MAGNETO-ELECTRIC NANOPARTICLES COBALT FERRITE ($CoFe_2O_4$) – BARIUM TITANATE ($BaTiO_3$) FOR NON-INVASIVE NEURAL MODULATIONS

Tyler Nguyen

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Doctoral Committee

_____
Fletcher White, Ph.D., *co-Chair*

_____
Armin Blesch, Ph.D., *co-Chair*

July 15, 2020

_____
Xiaoming Jin, M.S., Ph.D., *Primary Advisor*

_____
Richard Rodgers, M.D.

_____
Sakhrat Khizroev, M.S., Ph.D.

DEDICATIONS

For ***Mom and Dad***. Thank you for teaching me that the really great adventures happen outside of your comfort zones.

For my dear ***Natalie***. In the vastness of space and the immensity of time, somewhere within an undistinguished part of this universe (the outskirts of the Virgo Supercluster), in an undistinguished galaxy (The Milky Way), in an undistinguished region (the Orion Arm), revolving around an undistinguished star (the Sun), it is my joy to share a planet and an epoch with my amazing *G-nat*…

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MAGNETO-ELECTRIC NANOPARTICLES COBALT FERRITE ($CoFe_2O_4$) – BARIUM TITANATE ($BaTiO_3$) FOR NON-INVASIVE NEURAL MODULATIONS

Non-invasive brain stimulation is valuable for studying neural circuits and treating various neurological disorders in human. However, current technologies of non-invasive brain stimulation usually have low spatial and temporal precision and poor brain penetration, which greatly limit their application. A new class of nanoparticles known as magneto-electric nanoparticles (MENs) is highly efficient in coupling an externally applied magnetics wave with generating local electric fields for neuronal activity modulation. Here, a new type of MENs was developed that consisted of CoFe2O4-BaTiO3 and had excellent magneto-electrical coupling properties. Calcium imaging technique was used to demonstrate their efficacy in evoking neuronal activity in organotyic and acute cortical slices that expressed GCaMP6 protein. For in vivo non-invasive delivery of MENs to brain, fluorescently labeled MENs were intravenously injected and attracted to pass through blood brain barrier to a targeted brain region by applying a focal magnet field. Magnetic wave (~450 G at 10 Hz) applied to mouse brain was able to activate cortical network activity, as revealed by in vivo two-photon and mesoscopic imaging of calcium signals at both cellular and global network levels. The effect was further confirmed by the increased number of c-Fos expressing cells after magnetic stimulation. Histological analysis indicated that neither brain delivery of MENs nor the subsequent magnetic stimulation caused any significant increases in the numbers of GFAP and IBA1 positive astrocytes and microglia in the brain. MENs stimulation also show high efficacy in short-term pain relieve when tested with a tibial nerve injury mouse

model. The study demonstrates the feasibility of using MENs as a novel efficient and non-invasive technique of brain stimulation, which may have great potential for translation.

Fletcher White, Ph.D., co-Chair

Armin Blesch, Ph.D., co-Chair

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANEPP	Aminonaphthylethenylpolypyridinium
ATP	Adenosine triphosphate
A_1R	Adenosine 1 Receptor
aCSF	artificial Cerebral Spinal Fluid
BBB	Blood Brain Barrier
BME	Basal Medium Eagle
CNS	Central Nervous System
DAPI	4', 6-diamidino-2-phenylindole
DBS	Deep Brain Stimulation
FBS	Fetal Bovine Serum
fMENs	fluorescence Magneto-Electric Nanoparticles
FUS	Focus Ultrasound Stimulation
G	Gauss
GABA	Gama aminobutyric acid
GCamP6	Green Fluorescence Calmodulin M13 calcium indicator 6
GFAP	Glial Fibrillary Acidic Protein
GMO	Glycerol Mono-Oleate
HIFU	High Intensity Focus Ultrasound
IBA1	Ionized calcim Binding Adaptor protein 1
IV	Intravenous

LDP	Long-term depression
LTP	Long-term potentiation
MENs	Magneto-Electric Nanoparticles
MRI	Magnetic Resonance Imaging
MS	Magnetic stimulation
Nd	Neodymium
NMDA	N-methyl-D-aspartate
NP	Neuropathic pain
OCT	Optimal cutting temperature
PAP	Prostatic acid phosphatase
PBS	Phospho-Buffer Saline
PFA	Paraformadehyde
SSNRI	Selective-serotonin and norepinephrine reuptake inhibitors
T	Tesla
tACS	Transcranial Alternating Current Stimulation
TCDS	Transcranial Doppler Sonography
tDCS	Transcranial Direct Current Stimulation
TES	Transcranial Electrical Stimulation
TMS	Transcranial Magnetic Stimulation
TTX	Tetrodotoxin
VGCC	Voltage-gated Calcium Channel
VGSC	Voltage-gated Sodium Channel

Chapter 1 Introduction

Non-invasive neural stimulation, a paradigm shift in understanding, diagnosing, and treating neurological diseases

For more than a century, neuroscientific research has raced to understand the mechanism and to find treatment approach behind the manifestation of neurological related diseases. Routinely, investigators are limited to observe the changes of disease progression through behavior or record and interpret correlates of brain activity. For many decades, the same principle is usually followed by giving a diseased brain a specific task, recording its activity, analyzing its activity and compare to a normal healthy brain. We can then develop a theory of how the changes in a diseased brain activity compare to a normal brain, thus implicating the disease's effects. In the context of a more global study, pharmacological applications were used to block or enhance the neural network and observe their functional outcome. However, for more in depth understanding of which *specific* part of a neural pathway is affected by certain disease, investigators had to manipulate the cause and observed the predicted effect, in order to develop correlational evidence. In order to do so, historically, investigators usually relied upon interfering with the activity of the interest region via targeted pharmacological treatment or lesioning. The co-occurrence of a loss of function with inhibiting the activity (targeted pharmacological treatment) or interfering the signaling pathways (a lesion) of a *specific* part of neural region can be then interpreted as a causal relationship. This paradigm of investigation works well in certain animal disease models but not feasible in human studies[4].

In the example of neuropathic pain, which is defined as pain occurring in an area of body affected by neurological diseases or injury that results in central and peripheral sensitization. Historically, therapeutic approaches to neuropathic pain have focused on reducing or blocking neuronal activity within the pain pathway. Currently clinical approach includes pharmacological treatments with various receptors inhibitors (Voltage-gated Calcium channels inhibitors, serotonin and norepinephrine reuptake inhibitor) to more popular but riskier opioid-based analgesics (codeine, fentanyl, tramadol, morphine, etc...)[5]. A central issue with drug treatments for pain involves dangerous side effects, non-regional specific targeting (receptor inhibitors), and more importantly chronic sensitization to the substance (addiction) that are often fatal. Surgical techniques have also been attempted to interfere with pain pathway via lesioning regions of brain or neural tracts in spinal cord[6]. Surgical intervention via lesioning produced highly contradictory outcomes among different studies of pain management[6]. The reasons are followed:

First, it is extremely difficult to treat the mechanistic manifestations of refractory neurological diseases such as epilepsy, pain, various type of traumatic brain injuries because the injured nervous system (brains, spinal cord), on top of the effects caused by the disease itself, may have developed individual strategies to compensate for the defect[4]. Second, studies done by interfering with the neural circuitry via lesioning presents an extremely invasive approach in studying disease in human patients. Even with approval, the number of patients (and their health background) in a study are often small and inhomogeneous with respect to their individual anatomy and lesion, thus interpretations about the disease relevance of the lesion and treatment efficacy must be taken with a

grain of salt[4]. Third, lesioning is usually the last resort in both diagnostic and treatment approach for neurological disease.

A solution to this problem came with the invention of non-invasive brain stimulation (NIBS) techniques. The idea of stimulating neuronal activity wirelessly by external forces has only been applied as a treatment approach towards some diseases in human within the past 30 years and as a tool for research in neuroscience only relatively recently[7, 8]. The most established techniques are Transcranial Magnetic Stimulation (TMS) and Transcranial Electrical Stimulation (TES), which also are the only FDA-approved non-invasive stimulation techniques used in the clinics. These techniques utilize strong external magnetic field (TMS) or electric field (TES) applied for short duration to elicit action potentials thus enhancing neuronal activity[8, 9, 10]. With full manipulation of the applied force parameters (i.e. strength, duration, frequency), investigators can either functionally inhibit a certain brain area or excite a structure and measures the outcome. All of this can be done harmlessly while the subject is fully awake, thus provide incredible tools for human applications.

Non-invasive brain stimulations, powerful approaches that are not without their flaws

For a non-invasive brain stimulation technique to be fully effective, four criteria have to be met: ***spatial resolution, temporal resolution, non-invasiveness, and clinically feasibility***. Spatial resolution is defined as how small (or specific) an area is being stimulated. As the treatment is applied, if it only stimulates a specific region of the brain or even a subpopulation of neurons, it is said to have a high spatial resolution. Temporal resolution is defined as the delay period between treatment is applied and the effects observed. If the effects observed immediately after treatment application, it is said to have high temporal resolution[8]. Figure 1 (adapted from Polania et al. 2018) shows eloquently diverse types of non-invasive brain stimulation techniques (both clinical and research) with their corresponding spatial and temporal resolution effects and their increasing adaptation in scientific studies. These effects determine the technique's level of efficacy. In combination with its non-invasiveness mode of treatment and clinical feasibility, these are four primary determinants for the transition of the technique from research to clinical. Furthermore, other important factors should also be considered for this transition ranges from the toxicity, off target side effects, to accessibilities and cost. The two most commonly used forms of noninvasive brain stimulation clinically are Transcranial Magnetic Stimulation (TMS) and transcranial Direct & Alternating Current Stimulation (tDCS & tACS)[11, 12, 13]. However, they have relatively low spatial and temporal precisions, which greatly diminish their efficacy and result interpretation. For example, regular TMS require high intensity of external field applied (~20k-50k Gauss, G, or about 2-5 Tesla, T,) but have a spatial resolution of 3-5 cm and depth of penetration

of ~1-1.5 cm, which allows for stimulating cortical gyrus[14, 15]. tDCS also has very low spatial resolution and it is used to target larger superficial cortical area but has limited effects on deep brain areas[14, 16, 17]. In the example of neuropathic pain, TMS has been used in clinical trials for pain management but produces unreliable results. A meta-analysis study of 23 clinical trials that use TMS in patients who suffered from long term pains reported only 5 out of 23 trials yield more than 50% of patients reported pain relieve[18]. Among these five effective trials, all patients who reported to experience less pain only do so partially and in very short-term. The treatment had to be applied at high frequency and high magnetic intensity to consistently achieve lower pain threshold.

For the past 15 years, recent approaches like optogenetics[19, 20] have improved in ways that were limited in tDCS and TMS (i.e. cellular specificity and temporal resolution). Optogenetics utilize light to stimulate with extremely high spatial and temporal resolutions (i.e. a subpopulation of neurons in millisecond timescale) by taking advantage of the light-sensitive proteins introduced to the subject. This technique, however, presents huge drawbacks of required genetic modifications in the subject. Very recently, acoustic-based transcranial stimulations such as High Intensity Focused Ultrasound (HIFU) and Transcranial Doppler Sonography (TCDS) were used to study TBI[13, 21], Parkinson disease[22], tremor[23], and neuropathic pain[22] with improved spatial resolution and penetration. Investigators also tried to use focused ultrasound in low intensities for brain stimulation[24] with Legon's group done tests on human subjects[25]. Although the stimulation size of 4.9 mm in depth and 18 mm in diameter shown to be effective in evoking neuronal activity, high volume release of thermal energy due to continuous oscillating sound waves could decrease synaptic transmission, and results in tissue

homogenization, protein denaturation, and DNA fragmentation. This remains a critical hurdle of HIFU research to pass in optimizing its safety and efficacy[26, 27].

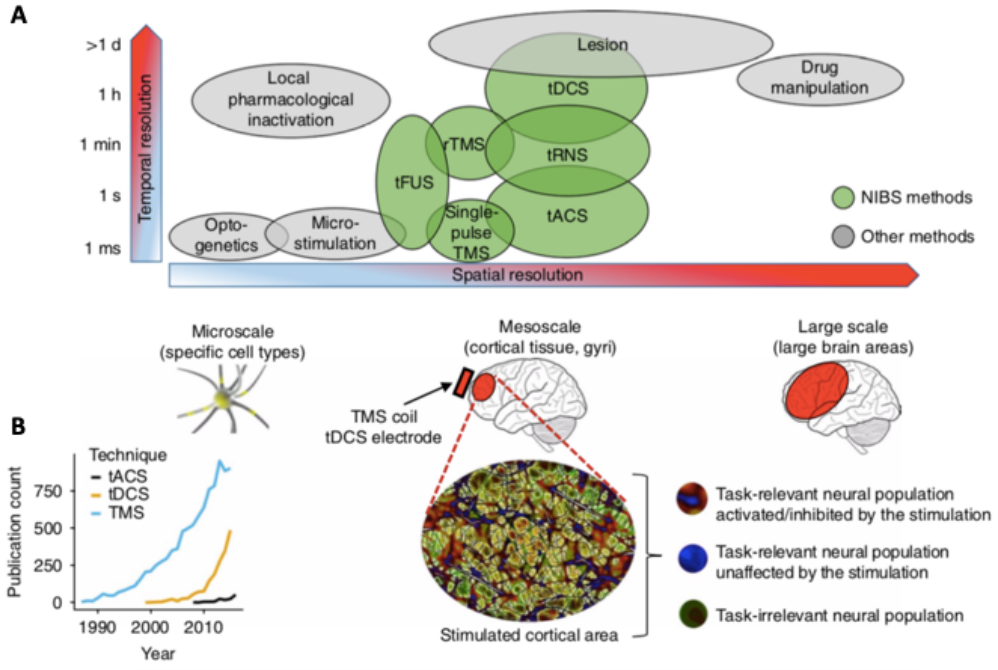


Figure 1. Overview of non-invasive brain stimulation approaches (adapted from Polania et al. 2018). A. The temporal and spatial resolution at which different causal brain interventions work. Non-invasive brain stimulation methods work at the mesoscale level, and the temporal resolution varies between high and low depending on the specific stimulation protocol. Non-invasive brain stimulation necessarily involves the relatively indiscriminate activation of large numbers of neurons; the apparent temporal and spatial specificity seen in studies is thus unlikely to reflect the anatomical and temporal specificity of the stimulation. Instead, it may indicate disruption of behaviorally relevant operations that are carried out by a relatively small number of cell groups within larger brain regions. **B**. The exponentially growing number of citations per year for TMS, tDCS and tACS.

Magnetic-electric nanoparticles-based materials, a potentially promising new vehicle for neural stimulation

In the last decade, nanoscale neural interfaces have become a valuable tool for drug delivery, monitoring and modulating neuronal activity[28]. Developments such as state of the art nanoelectrodes was used to localize epileptic foci in mice[29], while various types of nanoparticles were used in drug delivery[3, 30] or neuronal activity modulations[31, 32]. Within this new realm of nanoparticle-based neural modulation, diverse types of nanoparticles with diverse properties have been shown, at least in research stage, to have the abilities to efficiently stimulate neurons. Two ways of nanomaterials that have been shown to be very effectively stimulate neuronal activity are through increase in surface temperature[31, 32] or induce local magnetic field[33]. Magneto-thermal brain stimulation takes advantage of magnetic nanoparticles that generate heat when an external magnetic field is apply. While magnetic ferroelectric nanoparticles can generate its own micro magnetic field in response to external applied field to activate neurons *in vitro*. These approaches, however, face moderate problems of toxicity and off target effects. In the case of magneto-thermal nanoparticles, introduction of nanoparticles to the animal requires an invasive step direct delivery of particles into the brain. While magnetic ferroelectric nanoparticles stimulation required application of a very high intensity of external field (~8-15k G), very close to the strength require for TMS, thus present a possibility of off-target activation. Despite these drawbacks, nanoparticle-based technique presents a tremendous potential of being the next frontier for neural modulation due to its limitless manipulation of the particles and the stimulation modes to obtain desired effects.

My dissertation will focus on a type of magnetic nanoparticles called magneto-electric nanoparticles (MENs) as a novel and more effective mode of neuronal stimulation. The concept of MENs was first proposed by Guduru et al. as a way to delivery drug to a target area by taking advantage of the magnetic-electric coupling between the particles and the drug of interest[3]. MENs composed of a core-shell cobalt ferrite ($CoFe_2O_4$) – barium titanate ($BaTiO_3$) structure size ranges between 25 ~ 35 nanometers (nm), in which cobalt ferrite ($CoFe_2O_4$) cubic core is tightly enveloped by spherical barium titanate ($BaTiO_3$) shell (Figure 2A-B). The ferrite core is the only FDA-approved magnetic nanoparticle that has been used as an active ingredient in radioactive dyes for functional magnetic resonance imaging (MRI)[34], although the core compound itself is non-radioactive. Depending on the type of external magnetic field is applied, the $CoFe_2O_4$ generates different physical properties. When a moderately strong external field is applied (~3-6k G or 0.3-0.6 T), the core becomes ferromagnetic and becomes attracted towards the field. However, when a weak field is applied at a particular frequency (~300-600 G or 0.03-0.06 T), the core vibrates (micro-vibration) in space and its cobalt-iron oxide bonding becomes stretch out ever so slightly. The physical stretching of the core induces mechanical stress on the barium titanate (BaTiO3) piezoelectric perovskite shell. Because of its nature as a piezoelectric perovskite shell, any mechanical stress will induce surface charge separation (in this case, charge redistribution) that allow each individual shell to generate its own micro local electric field (on the order of 1000V/m or higher)[35]. This precise core-shell magnetic-electric couplings of these MENs enable Nair et al. group to bind antiretroviral HIV drug via electrostatic interaction, localize the drug-

carrying MENs to the targeted site and release them, all done wirelessly via controlling the applied external magnetic field[3].

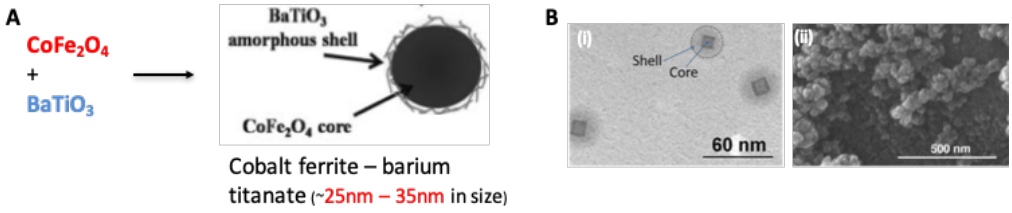


Figure 2. Cobalt ferrite ($CoFe_2O_4$) – barium titanate ($BaTiO_3$), MENs composed of core-shell structure. A. MENs are synthesized via coordination chemistry in which cobalt ferrite ($CoFe_2O_4$) is made from the reaction of Cobalt-2-nitrate-hexahydrate with Iron-2-nitrate nonahydrate and barium titanate ($BaTiO_3$) is made from reaction of barium carbonate with titanium isoporoxide[1]. The combination reaction of cobalt ferrite ($CoFe_2O_4$) and barium titanate ($BaTiO_3$) generates the MENs[1, 2, 3]. **B.** Electron microscopic images of core-shell MENs (adapted from Nair et al. 2013)[3]

These MENs provide many advantages over other nanoparticle-based stimulation approach: first, its relatively small size allows easy traverse across the blood brain barrier (BBB). Second, its ferromagtically attractiveness towards moderately strong magnetic field allow precise localization of particles to a region of interest. Third, the sub-threshold low intensity magnetic field required for both localization and stimulation ensure no off-target activation. Fourth, most importantly, the magnetic-electric coupling increase efficiency and latency period of stimulation, which allow effective enhancement of neuronal activity. And fifth, because these particles have been used for drug delivery, modification of MENs by attachment of antibodies can potentially improve its specificity and enhance its stimulatory efficacy. The unique properties of MENs, due to their small size and the ME effect not displayed by any other nanoparticles known to date, may provide significant improvements over currently used techniques in in efficacy, precision, and tissue penetration for noninvasive brain stimulation. However, to date, no direct effect of MENs on stimulating individual neurons in a focal brain region and/or a large neural network has been demonstrated.

Overview of dissertation chapters

The central questions in this dissertation is: Do magneto-electric nanoparticles (MENs) cobalt ferrite ($CoFe_2O_4$) – barium titanate ($BaTiO_3$) stimulation enhance neuronal activity (specifically cortical neurons) both *in vitro* and *in vivo*? If so, what is the spatial and temporal profile of the stimulatory effects? And what type of effects do MENs stimulation have, in the context of therapeutic approach, on refractory disease animal models, specifically neuropathic pain?

The first question of does MENs stimulation enhance neuronal activity can be broken down into several critical components that need to be validated: (i) Can MENs magnetic and electric effects be measured prior to biological testing? (ii) What effects does MENs stimulation have on cortical neuronal activity *in vitro*? (iii) Can MENs be direct through BBB and localized in the brain *in vivo*? And (iv) What effects does MENs stimulation have on neuronal activity *in vivo*?

In Chapter 2, I address the question of how to measure MENs magnetic and electric effects before testing on neurons by describe how MENs are prepared and the evaluations of their magnetic and electric properties in an aqueous solution. I then tested their effects on a cortical neuronal culture *in vitro* and test how different types of the applied external magnetic fields induce different neuronal responses. Chapter 3 moves on to determine whether MENs can be drawn through BBB after treatment and localized in a brain region *in vivo* by assessing MENs spatial and temporal profile both in the blood stream and brain motor cortex. In the same chapter, I also examine particle toxicity by assessing whether the presence of MENs in the brain causes any short-term neural inflammation. Chapter 4 describes the effects of MENs stimulation on neuronal activity

in vivo validated via various imaging techniques and characterizes both the temporal and spatial profile of the effects. Finally, Chapter 5 examines the effects of MENs stimulation on a neuropathic pain animal model of tibial nerve transection injury. Hypothesis on the delivery and targeting of MENs to the brain in a live biological system, its stimulatory effects on neuronal activity (i.e. brain function), and its promising application as a therapeutic approach to neuropathic pain are formulated based on the above results in the Chapter 6 of this dissertation. Also, in this last Chapter, I report more recent efforts in improve the specificity of the MENs targeting and its effects efficacy by conjugating particles with various antibodies.

Chapter 2 - MENs exhibit unique magnetic-electric coupling, in response to external magnetic field, which enhances cortical neuronal activity *in vitro*

Introduction

To test the effects of MENs stimulation on neurons, I first focused on how to make MENs biocompatible and test its magnetic and electric properties. As noted in last chapter, MENs were fabricated by our collaborators Khizroev group at University of Miami[2] via hydrothermal method, i.e. in a high-pressure chamber at high-temperature for extended period of time to induce product crystallization. This core-shell structure particle ranges between 25 nm to 30 nm in size. MENs are multiferroics (exhibit both magnetic and electric polarizations) that consist of a magnetostrictive core and piezoelectric perovskite shell[36]. The magnetostrictive core responds to the applied external magnetic field by contracting and expanding (for weak applied field <2000 G) and becomes ferromagnetic (for stronger applied field above 3000 G)[37]. The contracting and expanding core put a physical strain (i.e. mechanical stress) on the piezoelectric shell constituent, which induce electrical polarization of the shell. This electrical polarization manifests through surface charge redistribution of the outer shell that in turn generates an electrical dipole, i.e. currents[36] (Figure 3 illustration).

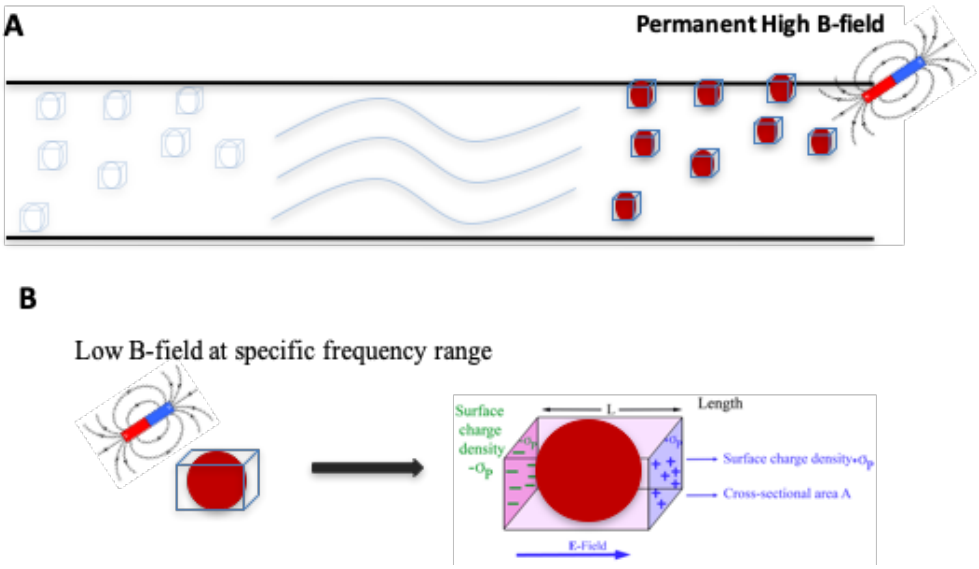


Figure 3. MENs produce different properties depends on the type of the applied external magnetic field. A. Application of a strong permanent external magnetic field can draw and localize MENs towards the field source. **B.** Weaker oscillating magnetic field at specific frequency ranges can induce core-shell's magnetic-electric coupling property that resulted in generation of electric field.

Due to its nature as core-shell magnetic nanoparticle, aggregations (or agglomerations) are common occurrence in any aqueous suspension. To understand core-shell particle agglomerations, one needs to understand the physics of how agglomerations occurs. In brief, for core-shell complex such as ($CoFe_2O_4$) – ($BaTiO_3$), the particle surface that interact with surrounding liquid, i.e. the surface of the $BaTiO_3$ shell, is called the **Stern Layer**. The ions of the outer shell continuously interact with ionic charges of the surrounding liquid (i.e. water) solution. As particle continue to interact with ions from the surrounding solution, this creates an interface between the outer shell and the surrounding water called the diffuse layer. So, the electrical potential between the Stern Layer and the diffuse layer is called the **zeta** (ζ) potential[38]. The higher zeta potential, the more stable the dispersion, the better the particles will repel each other. The lower the zeta potential, the less stable the dispersion, particles will tend to agglomerate.

Agglomeration of particles has detrimental effects to living cells and tissues[34]. To prevent MENs agglomerations, particles are coated with a biocompatible, low viscosity monoglycerides that increase particles zeta potential while maintain the integrity of the magneto-electric properties. Its magnetic and electric properties were tested in a polar aqueous solution.

Magnetic fields are ideal for noninvasive manipulations of neural activity as they can achieve deep tissue penetration and localization by tuning force-inducing field gradients. To overcome the weak interaction between sub-threshold magnetic field and biological molecules, magnetic fields should be translated into other forces. Previous efforts were made in developing magnetic-based nanoparticles that translate external magnetic field into other forces for neuronal stimulation[31, 33]. *In vitro* experiments done by Tay et al. with silicone-coated Nickle-Iron (NiFe) particles and Chen et al. with Iron-Oxide-Oleate ($Fe_3(C_{18}H_{33}O_2)_8$ showed that by applying an external magnetic field and taking advantage of the enhanced magnetic coupling[33] and magnetic-thermal coupling[31], they were able to stimulate neuronal activities. Their techniques encountered several drawbacks including demagnetization of the particles[33] and over-heating of particles causes cellular dysfunction if external field is applied for long period of time[31]. Derived from similar concept, these MENs take advantage of the magnetic-electric coupling to produce its effects. Our aim is to activate MENs in an *in vitro* setting and with different types of external magnetic field to determine its effects on neuronal activity.

Methods

MENs functionalization with Glycerol Monooleate (GMO). MENs were synthesized using the hydrothermal method as described in Guduru et al.[39]. It composes of an-almost perfect lattice matched surface interface between the magnetostrictive core and the piezoelectric shell (Figure 3 & Figure 4). To eliminate aggregation and potential toxicity, MENs were surface functionalized by a 2-nm thick coating of glycerol monooleate (GMO) as followed: MENs were washed twice with phosphate-buffered saline, PBS (pH – 7.4) and sonicated for 2 mins between washes for uniform dispersion in solution. MENs were then suspended in glycerol mono-oleate (GMO) solution (8 μL GMO/1 g MENs) and mixed thoroughly on a rotator for 12 hours. Excess GMO were then removed by extraction with 1 ml of 70% ethanol:phosphate-buffered saline (PBS) and two subsequent washes with PBS. Finally, the MENs-GMO mixture were suspended in saline and store at -20 ^{o}C (for short-term storage of 3-5 days) or freeze dried in liquid nitrogen for long term storage (more than 1 week). ***To prepare $CoFe_2O_4$-$BaTiO_3$ -Texas Red***, fluorescent MENs (fMENs), Texas Red NHS ester was first reacted with oleylamine (2:1 molar ratio) for 18-24 hours on a rotator in a dark environment. Hexane was added to help dissolved the oleylamine-Texas Red mixture. The solution of oleylamine-Texas Red NHS ester was then combined with the GMO-MENs mixture (1:3 volume ratio of GMO-MENs: oleyamine-TexasRed). This mixture was then rotated for 12 hours to allow coating. After removal of excess GMO through multiple washes in PBS, the final product was store at -20 ^{o}C (short-term storage).

Assessment of MENs ferromagnetic properties. 2 μL of fMENs (of concentration 5 mg/ml Saline) were mixed with 0.1% Agarose solution on a 10mm cell

culture dish. The solution was then sonicated with Branson 1510 sonicator (NIST, Gaithersburg, MD) for 30 seconds to ensure uniform distribution of fMENs in Agarose. A 3500 G neodymium (Nd) conical shape permanent magnet was then placed with the tip at the center of the culture dish for 15 minutes. Images where taken both before magnetic application and after magnetic application with a 5x objective using an Olympus IX71 (Olympus America, Merryville, NY) inverted microscope.

Assessment of MENs electric properties. A mixture of 3 µl of 10 mM di-8-ANEPPs (di-8-butyl-amino-naphthyl-ethylene-pyridinium-propyl-sulfonate; Sigma Aldrich) and 2.5 µl of 20% pluronic: DI water was combined with 1ml organically modified silicate (ormosil) in a 1.5 ml Eppendorf tube. This yielded a "loading solution" contains approximately 30 µM ANEPPs and 0.05% pluronic. 3 µl of MENs and 10 µl of ANEPP-ormosil dye solution was combine in a centrifuge tube and sonicated. A drop ~1-2 µl of mixture was then place onto a microspic slide topped with cover slip. A continuous wave of ~450-600 Oe magnetic field was applied to the top of the dish during fluorescence imaging process. Dye signals was captured using a fluorescence microscope (Zeiss AxioVert 200, Zeiss, Germany) equipped with an oil immersion objective (20x), a monochromator and a cooled CCD camera. Applied excitation wavelength for ANEPPs was done at 485 nm with observed emissions at 595 nm and 700 nm. Images was obtained with MetaFluor 7.11. Two addition control groups were done with ANEPP-ormosil and magnetic field (without MENs) and ANEPP-ormosil and MENs (without applied magnetic field).

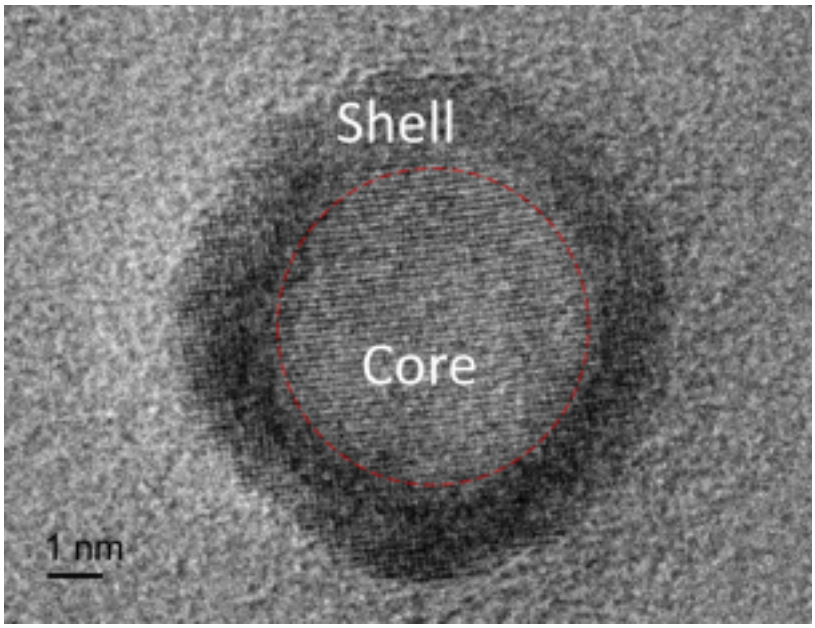


Figure 4. Transmission electron microscopy (TEM) image of a MEN. The almost-perfect lattice matched surface interface between the magnetostrictive core and the piezoelectric shell (shown with broken line) is vital for enabling MENs with record high ME values on the order of 5 V/cm/Oe in the frequency range, 0 to 200 Hz, under study.

In vitro calcium imaging of cortical neurons in organotypic GCaMP6 brain slices. *Cortical culture slice preparation:* Brain tissue was obtained from 3-5 days old C57BL mice (n=5). The animals were quickly decapitated and the brain was removed and immediately immersed in an ice-cold cutting aCSF solution (2 mM KCl, 1 mM $MgCl_2$, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1 mM $CaCl_2$, 10 mM D-Glucose, 206 mM Sucrose). The cerebellum was then removed, and the brain was fixed onto the cutting stage with glue at the caudal end. Vibratome VT1200 (Leica Biosystems, Buffalo Grove, IL.) sectioning apparatus was used to section the brain into coronal slices of 350 µm thick at a cutting velocity of 0.75 mm/minutes. The slices were then collected and stored in a cold oxygenated aCSF solution (126 mM NaCl, 2.5 mM KCl, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 10 mM D-Glucose). Only intact slices were then arranged carefully onto semi-porous membranes (4 slices/membrane).

The slices were cultivated at ~37 ^{o}C and 5% CO_2 in a culturing medium consisting of 46% Basal Medium Eagle (BME), 25% Earle's Salt Solution, 25% fetal bovine solution (FBS), 3% of 22% Glucose solution, and 1% L-Glutamine-Penecilin-Streptomycin solution. This medium was micro-filtered every time prior to addition into slice culture. 75% of medium were change every three days. *Magnetic field application:* Prior to imaging, a culture slice was taken from the culture well via cutting the membrane surround the slice then place in a custom-made recording chamber filled with 37 ^{o}C aCSF. The recording chamber is a 2-cm diameter well with glass bottom in which the whole chamber is position between two electromagnets for stimulation. 5 μL of MENs (at a concentration of 5 mg/mL aCSF) was added to the recording chamber. A 5000 G conical magnet was applied for ~15 minutes under the glass to draw MENs to a small area within the slice. For stimulation, the recording chamber with diameter of ~10 mm contains the cultures were place between two electromagnets. A sinusoidal magnetic field of ~450 G with 50 ms pulse width at 10 Hz frequency was applied to stimulate the MENs during the imaging process. *Two-photon imaging: In vitro* two-photon imaging was performed using a Prairie Technologies Ultima 4423 two-photon system (Bruker Inc., Middleton, WI) equipped with a MaiTai Ti:Sapphire laser (Newport, Mountain View, CA) tuned to 900 nm. Band-pass filtered fluorescence (560-600 nm) was collected by photomultiplier tubes of the system. The average laser power on the sample was ~20-30 mW. All experiments were acquired at a resolution of 512 x 512 pixels using a 20x water-immersion objective (Nikon Instruments Inc., Melville, NY). Images were capture at a rate of 4-5 fps. Typically, each recording session consisted of 30 s of baseline recording, 40s with magnetic field application, and 30 s of post-magnetic application

period.

Ex vivo calcium imaging of acute GCaMP6 cortical slices. *Cortical slice preparation:* Thy1-GCaMP6s transgenic mice, in which layers II/III and V pyramidal neurons express GCaMP6 calcium sensor protein, were purchased from Jackson's Laboratory and bred locally. The mice (n=4) at the age of ---were anesthetized with 78 mg/kg ketamine and 22 mg/kg xylazine (intraperitoneally) and decapitated. The brain was removed and immediately placed in an ice-cold oxygenated sucrose artificial cerebral spinal fluid (s-ACSF) solution (206 mM sucrose, 2 mM KCl, 1 mM MgCl2, 2 mM MgSO4, 1.25 mM NaH2PO4, 26 mM NaHCO3, 10 mM D-glucose, 1 mM CaCl2). After 1-2 mins, the brain was situated on a cutting stage such that the cortices faced the approaching blade. Agarose gel was used as cushion against the ventral part of the brain to prevent movement while cutting. Slices of 350 μm thick were cut with a Vibratome (Leica VT1200S; Leica, Nusslock, Germany) when the brain was submerged in the cutting solution. The slices were then incubated at 37 ^{o}C for 1 hour in a gridded chamber filled with oxygenated ACSF (124 mM NaCl, 3 mM KCl, 2 mM MgSO4, 1.25 mM NaH2PO4, 26 mM NaHCO3, 10 mM D-glucose, 1 mM CaCl2). Each slice was on its own grid for up to 4 hours. *Calcium imaging:* A custom-built recording chamber was made by attaching a temperature control heated well (Thermal Well Temperature Controller TC-100, BioScience Tools, San Diego, CA) onto a clear cover slip glass. A liquid inflow and a vacuum outflow tubing were installed to generate aCSF current through the chamber. A cortical slice was placed in the chamber and secured with a metal ring. Calcium activities of these slices were imaged with a system consisting of a Leica DM6000 FS upright microscope with a fluorescence light source directed through a 10x

water submerge objective (Leica, Nusslock, Germany). Images were capture with an iXON EMCCD DU-88U camera system (Andor USA, Concord, MA) at ~45-50 fps. Cortical slices were imaged at ~35 ^{o}C in ACSF with 20 µM bicuculline. *MENs treatment and stimulation parameters:* After the liquid flow of the recording chamber was turn off briefly, 20 µL of MENs (concentration of 5 mg/mL in aCSF) was added to the chamber. A 5000 G conical magnet was applied under the glass for ~5-8 mins to draw MENs to a small area within the slice. Liquid flow was turned back on prior to stimulation and recording. For stimulation, the recording chamber with diameter of ~10 mm containing the slice was placed between a pair of electromagnets. A unipolar magnetic field of 750-875 G with 200 ms pulse-width was applied to stimulate the MENs during the imaging process.

Data analysis of Ca imaging. videos of calcium signals from cultured slices and *in vitro* imaging were analyzed post hoc using ImageJ software (NIH) with a Time Series Analyzer plugin (Balaji J. UCLA). For all calcium imaging videos, only active cells (cells that produce calcium fluorescence flashes throughout all frames) were analyzed. To analyze individual cells, three separate background points was chosen by drawing circles with the same diameter as the respective cell body in the surrounding area within the cell body vicinity. The background areas selected did not include dendrites and other visible neuronal structures. Data obtained includes cell body response and the three background fluorescent profiles. Fluorescence calcium signal of a cell ΔF/F was calculated by equation (1) excerpt from Chen et al. group [40] that was developed by Kerlin and his colleagues [41]:

$$\frac{\Delta F}{F} = \frac{(F_{true} - F_B)}{F_B} \qquad \text{and } F_{true} = F_{cell} - r * F_B \qquad \textbf{(Eq.1)}$$

where F_B is the average baseline fluorescence over three different regions surrounding the analyzed cell of interest and r is the contamination ratio standard constant 0.7. These data were then used to construct a calcium peak profiles using OriginPro 9.1 (OriginLab, Northampton, MA). Analyses of peak amplitude, frequency, and durations were done with Peak analysis toolbox of OriginPro9.1.

Statistics. Mean values and final plots were developed in Microsoft Excel, Jmp Analysis 11 (SAS Institute Inc. 2013. Cary, NC), and GraphPad Prism 6 (GraphPad Software, La Jolla, California). All statistical analyses were done with Jmp Analysis 11 and GraphPad Prism 6. ANOVA analyses were used for comparing calcium amplitudes of the same group across different time points (Repeated measures Anova), amplitudes and frequencies of calcium imaging data (one-way). For comparisons that yield statistical significance, Tukey's HSD post-hoc analyses were applied for further comparisons between specific groups.

Results:

MENs could be localized with a strong applied magnetic field and produce electric field with a weaker applied field. To confirm the attraction property of MENs towards a strong external Nd magnetic field, a uniform mixture of 0.1% agarose with 5 mg/ml fluorescent fMENs-Saline solution was prepared. This particular agarose concentration ensures the similarity in texture to brain tissue. We observed a uniform distribution of MENs throughout the mixture (Figure 5-top) prior to magnet application. An external Nd magnet of ~4500 G was placed at the center of the solution surface and found there was localization of fMENs surrounding the center of the applied field (Figure 5A-bottom). Furthermore, the localization appeared to be within 2-3 mm in diameter with respect to the 1 mm tip of the conical-shaped Nd magnet. This suggests MENs can be attracted to a relatively small area with a strong magnetic field.

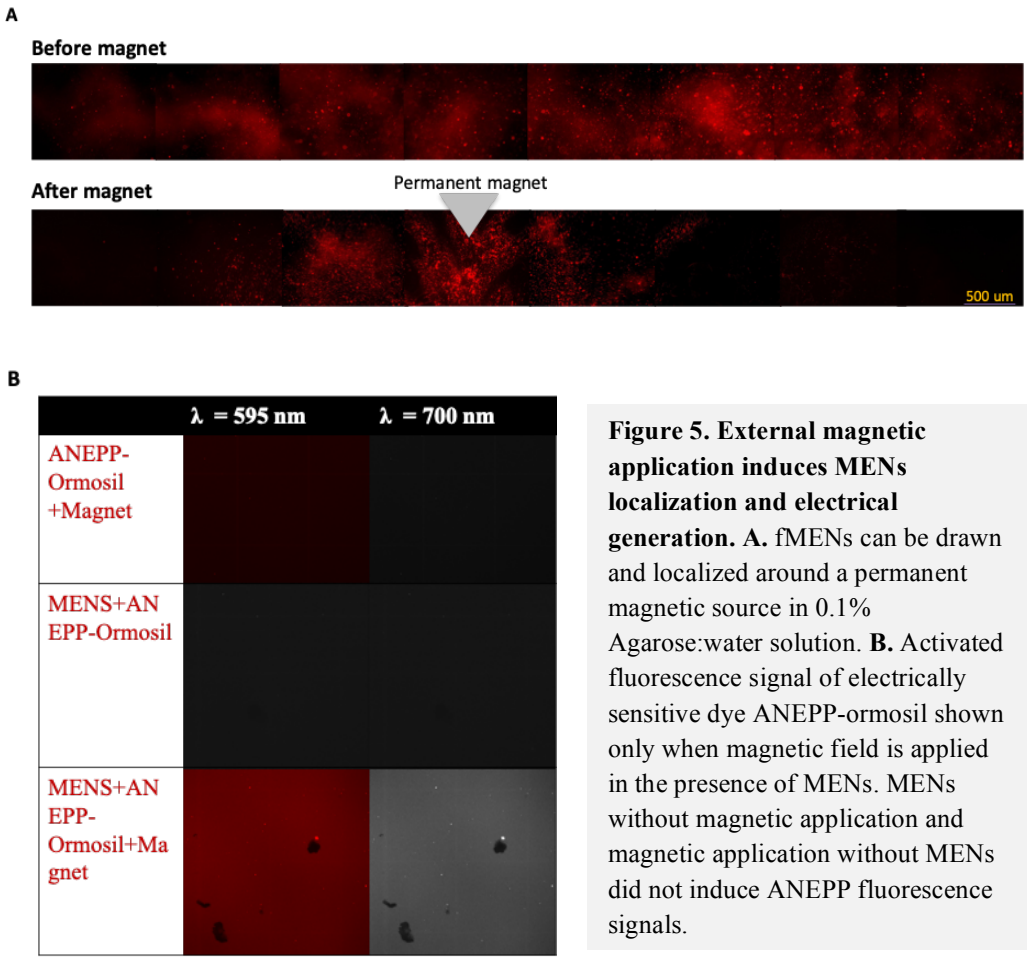


Figure 5. External magnetic application induces MENs localization and electrical generation. A. fMENs can be drawn and localized around a permanent magnetic source in 0.1% Agarose:water solution. **B.** Activated fluorescence signal of electrically sensitive dye ANEPP-ormosil shown only when magnetic field is applied in the presence of MENs. MENs without magnetic application and magnetic application without MENs did not induce ANEPP fluorescence signals.

In a separate experiment, a uniform mixture of 5 mg/ml MENs-saline solution was prepared with an electrically sensitive fluorescent dye ANEPP-ormosil. We observed an increase in ANEPP fluorescent signals at both emission wavelengths 595nm and 700nm when a ~450 G external field was applied (Figure 5B). No fluorescent signals were observed in solution that only contain ANEPP-ormosil dye with magnetic application and solution that contain ANEPP-ormosil dye plus MENs without magnetic application (Figure 5B). This suggests a weak external field can be used to induce MENs generation of electric field.

Magnetic stimulation of MENs enhanced neuronal activity in cultured and acute cortical slices. We first assessed the effects of magnetic stimulation of MENs on neuronal activity in organotypic cortical slices prepared from GCaMP6 transgenic mice. After the slices reached 5 days old *in vitro*, they were loaded with MENs, application of continuous magnetic wave (10 Hz for 10s sinusoidal waves at 450 G) resulted in a near 200% increase in peak amplitude of calcium transients from individual neurons (Figure 6A and B, F/F_o of 0.809 for baseline vs 3.586 for magnet on, $p < 0.005$, Repeated ANOVA, Tukey's HSD). In slices that were initially active, there was a significant increase in calcium signals; whereas in slices that were not active, magnetic stimulation caused activation of calcium activity (Figure 6A and B). We also found dramatic gains in spike frequency during period when the magnet was turned on (2.6 spikes/cell) compare to baseline (1.43 spikes/cell) and after magnet was turned off (1.46 spikes/cell) (Figure 6C and D, $p < 0.01$, Repeated ANOVA, Tukey's HSD).

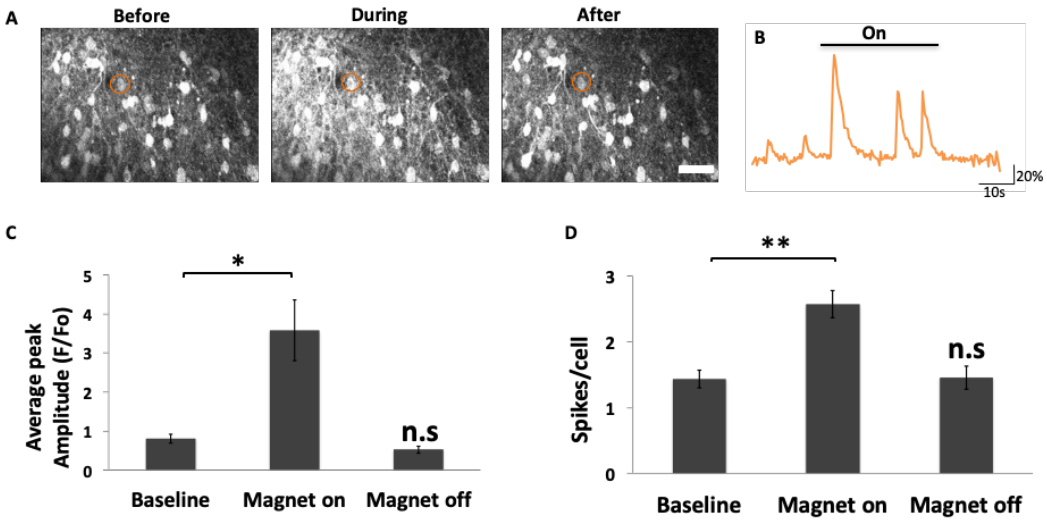


Figure 6. Magnetic stimulation of MENs-loaded cortical slice cultures induced neuronal activity. Cultured cortical slices that expressed GCaMP6 in pyramidal neurons were loaded with MENS by adding 20 µl of 5 mg/mL MENs solution, followed by applying a conical permanent Neodymium (Nd) magnet for ~15 minutes. Magnetic stimulation was made with a pair of electromagnets at 10 Hz. A. Representative two-photon images of calcium signals in periods before, during, and after magnetic stimulation. B. A sample trace of calcium transients measured from a neuron labeled with red circles in (A). **C, D**. Increases in average calcium spike amplitude (C) and number of spikes per cell (D) during the stimulation period. Calcium activity returned to baseline level after the magnet was off. *Scale bar*: 50 µm. *: $p<0.05$, **: $p<0.01$. Repeated measured ANOVA, Tukey's HSD, n = 9 slices.

In order to improve the temporal precision of the stimulation, asingle pulse (unipolar square pulse at 750-875 G with 200 ms duration) of magnetic wave was applied and tested the effect in acutely prepared cortical slices from GCaMP6 transgenic mice (Figure 7A-E). We found that evoked calcium response had much shorter latency period, with over 80% of responses occurring in less than 1 second, with the quickest response being ~75 ms (Figure 7A, C, D, and E). The mean amplitude of calcium spikes at baseline was 0.127 ± 0.035, which became 0.541 ± 0.056 during magnetic stimulation, and returned to magnet was turned off ($p < 0.005$, Repeated ANOVA, Tukey's HSD; Figure 7A, B). This suggest unipolar magnetic wave can shortened neuronal response latency to MENs stimulation.

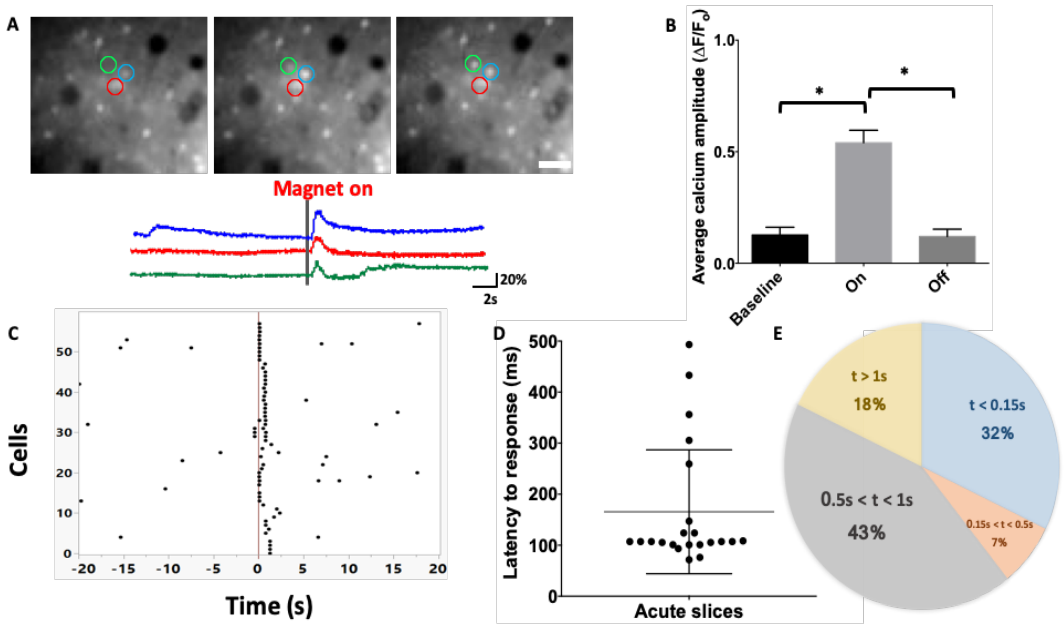


Figure 7. Single unipolar magnetic pulses induced neuronal activity in MENs-loaded cortical slices. Cortical slices prepared from GCaMP6 transgenic mice were loaded MENs similar to Fig. 2 and stimulated with single pulses of magnetic field (~750-875 Oe at 200 ms pulse-width). **A.** Sample images of neurons in a slice and traces of calcium transients measured from the circled neurons. **B**. Average peak amplitudes of calcium spikes measured at baseline and after the magnets were turned on and off. **C.** Temporal response profile of all responding cells. **D**. Response latency of cells that between 0 and 500 ms after magnet was turned on. **E.** Percent distribution of response time from active cells ranges from less than 150 ms ($t < 0.15s$), to between 150ms to 500ms ($0.15s < t < 0.5s$), between 500ms and 1s ($0.5s < t < 1s$), and over 1s ($t > 1s$). *Scale bar:* 50 µm, *p<0.005, Repeated-measured ANOVA, Tukey's HSD. n = 13 slices (total 4 animals)

Discussion

To examine the magnetic and electrical properties of MENs, functionalization of MENs with GMO was done to prevent particle agglomeration then applied strong magnetic field to an aqueous solution of fluorescently labeled MENs to assess its localization with respect to the magnet location. We found the localization of MENs towards a strong permanent magnetic field in the order of ~3500 G could be as small as 2-3 mm in diameter surround the center of the field. Its electrical output was measured by applying a weaker external field in the presence of an ANEPP electrically sensitive dye and found ANEPP fluorescent signal increased only when during magnetic application. The ultimate goal of this study is to determine the effects of MENs stimulation, by a weak external magnetic field at the right frequency, on neuronal activity. We set out to test this hypothesis by first applying MENs to cultured organotypic GCaMP6 mice brain slices *in vitro*. Due to high amount of electromagnetic noise generated by the stimulating electromagnets, calcium imaging gave us the advantage of monitoring neuronal activity without any interference that would not be possible with electrical recording. Upon magnetic application, we observed dramatic increases in neuronal calcium response. This is a clear indication of the stimulatory effects of MENs on neuronal activity. Our results are consistent with previous reports of using $BaTiO_3$ to activate neurons by either complexing this piezoelectric compound with a vibratory sensitive core of gum Arabic then stimulate with high intensity ultrasound[42] or by direct stimulation with focus ultrasonic wave[43]. Furthermore, Marino et al. also performed sodium imaging and found the observed gain in activity was TTX-sensitive. With their ultrasound stimulation parameters however, their neuronal response times to the stimulation were in the order of

10th of seconds. Whereas the observed response time were sub-hundredth of milliseconds when a unipolar field is applied. By applying unipolar field, this enabled the resulted polarization to match more closely with the depolarization phase of an action potential thus resulted in quicker neuronal firing. Since calcium imaging signals are inherently limited by its slower kinetics than electrical signals and GCaMP6s signal is known to have a temporal resolution of 100-150 ms[40], the actual latency is estimated to be 50 ms and less. This is a good temporal resolution and a clear indication of the stimulatory effects of MENs on neuronal activity.

A big advantage of this technique compare to others is these MENs complex-containing the robust piezomagnetic core material $CoFe_2O_4$, which enables full control of particles localization. My next aim is to assess whether MENs can be directed through the BBB and localize to a brain region with minimal toxicity. Then further evaluation of its stimulatory effects *in vivo* along with its potential application to a disease model can be done.

Chapter 3 – MENs can be drawn across BBB and localized in brain cortex with minimal toxicity

Introduction

The blood brain barrier (BBB) represents an important physiological barrier that serves as a defense mechanism against harmful foreign pathogens/various toxic chemicals. However, the BBB also represent a physical barrier preventing effective targeting of the brain parenchyma with diagnostic and therapeutic agents. Targeting through BBB is a clinically important, yet difficult goal to achieve in developing therapeutic technologies[44].

Nanoparticle-based molecular transport has been the focus of recent strategies to enhance delivery and reduce toxicity in many diverse fields[34, 45, 46]. Magnetic-based nanoparticles in particular have been an important focus of these developments. This is due to their unique properties to respond to magnetic fields as well as their safety, i.e. their usage as MRI contrast agents[34, 47]. However, magnetic nanoparticles applications to the central nervous system, particularly the brain, have been very limited, due in part to the adverse effects of nanoparticles when subject to external field for long period of time, for example the overheating effects[31] or the demagnetization (i.e. loss in magnetic response)[33, 48] of particle itself. There are also the FDA limitations of applied magnetic field strength on human patients (~80k G or 8 T for adults and 40k G or 4 T for children)[49]. Therefore, more efforts have focused on developing magnetic nanoparticles that can withstand long duration of applied magnetic field without alteration of their intrinsic properties as well as particles that can be localized efficiently with a weaker applied field[50, 51]. Majority of these studies have focused on cancer animal models in the

periphery or drug targeting to various organs[52, 53, 54].

MENs with the iron oxide ferromagnetic core, which complexed with a central cobalt atom, enhancing their magnetorestrictive response to a weaker applied magnetic field while enable it to retain its properties without demagnetization[35, 36]. This makes MENs a good candidate for targeting across BBB using weaker magnetic field and localize it to a brain area. Our aim is to determine whether MENs can be directed across BBB to the brain and to characterize its spatial and temporal profile. We also assess their short-term toxicity, in the context of neural inflammation, in the brain tissue.

Methods

***In vivo* vascular fMENs imaging.** *Animal preparation:* Thy1-GCaMP6s mice (n=5) were anesthetized with (87.3 mg/kg / 13.7 mg/kg) Ketamine/Xylazine mixture and the scalp was removed. A cranial window (3 mm in diameter) was made, in one cortical hemisphere, of an area 1.5 mm lateral from midline and 1 mm posterior from bregma. Two pieces of cover glass (a 3-mm diameter glass adhered to a 5-mm diameter glass) were glued together with cyanoacrylate glue with the 3-mm glass facing the brain surface. After the glass was installed on the cranial window, an L-shaped titanium head-plate was also glued on the posterior region of the head for head fixation during imaging. The animals were allowed to recover for 7 to 10 days. *MENs injection:* single injection of 200 µL fMENs at 5 mg/ml retro-ortibally was performed. Two-photon imaging (as describe in method section of chapter 2) was done of cerebral blood vessels above the cortical area were taken at baseline, and 0, 5, 10, 20, and 30 minutes after fMENs injection.

***In vivo* fMENs imaging to determine particle localization.** Using GCaMP6 mice with prepared-windows as described above, a single injection of 200 µL fMENs at 5 mg/ml retro-orbitally was performed. Two sets of stacked cylindrical magnets of 6000 G field strength were applied to draw the MENs towards windows area for 10 minutes. The cylindrical magnets were then replaced by a 5000G conical magnet to further localize the MENs. Images were taken at various time points: before fMENs injection, after fMENs injection, after fMENs injection with drawing magnet application, and 24 hours post treatment. Animals were then euthanized and brain were dissected. Brain were then section into 250 µm thick slices and imaged immediately to assess nanoparticles

localization. All imaging was done with two-photon microscopy, procedure similar to described above.

Immunofluorescence analysis of astrocytes and microglias. To determine potential glial reactivity and neuroinflammation induced by the MENs, we used an antibody against ionized calcium binding adaptor protein (IBA1) to label microglia, and an antibody against Glial Fibrillary Acidic Protein (GFAP) to label astrocytes[55, 56]. *MENs treatment:* C57BL6J mice (n = 4 animals/group) were assigned into three groups: control (saline injection + magnetic stimulation), MENs-treated (MENs without magnetic stimulation), and MENs-treated with magnetic stimulation (MENs+ms). MENs-treated only group was subjected to ~6000 G Neodymium (Nd) magnets placed above and below one brain hemisphere to draw the particles to that hemisphere. MENs-treated with magnetic stimulation group was subjected to both the Nd drawing magnets and also two electro-magnetic magnets for five 2-minutes duration stimulation period at 10 Hz, with each period separated by five-minutes break gap. Within the MENs-treated+ms group, subgroups of mice were then euthanized at various time points after treatments (4 hours, 24 hours, 3 days, and 1 week). *Animal perfusion/ Brain sectioning:* mice were deeply anesthetized with (87.3 mg/kg / 13.7 mg/kg) Ketamine/Xylazine and perfused transcardially with PBS buffer (0.01 M phosphate saline PBS, 80ml) followed by 4% formaldehyde PFA (150 ml) at room temperature. The brains were then dissected and placed in 30% sucrose for 48-72 hours at 4^{o}C. The tissue was then frozen in O.C.T Compound (Sakura Finetek, Torrance, CA) at -80 ^{o}C, and sectioned at 40 μm thickness coronally with a cryostat. Immunofluorescence was performed on free-floating sections as followed: After the brain sections were washed four times with PBS, then incubated

with blocking solution (5% Normal Goat Serum, 0.1% Triton, 1% BSA) for 1 hour at room temperature, they were incubated overnight with a primary antibody. Brain sections were then washed the next day and incubated with fluorescent goat anti-rabbit secondary antibody for two hours. For nuclear staining, 4', 6-diamidino-2-phenylindole (DAPI; 1: 10,000) was added to the solution for a final 5 minutes. Finally, slices were washed three times with PBS and mounted and imaged using a Neurolucida confocal imaging system. Primary anti-GFAP (mouse; 1:800, Sigma Aldrich G3893) and anti-IBA1 (goat; 1:200, ABCam ab5076) antibodies were used to labeled astrocytes and microglias respectively, which were followed by secondary antibodies of goat anti-mouse Cy5 (1:500, Jackson Immuno) and donkey anti-goat 488nm (1:1000, Fischer Scientific).

Statistics. Mean values and final plots were developed in Microsoft Excel. All statistical analyses were done with Jmp Analysis 11 (SAS Institute Inc. 2013. Cary, NC), and GraphPad Prism 6 (GraphPad Software, La Jolla, California). One-way ANOVA analyses were used for comparing positive stained cells between groups of IBA1 and GFAP immunohistochemistry experiment. For comparisons that yield statistical significance, Tukey's HSD post-hoc analyses were applied for further comparisons between specific groups.

Results

MENs remained in the blood stream up to 30 mins post-injection. After injecting fMENs into the tail vein of mice, repeated two-photon imagings were done to track the changes in fluorescence intensity in the cortical vascular network. There was high level of fMENs in blood stream immediately after injection, which decreased rapidly over a short period of time (Figure 8A-B). The fluorescence intensity in blood stream dropped to about 50% in less than 15 minutes and was completely abolished in about 30 minutes post injection. This suggest that the optimal time window for applying a drawing magnet is within 20 minutes after intravenous injection of MENs.

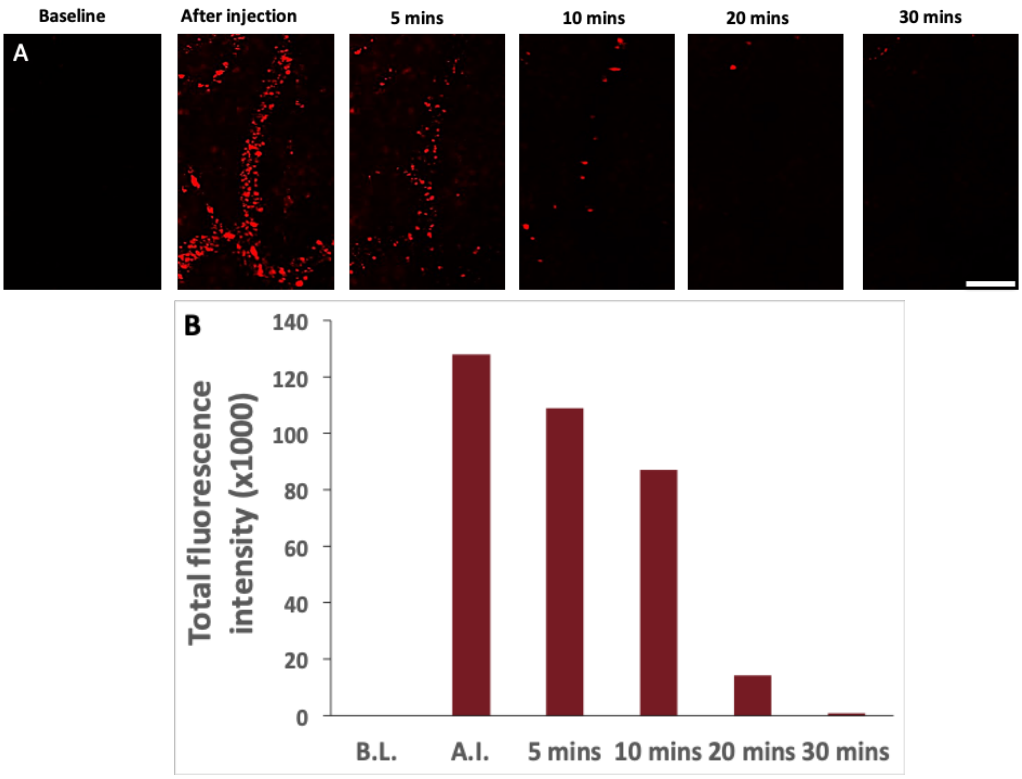


Figure 8. *In vivo* delivery of fMENs into blood vessels. **A.** Vascular MENs level reduced after 10 mins post injection. *In vivo* two-photon images of cortical blood vessels were taken at baseline (B.L.) and at 5, 10, 20, and 30 mins after intravenous injection of 10 µg fluorescence labeled MENs (fMENs). No magnetic field was applied. B. Measured fluorescence signal of fMENs in blood vessels. *Scale bar:* 100 um.

Magnetic delivery of MENs to brain. To assess the effectiveness of using strong permanent magnet to deliver MENs to the brain, fluorescence-labeled MENs (fMENs) were delivered by injecting through retro-orbital vein to mice with cranial windows, then applied a cone- shape Nd magnets of strength ~3500-4500 G above and below the head for 20 minutes. *In vivo* two-photon imaging showed that fluorescence signals were visible through cranial windows in mice with fMENs and magnet attraction, but not in mice with MENs injection only, suggesting that the fMEN were drawn by a magnetic field to cross BBB and entered brain parenchyma *in vivo* (Figure 9A-B). When the same mice were imaged 24 hours later, there remained about 70% fluorescence signal in the same brain

region, suggesting that a significant amount of fMENs remained in cortical tissue at 24 hours after delivery. Finally, when the same animal was perfused after last imaging time point and cortical coronal slices were section, we observed significant level of fMENs at the upper cortical surfaces (Layers 1-4 of cortex) with some detected at deeper layer, mainly in the hemisphere where the magnet applied (Figure 9C). The opposite hemisphere of the same brain slice showed only minute traces of fMENs. This suggest magnetic localization parameters can efficiently direct fMENs close to one brain hemisphere and particles retain at the area for at least 24 hours post treatment.

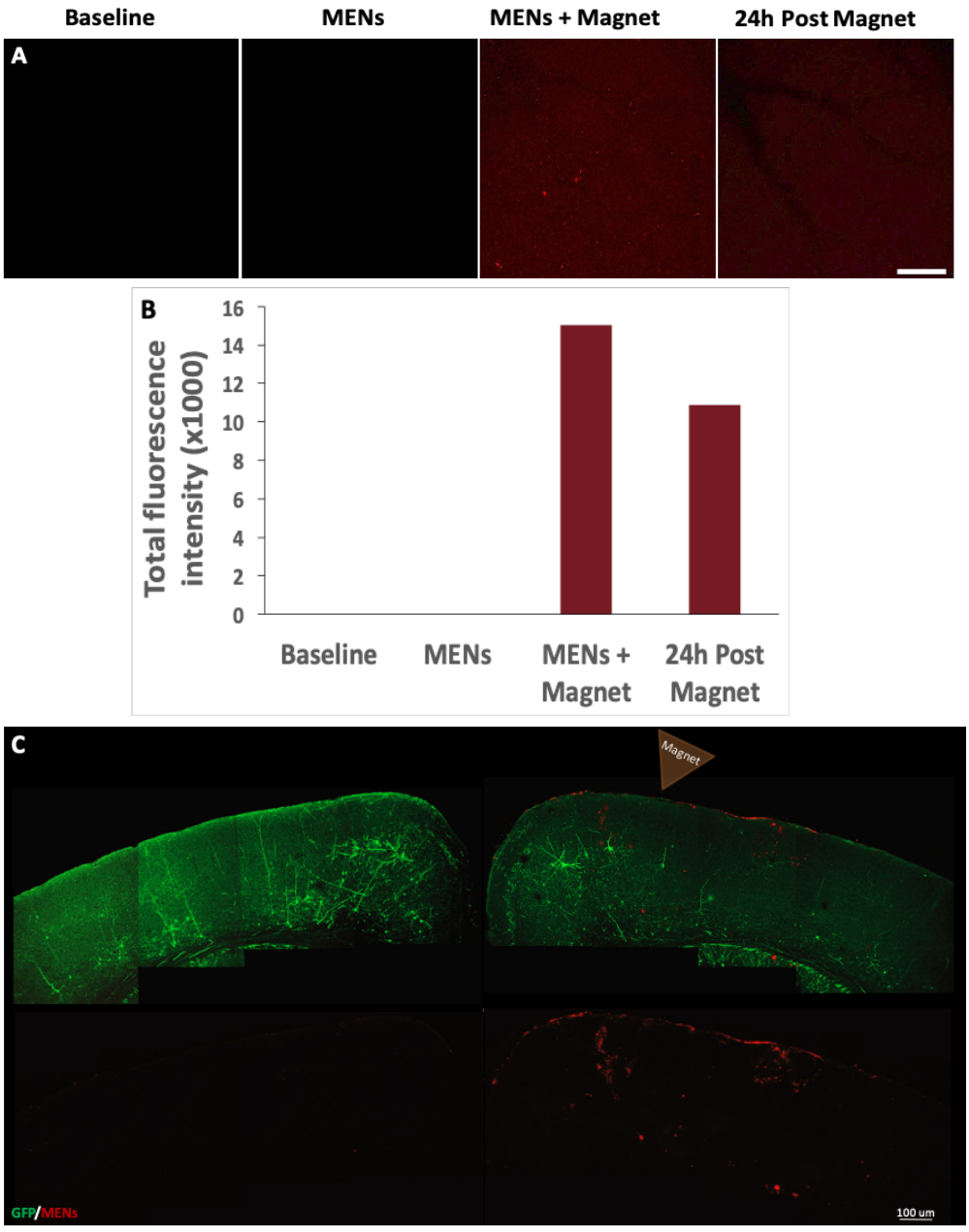


Figure 9. *In vivo* delivery of fMENs into mouse cortex. A. *In vivo* two-photon images of layer 2/3 sensorimotor cortex were taken at baseline (C1) and after (C2) intravenous injection of 10 µg fMENs, after applying ~4000-5000 Oe magnetic field for 45 minutes (C3), and on the second day after injection (C4) indicates successful delivery of MENs to cortical tissue after magnet application, which MENs remained up to at least 24 hours after delivery. B. Measured fluorescence signal of fMENs in brain tissue. C. Confocal images of mous cortex showed high level of fMENs in cortical hemisphere where magnet is applied *Scale bar:* 100 µm.

MENs treatment with magnetic stimulation resulted no change in astrogliosis and microglial activation. Delivering MENs to a specific brain region may initiate inflammatory responses of glial cells, particularly astrocytes and microglias (Figure 10A-H). To assess whether astrogliosis and microglial activations occurred, we made intravenous injection of 200 µl of MENs at 200 µg/ml and magnetic delivery of MENs. Mice that received MENs delivery and MS were also assessed at various time point after the stimulation. There were no significant differences in the densities of both IBA1 and GFAP positive cells up to one week among all the groups (Figure 10 A-C, G, H). Considering these results together, MENs-MS did not induce any apparent inflammatory response in mice.

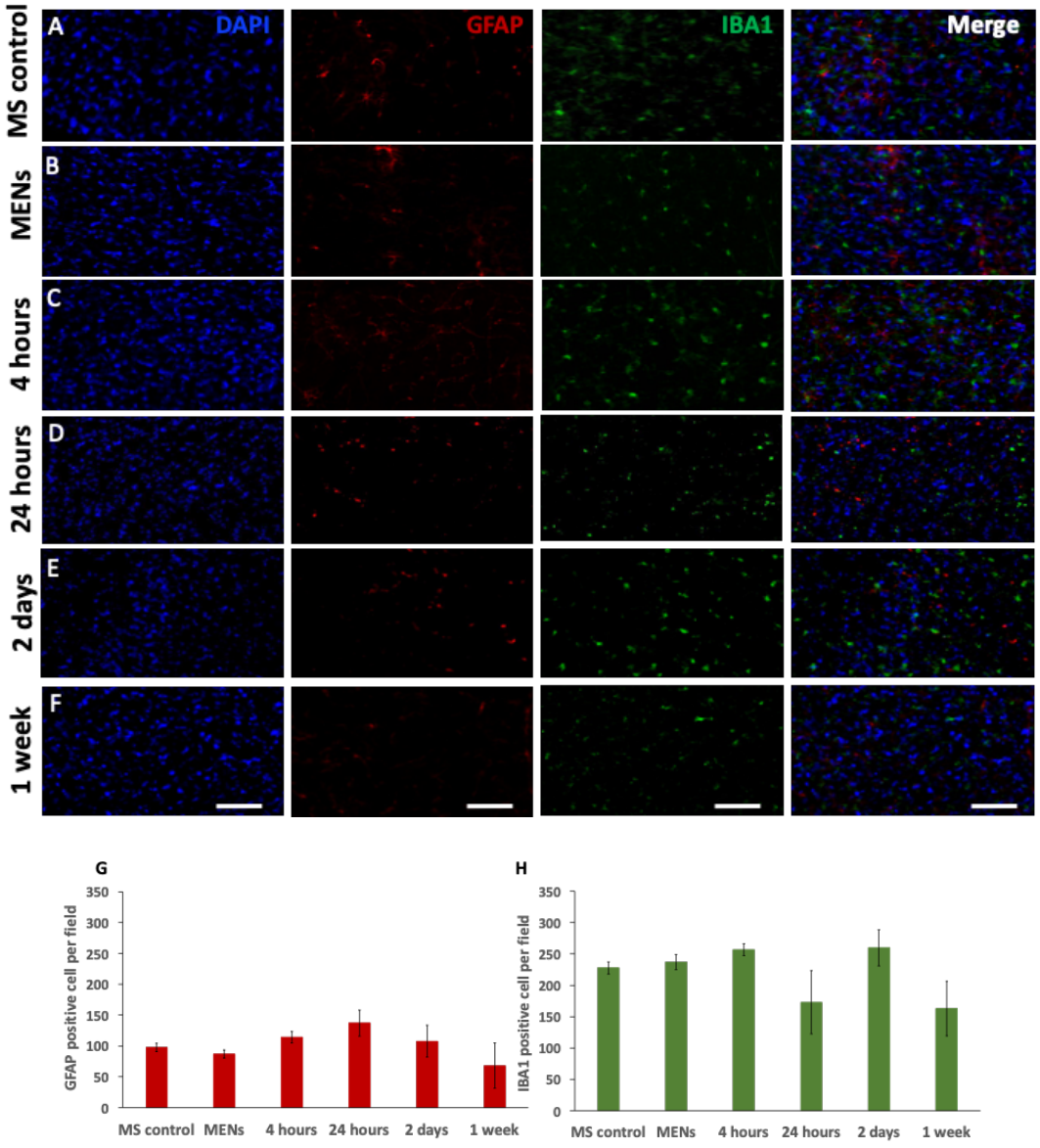


Figure 10. No significant changes in the numbers of GFAP and IBA1 positive cells after MEN delivery and MENs-MS. Brain sections were obtained from six groups of C57BL mice including MS control, MENs-delivery (MENs), and MENs delivery followed by MS at 4h, 24h, 2 days, and 1 week after. **A-F.** Sample confocal images of coronal cortical slices stained for DAPI, GFAP, and IBA1 at different time points. **G, H**. Quantifications of GFAP (G) and IBA1 (H) positive cells showed no significant differences in the numbers of these cells after MEN delivery or followed by MS. n = 4 mice/group. *Scale bar:* 50 µm

Discussion

MEN's ability to be attracted to a strong permanent field was further tested *in vivo* by injecting the nanoparticles into the blood stream and drawn them towards one brain hemisphere with the permanent magnet. MENs can be drawn across BBB and strictly localized to one hemisphere where the magnet is applied above the skull area of that hemisphere. This presence of MENs remained in the brain tissue up to at least 24 hours after treatment. Furthermore, earlier reports have also shown that these MENs, after being drawn across BBB, appear to localize mainly on the extracellular side both in the CSF[57] and binding to extracellular membranes of various type of CNS residential cells[35, 57]. However, there are also evidence of MENs distribution in cell cytosol[57]. It was also found that the chemical structure and integrity of MENs were not compromised during this process of navigation from the blood stream across the endothelial cell layers of the BBB to be localized in the brain[57]. After a single IV injection of MENs without magnetic field application, MENs retained in the vasculature up to 30 minutes but dissipated quickly. This suggests immediate application of permanent magnetic field right after MENs injection for at least 30 minutes is required to ensure maximum delivery of MENs to the brain tissue.

In CNS, we assessed neuro-inflammatory response to MENs by treating animals with MENs and evaluate their microglial and astrocytic activations at multiple times point after MEN treatment and magnetic application. No significant changes in microglial activation nor astrogliosis were observed in mice injected with MENs then drawn with magnets compare to mice that were only injected with MENs without magnet application as well as control mice with only saline treatment combine with magnetic application.

Furthermore, IBA1 and GFAP levels remained at similar level, up to 1 week, compared to control group. In addition to our findings, Kaushkik et al. also reported no changes in peripheral immune response, liver and kidney toxicity, nor abnormal behavioral changes up to one week after MENs treatment with magnetic drawing towards the brain cerebral cortex[57]. Taken together, these results provide encouraging information on the safety of MENs to the biological system that therefore can potentially be applied for translational research and clinical treatment.

Understanding the spatial and temporal distribution of MENs in an *in vivo* system in combination with the observed stimulatory effects on *in vitro* cultured neurons, the next aim is to assess MENs effects on neuronal activity in a living animal. Our observation of MENs targeted to one cortical hemisphere and remained there for at least 24 hours raises additional questions of whether the stimulatory effects of MENs *in vivo*, if true, will last for at least 24 hours after single treatment? And will the effects be only localized to the hemisphere where particles presence was observed? Chapter 4 will go into detailed of the experiments we conducted to answer such questions.

Chapter 4 MENs stimulation enhance cortical neuronal activity *in vivo* that lasted up to at least 24 hours

Introduction

In the past 30 years, modern noninvasive brain stimulation techniques have made remarkable contributions to the research, diagnosis, and treatment of many neurological diseases. Ranging from clinically used transcranial electrical stimulation (TES) to magnetic stimulation (TMS) to recently developed research-only optogenetics and focus ultrasound stimulation (FUS), non-invasive brain stimulations have made great strides in advancing neuroscience. These, however, are often limited by the relatively low spatial and temporal resolution (TES, TMS)[14, 15, 58], required genetic modification (optogenetics)[19], scarce penetration into the deeper brain regions (TES, optogenetics), and unquantified detrimental adverse effects (FUS)[26, 27]. Another approach that well-characterized and well-used in the clinical settings are represented by deep brain stimulation (DBS). This has shown to be very effective in a targeted, localized modulation of functions of a brain region. But the technique comes with the cost of risk due to its invasive surgical operation followed by long-term inflammation and gliosis at the surgical site[59].

The landscape of nanoparticles applications in neuroscience, particularly neural stimulation, is still in its infancy with many optimizations needed to be done. Specially, non-invasive neural stimulation with nanoparticles are still in its early stages of *proof-of-concept* studies and *in vitro* demonstrations, which are promising but not yet translatable[33, 42, 60]. MENs hold promising potentials observed both in our study and others[35, 57], to be targeted across BBB and localized to a relatively small brain region with

minimal toxicity. Furthermore, promising MENs stimulation of neuronal activity observed *in vitro* (Chapter 2) encourages us to examine its effects in an *in vivo* system as well as characterize temporal and spatial profiles of the response.

Methods

In vivo calcium imaging of cortical neurons in GCaMP6 transgenic mice.

Animal preparation: Thy1-GCaMP6s mice (n=5) were anesthetized with (87.3 mg/kg / 13.7 mg/kg) Ketamine/Xylazine mixture and the scalp was removed. A cranial window (3 mm in diameter) was made, in one cortical hemisphere, of an area 1.5 mm lateral from midline and 1 mm posterior from bregma. Two pieces of cover glass (a 3-mm diameter glass adhered to a 5-mm diameter glass) were glued together with cyanoacrylate glue with the 3-mm glass facing the brain surface. After the glass was installed on the cranial window, an L-shaped titanium head-plate was also glued on the posterior region of the head for head fixation during imaging. The animals were allowed to recover for 7 to 10 days. *MENs treatment:* After the animals were sedated via intraperitoneal injection of chloprothixene (0.04 mg/ml) and isofluorane, they were injected retro-orbitally with 200 µl $CoFe_2O_4$-$BaTiO_3$ –Texas Red (fMENs) at 5 mg/ml. MENs localization paradigm is similar to described above: two sets of stacked cylindrical magnets of 6000 G field strength were applied to draw the MENs towards windows area for 10 minutes. The cylindrical magnets were then replaced by a 5000G conical magnet to further localize the MENs for 15 minutes. Following the delivery of the MENs, the animal's head was stabilized by fixing the L-shaped titanium metal plate to a custom-made base and the body temperature was maintained. Magnetic stimulation was made by placing two electromagnets (~500 ms pulse-width at ~300-450 G) closely on each side of the head. Low power images show the pattern of blood vessels on brain surface were captured. Two-photon images of calcium transients of layer II/III neurons were taken at baseline, during magnetic stimulation, and after magnetic stimulation was turned off. We tested a

combination of different stimulation frequencies (5, 10, 20, 50, and 100 Hz). In each imaging field, two optical planes in layer II/III were imaged for 2 minutes at 4-5 frames per second (fps).

Mesoscopic whole brain imaging windows preparation. Thy1-GCaMP6s transgenic mice were anesthetized with (87.3 mg/kg / 13.7 mg/kg) Ketamine/Xylazine mixture and the scalp was removed. After a large cranial window (approximately 10 x 8 mm) was made by removing the skull, a piece of curved cover glass was installed and tightly sealed with super glue and dental cement. The animals were allowed to recover for 7 to 10 days prior to imaging. Mesoscopic imaging set-up used is similar to previous developments[61, 62]. In brief, whole brain calcium activity was imaged with a system consisting of a Leica DM6000 FS upright microscope with a Leica EL-6000 120W mercury fluorescence light source directed onto the prepared glass skull surface. Images were capture with a two-lens system composes of a top zoom Nikkor 70-300mm of aperature f3.5-5.6 set for 70mm at f3.5 couple through an inverted lens adapter to a bottom Nikkor 50mm prime at f1.4. Videos were capture with an iXON EMCCD DU-88U camera system (Andor USA, Concord, MA). Delivery of MENs and magnetic stimulation paradigm are done exactly as described in two-photon imaging.

C-fos staining of cortical neurons. C57BL6J mice (n = 4 animals/group) were assigned into three groups: control (saline injection + magnetic stimulation), MENs-treated (MENs without magnetic stimulation), and MENs-treated with magnetic stimulation (MENs+ms). MENs-treated only group was subjected to ~6000 G Neodymium (Nd) magnets placed above and below one brain hemisphere to draw the particles to that hemisphere. MENs-treated with magnetic stimulation group was

subjected to both the Nd drawing magnets and also two electro-magnetic magnets for five 2-minutes duration stimulation period at 10 Hz, with each period separated by five-minutes break gap. Brain tissues of these mice were stained for c-Fos protein, an indicator of action potential firing[63]. *Animal perfusion/ Brain sectioning:* Four hours post treatment, mice were deeply anesthetized with (87.3 mg/kg / 13.7 mg/kg) Ketamine/Xylazine and perfused transcardially with PBS buffer (0.01 M phosphate saline PBS, 80ml) followed by 4% formaldehyde PFA (150 ml) at room temperature. The brains were then dissected and placed in 30% sucrose for 48-72 hours at 4^{o}C. The tissue was then frozen in O.C.T Compound (Sakura Finetek, Torrance, CA) at -80 ^{o}C, and sectioned 40 μm thickness coronally with a cryostat. Immunofluorescence was performed on free-floating sections as followed: After the brain sections were washed four times with PBS, then incubated with blocking solution (5% Normal Goat Serum, 0.1% Triton, 1% BSA) for 1 hour at room temperature, they were incubated overnight with a primary antibody for c-Fos (rabbit; 1:800, Sigma Aldrich G3893). Brain sections were then washed the next day and incubated with fluorescent goat anti-rabbit secondary antibody (1:200; Invitrogen, Carlsbad, CA) for two hours. For nuclear staining, 4', 6-diamidino-2-phenylindole (DAPI; 1: 10,000) was added to the solution for a final 5 minutes. Finally, slices were washed three times with PBS and mounted and imaged using a Neurolucida confocal imaging system.

Statistics. Mean values and final plots were developed in Microsoft Excel, Jmp Analysis 11 (SAS Institute Inc. 2013. Cary, NC), and GraphPad Prism 6 (GraphPad Software, La Jolla, California). All statistical analyses were done with Jmp Analysis 11 and GraphPad Prism 6. ANOVA analyses were used for comparing calcium amplitudes

of the same group across different time points (Repeated measures Anova), amplitudes and frequencies of calcium imaging data (one-way), compare of calcium amplitudes and frequencies across different tested magnetic frequencies and different cortical region in mesocopic imaging (one-way), and compare of positive stained cells between groups of c-Fos immunohistochemistry experiment (one-way). For comparisons that yield statistical significance, Tukey's HSD post-hoc analyses were applied for further comparisons between specific groups.

Results

MENs treatment with magnetic stimulation increased GCaMP6 cortical neuronal calcium activity *in vivo*. *in vivo* two-photon imaging in GCaMP6 transgenic mice was done to assess whether MENs-MS increases cortical neuronal activity. There was a dramatic increase in the mean total amplitude of somatic calcium spikes (F/F_o of 2.37 vs 1.50 for baseline and 1.36 for magnet off, Figure 11A, C) with $p<0.0001$, both cases and response frequency (3.08 spikes/cell vs. 1.96 for baseline with p=0059 and 1.54 for magnet off with $p<0.0001$, repeated ANOVA, Tukey's HSD, Figure 11D). Dendritic calcium transients also showed similar increased fluorescence amplitude and cell spiking patterns with MENs stimulation (Figure 11A, E, and F). These data provide strong evidence that MENs-MS can activate and enhance neuronal activity *in vivo*.

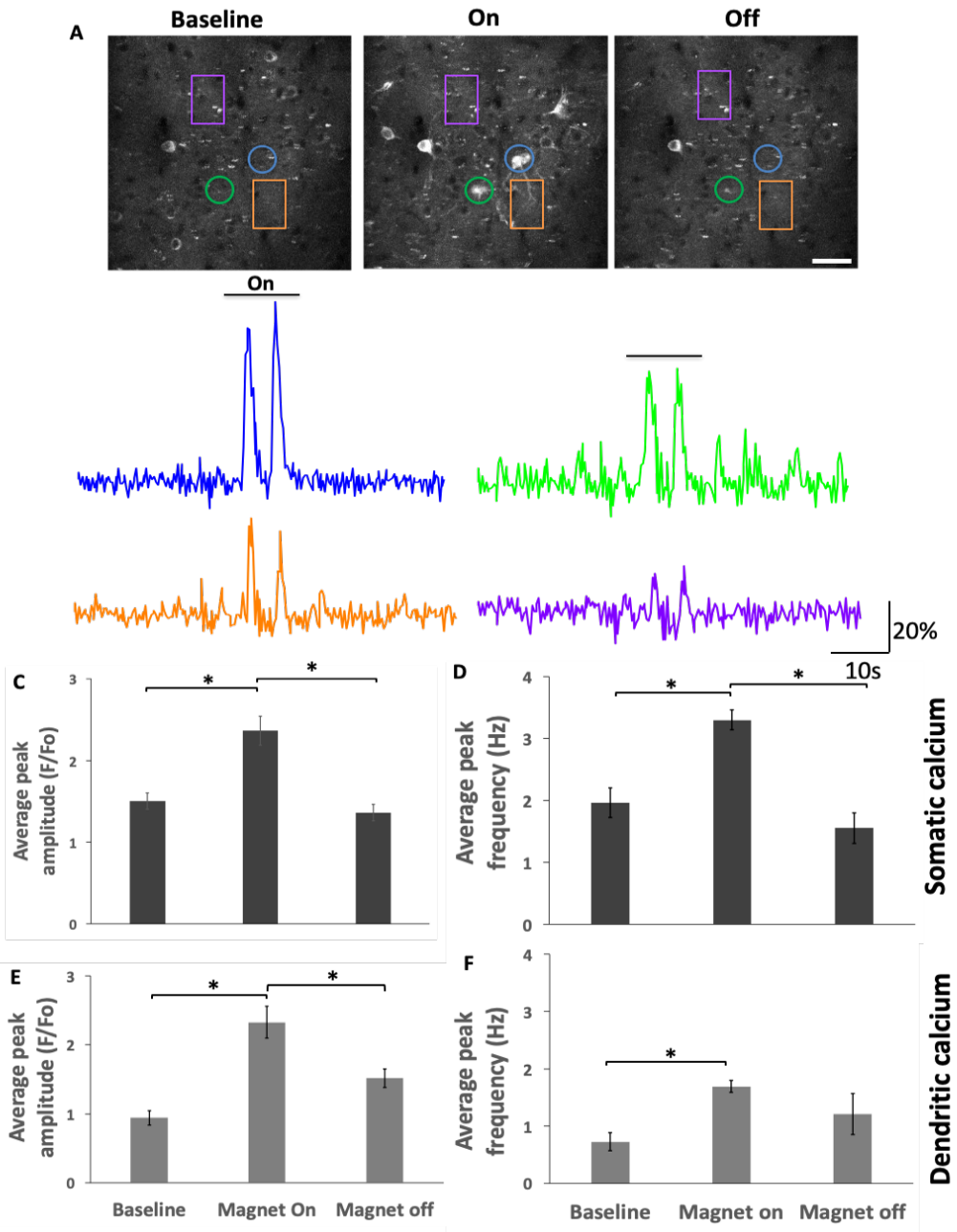


Figure 11. Magnetic stimulation activated cortical layer II/III neuron *in vivo*. A-B. Representative images (A) and traces (B) of calcium transients measured from layer II/III cortical neurons. **C-D.** Significant increases in average calcium spike amplitude (C) and mean spike number (D) of cortical neurons after magnet stimulation was turned on. **D-F.** Significant increases in average calcium spike amplitude (E) and mean spike number (F) of cortical dendritic arean = 5 mice, *p <0.05, one-way ANOVA, Tukey's HSD.

Furthermore, various magnetic frequencies of 5, 10, 20, 50, and 100 Hz were used during the stimulation period, after the magnets were turn off, and ten minutes after they were off. For every magnetic frequency applied during the stimulation period, there were significant increases in both somatic and dendritic calcium spike amplitude (Figure 6D, Figure 7D) and spike frequency (Figure 12A). Interestingly, it appeared that the highest neuronal response (i.e. highest average spike amplitude and frequency) to the stimulation were at 5, 10 and 20 Hz stimulation frequencies (Figure 12A-B). 10 Hz frequency was picked as the standard for *in vivo* stimulation in most of experiments of our study.

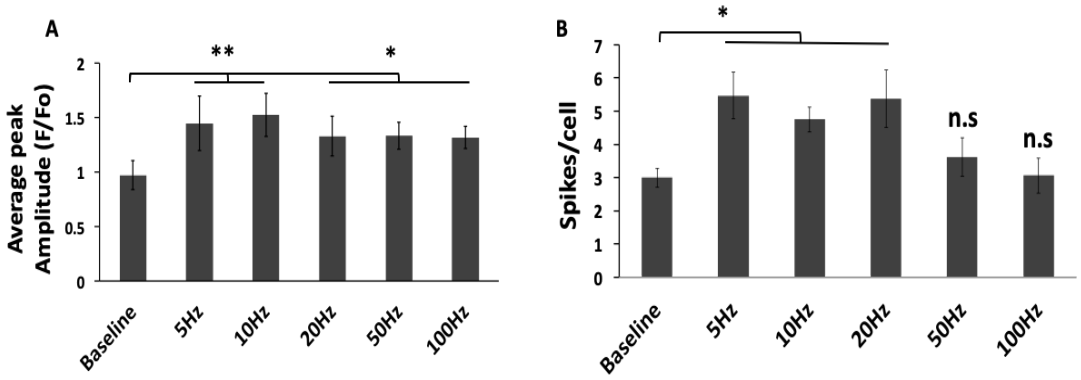


Figure 12. Magnetic stimulation efficiently activated cortical layer II/III neuron *in vivo* between 5-20Hz of applied magnetics frequencies. A-B. Increase in average calcium spike amplitude (A) and total spikes per cell (B) of cortical neurons when magnet was turned on at each frequencies of stimulations. Changes in the amplitudes and spike numbers of calcium transients at electromagnetic stimulation frequencies of 5, 10, 20, 50, and 100 Hz. The data indicate that magnetic waves between 5-10 Hz were most effective in stimulating neurons *in vivo*. $n = 5$. *$p < 0.05$, **$p < 0.01$, *two-way* ANOVA, Tukey's HSD. *Scale bar* – 100μm.

Single MENs treatment with magnetic stimulation increased *in vivo* GCaMP6 cortical neuronal calcium activity at targeted areas for up to at least one day after treatment. Multiple brain stimulations over a period of time may be required to achieve treatment effect in certain neurological diseases. To evaluate this, we gave animals a single MENs delivery, and then performed MS and calcium imaging at

different time points (Figure 12,13). When comparing calcium responses across all time point during period of magnetic application, there was a large increase in the amplitude of calcium spikes when MS is applied immediately after MENs delivery, when compared with baseline where magnet was applied without MENs treatment (mean F/Fo of 2.7 ± 0.27 vs 1.13 ± 0.07 of baseline, $p < 0.05$, one-way ANOVA, Tukey's HSD, Figure 12B, D). Neuronal activity could still be stimulated with similar parameters of MS at 24 hours after MENs delivery (F/Fo of 2.35 ± 0.28, $p<0.05$, one-way ANOVA, Tukey's HSD), but not at 72 hours after (Figure 13D. F/Fo of 1.21 ± 0.18, $p>0.05$, one-way ANOVA). There also was higher number of active cells after MENs treatment up to 24 hours (~90 cells of MENs+ms and 82 cells of 24 hours vs. 62 of ms-only baseline, Figure 13C), and recover to baseline level at 72 hours (~55 cells, Figure 13C). Individual time point results also showed increased in calcium amplitude when magnetic field was applied only in MENs+ms group and 24 hours after (Figure 14A-D). The result suggests that a single delivery of MENs to brain is effective for non-invasive brain stimulation for at least 24 hours.

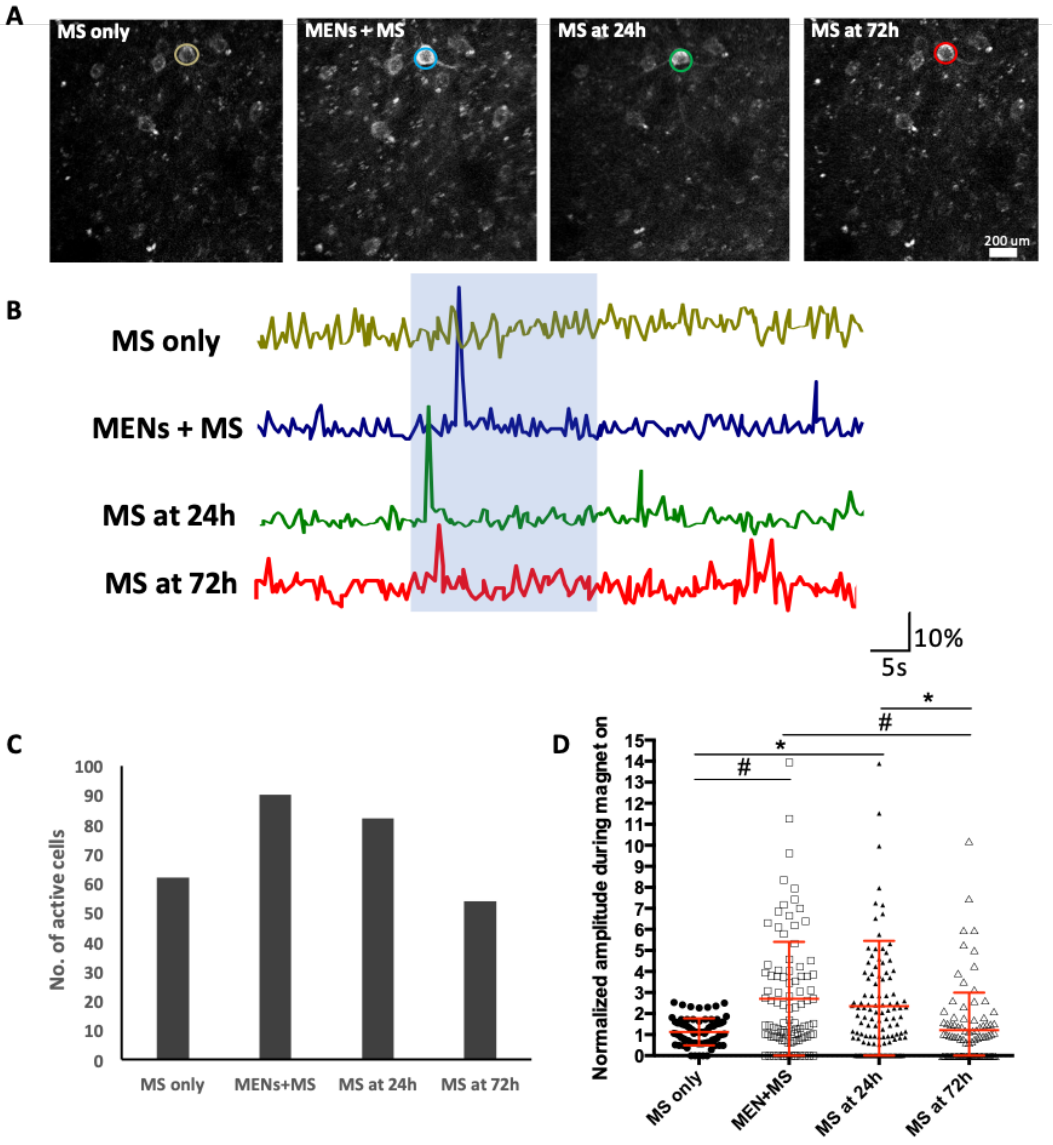


Figure 13. MENs-MS was effective in activating neurons in 24 hours after a single delivery of MENs. A-B. Representative two-photon images and traces of calcium signals of same cortical layer II/III neurons at baseline (MS only) and immediately after MENs delivery followed by MS (MEN+MS), MS at 24 hours after, and MS at 72 hours after. 10Hz magnetic stimulation was applied for 20s in the middle of recording period at each time point. **C**. There was an increase in number of active neurons after MENs-MS, which remained higher up to 24 hours after initial delivery. **D.** There were increases in response amplitude immediately after MENs-MS and at least 1 day after initial delivery, which returned to baseline level at 3 days post-MEN delivery, one-way ANOVA, Tukey's HSD, n=5, *p <0.05, #p <0.005.

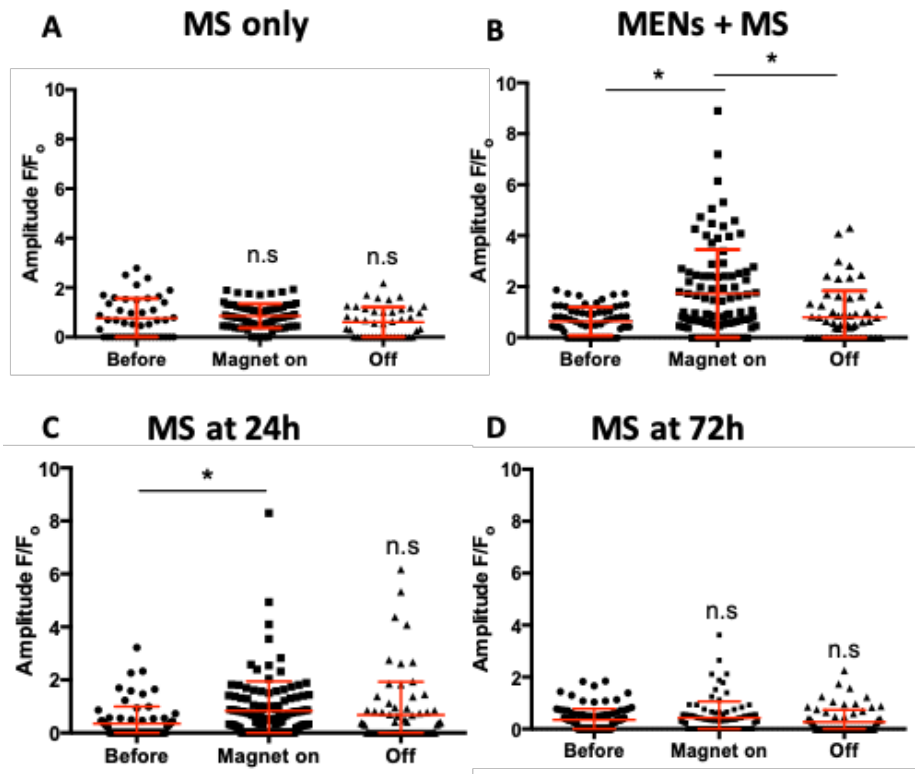
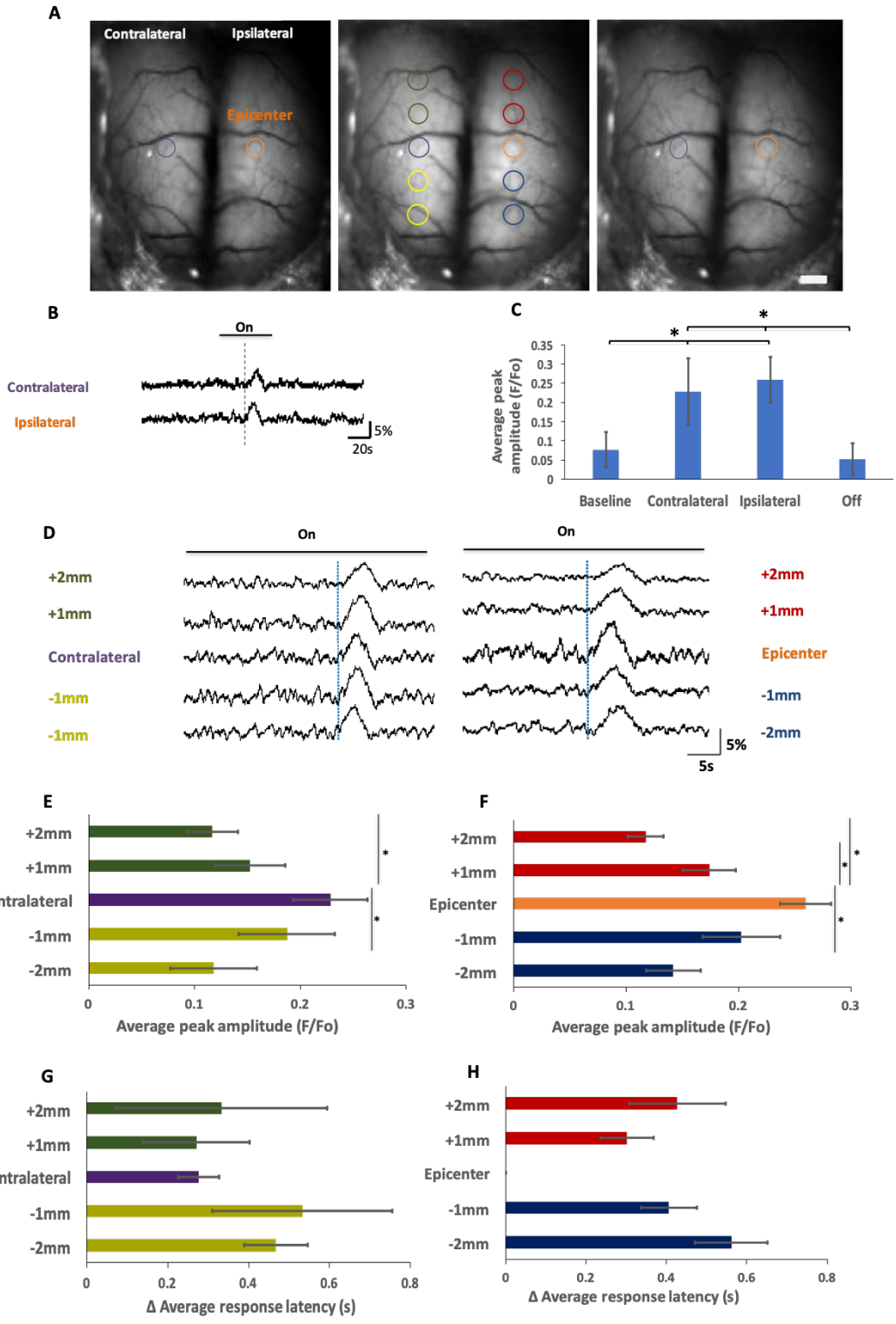


Figure 14. Increased in neuronal activity observed only in MENs treated group immediately after treatment and 24h later. A-D. No change in calcium response amplitude in magnetic stimulation group without MENs treatment (A). Increase in response amplitude immediately after magnetic stimulation only immediately after treatment (B) and at 24 hours later (C). At 72 hours after treatment, no significant increase in response amplitude. one-way ANOVA, Tukey's HSD, n=5, *p <0.05.

To assess the effect of MENs-MS on global network activity, we applied mesoscopic brain imaging technique to evaluate hemispheric calcium response in GCaMP6 transgenic mice (Figure 15A-F). The epicenter region was defined as the location where a permanent magnet was applied to draw MENs to a local cortical region (Figure 15A). Application of 10 Hz magnetic wave induced cortical calcium spikes with significantly higher amplitude and varying latencies in the epicenter and contralateral cortex ($p<0.05$, repeated ANOVA, Tukey's HSD, Figure 15B, C). Comparing the responses among the epicenter and different ipsilateral and contralateral cortical regions revealed a highest peak amplitude at the epicenter and gradual reduction in amplitude

($p<0.05$, one-way ANOVA, Tukey's HSD, Figure 15D, E, and F) and increase in latent period (Figure 15D, G, and H) at locations further away from epicenter. The results suggest that neuronal activity was initiated at the epicenter where MENs are localized and spread to more distal brain regions and the contralateral cortex. These data provide strong evidence that MENs-MS can activate and enhance neuronal activity *in vivo*.


A
Contralateral
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B
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Average peak amplitude (F/Fo)
0.35
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Average peak amplitude (F/Fo)
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+2mm
+1mm
Epicenter
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0.3
Average peak amplitude (F/Fo)
G
+2mm
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Contralateral
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0.2
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0.6
0.8
Δ Average response latency (s)
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+2mm
+1mm
Epicenter
-1mm
-2mm
0
0.2
0.4
0.6
0.8
Δ Average response latency (s)

Figure 15. Mesoscopic activity imaging revealed initiation and spreading of MENs-MS induced cortical activity *in vivo*. A-B. Representative images (A) and traces (B) of calcium transients measured from both hemispheres at baseline (left image) and after turning on and off magnetic stimulation (middle and right images). The epicenter was a cortical region where a conical magnet was applied to attract MENs. Dotted lines indicate the start of calcium spike. **C.** There were significantly higher amplitudes of calcium signals on cortex ipsilateral (at epicenter region) and contralateral (same region on opposite hemisphere) to MENs delivery induced by MS (10 Hz at 450 Oe for ~30 seconds). n = 5, *p <0.05, repeated-measured ANOVA, Tukey's HSD. **D.** Sample traces of calcium transients measured from epicenter, cortical regions anterior and posterior from epicenter, and areas of contralateral cortex. **E, F.** The mean amplitude of calcium signals in the epicenter was higher than that of cortical regions more anterior or posterior to it; the contralateral cortex had similar differences. **G, H.** The latency period at the epicenter was the shortest than all other cortical regions. n = 5, *p <0.05, one-way ANOVA, Tukey's HSD. *Scale bar:* 1 mm.

MENs treatment with magnetic stimulation increased cortical c-Fos expression.

There was an increase in cortical c-Fos-positive cells only for animals that has both MENs treatment and magnetic stimulation, average of 176±27.5 cells/counted region vs 70.5±15.25 for MENs only group and 51±8 for control. MENs treatment-only animal and magnetic stimulation-only naïve retained similar levels (Figure 16A, B). Taken together, this further supports the MENs+ms stimulatory effect findings observed in calcium imaging experiments.

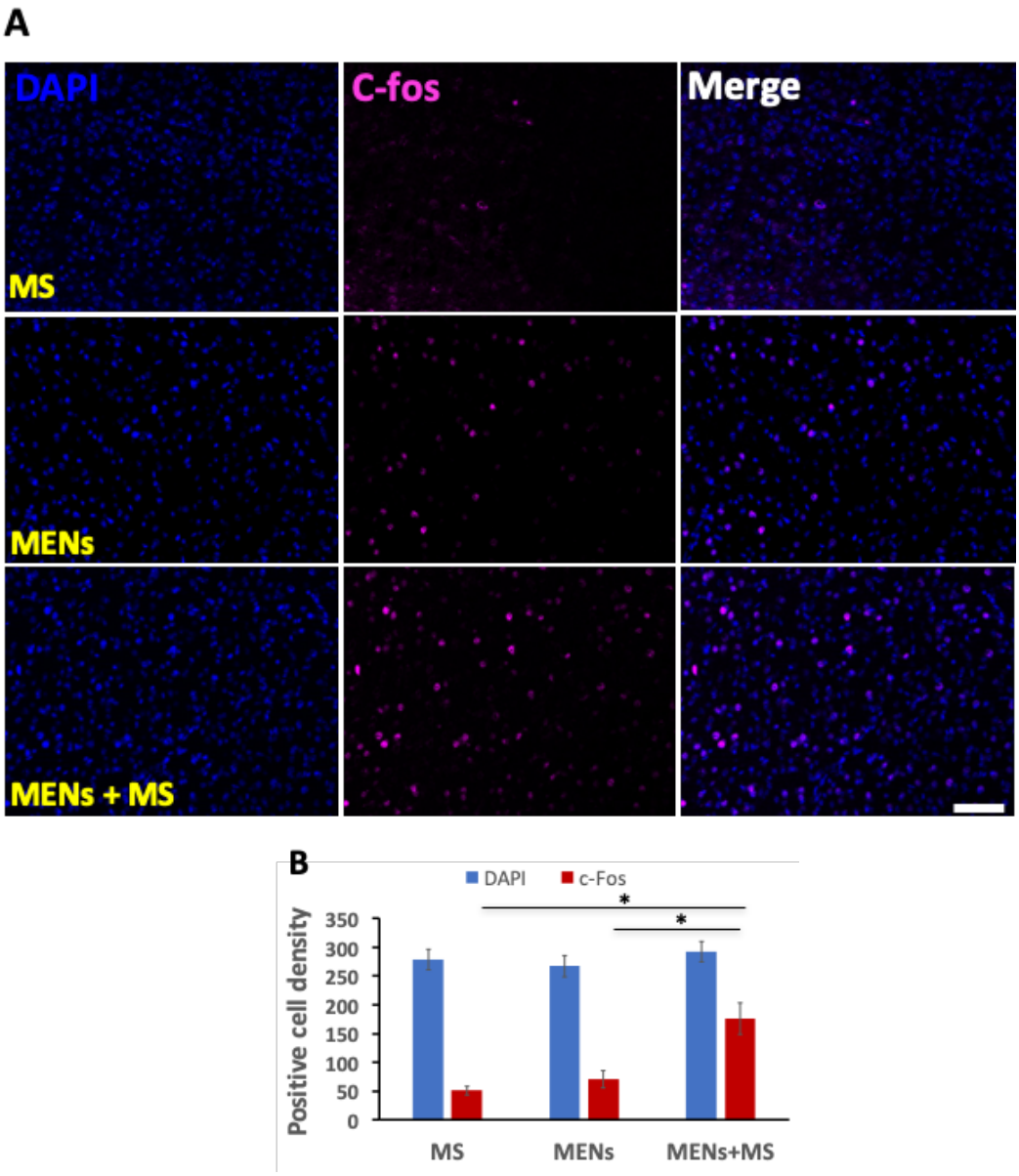

Figure 16. MENs-MS increased the number of c-fos expressing cells. A. Representative images of immunofluorescence from mice that received magnetic stimulation (MS, top row), MENs delivery only (middle row), or MENs+MS (bottom row). Images were taken with an 20x objective. **B.** A significantly higher density of c-Fos expressing cells in MENs+MS group than either MS only or MENs only group. *Scale bar:* 20 µm. n=3 mice/group, *: $p<0.05$, Two-way ANOVA, Tukey's HSD.

Discussion

We further tested MENs stimulatory effects *in vivo* by injection of particles into mice then applied a strong permanent magnetic field to draw them to a cortical area. A weaker electro-magnet for stimulation was applied. Because magnetic stimulation using a pair of electromagnets generates a high level of electromagnetic noise, calcium imaging technique was used to record neuronal activities instead of using electrophysiological recording. Furthermore, Calcium imaging also allow us to efficiently examine neuronal activities not only in individual neurons *in vivo* and *in vitro* but also in both cortical hemispheres. By repeating the calcium imaging experiment, there was significant increase in calcium transient amplitude and response frequency only during the period in which the magnet was turned on. This increase was not only observed at somatic area but also at dendritic area as well. Furthermore, the gain in calcium transient amplitude retained significantly above baseline level for every magnetic stimulation frequency. Calcium response frequency also increased, where the most dramatic gains were seen between 5 and 20 Hz magnet frequencies. This suggests the stimulatory effects of MENs on cortical neurons not only exists at cell body's activity level but possibly also at the synaptic activity level. Whole brain mesoscopic imaging revealed activity changes in a spatially dependent manner relative to the location of MENs. We further assessed the longitudinal profile of MENs effects on neuronal activity and found their stimulatory effects lasted for at least one day post single treatment and dissipated after three days. In addition to functional assessments, stained mice cortical brain slices were also stained for c-Fos expression, a classical indicator of excitability (e.g. action potential firing)[63] that would further support the MENs's stimulatory effect on neuronal activity. Comparing to

sham and MENs-treated only animals, there was a very large increase in cortical c-Fos expression level only in animals that were subjected to both MENs injection and magnetic stimulation. In contrast, there was no change in c-Fos level in MENs-treated only animals compare to sham.

To our knowledge, this study is the first study that reports the stimulatory effects of magnetic-electric based nanoparticles on neuronal activity *in vivo* both at single-cell level as well as large brain regions via calcium imaging. Previous reports by investigators at FIU also found increase in EEG responses during magnet-on period in animals treated with MENs but return to baseline when magnet was turn off[35]. When testing with a control non-electric nanoparticles, in which, the particles only consist of the $CoFe_2O_4$ core but the electric-generative $BaTiO_3$ perovskite shells were absent. These nanoparticles retained their ability to be attracted to a strong magnetic field but lacking their electrical current-generated property. It was reported that there is no change in EEG response when magnet was turn on at various stimulation frequencies. Taken together, these results demonstrate a very useful and potentially ground-breaking ability of MENs to enable precise control of neuronal activity both *in vitro* and *in vivo*.

The study's goal was to demonstrate as a proof-of-concept the ability of MENs to be localized in the brain and efficiently activated and enhanced neuronal activity. Although MENs stimulation can efficiently enhances cortical neuronal activity, it was not sufficient to induce down-stream motor response when we preliminary tested on 3 animals. This is partially due to the required synchronized large population firing of motor cortical neurons in order to evoke muscle movements[64]. Additionally, once MENs is drawn to a cortical hemisphere, applied external magnetic field facilitated MENs

stimulatory effects all neurons in the area. This include both excitatory and inhibitory neurons. Furthermore, the act of imaging the calcium activity in Thy-1-GCaMP6 expressing neurons only allow us to visualize activity of excitatory synapses. Thus, the enhancement in cortical neuronal activity observed should not be expected to produce down-stream motor response due to the possible enhancement of inhibitory neuronal activity by MENs that off-set total cortical response. In order to facilitate down-stream evoked motor response, we need to further exploit the potential of MENs to improve its stimulatory efficacy. Thus, additional effort is being made to precisely localize more MENs to a smaller area of the brain, even towards cell-type specific localization to enable extreme focal neural modulation. This recent work will be discussed in the last chapter of this dissertation. Due to MENs promising potential as the next non-invasive stimulation approach, we proceed with testing its effects on a neuropathic pain disease model. The following chapter 5 focuses on the first confirmed ability of MENs stimulation in relieving neuropathic pain, its potential application to other disease types, and further optimizations for improve therapeutic efficacy.

Chapter 5 MENs stimulation at acupuncture points diminishes nociceptive responses in tibial nerve transection mouse model

Introduction

The International Association for the Study of Pain (IASP) classifies neuropathic pain (NP) as pain "initiated or caused by a primary lesion or dysfunction in the nervous system"[65]. NP afflicts millions of people worldwide. This is due to many common diseases, injuries, and adverse effects from medical procedure cause NP by producing lesion in somatosensory pathways of the central or peripheral nervous system[66, 67]. Current pain management of chronic NP is complex and existing treatments are often inadequate.

Classically, NP results from a primary lesion induce central and/or peripheral sensitization of the nervous system[6]. This, in turn, produces spontaneous, random, and uncontrollable hyper-excitability of neurons. Therefore, traditional approach to NP therapeutics has mostly aimed to reduce or block neuronal activity. Treatments ranging from blocking voltage-gated sodium (VGSCs) or calcium channels (VGCCs) with carbamazepine and gabapentin, respectively, to specific receptors inhibitors like serotonin and norepinephrine (selective serotonins and norepinephrine reuptake inhibitors, SSNRIs) are used widely[5]. A riskier approach of targeting other modulating pathways has shown effective in pain management but come at great cost of increase in drug tolerance, which often lead to misuses and addiction (i.e. opioid-based analgesics such as codeine, fentanyl, tramadol, morphine)[5]. Surgical approaches (thalamotomy and various tractotomies) aim to interfere with the pain signaling pathway has also been increasingly recommended by health-care provider, though as last resorts[6].

An emergent advancement in understanding pain mechanism hypothesizes that overcompensation of an innate nervous system response to lesions (a process called homeostatic plasticity) that may give rise to NP[68, 69]. Synaptic plasticity involves as a regulator for activity by either strengthening the synapses (long-term potentiation, LTP) or weakening the synapses (long-term depression, LTD)[70]. For example, in a healthy individual, a homeostatic response is elicited when two electrical stimulations are applied at short intervals to the cortex[71]. This phenomenon is observed as an initial increase in cortical excitability after the first stimulation (synaptic strengthening) that is then followed by a decrease in excitability and reverse towards inhibition (synaptic weakening) when the second stimulation is applied. In this way, the nervous system corrects for exposure to excessive level of excitation and prevent aberrant synaptic plasticity[71, 72].

Evidence from animal models of pain and conditions such as migraine and muscular dystonia after injury in human suggest a link between impaired homeostatic plasticity and symptoms. In injured animals, the primary lesion caused by the injury produces acute loss in neuronal activity, which resulted in chronic hyper excitability that contribute to long-term pain[69, 73, 74]. An abnormal nervous system after trauma gives rise to an impaired homeostatic plasticity regulation, which resulted in inappropriate and excessive compensations. This is evidenced by more recent counterintuitive approaches that shown to effectively reduce hyperalgesia via stimulations[6, 18]. Electrical stimulations of multiple locations along the pain signaling pathways[6] or cortical brain stimulation with magnets, TMS[18] produce promising results in patients reporting pain relief. Optogenetics stimulation in animal models was also shown to very effectively increased nociceptive

threshold[69]. Unfortunately, these reliefs are partial and short-term. Eastern traditional approaches like acupuncture has also been shown to partially relieve both central and local pain, with electro-acupuncture results in even better efficacy in both animal models and human patients[75, 76, 77, 78, 79].

Due to MENs promising potential, we sought to determine the effects of MENs treatment combining with magnetic stimulation of tibial nerve transection mouse model. Tibial nerve injury reliably produces local and long-term pain in rodents[69, 80, 81, 82] and past efforts have shown that local treatment using electroacupuncture[76] and injection of drug into acupuncture point[83] seems to effectively reduce stimulus-dependent hyperalgesia. Our plan was to inject MENs to a well-known acupuncture point, which is often used to reduce nociception in human and perform the magnetic stimulation to assess the animal's behavioral response.

Methods

Fluorescent MENs (fMENs) treatment to acupuncture point. Preparation of *$CoFe_2O_4$-$BaTiO_3$ -Texas Red, fluorescent MENs (fMENs):* Texas Red NHS ester was first reacted with oleylamine (2:1 molar ratio) for 18-24 hours on a rotator in a dark environment. Hexane was added to help dissolved the oleylamine-Texas Red mixture. The solution of oleylamine-Texas Red NHS ester was then combined with the GMO-MENs mixture (1:3 volume ratio of GMO-MENs: oleyamine-TexasRed). This mixture was then rotated for 12 hours to allow coating. After removal of excess GMO through multiple washes in PBS, the final product was store at -20 ^{o}C (short-term storage). *Injection of fMENs in muscles:* C57BL6J mice (n = 5) intramuscularly injected with 50 µl fMENs (20 mg/ml) at the acupuncture point number 34 Yanglingquan (near the depression of anterior and inferior to the head of fibular, on the lateral portion of gastrocnemius, part of the peroneus longus, and at the distal end of vastus lateralis). This point has been shown to be effectively relieve lower leg pain in human patients and animal models (Figure 17) [76, 77]. *Animal perfusion/ Brain sectioning:* Mice were then euthanized at 1 hour, 1 day, and 3 days post treatment and muscles was sectioned. In brief, mice were deeply anesthetized with (87.3 mg/kg / 13.7 mg/kg) Ketamine/Xylazine and perfused transcardially with PBS buffer (0.01 M phosphate saline PBS, 80ml) followed by 4% formaldehyde PFA (150 ml) at room temperature. Whole skin-muscle group section (gastrocnemius, part of the peroneus longus, and at the distal end of vastus lateralis) at the injection area were then dissected and placed in 30% sucrose for 48-72 hours at 4^{o}C. Tissue preparation and section was done similar to previous report (Meng et al. 2014). In brief, tissues were then thoroughly dried with paper towel to remove

excessive moisture that could produce freezing artifacts. O.C.T Compound (Sakura Finetek, Torrance, CA) was placed in the bottom of a shallow cryomold, enough to supply the foundation for oriented muscle. Tissue was oriented with the skin facing the OCT base. Once the tissue was placed in the mold with majority of the muscle protruded from the OCT so the sectioned muscle of interest is not in contact with OCT. The OCT foundation was then freezed with dry ice at ~ -80^{o}C. Isopentane was then used to fill into the rest of the mold for fast freezing to prevent muscle fiber degradation during sectioning. The whole solution with muscles and OCT was then fast-freezed with liquid nitrogen (~ -140^{o}C to -150^{o}C). Muscle tissues were sectioned at 80 µm thickness with cryostat. Tissues were mounted onto glass slides and imaged using using a Neurolucida confocal imaging system.

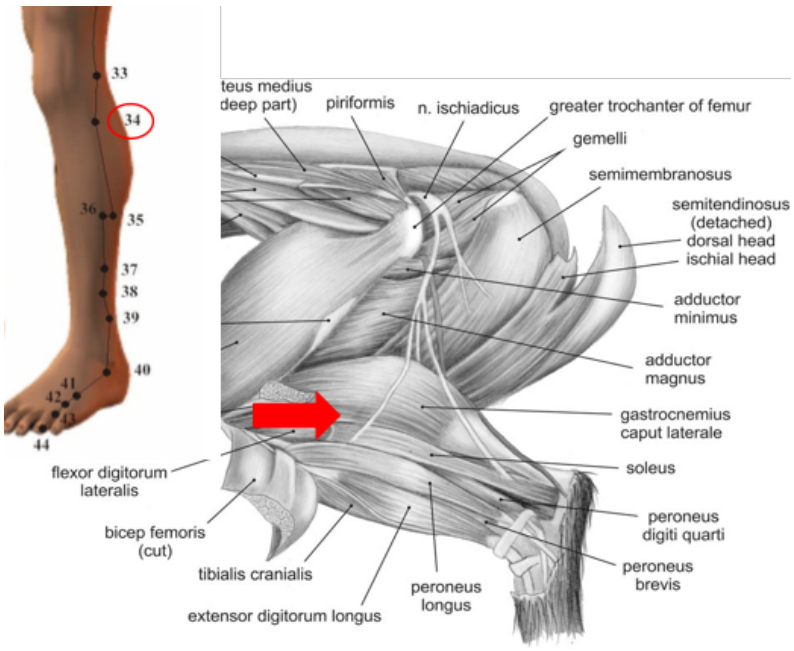

Figure 17. Injection of fMENs into a well-established acupuncture point 34 (Yanglingquan) for treating chronic pain. Yanglingquan acupuncture point exists in a depression anterior and inferior to the head of fibula in human (red circle) and its corresponding region in rodent (red arrow)

Tibial nerved transection injury (TNI) and MENs treatments. *TNI injury:* C57BL6J mice (n = 20 animals / groups) at approximately 30-45 days old were used. For injury, the mice were anesthetized with (87.3 mg/kg / 13.7 mg/kg) Ketamine/Xylazine. A small incision was made on the left leg to expose the tibial nerve, a silk thread was used to make three consecutive loose ligations around it and the nerve was transected distal to the ligation. The incision was then sutured and animals were returned to their housing. *Treatment paradigm:* All injured animals were then randomized and divided into four groups: Saline treatment, MENs treatment, Saline + magnetic stimulation (ms) treatment, MENs + magnetic stimulation (ms) treatment. While animals were under 2% isoflurane, 50 µl of Saline or MENs-saline (20 mg/ml) were injected into the acupuncture point of their respective groups. The leg was then taped to an elevated stage securely between two electromagnets. For magnetic stimulation, a 10 Hz continuous magnetic waves of ~850 Gauss were used. The stimulations were done with duration of 5 minutes interval with 2 minutes break in between for total of 40 minutes stimulation-time and 10 minutes break-time. Animals are then subject to behavioral test after stimulation. For 1 day and 3 days time-point, animals were only subjected to magnetic stimulation. No further saline or MENs injection were done at these two time-points.

Von Frey pain behavioral assessment. Mechanical hypersensitivity was measured using a mechanical Von Frey anesthesiometer. Calibrated Von Frey filaments measuring 100 µm in diameter capable of exerting bending forces of 5, 10, 20, 40 mN were applied to the plantar surface of the animal hind paws. Each stimulus lasted approximately 1 second and had a 10–15 second intermission between applications. Increasing Von Frey filaments were applied until a paw withdrawal was observed and the

corresponding stimulation intensity was recorded. Von Frey test was done at multiple time points: baseline (before injury), 10 days after injury (to assess pain development), 1 hour after treatments, 1 day after treatments, and 3 days after treatments. The test was done to both ipsilateral hind paw as well as the contralateral hind paw. The test was conducted by a research analyst blinded to treatments and group assignment.

Statistics. Mean values and final plots were developed in Microsoft Excel. All statistical analyses were done with, Jmp Analysis 11 (SAS Institute Inc. 2013. Cary, NC), and GraphPad Prism 6 (GraphPad Software, La Jolla, California). Two-way ANOVA analyses were used for comparing pain response threshold of the treatment groups across different time points. For comparisons that yield statistical significance, Tukey's HSD post-hoc analyses were applied for further comparisons between specific groups.

Results

MENs levels in muscle decrease drastically over time after a single injection. After injecting fMENs into acupuncture point, repeated confocal imaging of the muscles sections that were dissected at various time points were performed to tracked the changes in fluorescence intensity in the muscular network. There was high level of fMENs in deeper muscular region at 1 hour after injection, which decreased rapidly over 3 days (Figure 18A-C). The fluorescence intensity in muscle area dropped to about half after 1 day and was almost completely abolished at day 3. This suggest that the fMENs clearance from muscles happened relatively quick after the initial injection.

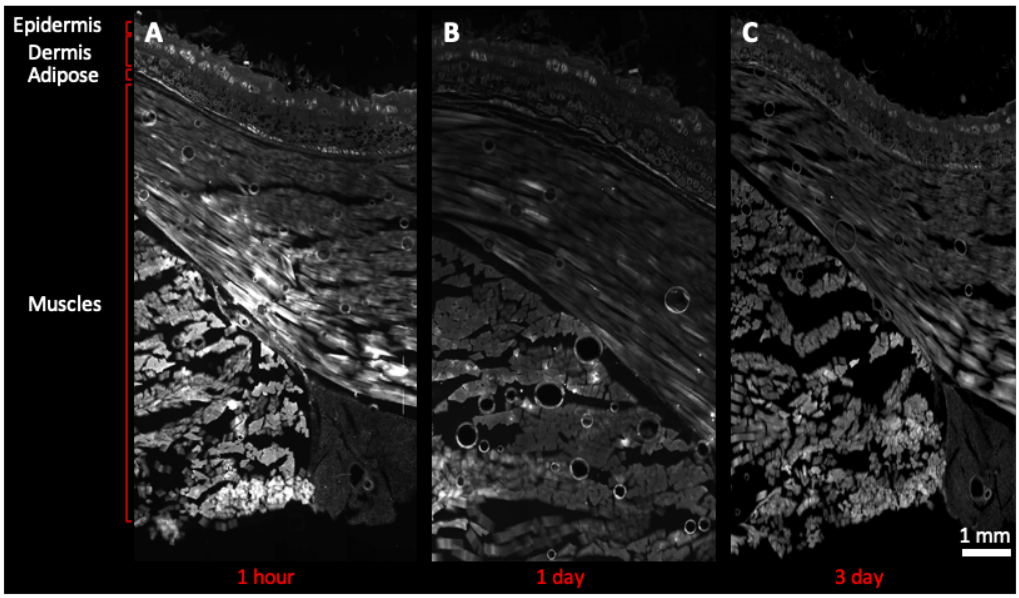

Figure 18. fMENs distribution in muscles decrease significantly 1 day and 3 days after treatment. A-C. fMENs fluorescence signals in lateral portion of gastrocnemius muscles right at 1 hour after injection (A) and decrease significantly at 1 day (B) and 3 days post treatment (C).

MENs with magnetic stimulation at acupuncture point resulted significant improvement in pain response at the ipsilateral hind paw of tibial nerve injured

mice. In order to ensure our injury model resulted in long term pain, we conducted Von Frey assessment at baseline and 10 days after injury. There was, in all groups, pre-treatment, pain response threshold dramatically reduced only in the ipsilateral hind paw (Figure 19A). No significant change was observed at the contralateral hind paw (Figure 19B). This suggests pain has developed in these injured animals. We then treated these animals according to their group treatment paradigm. The subsequent MENs-magnetic stimulation (ms) significantly increased the threshold of Von Frey test of the ipsilateral hind paw almost 3-fold at 1 hour after injection ($p > 0.05$ compare to control vs. $p < 0.01$ of saline group, MENs-only group, and saline-ms group, Two-way ANOVA, Tukey' Figure 19A). The increased threshold was found to be short-lived as it returned to post-injury level at 1 day and 3 days after treatment (Figure 19A). At the contralateral hind paw, there was no changes in pain response at 1 hour and 3 days after treatment, but there was a brief increase in pain threshold in the MENs-only and Saline-ms groups (Figure 19B). This could be due to random chance as there was no change in pain threshold observed at this time point for the other remaining two groups: saline-only and MENs-ms (Figure 19B). Together, this data suggests MENs treatment combine with magnetic stimulation greatly reduce hyperalgesia, even though acutely, in TNI mouse model.

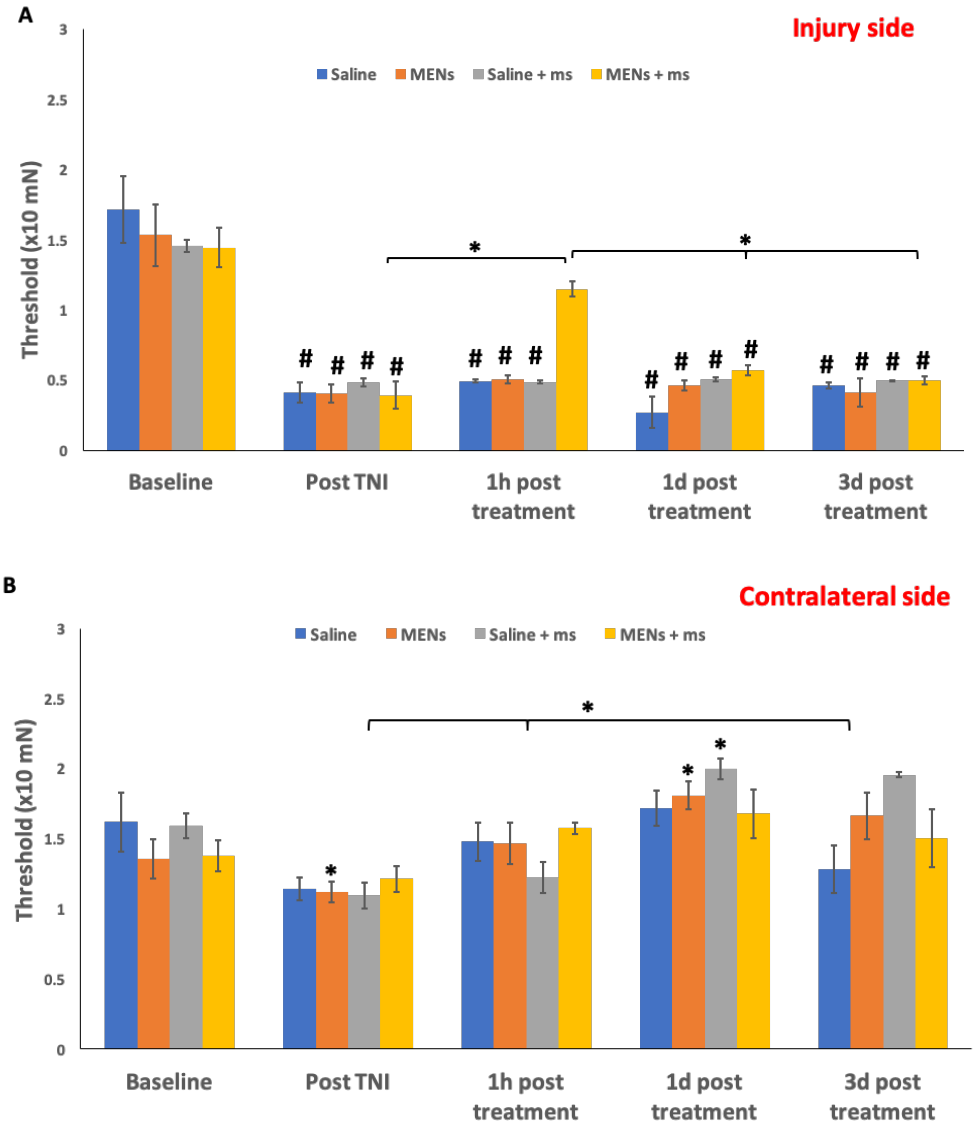

Figure 19. Significant pain relief in MENs magnetic stimulated animals suffered from tibial nerve injury (TNI). A. Von Frey assessment of the ipsilateral hind paw (injury side) show decreased in mechanical pain threshold of all treatment groups after TNI with most dramatic increased in mechanical pain threshold at 1 hour after injury only in MENs with magnetic stimulation treatment group (MENs+ms). No change in pain threshold in saline-treated, MENs-treated, and saline-magnetic stimulation only groups. Two-way ANOVA, Tukey's HSD, *$p < 0.05$. **B**. No change in pain threshold at contralateral hind limb of saline, saline+ms and MENs+ms treatment groups with slight decrease in pain threshold in MENs-treated group. Increased in pain threshold of saline+ms group at 1h post treatment, MENs and saline-ms groups at 1 day, and MENs treated group at 3 days post treatment. Two-way ANOVA, Tukey's HSD, *$p < 0.05$

Discussion

Numerous studies have shown that acupuncture is effective for treating certain types of pain, particularly local muscular pain[84, 85, 86]. This is mainly due to the close proximity of acupuncture point in the muscle to the peripheral nerves, an anatomical location that is ideal for intercepting pain signals before they reach the brain. Taking advantage of this fact, we employed MENs-magnetic stimulation at these acupuncture points and found this greatly reduce nociceptive response in tibial nerve injured animal. The presence of MENs in the muscles after injection were abundant, however, reduced rather quickly in the days following the treatment. This could be due to the nature of skeletal muscles, being highly vascularized, which enable the quick clearance of MENs. This highly correlated with the observed short-term, but significant, pain relieve in Von Frey assessments. More importantly, the effects that were observed are extremely promising, it opens a window of possibility in developing pain therapeutics with MENs stimulation. Previous study aimed at injecting prostatic acid phosphatase (PAP) into the same acupuncture point also shown pain relieve in various mouse models of acute and chronic pains[83]. Mechanistically, this is due to the levels of extracellular adenosine monophosphate (AMP) surrounding the peripheral nerve[87]. Excess extracellular AMP are converted to either adenosine triphosphate (ATP) in the synapse to aid with neurotransmitter release or back-converted to adenosine. Adenosine then activates adenosine 1 receptor (A_1R), that resulted in increase in excitatory signal of glutamatergic synapses and decrease excitatory signal of gamma (γ)-aminobutyric (GABA)ergic synapses at lamina I neuron on the spinal cord[88]. Thus, injecting PAP at acupuncture point manually increase the pool of excess AMP. This enhance in local synaptic activity

signals for homeostatic regulation of the signaling pathway all the way up to the motor-sensory cortex that resulted in reduced nociceptive response[83]. By injecting MENs directly to the acupuncture point and stimulate them with magnet, this directly enhance excitatory synaptic response thus direct the homeostatic response back to the proper compensation, thus reducing pain.

Although a promising first step in MENs application in pain treatment, there are major drawbacks that needed to be addressed. First, with a single injection, MENs level in acupuncture point muscular area dropped greatly with time thus reduce long term efficacy of the treatment. This can be addressed by performed injections of MENs at later time point before magnetic stimulation, the cumulative effects from multiple injections may enhance the pain relief response. Second, to ensure prolong presence of MENs in the muscle, even after a single injection, MENs can be conjugated with neural muscular junction specific antibodies to MENs. This will direct MENs more specifically towards the muscle-nerve interphase and antibody binding will ensure long lasting presence of MENs at the area. This is a clear advantage of this MENs approach over traditional acupuncture or injecting pharmacological molecules into the acupuncture site. Modifications to MENs via conjugations with antibodies or small molecules enable diverse modes of delivery that can tremendously improve its therapeutic efficacy.

Chapter 6 Conclusions

In this final chapter, I discuss the novel contributions of this dissertation to the field of non-invasive neuromodulation and its implications for a potentially promising therapeutic approach to neuropathic pain. Next, I highlight the strengths and weakness of the study, and finally, discuss current efforts as well as recommended future directions to bring this work eventually to clinical stage.

Implications for neuromodulation.

In my dissertation, I set out to explore the potential of magneto-electric nanoparticles cobalt ferrite ($CoFe_2O_4$) – barium titanate ($BaTiO_3$) in stimulating neuronal activity. Specifically, my dissertation focuses on testing its ability to activate neurons both *in vitro* and *in vivo*, understanding its spatial and temporal distributions once introduced into a physiological system, and assessing its toxicity to the brain tissue. Finally, I tested MENs stimulatory effects on a neuropathic pain animal model. Eventually, I sought answers to these central questions: Do magneto-electric nanoparticles (MENs) cobalt ferrite ($CoFe_2O_4$) – barium titanate ($BaTiO_3$) stimulation enhance neuronal activity (specifically cortical neurons) both *in vitro* and *in vivo*? If so, what is the spatial and temporal profile of the stimulatory effects? And what type of effects do MENs stimulation have, in the context of therapeutic approach, on refractory disease animal models, specifically neuropathic pain?

This dissertation provided evidence that MENs treatment combining with external magnetic application were able to induce and enhance neuronal activity both *in vitro* and *in vivo*. I, first reported that by using different external magnetic field, we can produce vastly different response of the MENs. With strong applied field, MENs can be localized

while with weak applied field at specific frequency range could induce electric field generation by MENs (Chapter 2). Through calcium imaging, I found MENs stimulation can efficiently activate cultured cortical neurons and by modifying the applied magnetic field, we were able to improve the neuronal response (Chapter 2). This produced a foundation for future subsequence tests in live animals.

When introduce to a living system, without any magnetic application, MENs was found to be circulating for short period of time in the vasculature, thus established the critical period for drawing MENs to the brain (Chapter 3). It was then showed MENs can be drawn from the blood stream across BBB into the brain with moderately strong magnetic field, localized mainly in one cortical hemisphere where magnets were applied, and remained at high level for at least 24 hours after delivery (Chapter 3). The presence of MENs in the brain tissue resulted in no inflammatory toxicity (i.e. microgliosis or astrogliosis) up to one week after treatment (Chapter 3). Through calcium imaging and staining for c-Fos protein, MENs stimulatory effects were tested on live animals and found by applying weak magnetic field at frequency range between 5 to 20 Hz, MENs could efficiently enhance cerebral cortical activity (Chapter 4). Activity enhancement of neurons lasted for at least one day and recover within three days after the first treatment (Chapter 4). This is highly consistent with the observation of MENs presence in brain tissue up to at least one day post injection (Chapter 3). The enhanced activity started mostly at the region within the cortical hemisphere where the MENs localized and then permeated outward to the surrounding areas (Chapter 4). This is consistent with the one-hemispherical localization of MENs, after been drawn to the brain (Chapter 3). Furthermore, this dissertation also reports the testing of MENs stimulation on a

neuropathic pain animal model, tibial nerve transection injury (TNI). After injecting MENs intramuscularly into an acupuncture point (well-known for lower muscle pain treatment), stimulation of MENs with applied external magnetic field significantly increased injured animal nociceptive response threshold (Chapter 5). This relief in pain was found to be short term due to MENs level at the treated muscular areas reduced greatly over time (Chapter 5). However, these results provide a promising outlook on the potential of MENs as a therapeutic approach, not only to pain, but also to other refractory neurological diseases.

Finally, the systematic development of a stimulation protocol, the studies of MENs effects *in vitro*, the spatial and temporal profiles of their presence in living system, the spatial and temporal profiles of their effects *in vivo*, and their promising therapeutic potential to neuropathic pain pave the way for future research in applying of particles engineering in solving biological problems. While this dissertation emphasizes the discovery of the very useful effects of MENs on neuronal activity and its potential disease application, further studies in improving its efficacy would greatly solidify its role in future trials in humans.

Strengths and weaknesses of the dissertation.

Due to its novel characteristic of exhibiting different magnetic and electric properties, depending on the type and strength of magnetic field is applied, a top down assessment of its effects was necessary to validate its potential. Comparing with other non-invasive neuromodulation techniques, major strengths of MENs are their ability to be drawn through BBB and localized to small region of interest and their activity can be completely controlled by external magnetic field. A strength of this dissertation comes from its top-down assessments of not only MENs effects, but also the effects' spatial and temporal profiles as well as its toxicity in living animal. We also tested its effects in treating pain. This set the study's work apart from previous attempts in validating nanoparticles roles in neuromodulations[31, 33, 48], where their work did not fully test on an *in vivo* system. All of this improved the credibility of our findings.

The promising effects of MENs, however, are not without its flaws. First, MENs were shown to activate cortical neuronal activity *in vivo*, but not sufficient to evoke downstream motor response, as other techniques such as electrical stimulation, transcranial magnetic stimulation, and optogenetics have demonstrated[11, 12, 13, 19, 20]. Second, MENs pain relieving effects were promising but short-term. Our approach produces similar pain-relieving effects as other non-invasive brain stimulation techniques that were tested to treat pain[6, 18, 75, 76, 77, 78, 79], but it is still behind traditional pharmacological and surgical approaches[5, 6]. Future work to improve MENs stimulation efficacy and specificity will help address these issues

Current efforts and recommended future directions.

By the time this dissertation is written, preliminary experiments were conducted try to improve the efficacy and the specificity of MENs stimulations. As mentioned before, although MENs stimulation can efficiently enhance cortical neuronal activity, it was not sufficient to induce down-stream motor response. This is partially due to the required synchronized large population firing of motor cortical neurons in order to evoke muscle movements[64]. Once MENs are drawn to a cortical hemisphere, applied external magnetic field facilitated MENs stimulatory effects all neurons in the area. These include both excitatory and inhibitory neurons. Furthermore, the act of imaging the calcium activity in Thy-1-GCaMP6 expressing neurons only allow for observing activity of excitatory synapses. Thus, the enhancement in cortical neuronal activity observed should not be expected to produce down-stream motor response due to the possible enhancement of inhibitory neuronal activity by MENs that off-set total cortical response. In order to facilitate down-stream evoked motor response, there is a need to further exploit the potential of MENs to improve its stimulatory efficacy. Thus, additional effort is being made to precisely localize more MENs to a smaller area of the brain, even towards cell-type specific localization to enable extreme focal neural modulation.

One potential solution is to test whether conjugation of antibody to MENs can direct the particles to a specific neuronal cell-types. Preliminary, MENs were conjugated with an antibody that target the extracellular subunit GluR2 of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. Because AMPA receptors mainly express in glutamatergic excitatory neurons, we hypothesize that directing MENs towards this cell type should improve its stimulation efficacy towards this cell type. First through

histological assessment, the localization of MENs were observed only to extracellular membrane of cortical neurons and hippocampal neurons (CA1 area and dentate gyrus) when drawn to cortex and hippocampus, respectively (Figure 20A-C). This is highly correlate with GluR2 distribution done in mice of similar age in Allen Institute Brain database (Allen Brain Atlas).

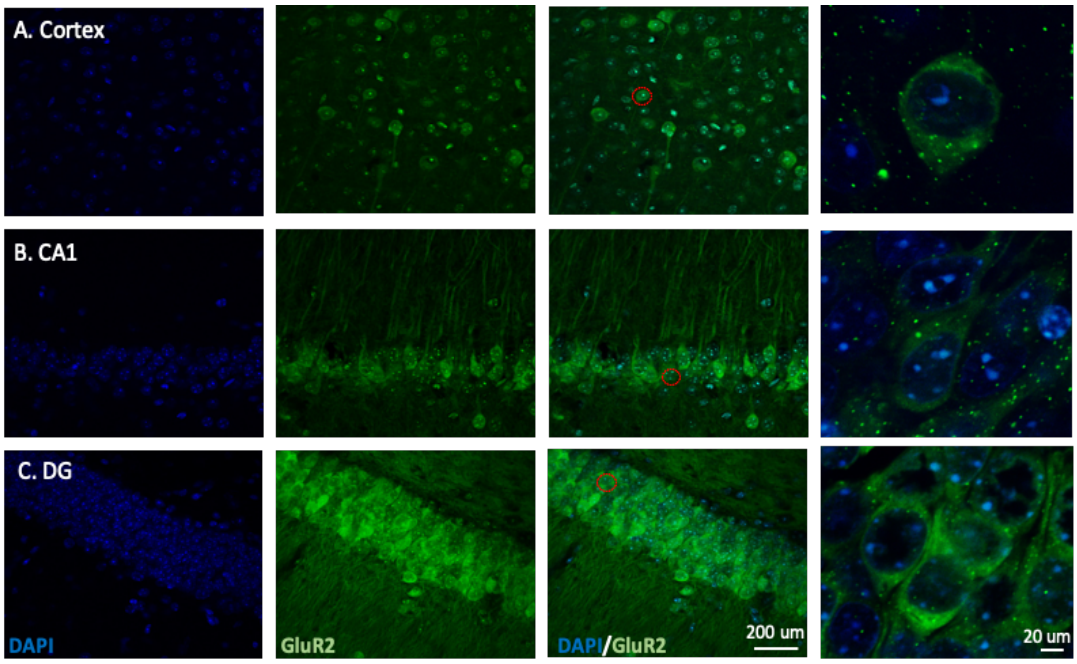

Figure 20. MENs-GluR2 binds to neurons in both cortex and hippocampus. A-C. Representative images of immunofluorescence staining of conjugated MENs-GluR2 to mice brain slices show binding of particle-antibody complex to neuronal cell membranes in cortex (A), CA1 (B) and dentate gyrus (C) regions of hippocampus. No colocalization of MENs-GluR2 with DAPI nucleus stains.

We then tested its effects *in vivo* by treating animals with MENs-GluR2 conjugated particles and perform the same calcium imaging experiment as reported in Chapter 4. Interestingly, MENs-GluR2 were found to enhance greater neuronal response at smaller delay compare to MENs only (Figure 21A-B). The 2 to 3-fold decreased in respond latency was not only observed at single cell level but also at populations level as well (Figure 22A-B). Even though results are promising, further experiment needs to be

done to reliably confirmed this improve effects. Furthermore, MENs can also be conjugated with neuromuscular junction specific antibody to prolong MENs presence in the acupuncture points thus may greatly enhance its pain relief efficacy.

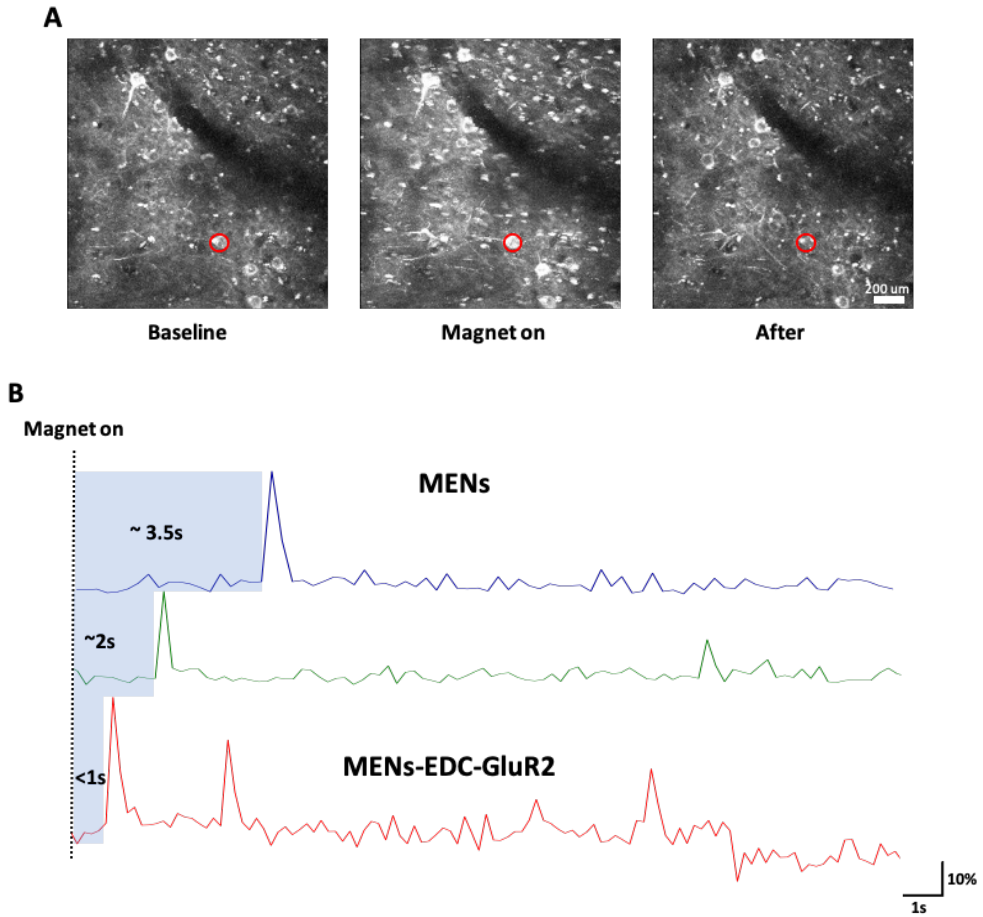

Figure 21. MENs-GluR2 was effective in activating neurons with relatively quicker response *in vivo*. A. Representative two-photon images of calcium signals of cortical layer II/III neurons at baseline (MS only) and immediately after MENs-GluR2 delivery followed by MS (MEN+MS) and after MS stimluation. 10Hz magnetic stimulation was applied for 20s in the middle of recording period at each time point. **B.** Representative traces of calcium signals of MENs-GluR2 conjugate treated mouse show shorter response latency (i.e. faster response time) compare to MENs treated mouse during magnetic stimulation.

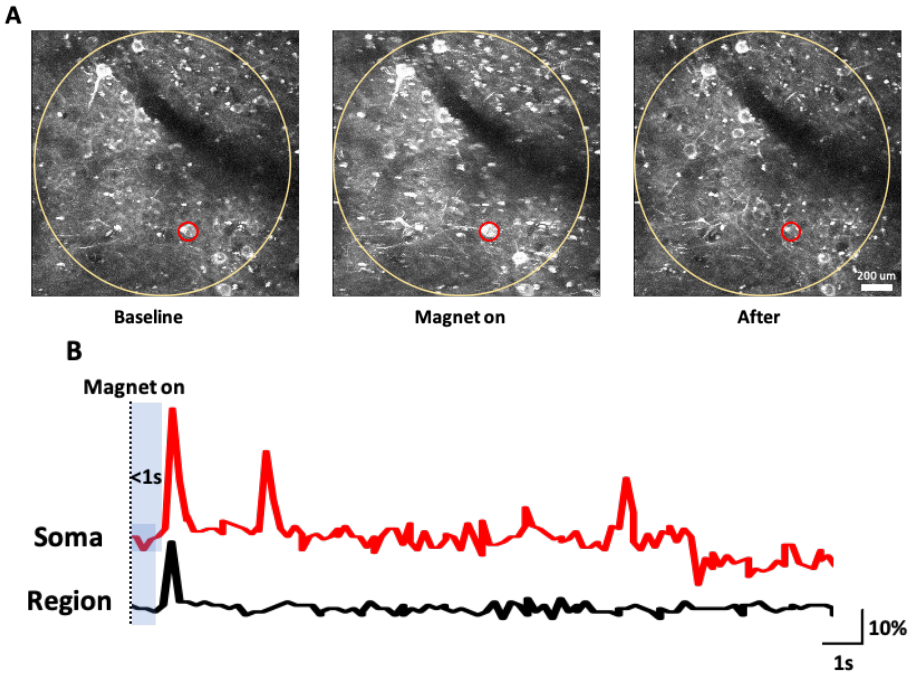

Figure 22. MENs-GluR2 was effective in activating neurons with relatively quicker response *in vivo* observed in both somatic body and population level. A. Representative two-photon images of calcium signals of cortical layer II/III neurons at baseline (MS only) and immediately after MENs-GluR2 delivery followed by MS (MEN+MS) and after MS stimluation. 10Hz magnetic stimulation was applied for 20s in the middle of recording period at each time point. **B.** Representative traces of calcium signals of MENs-GluR2 conjugate treated mouse show enhanced response in both neuronal soma and large population.

Another informative approach to enhance MENs efficacy for brain stimulation is to increase MENs crossing through the BBB. As was shown in Chapter 3, once injected to the blood, MENs level dissipate within 20 to 30 minutes. Strong magnets used to force MENs through BBB may not be sufficient to ensure higher level of MENs in the brain. One solution to this is to combine MENs drawing to the brain with ways to either help transporting MENs through BBB or open up the BBB for MENs to cross. The transport issue can be addressed by conjugating MENs with antibody to target for BBB receptor mediated transport proteins such as transferrin, lactoferrin, or low-density lipoprotein receptor[89, 90]. The second approach of open up BBB for more MENs penetrations can be addressed by combining clinically approved pre-treatment of BBB-opening compounds

such as mannitol[91] and borneol[92] with MENs localization to increase level of MENs crossing BBB towards the central nervous system.

In summary, this dissertation demonstrates the ability of magneto-electric nanoparticles cobalt ferrite ($CoFe_2O_4$) – barium titanate ($BaTiO_3$), to enhance neuronal activity efficiently and effectively. By controlling the magnetic field, we can control both the delivery and activity of MENs in the brain. This also provide promising results in therapeutic potential of MENs towards treating neuropathic pain. With many drawbacks of current clinical non-invasive brain stimulation techniques, MENs stimulation offers a new and improve way to modulate brain activity. Combine with diverse ways of nanoparticle modifications, this could potentially open new doors to a more robust and precise brain control that may currently not be possible.

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88. Imlach W. BR, May L., Christopoulos A., Christie M. . A positive allosteric modulator of adenosine A1 receptor selective inhibits primary afferent synaptic transmission in neuropathic pain model. *Molecular Pharmacology* **88**, 460-468 (2015).

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Curriculum Vitae
Tyler Nguyen

EDUCATION

Doctor of Philosophy (Ph.D.) in Medical Neuroscience, Indiana University, Indianapolis, IN, 2020 (GPA 3.95/4.00)
Master of Science (M.S.) in Anatomy & Cell Biology, Indiana University, Indianapolis, IN, 2018 (GPA 3.85/4.00)
Bachelor of Science in Chemistry (ACS certified), Purdue University, Indianapolis, IN, 2012 (GPA 3.60/4.00)
Bachelor of Science in Applied Mathematics, Purdue University, Indianapolis, IN, 2012 (GPA 3.60/4.00)

RESEARCH EXPERIENCE

Graduate Research:
Ph.D. Thesis, "Magneto-Electric Nanoparticles cobalt ferrite-barium titanate ($CoFe_2O_4$–$BaTiO_3$) for non-invasive neural modulations." Advisor: **Dr. Xiaoming Jin**, Department of Neurological Surgery & Department of Anatomy and Cell Biology, IU School of Medicine, Spinal Cord and Brain Injury Research Group (SCBIRG), Stark Neurosciences Research Institute; Clinical advisor: **Dr. Richard B. Rodgers,** Department of Neurological Surgery, Goodman Campbell Brain & Spine Institute, IU Health, Indianapolis, IN.
Particle engineer advisor: **Dr. Khizroev Sakhrat,** Fellow of National Academy of Inventors, Department of Electrical and Computer Engineering, College of Engineering and Computing; Department of Cellular Biology and Pharmacology, College of Medicine, Florida International University. May 2015 – September 2020
Thesis Committee members: Xiaoming Jin Ph.D., Richard B. Rodgers M.D., Armin Blesch Ph.D. ***(co-Chair),*** Fletcher White Ph.D. ***(co-Chair)***, Khizroev Sakhrat Ph.D.

- Develop a non-invasive stimulation technique using magneto-electric nanoparticles (MENs) cobalt ferrite-barium titanate ($CoFe_2O_4$–$BaTiO_3$) that is capable of both inducing and enhancing cerebral cortical activity with high spatial and temporal resolutions, deep penetration, and minimal toxicity, which will potentially be a new approach towards treating neural dysfunction in traumatic brain injury (TBI) and spinal cord injury (SCI).
- Characterization of nanoparticle density in brain tissue via electron microscopy and confocal microscopy.
- Assessment of the MENs effect on the integrity of blood brain barrier (BBB) by assessing BBB leakage, MENs clearance form blood stream and internal organs, and their toxicity to vital organs.
- Performing in vivo calcium imaging GCaMP-6 mice to determine the effect of MENs on activating cortical neurons.
- Performing mesoscopic whole brain imaging of GCaMP-6 mice to determine the global effect of MENs on activating cortical neurons.
- Performing patch clamp recording and ex-vivo calcium imaging on cortical slices to determine MENs effect on neuronal membrane properties, synaptic transmission, and dendritic excitability.

- Conjugation of various antibodies to MENs for cell-type specific localizations.
- Apply MENs stimulation in various TBI models to assess its stimulatory effects in treating post-traumatic neural dysfunction and neuropathic pain.
- Assessing MENs effect on TBI animals via in vivo imaging, electro encephalopathy (EEG), electromyography (EMG) recordings, and behavioral tests

M.S. Thesis, "Mechanism of Depressed Responsiveness of motor cortex after mild Traumatic Brian Injury (mTBI): Quantitative optogenetic mapping analysis and calcium imaging of mice motor cortex dynamics post mTBI." Advisor: Dr. Xiaoming Jin, Department of Neurological Surgery & Department of Anatomy and Cell Biology, IU School of Medicine, Spinal Cord and Brain Injury Research Group (SCBIRG), Stark Neurosciences Research Institute, May 2014 - May 2016.

- Investigating the animal functional changes, neurons' morphological, and neuronal plasticity after mTBI
- Performing cranial preparation for optogenetic EEG recording.
- Performing craniotomy for optogenetic stimulation and motor cortex mapping
- Preparing brain slides for investigating the pathological changes using two-photon microscopic analysis.
- Performing optogenetic mapping and electrophysiological recording and analysis via electromyography (EMG) and electroencephalography (EEG).
- Performing Calcium imaging with GCaMP transgenic mice to investigate changes of neuronal firing after mTBI via analyzing fluorescence response to investigate intracellular calcium dynamics.
- Performing whole-cell patch clamp recording to investigate ion channel plasticity after mTBI by analyzing Evoke Post-Synaptic Current (EPSC).
- Performing craniotomy to make cranial windows for in vivo calcium imaging.
- Performing tissue perfusion with to preserve animal brain for morphological imaging.
- Investigating neuronal morphological changes after injury via two-photon excitation microscopy.
- Performing various behavioral testing including: Rotarod test, cylinder test, grid-walk analysis, and sensory tape test.
- Investigating the pathological, behavioral, and functional changes in motor cortex after two types of spinal cord injuries (unilateral hemisectional injury and bilateral pyramidotomy injury) in mice. (Collaboration projects with Dr. Xiaoming Xu, Neurosurgery Department, IUSM)

Undergraduate Research:

"A stochastic model for bacterial dynamics toward point food sources with emergent run-and-tumble." Advisor: **Dr. Steven Presse**, Department of Physics (IUPUI) & Department of Cellular and Integrative Physiology and Department of Medical and Molecular Genetics (adjunct faculty), IU School of Medicine and Advisor: **Dr. Elliot Rosen**, Department of Medical & Molecular Genetic, IU School of Medicine. February 2013 – February 2015

- Investigating the predatory dynamics of Bdellovibrio bacteriovorous (B.V) towards Escherichia Coli (E.Coli).
- Grown bacterial culture by suspension and plating.
- Characterized bacteria with Particle Sizing Technique, Ultraviolet Analysis, and Microscopy.
- Characterized the genetics of Bdellovibrio host dependent and host independent strands by Gel Electrophoresis and Polymerase Chain Reaction (PCR).
- Tracking the bacterial movement by obtaining microscopy (fluorescence and confocal) images and using Particle Tracking software.
- Developing microfluidic chambers for investigating bacterial kinetics towards point food source
- Investigate bacterial hydrodynamic, chemotactic, and electrostatic interaction toward different surfaces such as charged/uncharged beads, polar/nonpolar surfaces, and high flow environment.
- Developed an *infotaxis* kinetic mathematical model to describe B.V dynamics and studied B.V motion by simulating model prediction on MathLab and C++.
- Apply this model to study the various cell type predatory behaviors towards prey source.

"Synthesis of Di-substituted Chalcones, kinetic study of Hydrogenation reaction, and kinetic study of Chalcone-based Lycopene Cyclase inhibitors." Department of Chemistry and Chemical Biology, IUPUI, April 2010- June 2013. Advisor: **Dr. Ryan Denton**

- Synthesized Non-substituted Chalcones, Mono-substituted Chalcones, and Di-substituted Chalcones on small scale using heating under reflux as well as a greener reaction by solvent free synthesis, and reduced the complexes by Hydrogenation reactions with presence of various metals
- Purified products using various Chromatography techniques, Crystallization.
- Characterized products with Nuclear Magnetic Resonance (1H NMR), Infrared (IR) spectroscopy, Ultra-violet (UV-vis) Spectroscopy, High performance Liquid Chromatogrpahy (HPLC), and Gas Chromatography-Mass Spectrometry (GC-MS).
- Studied hydrogenation rates of each Chalcone's substituents by model discrimination using GC.
- Studied Chalcone's reaction properties in form of enzymes in Biological organism
- Computational research on Lycopene Cyclase and possible Inhibitors for the enzyme.
- Using Swiss-Model for Homology Modeling, Argus Lab and Chimera for docking and enzyme kinetics studies.
- Developing assay for lycopene cyclase with lemons and bell peppers.
- Synthesized Chalcone-based inhibitors to test on Lycopene cyclase.
- Implemented part of this research into Organic Chemistry Undergraduate Laboratory

"Kinetic mathematical modeling of reductive hydrogenation of Chalcones derivative: 4-Benzyloxy-4-Chloro Chalcones." Department of Mathematics, IUPUI, February 2012 - June 2013. Advisor: **Dr. Julia Arciero**

- Investigate the kinetic rate of reduction of Multifunctional Chalcones by developing a modified Michealis-Menten mathematical model.
- Utilizing Maple for model building and analysis
- Utilizing Matlab for model simulation and experimental data fitting.

CURRENT RESEARCH INTERESTS

- Non-invasive treatment approaches to post-traumatic dysfunction of brain, neuropathic pain, and post-traumatic epilepsy (e.g. nanoparticles, focus ultrasonic stimulation, etc…
- Neural plasticity after Spinal Cord (SCI) and Brain (TBI) Injuries, specifically reorganization of cortical spinal tract after SCI and changes in glutamate receptors function after TBI.
- Using novel imaging and electrophysiological techniques such as in vivo Optogenetic Mapping, in vivo and in vitro calcium imaging, patch-clamp recording to study mechanisms of post-traumatic neuronal plasticity.
- Investigating biological problems using mathematical and physics modeling.

PUBLICATIONS

- **Tyler Nguyen**, Julia Arciero, Joshua Piltz, K. Danielle Hartley, Timothy Rickard, Ryan Denton. "Kinetic study of competitive catalytic transfer hydrogenation of a multifunctional molecule: 4-benzyloxy-4-chlorochalcone." Reaction Kinetic, Mechanisms and Catalysis. Vol. 109. No. 2. Springer (2013)
- Hossein Jashnsaz**, Tyler Nguyen**, Horia I. Petrache, Ph.D, Steve Presse Ph.D. "Inferring models of bacterial dynamics toward point sources." PLOS ONE (2015).
- Hossein Jashzsaz, Mohammed Al Juboori, Corey Weistuch, Nicholas Miller, **Tyler Nguyen**, Viktoria Meyerhoff, Bryan McCoy, Stephanie Perkins, Ross Wallgren, Bruce D. Ray, Konstantinos Tsekouras, Gregory G. Anderson, Steve Presse. "Hydrodynamic Hunters" Biophysical Journal. Vol. 112, No. 6: 1282-1289. (2017).
- Nagesetti, A.; Stewart, T.; Liang, P.; **Nguyen, T**.; Horstmyer, J.; Khizroev, S. "Three-dimensional Navigation of Magnetic Nanoparticle via Enabling metastable diamagnetic response." Human Brain Mapping (2018).
- Wu, W.; **Nguyen, T**.; Ordaz, J.; Zhang, Y.; Liu, N.; Hu, X.; Ping, X.; Wu, X.; Liu, Y.; Qu, W.; Gao, S.; Shields, C.; Jin, X.; Xu, X. "Activity-mediated rebuilding of an alternative corticospinal circuitry in mouse model of Brown-Sequard syndrome." Nature communications (2020) – *in revision.*
- **Nguyen, T.;** Walerstein, J.; Haider Al-Jaboori, M.; Xiong, W.; Jin, X. "Changes in ionotropic glutamate receptor functions underlies loss of activities of motor cortex after concussive type mild traumatic brain injury (mTBI)." Journal of Neurotrauma (2020) – *in revision.*
- **Nguyen, T.**; Gao, J.; Wang, P.; Negessetti A.; Andrews P.; Masood S.; Vriesman Z.; Liang P.; Khrizroev S.; Jin X. "Magnetoelectric nanoparticles for magnetic brain stimulation with higher spatial and temporal resolutions in vivo." Nature Methods (2020) – in revision

AWARDS

- CTSI Predoctoral Trainee Award Grant # UL1TR002529 (A. Shekhar, PI), 5/18/2018 – 4/30/2020**. Grant Number UL1TR002529,** National Institutes of Health, National Center for Advancing Translational Sciences (NIH), Clinical and Translational Sciences Award and the Indiana University Department of Medicine.
- CTSI Predoctoral Trainee Award Grant # TL1TR002531 (A. Shekhar, PI), 5/18/2020 – 4/30/2021**. Grant TL1TR002531,** National Institutes of Health, National Center for Advancing Translational Sciences (NIH), Clinical and Translational Sciences Award and the Indiana University Department of Medicine.
- IUSM travel awards Fall 2018 (Indiana BioMedical Gateway, IBMG, program)
- Grant recipient of 2007-2009 Indiana University-Purdue Univeristy Indianapolis First Generation Scholarship (FGS)
- Grant recipient of the Undergraduate Research Opportunity Program (UROP) scholarships for "Homology Modeling, Docking, and Biological Assay Development of Lycopene Cyclase in Fruit.". Proposed the development of a crude homology model of plant lycopene cyclase, which would be utilized in preliminary docking experiments, along with the development of a whole fruit assay to test a series of inhibitors. (August 1, 2011 – May 30, 2012)
- IUPUI School of Science Dean's List 2007-2011

CONFERENCES AND PRESENTATIONS

- "Multifunctional Chalcone – A competitive reduction studies." **Nguyen, Tyler**; Iqbal, Tumare; Rickard, Tim; Denton, Ryan. IUPUI Chemistry and Chemical Biology Departmental Research Seminar (2010). (Oral Presentation)
- "Multi-Functional Chalcones Under Reductive Conditions: A Relative Rate Study." **Nguyen, Tyler**; Rickard, Tim; Hartley, Danielle; Denton, Ryan E. Butler Undergraduate Research Conference (2011).
- "Homology Modeling, Docking, and Biological Assay Development for Lycopene Cyclase in Fruit." Undergraduate Research Program Conference at IUPUI (2011).
- "Catalytic transfer hydrogenation of multi-functional Chalcones with ammonium formate." **Nguyen, Tyler**; Rickard, Tim; Hartley, Danielle; Iqbal, Tumare; Denton, Ryan. 42nd Central Regional Meeting of the American Chemical Society, Indianapolis, IN, June (2011).
- "Mathematical modeling on hydrogenations reduction rates of multifunctional Chalcones." **Nguyen, Tyler**; Piltz, Joshua; Arciero, Julia; Denton, Ryan. Indiana University Undergraduate Research Conference (2012)
- "Tracking Study of Escherichia Coli and Bdellovibrio bacteriovorus towards food source using microfluidics." **Nguyen, Tyler;** Rosen, Elliot; Johnsons, Merrell; Petrache, Horia; and Presse, Steven. 2014 Indiana Academy of Science Midwest Symposium. (March 2014). (Oral Presentation)
- "A model for bacteria decision making based on stochastic event detection." **Nguyen, Tyler;** Jashnsaz, Hossein; Presse, Steven. 2014 Indiana Academy of Science Midwest Symposium. (March 2014).
- "Identifying the role of hydrodynamic interaction son predation of the predation of Bdellovibrio bacteriovorus." **Nguyen, Tyler;** Jashnsaz, Hossein; Al Juboori,

Mohammad; Miller, Nick; Anderson, Gregory; Presse, Steven. *IUPUI Nanotechnology Research Forum and Poster Symposium*. (2015).

- "Optogenetic mapping revealed longitudinal changes in mouse motor cortex after closed-head mild Traumatic Brain Injury (mTBI)." **Nguyen, Tyler**; Jin, Xiaoming. Indiana University School of Medicine Spinal Cord and Brain Injury Research seminar (November 2014). (Oral presentation)
- "Optogenetic mapping revealed longitudinal changes in cortical motor map following closed-head mild traumatic brain injury (mTBI)" **Nguyen, Tyler**; Ping, Xingjie; Jin, Xiaoming. *Kentucky Spinal Cord and Brain Trauma Midwest Symposium*. Louisville, Kentucky. (Jun 2015)
- "Mechanism of mild Traumatic Brain Injury (mTBI)." **Nguyen, Tyler**. Indiana University School of Medicine Spinal Cord and Brain Injury Research seminar (September 2015). (Oral presentation)
- "Transcranial optogenetic mapping revealed longitudinal changes in motor maps of ipsi-lesional and contra-lesional cortex following mild traumatic brain injury." **Nguyen, Tyler**; Jin, Xiaoming. *Society for Neuroscience* 2015 National Conference. Chicago, Illinois. (Oct 2015)
- "Changes in Glutamate receptor functions following mild Traumatic Brain Injury (mTBI)" **Nguyen, Tyler**. *2nd Signature Center Initiative Symposium for Indiana Spinal Cord and Brain Injury Research*. Indianapolis, Indiana. (Oct 2016), (Oral Presentation)
- "Magneto-electric nanoparticle for non-invasive brain stimulation." **Nguyen, Tyler;** Vriesman, Zoe; Stewart, Tiffanie; Khizroev, Sakhrat; Jin, Xiaoming. *2017 Jack & Linda Gill Symposium & Awards*. Indiana University. Bloomington, Indiana. (Sep 2017), (Poster presentation).
- "Activation of cortical neuron by magneto-electric colbalt-ferrite barium titanate nanoparticle based non-invasive brain stimulations." **Nguyen, Tyler**. Indiana University School of Medicine Spinal Cord and Brain Injury Research seminar (October 2017). (Oral presentation)
- "Colbalt-Ferrite Barium Titanate nanoparticles for brain stimulation." **Nguyen, Tyler**. Stark Neuroscience Research Seminar (Feburary 2018). (Oral presentation)
- "Magneto-electric nanoparticle activates cortical neuronal cell in vitro." **Nguyen, Tyler.** *Indiana University/Purdue University Brain and Spinal Cord injury symposium* (September 2018). (Poster Presentation).
- "Magneto-electric nanoparticle for non-invasive brain stimulation, an *in vivo* study." **Nguyen, Tyler;** Vriesman, Zoe; Andrews, Peter; Masood, Sehban; Stewart, Tiffanie; Khizroev, Sakhrat; Jin, Xiaoming. *2018 Jack & Linda Gill Symposium & Awards*. Indiana University. Bloomington, Indiana. (Sep 2018), (Poster presentation).
- "Non-invasive neuronal activation by magneto-electric nanoparticles." **Nguyen, Tyler;** Vriesman, Zoe; Andrews, Peter; Masood, Sehban; Stewart, Tiffanie; Khizroev, Sakhrat; Jin, Xiaoming. *2019 Greater Indiana Society for Neuroscience*. IUPUI. Indianapolis, Indiana. (March 2019). (Poster presentation).
- "Effects of magneto electric nanoparticle stimulation on neuronal activity, an in vitro study." **Nguyen, Tyler**. *2019 Stark Neuroscience summer symposium*. (July 2019), (Oral presentation).
- "Magneto-electric nanoparticles for non-invasive brain stimulation, in vivo mouse imaging study." **Nguyen, Tyler**; Vriesman; Zoe, Andrew, Peter; Masood, Sehban;

Sakhrat, Khizroev; Jin, Xiaoming. *2019 Indiana CTSI Annual Meeting*. (September 2019), (Poster Presentation).

- "Cobalt Ferrite-Barium titanate magnetic electric nanoparticles, a new frontier in neuromodulation." **Nguyen, Tyler**; Andrew, Peter; Sakhrat Khizroev; Jin, Xiaoming. *2019 Gill Symposium & Awards*. (September 2019), (Poster Presentation).
- "Effects and Mechanism of cobalt ferrite-barium titanate nanoparticles non-invasive neural modulation." **Nguyen, Tyler**; Vriesman; Zoe, Andrew, Peter; Masood, Sehban; Stewart, Tiffanie; Nagasetti, Abhinegan; Sakhrat, Khizroev; Jin, Xiaoming. *2019 Society for Neuroscience meeting (SfN)*. (October 2019), (Poster Presentation).
- "Non-invasive neuromodulations, history & current frontiers." **Tyler, Nguyen**. *Indiana Spinal Cord and Brain Injury Fall Seminar Series*. (November 2019), (Oral Presentation).
- "Magneto-Electric nanoparticle – the next frontier in neural modulation." Tyler, Nguyen. *Association for clinical and translational science (ACTS) conference*. Washington D.C (April 2020), (Oral Presentation)

TEACHING EXPERIENCE

Teaching Assistance, General Chemistry (C125), IUPUI, May 2012- June 2015

- Lecture over General Chemical theories, Chemistry Lab techniques, and conduction reactions.
- Instruct students on how to carry out a chemical reaction, isolate, purify, and analyze the reaction products.

Teaching Assistance, Organic Chemistry Laboratory II (C344), IUPUI, August 2011-May 2013

- Lecture over Organic Chemistry Reactions, theories, and Instrumental techniques.
- Instruct students on how to carry out organic chemistry experiment.
- Analyzed students' experimental products with various analytical instruments (Infrared Spectrometer, Gas Chromatography and Liquid Chromatography, Mass Spectrometer, H-NMR).

Laboratory instructor, Instrumental Analytical Chemistry (C411), IUPUI, August 2013 – December 2013

- Lecture over concepts, design, and functions of different analytical Instruments (such as Flame Ionizations, Gas and Liquid chromatography, Mass spectrometry, Fourier Transform Infrared Spectroscopy, Ultraviolet Spectroscopy).
- Instruct students on how to develop methods, run, trouble shoot, and maintain different instruments.
- Instruct students on how to choose the appropriate instrument(s) to analyze a sample of interest.

Laboratory instructor, Experimental Physical Chemistry of Bulk Matter (C363), IUPUI, January 2014 – May 2014

- Lecture over concepts, design, and functions of different physical chemistry related analytical instruments (such as Bomb Calorimeter, Magnetic susceptibility analysis, Viscometer, Tensiometer, C-NMR, IR, and Gas Chromatography)
- Instruct students on how to develop methods, run the instruments, and analyze data

- Instruct students on how to design experiments that utilize combinations of instruments and how these combinations can be modulate depends on the question of study

OTHER EXPERIENCE

- Business Developer, the Change for Change campaign and the Change for Change Benefit event, project manager: Mr. Aaron Christian at IUPUI, January 2010 – May 2010.
- Conducted Powerpoint presentation to local and national businesses to encourage for donations for the campaign and sponsorship of the event.
- Organizing the Change for Change Benefit event that resulted in over 5000 dollars donation to the Freedom Writer Foundation

Chemistry tutor at IUPUI Chemistry Resource Center (2012-2014)
Volunteer, IU Health University Hospital Dialysis Outpatient Center 2007-2010

COMPUTER EXPERIENCE

- Proficient in Excel, MS word, Powerpoint, Apple Numbers, Pages, and Keynote
- Scientific modeling analysis: SciFinder Scholar, ChemDraw, Cambridge Structural

Database, Time Series Analyzer software (Calcium imaging), and OriginPro Lab Imaging (optogenetic mapping)

- Homology Modeling and Biochemical analysis: Swiss-Model, Spartan, Argus Lab, and Chimera
- Programming languages for mathematical modelling: Python, C++, and Matlab

Statistical analysis: Jmp, Prism, and StatPlus (Mac OS)

- Electrophysiological data recording via pClamp Software for EMG and EEG, Metamorph Microscopy software for calcium imaging, and electrophysiological data analysis via Detector® software

CONTRIBUTION TO SCIENCE

- **Early Career**: My early career contributions were focused on two set of works: (a) Investigating the reduction reaction kinetics of a di-substituted chalcones-type molecules called 4-Benzyloxy-4-Chloro Chalcones. This molecule is of particular importance due to its natural existence in orange and yellow fruits and vegetables and has been shown to be an effective treatment in reducing prostate and ovarian cancer cysts. Within the plants, lycopene cyclase enzyme converts this chalcones into beta-carotenes that gives rise to the bright orange and yellow color of the fruits. By understanding the kinetics of this reaction, one can minimize the reduction and extract chalcones from the plants. I performed various types of reduction reaction of this multifunctional chalcones and use various spectroscopic technique to investigates the resulting products and the rate in which each product formed. I also, in collaboration with an applied mathematician, developed a kinetic mathematical model, derived from Laws of Mass Actions, to help predict the rate of products formations when given various types of function groups. (b) Investigate the predatory dynamics of Bdellovibrio bacteriovorous (B.V.) towards Escherichia Coli (E.Coli) and develop a stochastic mathematical model to predict the

searching behavior of a predator towards its prey, taken into account combinations of environmental factors such as chemotaxis, barotaxis, thimotaxis, and the subject's Poisson random movements. This model is able to not only predict movement behavior but also is the foundation of building an algorithm, in which, one can control an object movement based on information collected from surrounding before the movement is decided. I also developed the culturing protocol of B.V. and E.Coli and designed a "hunting chamber" using microfluidics to assess the predatory behavioral of B.V. in real-time. This helped tested the model predictions.

- **Graduate Career:** My graduate work focuses on set of directions: (a) Investigate the neural plasticity of the motorsensory cortices after traumatic brain injury (TBI) and spinal cord injury (SCI). For TBI, I utilized various electrophysiological, functional imaging, and behavioral assessment techniques: optogenetic mapping, calcium imaging, field potential recording, glutamate uncaging with two-photon imaging to investigate the change in activity of the sensory-motor pathway at both the systemic level and cellular level. I then assess the molecular mechanism, in which glutamatergic ion channel dysfunction gives rise to cellular and behavioral deficits after trauma. For SCI, I utilized optogenetic stimulation and optogenetic mapping to investigate the reorganization of the corticospinal tract (CST). I also showed, as proof-of-concept, that stimulation of the motor cortex induces CST reorganization in such a way that improved behavioral outcomes were observed. (b) The later part of my graduate work focused on developing a new and improve non-invasive brain stimulation technique using magnetic-electric nanoparticles (MENs) cobalt ferrite-barium titanate ($CoFe_2O_4$–$BaTiO_3$). By applying various functional imaging techniques, I demonstrated both in vitro and in vivo that MENs can be safely used to modulate neuronal activity. My research has shown MENs can be: injected into the blood stream intravenously, safely and wirelessly guided through the blood and cross the blood brain barrier to localize in a small brain region. A weak magnetic field can then be used to activate MENs in the brain and enhance neuronal activity. I then showed MENs stimulation is effective in reducing nociceptive responses in tibial
- **Postdoctoral Career:** As a postdoctoral fellow, my research will be focused on investigating the role of neuroinflammation in contributing to the pathology and behavioral deficits, particularly pain, after traumatic brain injury and ultimately long-term neurodegenerations.