzack and Wall 1965). Briefly, according to the gate control theory, small diameter fibers excite cells in the spinal cord that sends information to a higher center for the perception of pain. Large diameter fiber input reduces noxious input of nociceptor by activation of inhibitory neurons in the substantia gelatinosa of the spinal cord. Accordingly, stimulation of large diameter fibers by TENS inhibits nociceptive fiber evoked responses in the dorsal horn (Melzack and Wall 1965). Another theory is that TENS increases the release of endogenous opioids, subsequently blocking opioid receptors in the PAG-RVM pathway (Sluka et al. 1999; Kalra et al. 2001). Reports of clinical effectiveness of TENS for phantom limb pain even when applied to the contralateral side (Katz et al. 1989; Giuffrida et al. 2010) support the second theory – the release of endogenous opioids by TENS.

Aversive stimulation and habituation When the intensity of stimulation increases to the extent that subjects feel pain, the stimulation becomes aversive or noxious. Ironically, repetitive painful stimulation leads to reduced pain, i.e., habituation (Bingel et al. 2007; Rennefeld et al. 2010). In a recent study (Rennefeld et al. 2010), daily painful thermal stimulation of the left volar forearm for l week was delivered to healthy subjects. Significant pain attenuation was observed at the site of stimulation, the contralateral arm and the ipsilateral leg. In the same study, the authors also found that pain habituation to noxious stimulation was unaffected by the opioid antagonist naloxone. Thus, these results strongly support a central mechanism of pain habituation that does not directly involve the endogenous opioid system. *The naloxone non-reversible effect by aversive electrical stimulation suggested that the effect was mediated by a mechanism different from the release of endogenous opioids,* as described for TENS and acupuncture. Brain imaging studies after painful thermal (Bingel et al. 2007) and electrical (Christmann et al. 2007) stimulation revealed that, in addition to decreased activity in classical pain areas, including thalamus, insula, putama and somatosensory cortices, pain-related responses in the ACC significantly decreased over time. The ACC has been reported to selectively process the aversive quality of noxious stimulation (LaGraize et al. 2006), but does not influence sensation of the stimulation (LaBuda and Fuchs 2005). The brain imaging findings of decreased pain-related responses in the ACC after repetitive aversive stimulation indicates a negative effect on the affective processing of the stimulation, subsequently resulting in less unpleasantness over time, without changes in sensation of noxious stimuli.

Acupuncture and electro-Acupuncture for pain Acupuncture is a special kind of neurostimulation that is applied to acupuncture points (Acupoints). Acupoints are special points located in the periphery but are connected to internal organs including spinal cord and brain, following different channels, according to traditional Chinese medicine. Systematic investigation on mechanisms of acupuncture started in 1950s in China (Han 2004). Evidence on clinical effectiveness and mechanisms has accumulated over decades in Western Medicine literature (Hui et al. 2000; Han 2004; Yoo et al. 2004; Cho et al. 2006; Dhond et al. 2008). Acupuncture for pain relief via the release of endogenous opioids has been well accepted in Western Medicine. Modification to traditional acupuncture needle has been made (Han et al. 1981; Han 2004). It has been replaced by a surface electrode (or equivalent). When a specialized electrode is placed over traditional acupoints, electrical stimulation is delivered. This modification has been termed electroacupuncture. Needle acupuncture and electroacupuncture are both effective in analgesia (Wan et al. 2001; Huang et al. 2002). *The effect of electroacupuncture is usually reliable, but dependent on intensity and frequency of delivered electrical stimulation: different frequency of electrical stimulation generates different endogenous opioids, and the analgesic effect is naloxone-reversible* (Huang et al. 2002; Han 2004).

Aversive electrical stimulation to acupuncture points Theoretically, analgesic effects are anticipated to be strengthened if different mechanisms of treatment are delivered at the same time. Following this line, repetitive aversive electrical stimulation delivered to acupoints is expected to have combined effects: 1) habituation to aversive stimulation as described above; 2) analgesic effect secondary to the release of endogenous opioids secondary to stimulation of acupuncture points. Therefore, i*n combining different mechanisms (the release of endogenous opioids, habituation), aversive electrical stimulation to acupoints is theoretically anticipated to produce significant analgesic effects.*

Psychological/Cognitive coping /Cognitive behavioral therapy In addition to the above modalities, a variety of approaches is available for managing psychological and environmental contributions to pain and distress. Pharmacological strategies such as anxiolytic and antidepressant therapy and non-pharmacological strategies such as cognitive behavioral approaches may be used. The use of cognitive-behavioral therapy, including mirror therapy(Barbin et al. 2016) and virtual reality therapy(Ortiz-Catalan et al. 2016) has been reported to modify phantom limb pain. These techniques may act by modifying the central process involved in pain perception.

- ***Human breathing and possible effects on modulation of pain***

As briefly mentioned, respiration and pain are co-modulated at the sub-cortical level (The PAG) as part of vital surviving functions. Human breathing is a very unique motor act. It can be controlled reflexively (automatic breathing), e.g., during sleep, while humans are also able to breathe voluntarily when needed (voluntary breathing), e.g., singing, speech, etc. Automatic breathing is believed to originate in the brainstem via the ponto-medullary respiratory oscillator. A descending bulbo-spinal projection from the oscillator synapses with the spinal cord anterior horn cells with rhythmic projections to the respiratory muscles to cause automatic breathing. The oscillator can function automatically without any peripheral feedback, and only responds to changes in pH and P_{CO2}. (cf. review (Guz 1997)). In contrast, cortical inputs are required during voluntary breathing. Spinal motoneurons receive cortico-spinal inputs originating from discrete regions of the motor cortex where the respiratory muscles are represented. These cortical areas are clearly identified in humans (Gandevia and Rothwell 1987; Colebatch et al. 1991; Maskill et al. 1991; Sharshar et al. 2004). Clinical evidence strongly suggests that bulbo-spinal fibers project separately from the relevant corticospinal fibers. For instance, patients with brainstem lesions (Plum and Leight 1981) or very high cervical cord lesions (Davis and Plum 1972; Lahuerta et al. 1992) can breathe voluntarily, but lack automatic breathing when drowsy or asleep. Cortico-spinal pathways could bypass the brainstem respiratory centers and provide direct cortical control to the spinal respiratory motoneurons during voluntary breathing (Corfield et al. 1998). During normal functioning, spinal motoneurons are able to integrate these different sources, including descending cortico- and bulbo-spinal inputs and peripheral afferent inputs into a segmental interneuronal network (Aminoff and Sears 1971).

During voluntary breathing, humans need to voluntarily suppress autonomic control of breathing (Guz 1997; Haouzi et al. 2006) through voluntary cortical activation (the “cortical respiratory center”). Brain imaging studies (Colebatch et al. 1991; Maskill et al. 1991; Ramsay et al. 1993; Fink et al. 1995; Evans et al. 1999; Smejkal et al. 1999; Smejkal et al. 2000; Macey et al. 2003; Macey et al. 2004; Mazzone et al. 2007; Evans et al. 2009; Evans 2010) have demonstrated extensive involvement of

cortical areas bilaterally, including the primary motor cortex (M1), the premotor cortex, the supplementary motor area, the primary and secondary somatosensory cortices, the insula, the ACC and amygdala, and the dorsolateral prefrontal cortex. The insula is known to have strong connections to brainstem centers (Tsumori et al. 2006). Respiratory specific insular connections include the medullary respiratory chemoreceptors and pulmonary stretch receptors (Gaytan and Pasaro 1998; Hanamori et al. 1998). *Activity of the insular cortex is not modulated during automatic breathing, according to breath-by-breath analysis in a recent fMRI study* (Evans et al. 2009). *The insula and associated operculum, however, are consistently activated during various sensorimotor respiratory tasks* (Evans 2010). Change in respiratory status, e.g., urge-to-cough (Mazzone et al. 2007), needs a change in pulmonary stretch receptor or chemoreceptors (Gaytan and Pasaro 1998; Hanamori et al. 1998), leading to increased activity in the insula, thus possibly to provide respiratory interoception (Craig 2002).

- **Breathing-controlled electrical stimulation (BreEStim) for pain management – Preliminary data**

During autonomic breathing, inspiration is active while expiration is passive, mainly relying on the recoil force of the chest wall. Similarly, volitional inspiration activates more respiratory-related cortical and subcortical areas as compared to volitional expiration (Evans et al. 1999). We have discovered that there exist interactions between respiratory and motor systems during voluntary breathing. Specifically, there is a finger extension-inspiration coupling (Li and Laskin 2006; Li and Yasuda 2007; Ikeda et al. 2009; Li and Rymer 2011a). When electrical stimulation is delivered to the finger extensors during the inspiratory phase of voluntary breathing, a long-lasting effect of reduction in finger flexor spasticity (muscle tone) in chronic stroke patient is observed (Li and Rymer 2011a). We also observed interactions between voluntary breathing and pain modulation. In our pilot studies (Li et al. 2012a; Li 2013), shooting phantom pain in a patient who had an above-the-knee amputation disappeared after treatment with breathing-controlled electrical stimulation (BreEStim) to the ipsilateral forearm, but re-appeared 28 days later after receiving sustained electrical stimulation accidentally. This pilot case study provides a unique opportunity that the affective component of noxious stimuli for shooting phantom limb pain has been modified by the BreEStim treatment, but then re-triggered by the accidental stimulation. These observations of tone and pain reduction have led to a hypothesis that voluntary breathing, inspiration in particular, could be integrated into an electrical stimulation paradigm to improve its efficacy in neuropathic pain management. Though extensively used, efficacy of TENS in pain relief remains controversial (Sluka and Walsh 2003; Mulvey et al. 2010; Johnson and Bjordal 2011). Similar results of pain reduction after BreEStim treatment were also observed in a traumatic spinal cord injury patient with neuropathic pain (Li 2013).

In these two cases, BreEStim to acupoints on the forearm was not likely to modify the sources of noxious stimuli at the level of spinal cord injury (thoracic area) or at the residual limb (i.e., lower extremity). Rather, BreEStim modified how patients react to the noxious stimuli at the central level, i.e., the affective response to the same stimuli. Our case reports suggest that patients could tolerate the same noxious stimuli better after BreEStim. This is possibly realized by increasing pain threshold as a final result from the intervention, i.e., de-sensitization, or less sensitive to painful stimuli.

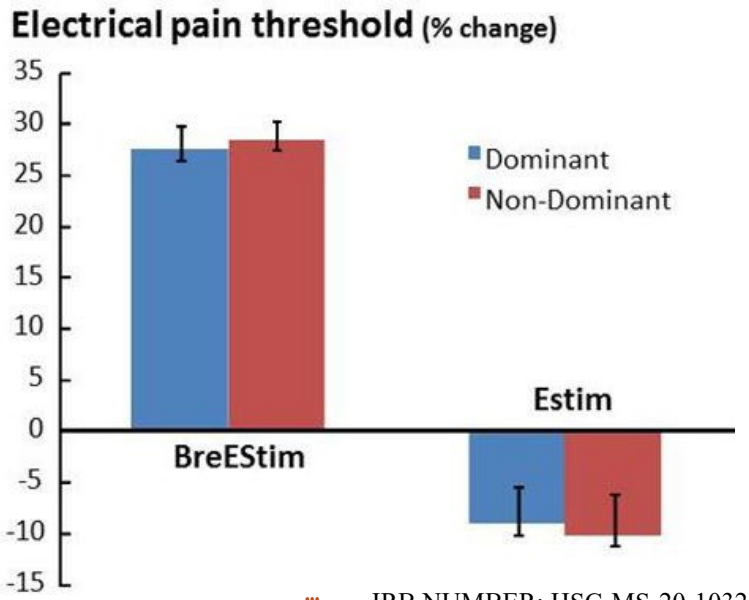

Figure 2 Change of electrical pain threshold as percentage of pre-intervention values after BreEStim and EStim in healthy subjects. Standard errors are presented (From Li et al. 2013(Li et al. 2013))

NOTE: The intensity of electrical stimulation was comparable between BreEStim and EStim, suggesting that the effect is not mainly mediated by electrical stimulation itself.

To test the hypothesis that BreEStim modifies pain threshold, subsequently resulting in reduction of neuropathic pain, we recently compared pain thresholds before and after BreEStim and EStim (Li et al. 2013). Two interventions were tested in the same healthy subjects in a randomized order, but at least 3 days apart in order to exclude any possible carry-over effect from the previous intervention. The results showed that BreEStim significantly increased electrical pain threshold, while EStim significantly decreased electrical threshold. Other thresholds (thermal, electrical sensation, and tactile sensation) remained the same after both interventions. Collectively, these findings suggest that BreEStim has selectively modified electrical pain threshold, i.e., affective component.

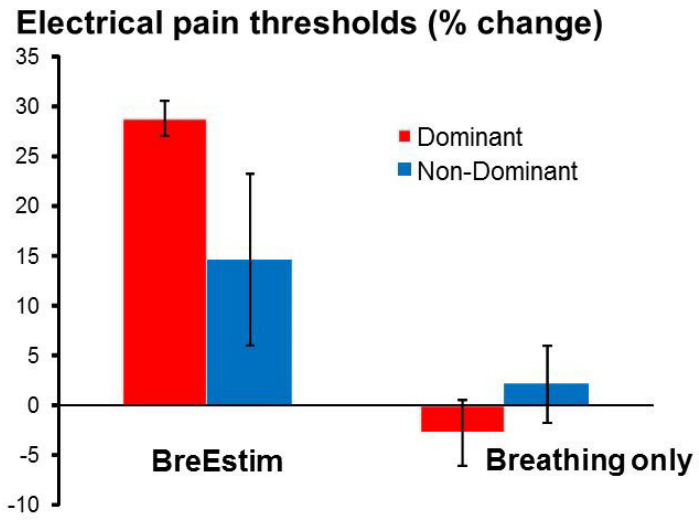

Figure 3. Changes of electrical pain threshold as percentage of pre-intervention values after BreEStim and Breathing-only. Standard errors are shown.

To further examine whether BreEStim-induced analgesic effect is primarily caused by voluntary breathing, we compared the effect of BreEStim and Breathing-only on experimentally induced pain in healthy subjects in another study in 2015(Hu et al. 2015). The same protocol was used. Electrical pain threshold increased to a similar extent as previously reported after BreEStim, but there was almost no change after Breathing-only (Figure 3).

- **BreEStim integrates multiple pain coping mechanisms**

Collectively, these findings suggest that BreEStim-induced analgesia is likely caused by internal pain-coping mechanisms activated during BreEStim, rather than by EStim or Voluntary Breathing alone. It is not the purpose of this project to investigate coping mechanisms of BreEStim. Rather, according to the above literature review, we speculate that could integrate various non-pharmacological coping mechanisms into one regime for PLP management. Here are possible mechanisms for BreEStim-induced analgesia and its de-sensitization effects.

Acupuncture effect Acupuncture-related analgesic effects, via the release of endogenous opioids, are triggered when acupoints are stimulated (Hui et al. 2000; Wu et al. 2002). We want to point out that it is not the purpose of the project to distinguish whether analgesic effects are related to electroacupuncture or just electrical stimulation, since both are possibly mediated by the release of endogenous opioids when electrical stimulation is delivered transcutaneously. The main purpose of the project is to investigate whether BreEStim could be translated into management of neuropathic phantom pain in the target patient population.

Habituation to aversive stimuli Pain-related responses decrease when painful stimulation is applied repetitively. It is important to mention that habituation to aversive stimuli is not naloxone-reversible, in other words, not mediated by the release of endogenous opioids (Rennefeld et al. 2010). Therefore, the above two mechanisms could have additive analgesic effects.

Influence of voluntary breathing. Effortful inspiration and expiration requires extensive cortical and subcortical activation, as mentioned above. The activation is more extensive during voluntary inspiration than voluntary expiration. The insular cortex is consistently activated during various sensorimotor respiratory tasks. Meanwhile, the insular cortex is critical in affective processing of pain. As such, unpleasantness of pain may be modulated during voluntary-breathing associated activation of the insular cortex. Furthermore, voluntary-breathing associated activation in the PAG may also enhance the ability of the PAG-RVM pathway in modulation of pain.

Anterograde amnesia to aversive stimulation When aversive stimulation is delivered during activation of the insular cortex, item-specific anterograde amnesia to the stimulation occurs (Stehberg et al. 2009). In other words, unpleasantness of peripheral noxious stimulation is not remembered

immediately after the stimulation, when the insular cortex is activated during stimulation. Expectedly, unpleasantness of peripheral painful stimulation, when triggered by voluntary inspiration (the insular cortex is activated), is decreased or not remembered. As such, it is expected to facilitate habituation to aversive stimulation even at higher intensity, subsequently increasing the analgesic effect of stimulation. A positive feedback loop could occur that patients request a higher intensity of electrical stimulation, even noxious, for a better analgesic effect.

The reward system is triggered As described above, this positive feedback loop is likely to occur by triggering the reward system, which is commonly associated with outcomes of aversive stimuli (Jensen et al. 2003). Expectedly, the intensity of aversive electrical stimulation is gradually driven higher during the course of stimulation.

B. Research Hypotheses and Specific Aims

The Main Research Hypothesis: In summary, BreEStim has advantages of integrating multiple internal pain-coping mechanisms to produce analgesia. We compared electrical pain threshold before and after BreEStim and EStim or Breathing-only in healthy subjects (Li et al. 2013; Li et al. 2014; Hu et al. 2015). Pain threshold was increased (i.e., de-sensitized) in both tested and contralateral hands after BreEStim, but decreased after EStim or Breathing-only without affecting other thresholds (thermal, electrical sensation, and tactile sensation). In other words, BreEStim has systemic de-sensitization effects. According to the literature, “unexplained” chronic pain after amputation, i.e., PLP, is a consequence of subsequent activation of "self-protection mechanisms" after amputationi, such as hyperamnesia and hyper-sensitization. Results from our preliminary study (Li et al. 2015) confirmed systemic sensitization in people with PLP. Therefore, we proposed the main hypothesized that **BreEStim could provide non-pharmacological analgesic effects for patients with chronic phantom limb pain after limb amputation via central desensitization.** Accordingly, we have the following specific aims.

Specific Aim 1: To examine whether BreEStim could have better analgesic effects on PLP

It is hypothesized that BreEStim has better analgesic effect than EStim, and the analgesic effect is accompanied by elevated electrical pain thresholds. Patients with PLP will receive both BreEStim and EStim in a randomized order with at least 3 days apart. The same amount of stimulation (120 electrical stimuli) at comparable intensities will be used as in our recent studies. According to our preliminary data, we expect that BreEStim has greater pain reduction and longer lasting effect. These results will parallel with increased electrical pain thresholds after BreStim, i.e., desensitization, as compared with EStim.

Specific Aim 2: To examine whether BreEStim-induced analgesic effect is dose-dependent

Similar to taking pain medications, it is hypothesized that there is a dose-response analgesic effect of BreEStim, i.e., an increased “dose” of BreEStim will have a greater impact on reduction of phantom limb pain. Two doses of BreEStim intervention (120 vs. 240 electrical stimuli) will be given to the same amputee with one week apart in a randomized order. According to our preliminary data from 3 subjects, we expect that that a high-dose BreEStim will produce a longer-lasting, but similar degree of pain reduction as compared to a low-dose BreEStim. The findings also suggest a possible cumulative effect of BreEStim, thus leading to a long term effect.

Specific Aim 3: to examine the long-term effect of BreEStim on neuropathic PLP

It is critical to know whether patients will tolerate electrical stimulation and the induced analgesic effect will diminish or the effect will accumulate if patients receive BreEStim repetitively over time, before it is translated into a therapeutic device. It is hypothesized that the BreEStim treatment will produce a greater analgesic effect than the convention electrical stimulation (EStim) treatment in patients after traumatic amputation over a course of 2 weeks (10 consecutive workdays). We will examine the hypothesis by comparing electrical pain threshold and effectiveness in pain reduction between BreEStim and EStim in patients after traumatic amputation of one limb. The analgesic effect will be assessed by pain reduction in visual analog scale and quantitative measurement of electrical sensation and pain thresholds. The effect on general well-being and change in pain medication will be assessed.

4. Research design and methods:

Location: Neurorehabilitation Research Laboratory at TIRR Memorial Hermann Hospital

C. Each sample population is appropriate and of sufficient size

Pilot data and sample size for Experiment 1 (Aim 1)

In Experiment 1, we plan to use our recent BreEStim and EStim protocols(Li et al. 2013; Li et al. 2014) to compare the induced analgesic effects in patients after traumatic limb amputation. The same experimental protocol for quantitative sensory testing (QST) will be adopted. QST, including mechanical sensation threshold, thermal thresholds (cold/warm sensation, cold/hot pain) and electrical sensation and pain thresholds will be measured in four limbs. In the pilot study we only measured electrical sensation and pain threshold in these areas to estimate the sample size.

We have tested the protocol in 3 patients with PLP after below-the-knee amputation (BKA) (Figure 4). On average, VAS scores decreased from 5.3 to 3.2 after BreEStim. The analgesic effect lasted 16.2 hours. In contrast, VAS scores changed from 4.9 to 4.1 after EStim. The effect lasted 2.0 hours. Electrical sensation detection threshold (EDT) remained similar for both BreEStim (4.9 vs 5.0 mA, pre vs. post) and EStim (4.9 vs. 4.8 mA, pre vs. post). However, electrical pain threshold (EPT) increased from 22.8 to 29.3 mA after BreEStim – a change of 22.1%, in contrast, EPT was very similar before and after EStim (23.4 vs. 23.6 mA, pre vs. post). No difference in tactile and thermal thresholds was found.

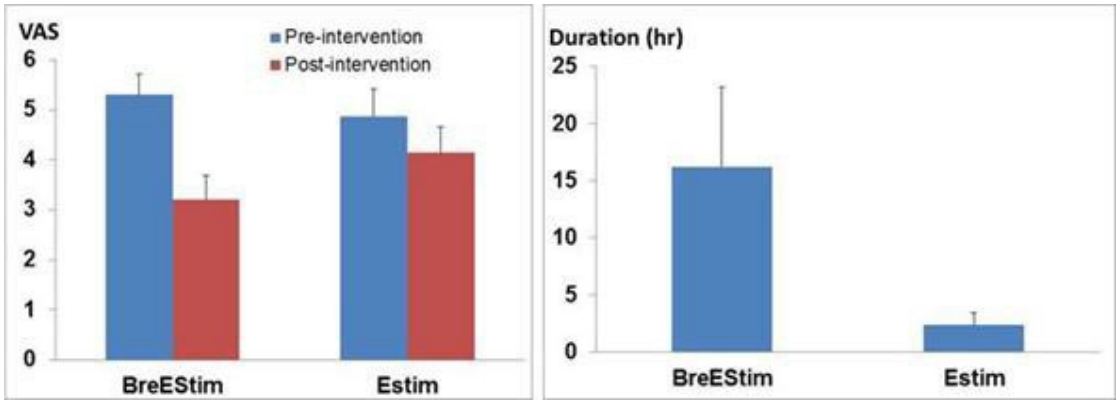

Figure 4 BreEStim produced a better and longer lasting analgesic effect than EStim in patients with phantom limb pain.

Power analysis and estimate of sample size for Experiment 1 Ms. Yang (our biostatistician consultant) has performed power analysis based on our pilot study and historical data from the literature. A two-way repeated measures ANOVA was used to determine whether any change in VAS score was due to the interaction between INTERVENTION and TIME (i.e. whether one of two interventions is more effective at reducing VAS scores). A power analysis using the G*power computer program (Faul et al. 2007) indicated that a sample of 12 people would be needed to detect a large effect size (> 0.8) with 85% power using a repeated measures two-way ANOVA with alpha at .05, a sample of 14 people would be needed for 90% power. To account for possible subject withdrawal (10%), we plan to recruit 16 subjects. Also to reduce the experimental bias and the order effect, the order of the interventions will be randomized.

Pilot data and sample size for Experiment 2 (Aim 2)

In Experiment 2, the same BreEStim protocol will be used, but at two different doses (120 stimuli vs. 240 stimuli). We have tested the protocol in 4 patients with phantom limb pain after BKA (Figure 5). On average, BreEStim120 and BreEStim240 had similar analgesic effects. VAS decreased from 6.4 to 3.5 after BreEStim120, and from 5.9 to 3.5 after BreEStim300. However, the analgesic effect lasted longer after BreEStim240 (26.1 hours) than after BreEStim120 (14.5 hours). Similar to Exp 2, these

analgesic effects were paralleled with similar degrees of change in EPT without changes in other thresholds.

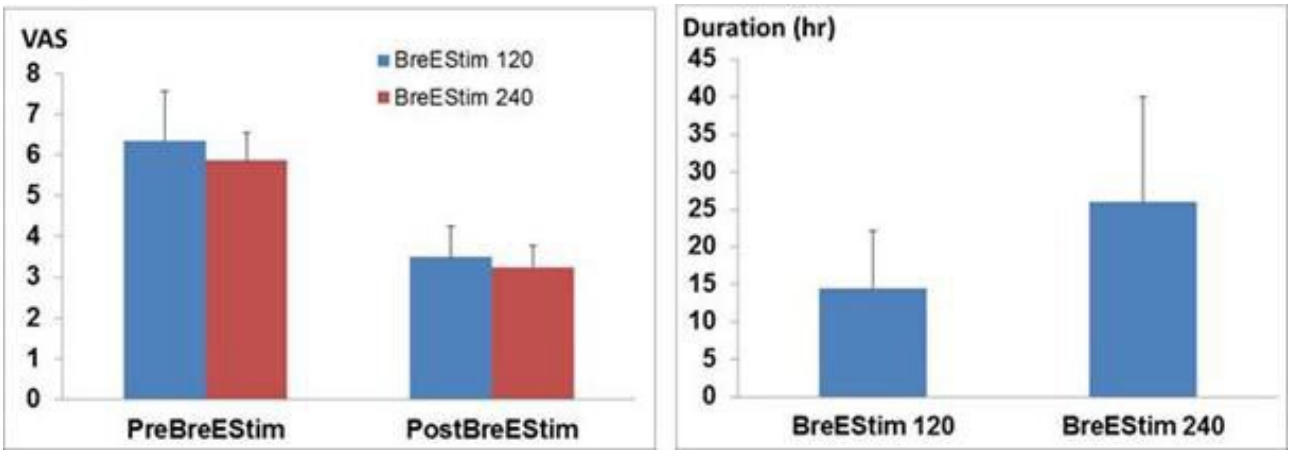

Figure 5 BreEStim has a dose-response effect on the duration, not the degree of analgesic effects on phantom limb pain.

Power analysis and estimate of sample size for Experiment 2: Ms. Yang (our biostatistician consultant) has performed power analysis based on our pilot data. One-group paired t-test was used to examine whether patients had longer duration of analgesic effects after BreEstim240 than BreEstim120. A power analysis using the G*power computer program (Faul et al. 2007) indicated that a sample of 13 people would be needed to detect large effects (d=0.8) with 85% power using a one-tailed paired t-test between two dependent means with alpha at .05, a sample of 16 people would be needed for 90% power (An effect size of 0.8 is estimate based on the large effect size observed in pilot studies). We plan to recruit 18 subjects in case some subjects could not come for the second session.

Pilot data and sample size for Experiment 3 (Aim 3)

We have tested two amputee subjects with chronic neuropathic PLP greater than 4 years. Subjects were young and medically stable. Both interventions were delivered in the same time of the day, and patients took the same medications during the course of treatment. BreEStim and EStim were delivered with 2 weeks apart for washout effects. The order of treatment was balanced between two subjects. Medications were or had been stable for at least 2 weeks prior to the first test. To demonstrate detailed responses after each treatment and the intensity of each treatment session, Figure 6 shows findings from one subject.

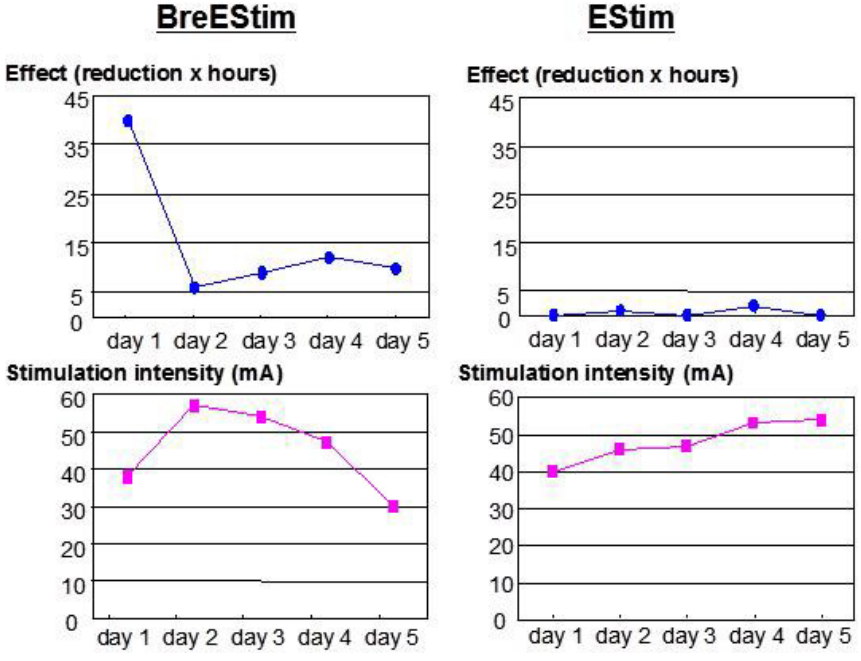

Figure 6 BreEStim and EStim in an amputee subject. The amputee subject (male, 31 years of age, Left above-the-knee amputation 5 years ago) received BreEStim first, waited for 1 week for washout, then received electrical stimulation only (EStim, without voluntary breathing). Surface electrodes were placed on the skin overlying the median nerve about 3 centimeters above the wrist joint for both BreEStim and EStim. A single electrical stimulus was triggered by voluntary breathing (BreEStim) or triggered randomly by a computer (EStim). The duration of single stimulus was 0.1 ms. The intensity of electrical stimulation (the lower panels, in pink) was

comparable among interventions (Figure 6). Overall, BreEStim had greater analgesic effect than EStim (the upper panels, in blue). Patient tolerated both interventions well. No side effect was reported.

Power analysis and estimate of sample size for Experiment 3 Ms. Yang (our biostatistician consultant) has performed power analysis based on our pilot study and historical data from the literature. Using nQuery Advisor® , we applied the t-test (ANOVA) for difference of VAS in 2 x 2 crossover design with a 0.05 two-sided significance level. To achieve 80% power of detecting a difference in VAS of 3.0 (the difference between the mean of group BreEStim, μ_1=4.0 and the mean of EStim group, μ_2=2.0) assuming that the Crossover ANOVA $\sqrt{MSE}$ is 3.4 (the Standard deviation of differences, σ_d, is 4.8), the sample size is 24. Assuming an expected dropout rate of 20% given the long-term intervention, we proposed to recruit 30 patients in total for Experiment 3.

D. The data collection and measurement techniques are appropriate and effective

- Experimental Design

The overall research design is a within-group, randomized controlled study to compare analgesic effects between EStim and BreEStim (single-session in Exp 1, long-term intervention in Exp 3), between BreEStim120 and BreEStim240 (Exp2). We prefer this within-group comparison because 1) pain, by nature, is a subjective perception, it is difficult to control (physical, social, psychological) multi-factors between two groups; 2) It will be fair that all subjects receive the innovative intervention, which is superior to regular electrical stimulation according to our pilot data; 3) according to our recent BreEStim study in patients with spinal cord injury, the duration of analgesic effect was 15 hours after one-session of BreEStim treatment. An interval of one week for washout should be sufficient for Exp 1 & 2. The washout interval will be 2 weeks for Exp 3. It is our experience with subjects with spinal cord injury that an interval of 7 days is sufficient for subjects to return to their baseline after BreEStim treatment. (Li et al. 2016; Karri et al. 2018b; Li et al. 2018) However, we did notice that one subject with post-amputation PLP had carry-over effect for one month(Li et al. 2012a). We plan to screen these patients with long-lasting carry-over effects. These patients will be excluded from the subject should this happen.

In Experiment 1, we plan to use our recent BreEStim and EStim protocols (Li et al. 2013; Li et al. 2014; Li et al. 2016) to measure mechanical sensation threshold, thermal (cold/warm detection, cold/hot pain) thresholds, and electrical sensation and pain thresholds in the treatment limb and contralateral limb. The same group of subjects will receive both BreEStim and EStim at a randomized yet balanced order with at least 7 days apart. The same amount of electrical stimulation (120 stimuli) will be given during both BreEStim and EStim. The purpose of this aim is to investigate whether BreEStim could have better analgesic effect than EStim in amputee subjects with chronic phantom limb pain as we observed in healthy subjects. We expect increased electrical pain thresholds in both limbs after BreEStim because of its central desensitization effects.

In Experiment 2, a group of subjects will receive BreEStim at two different doses (120 vs. 240 stimuli) on two different days with at least 7 days apart. The purpose is to compare the extent and duration of analgesic effects between two doses of BreEStim. We expect that BreEStim240 could have similar degree of pain reduction but with longer duration compared to BreEStim120, as shown in our pilot study. Therefore, we expect to use BreEStim240 in Experiment 3 for the long-term intervention.

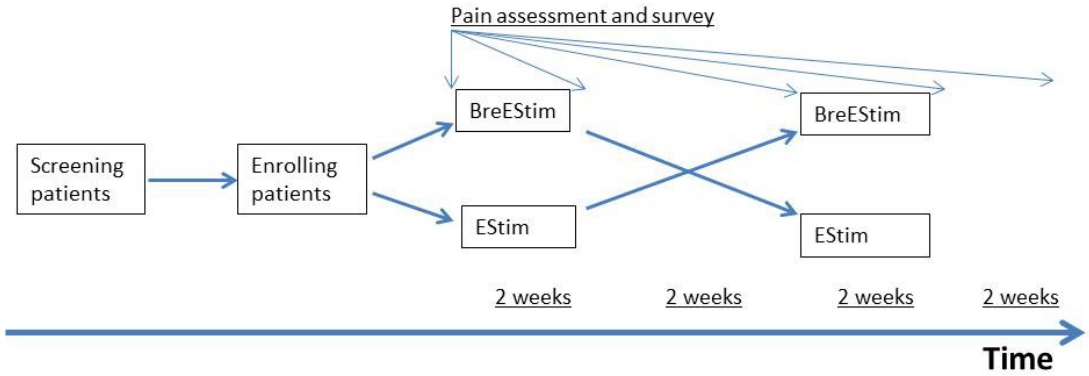

Figure 7: Illustration of study design for Experiments 3

In Experiment 3, the same established protocol will be used (Li et al. 2012a; Li 2013). The effect of two interventions (BreEStim and EStim) will be compared. Each intervention consists of 240 stimuli/session (about 40-50 min/session), 1 session/day for 10 consecutive sessions (over 2 weeks). After a follow-up (wash-out) period of two weeks, the same subject will be treated with the other intervention with a same follow up of 2 weeks. The intensity of stimulation will be adjusted as subjects could tolerate. Electrical stimulation will be delivered to the median nerve on the dominant side. The wash-out/follow-up period of 2 weeks allows removal of accumulative or carry-over effect from the previous intervention.

It is expected that electrical pain threshold will be further increased after BreEStim with parallel reduction in pain perception, while there is no or minimum change after EStim and no significant change in pain perception. Pain threshold will be measured using QST. Pain perception will be characterized by duration and amount of change in pain on visual analog scale (VAS), i.e., how much pain is reduced and how long it lasts. As such the effect of treatment is quantified by reduction x hours.

The secondary outcome measurement will be General Well-Being (GWB) Schedule Survey and Center for Epidemiologic Studies Depression Scale (CES-D), NIMH Survey to examine the possible effect of pain reduction on general well-being, depression and quality of life of the patients. These surveys have been well validated and commonly used (Sherman et al. 1984; Wartan et al. 1997; Kooijman et al. 2000).

The long-term BreEStim treatment is expected to have some cumulative analgesic effects. However, we decide to keep the same regime of medications, because change in medications will be a confounding factor. Therefore, the subjects will be explicitly instructed to maintain the same dose and frequency of pain medications after the BreEStim treatment even if pain is improved. The collaborating investigator Dr. Melton will screen and monitor their medications. This is exploratory at this time. If the baseline pain level improves after the 2-week BreEStim treatment, a clinical trial is warranted. Change in pain medications may be explored further in the future studies.

- **Experimental setup (BreEStim and EStim protocols and measurement)**

Experiment 1 We plan to use our recent BreEStim and EStim protocols (Li et al. 2013; Li et al. 2014). Briefly, subjects will be seated comfortably with the arm on the experimental table (Fig 8). Subjects will wear a facemask that will be connected to a Pneumotach system to record breathing signals and to prevent hyperventilation. Surface electrodes will be trimmed to be applied to the median nerve ipsilateral to the affected side proximal to the wrist line (see Fig 8). Note that important acupuncture points (Neiguang and Waiguan) are located beneath the electrodes. So these points will be stimulated during both BreEStim and EStim).

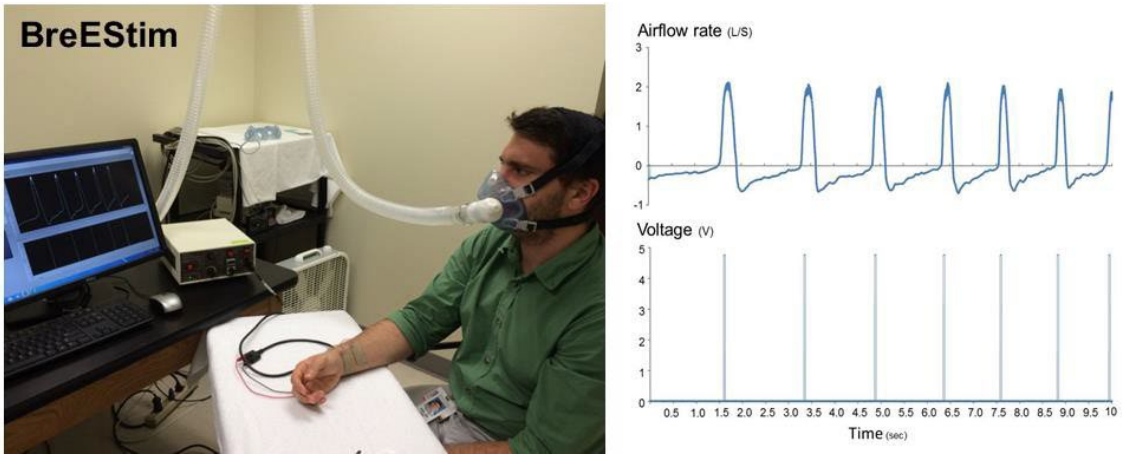

Figure 8 Experimental settings. On the right panel, an electrical stimulus (Lower) is triggered when the airflow rate (Upper) of a forceful inhalation reaches 40% of its maximum value.

In the BreEStim treatment, a single-pulse electrical stimulus is delivered to the nerve when patients are taking fast, strong, and deep inhalation, similar to a deep breath but faster and stronger. Voluntary inhalation plays an important role in this intervention. Voluntary inhalation is defined as effortful deep and fast inhalation. Subjects are instructed to take a deep breath, similar to routine deep breaths, but faster and stronger, usually involving obvious expansion of chest wall. It has been reported that there are respiratory specific connections between the insula and the activity of pulmonary stretch receptors (Gaytan and Pasaro 1998; Hanamori et al. 1998). Experimentally, the airflow rate is monitored online. When the airflow rate reaches 40% of its peak, an electrical pulse is triggered (Li et al. 2012a; Li 2013). The frequency of BreEStim generally varies. As in our previous series of BreEStim experiments, subjects are usually comfortable to continue voluntary breathing for 20 – 30 electrical stimuli without a break. As seen in Figure 8, BreEStim is delivered at about every 2 seconds. The single-pulse electrical stimulus is a square wave with a width of 0.1 ms. The intensity is set where subjects feel painful but yet tolerable, equivalent to 7 on the 0-10 VAS scale. When wearing a face mask, subjects usually tolerate such breathing very well. No hyperventilation has been reported (Li et al. 2012a; Li 2013).

In the EStim condition, subjects will also wear the face mask as in the BreEStim condition. The main difference is that subjects will be breathing automatically and comfortably and electrical stimulation pulses will be delivered randomly. The pulses will be programmed to randomly deliver at about every 2 seconds (between 1.9 seconds and 2.1 seconds). The pulse width will be the same at 0.1ms. Similarly, subjects control the intensity of electrical stimulation. For both BreEStim and EStim, they are strongly encouraged to increase the intensity to be painful yet tolerable gradually from their pain threshold. The level of pain with painful stimulation will be 7 on the 0-10 VAS scale. The purpose is to make the intensity of electrical stimulation comparable between two conditions. According to previous studies, the intensity is comparable between BreEStim and EStim(Li et al. 2013; Li et al. 2014).The intensity of electrical stimulation will be recorded during the course of treatment.

Two interventions (BreEStim vs. EStim) will be given to the same subject on a different day with at least 7 days apart. As in previous studies(Li et al. 2013; Li et al. 2014), a total of 120 electrical stimuli will be delivered for each intervention. The order of interventions will be randomized and balanced across subjects. Both interventions will be given at the same time of the day, as determined by subject's schedule. It is important to emphasize that subjects will be explicitly instructed to maintain the same pain regime even they feel that treatment has helped in pain reduction, such that pain reduction is mainly related to BreEStim or EStim.

Outcome measurement includes visual analogue scale (VAS) and Quantitative Sensory Testing (QST) and Hear rate variability (HRV). The VAS method has been extensively used and validated (McCarthy et al. 2005). Using VAS to rate pain intensity has been considered a standardized measurement to evaluate effectiveness of deep brain stimulation for pain management in a multi-center study by Medtronic (Medtronic, Minneapolis, MN). These results have been reported to US Food and Drug Administration for judgment on the efficacy of pain management(Coffey 2001). In the project, we plan to use a modified VAS to quantify pain reduction. As used in our pilot study (Li et al. 2012a; Li 2013), the effect of pain reduction is characterized by duration and amount of change in pain on VAS, i.e., how much pain is reduced and how long it lasts. As such the effect of treatment is quantified by reduction×hours. The effect and the intensity of stimulation will be recorded daily during the course of treatment. For VAS, the duration of analgesic effect from the intervention will also be recorded. VAS scores will be obtained before and 10 minutes after. Post-intervention VAS will be also assessed through follow-up phone calls every 4 hours except night time (10pm – 7am). It is important to emphasize that subjects will be explicitly instructed to maintain the same pain medication regimen even if they feel that treatment have helped in pain reduction.

QST includes tactile sensation threshold (TST), electrical sensation threshold (EST), electrical pain threshold (EPT), and thermal thresholds. VAS and QST will be recorded immediately before and 10 minutes after the interventions. In this experiment, we aim to test the central de-sensitization effect of BreEStim. To standardize QST (Li et al. 2015), all thresholds will be measured only on the residual limb at 5 cm above the distal residual limb. The symmetrical site on the contralateral limb will also be localized. After the target areas are localized and marked with a marker, subjects are instructed to stay relaxed in a chair with arms and legs comfortably supported in a symmetrical position. The order of QST will be randomized and balanced across testing sites. The following measurement will be made.

Tactile sensation threshold: Tactile sensation threshold will be tested using Von Forey filaments (Touch-Test Sensory Evaluator, North Coast Medical Inc.). The center of the thenar eminence/testing sites on the leg will be marked with a pen symmetrically on both hands. Subjects will be instructed to close their eyes. The experimenter will press the filament at a 90° angle against the marked area until it bows for approximately 1.5 seconds and then removed. Testing will begin with the thinnest 1.65 filament, then progresses to the next monofilaments. An explicit response of touch sensation is defined as tactile sensation threshold.

Electrical sensation and pain thresholds: The same trimmed electrodes will be used to examine electrical sensation threshold and electrical pain thresholds (electrical stimulator 7SA, Digitimer). A pair of electrodes is placed next to each testing site. For electrical sensation threshold, the intensity of electrical stimulation will start from zero and gradually increase in steps of 0.1 mA. Similarly, subjects will be instructed to close their eyes and to say "yes" when they explicitly feel electrical stimulation. Three repetitions will be made and the average will be used as the electrical sensation threshold. Electrical pain threshold is then measured. The intensity of electrical stimulation will start from the sensation threshold level and increase in steps of 1 mA. The electrical pain threshold is reached when subjects first feel electrical stimulation painful. To improve consistency among subjects, they are advised that the pain threshold level is equivalent to 1 on the 0-10 VAS scale. Similarly, the average of three tests will be used as the electrical pain threshold.

Thermal thresholds: Thermal thresholds (warm sensation, cold sensation, heat pain, cold pain) will be examined using a Medoc PATHWAY system. The established "Limits Full Series" protocol will be selected. Briefly, the protocol contains a series of tests in the following order: 4 tests of cold sensation threshold, 4 tests of warm sensation threshold, 3 tests of cold pain threshold, and 3 tests of heat pain threshold. The 30x30 ATS probe is secured with its center on the testing. Subjects will have an education session prior to the protocol. The averaged value will be used for each threshold.

Heart rate variability as objective outcome measurements: We have been searching for non-invasive objective pain outcome assessment. Heart rate variability has been recognized as a physiological marker for pain assessment (Appelhans and Luecken 2008; Karri et al. 2018b; Karri et al. 2019). We will follow our recent experimental procedures (Karri et al. 2018b; Karri et al. 2019) to collect 5 minutes of Heart Rate recordings before and after the interventions.

Experiment 2 The aim of this experiment is to explore this "dose-response" effect of BreEStim, by comparing the analgesic effects of two doses of single-session BreEStim. The same BreEStim protocol in Exp 1 will be used for Exp 2. In Exp 2, only BreEStim will be used. Two doses of BreEStim (120 vs. 240 electrical stimuli) will be given at least one week apart in a randomized order. Outcome measures, including VAS, QST thresholds, will be recorded pre- and 10 minutes post-intervention. Duration of analgesic effect (lasting hours) will also be recorded.

Experiment 3 The experimental set-up will be similar to Experiments 1&2. There are two different interventions – BreEStim and EStim. Each intervention consists of 240 stimuli/session (about 40-50 min/session), 1 session/day for 10 sessions (over 2 weeks, no treatment in the weekends). After a follow-up (wash-out) period of two weeks, the same subject will be treated with the other intervention with a same follow-up of 2 weeks. The intensity of stimulation will be increased gradually as patients could tolerate. Aversiveness of electrical stimulation is part of treatment strategy. During EStim, a single-pulse electrical stimulation is delivered to the forearm through surface electrodes randomly, while electrical stimulation is triggered by volitionally effortful inhalation during BreEStim as in Exp 1.

Specimens:

No specimens will be collected

5. Human Subjects

As mentioned above, we plan to recruit a total of 64 amputee subjects with phantom limb pain, including 16 subjects for Aim 1, 18 subjects for Aim 2, and 30 subjects for Aim 3. The number of subjects was estimated based on the pilot data.

- **Subject recruitment/assignment**

The collaborating physician (Co-I, Dr. Melton) will screen patients from the outpatient clinic of TIRR Memorial Hermann Hospital. We plan to register the project on www.clinicaltrials.gov. If patients are not currently seen by Dr. Melton, but are willing to participate in the project, they need to see Dr. Melton for screening and monitoring. In addition, Dr. Melton will monitor medical conditions of the patients and manage medical problems as needed. Patients will be recruited and enrolled in the project if they meet the following criteria:

Inclusion criteria: A patient who 1) has neuropathic PLP after amputation of one limb, upper or lower limb; NOTE that BreEStim has been shown to be effective for neuropathic pain management in patients after traumatic and non-traumatic spinal cord injury in recent studies(Li et al. 2016; Li et al. 2018). Therefore, we plan to recruit patients with chronic neuropathic PLP from both traumatic and non-traumatic limb amputation. 2) has chronic pain, >3 months; 3) is between 18 to 75 years of age; 4) is stable on oral pain medications at least two weeks. Patients are instructed to continue their pain medications as prescribed during the intervention period.

Exclusion criteria: Patients will be excluded if they 1) are currently adjusting oral pain medications for their neuropathic pain; 2) have pain, but not neuropathic, e.g., from inflammation at the incision wound of the residual limb; 3) have a pacemaker to avoid possible side effect of electrical stimulation; 4) have amputation in multiple limbs; 5) are not able to follow commands, or to give consent; 6) have asthma or other pulmonary disease; 7) are not medically stable.

Gender will be balanced

Potential subject populations and recruitment: Subjects will be recruited from TIRR outpatient clinic.

6. Data Collection and Analysis

Data collection details have been described above. Data analysis is described here.

Experiment 1 Repeated-measures two-way ANOVA tests with factors INTERVENTION (2 levels, BreEStim and EStim) and TIME (pre- and post-intervention) will be tested on dependent variables, including VAS, QST thresholds (tactile, electrical, and thermal), to determine statistical significance between two interventions. Paired t-tests will be used to compare the duration of lasting hours between two interventions. p value is set at 0.05. As observed in the pilot data, we expect that BreEStim could have central desensitization and analgesic effects on patients with phantom limb pain, but no such effect after EStim. These findings would suggest that the central desensitization effect of BreEStim could be translated to analgesic effects in patients with PLP. Furthermore, the findings of analgesic effects along with desensitization effects in patients with PLP support central sensitization as a plausible mechanism that mediates post-traumatic PLP.

Experiment 2 Similar to Exp 1, repeated-measures two-way ANOVA tests with factors INTERVENTION (2 levels, BreEStim 120 vs. 240) and TIME (pre- and post-intervention) will be tested on dependent variables, including VAS, QST thresholds (tactile, electrical, and thermal), to determine statistical significance between two interventions. Paired t-tests will be used to compare the duration of analgesic effect (lasting hours) between two interventions. p value is set at 0.05. As shown in the pilot data, we expect that BreEStim could have dose-dependent effects on the duration but not the degree of analgesic effects. These results also suggest that BreEStim could have cumulative effects if patients receive interventions repeatedly over a period of time.

In Experiment 3, Repeated-measures two-way ANOVA will be performed to determine statistical significance between two interventions. Factors include INTERVENTION (BreEStim vs. EStim) and TREATMENT (Pre- and Post-intervention, and Followup). Variables are VAS and modified VAS and QST parameters, GWB, and CES-D. It is hypothesized that the effect of pain reduction is greater in BreEStim than in EStim for amputation patients, manifested by pain reduction on VAS assessment and parallel change of increased electrical pain threshold as shown by pilot data in Experiments 3. Furthermore, pain reduction is accompanied by improvement in general well-being and mood.

Demographic data will be analyzed descriptively. Personal information will be de-identified.

7. Potential Risks/Discomforts:

The risks associated with this study are minimal. Electrical stimulation has been used extensively in clinical settings. Patients may feel uncomfortable, or even painful during electrical stimulation. The patients will be specifically instructed that they are encouraged to increase the intensity of electrical stimulation to the level they may feel comfortable, but they need to be able to tolerate at that level if given repetitively. They are also explicitly instructed to decrease the intensity of electrical stimulation if they can not tolerate it. Surface electrodes can cause skin irritation from the tape adhesive or the electrode paste. Breathing through a face mask may be make patients feel uncomfortable. All tested subjects in the previous studies (approximately 100 subjects, including patients) tolerated the mask well.

8. Benefits:

There are several potential benefits in this study: 1) direct benefit to subjects as their neuropathic pain may be relieved by the intervention; 2) benefit to the class of subjects: other patients in the same patient population may be benefit if the intervention is applied to them; 3) adding to the knowledge base: the findings will be definitely added to the knowledge base. Specifically, the study will provide evidence of an alternative and innovative intervention for neuropathic pain management after amputation.

9. Risk-benefit Ratio:

In view of the minimal risk, the knowledge to be gained far outweighs the risks.

10. Consent Procedures:

Informed consent will be obtained from the subject. After the subject is identified and is interested in participating, informed, written consent will be obtained by the investigator.

11. Confidentiality Procedures:

In order to minimize risk to confidentiality, all data will be de-identified, coded with a study-specific identification number, maintained on a password-protected server, and/or kept in a locked office. No findings will be released without written authorization by the subject or requests by law.

12. Costs:

The subject will not be expected to pay any costs.

13. Payments:

Subjects will receive a gift card of $40 for their participation in each session for Experiment 1 and 2. Experiment 3, each subject will have a total of 20 sessions (10 consecutive sessions of BreEStim, and 10 consecutive sessions of EStim, with 2 weeks apart). Each subject will receive a giftcare of $40 after the first session. Each of them will receive a total of $800 after the completion of all 20 sessions. No monetary compensation will be given if 20 sessions are not completed. This is to keep commitment and for subject retention.

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