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Magneto-mechanical Neuromodulation

by

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ABSTRACT

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Noninvasive control of the electrical activity in specific cells in the brain would transform fundamental neuroscience research and the development of therapeutic technologies. Current neural stimulation methods such as electrical or optogenetic stimulation achieve high levels of specificity, but are invasive. Magnetic stimulation is a potentially noninvasive stimulation modality because mammalian tissue is nearly transparent to magnetic fields. In this thesis we investigate a new neural modulation method based on magnetic fields that can potentially achieve similar levels of specificity with much lower invasiveness. Our method will use externally applied, uniform magnetic fields that induce dipole-dipole forces between superparamagnetic iron oxide nanoparticles bound to Piezo1, a mechanically sensitive ion channel. Based on our calculations and early preliminary results, these mechanical forces will be sufficient to open Piezo1, leading to cationic currents, that will alter cell activity. Expression of mutant Piezo1 protein can be targeted to genetically specific populations of cells by means of cell-type specific promoters in transgenic animals. This method is expected to achieve accurate control of genetically specific populations of cells, thereby enabling better research to answer fundamental biological questions and develop novel medical therapies.
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## Nomenclature

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<td>MSC</td>
<td>Mechanically Sensitive Channel</td>
</tr>
<tr>
<td>SPION</td>
<td>Superparamagnetic Iron Oxide Nanoparticle</td>
</tr>
<tr>
<td>MNP</td>
<td>Magnetic Nanoparticle</td>
</tr>
<tr>
<td>IONP</td>
<td>Iron Oxide Nanoparticle</td>
</tr>
<tr>
<td>TMS</td>
<td>Transcranial Magnetic Stimulation</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to Noise Ratio</td>
</tr>
<tr>
<td>BGTX</td>
<td>bungarotoxin</td>
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</tbody>
</table>
Chapter 1

Review of Relevant Literature

The novel magnetic neuromodulation method that I describe in this thesis is based on recent research advances in the field of molecular mechanotransduction and differs from current methods of neuromodulation. In the first section of this chapter, I will outline the motivation for developing tools to control neurons. In the second section, I discuss several types of neuromodulation methods and I review published literature related to each method. In the third section of this chapter, I review literature related to the discovery, characterization and significance of the mechanosensitive channel, Piezo1.

1.1. Motivation for controlling behavior of neurons

Eukaryotic cells maintain an electronic potential gradient and a concentration gradient across their lipid bilayer membranes. Many physiological properties and behaviors of cells depend on this transmembrane concentration gradient. Therefore any tool that facilitates manipulating this transmembrane potential is beneficial to biological
research as a way to probe the system under investigation. For example, voltage clamp electrophysiology, invented by Kenneth Stewart Cole and George Marmont (Marmont, 1949) can be used to simultaneously control the cell membrane potential and record ionic currents. It has enabled new research and discoveries that were not possible before its invention (Bauer, Lambert, & White, 2014). Hodgkin and Huxley (Hodgkin & Huxley, 1952) used voltage clamp techniques in their experimental investigations of the excitability of neurons, developing accurate models of neuronal action potentials which are still quite useful. In developing the novel neuromodulation tool I describe herein, we rely heavily on voltage clamp method.

More recently optogenetic proteins have shown to be extremely useful for research, particularly in neuroscience. Since Nagel et al. discovered the light-gated properties of the channelrhodopsin family of ion channels (Nagel et al., 2003), the field of optogenetics has made great advances. A variety of engineered variants and other naturally occurring optically gated channels (Gunaydin et al., 2010; Miri et al., 2014; Mukohata, Ihara, Tamura, & Sugiyama, 1999; Zhang et al., 2007, 2009) have all become part of the optogenetic stimulation toolkit. Optogenetics has enabled optical probing of neuronal circuitry in vitro (Boyden, Zhang, Bamberg, Nagel, & Deisseroth, 2005) and in vivo (Asrican et al., 2013; Cardin et al., 2010; Sohal, Zhang, Yizhar, & Deisseroth, 2009) and has been used in combination with optical recording for an all-optical recording and stimulation techniques (Hochbaum et al., 2014). Transgenic expression of optogenetic proteins may require an injection of lentivirus with DNA coding for the protein or this injection can be avoided by generating animal model (mice, rat) lines expressing the protein. Invasive surgery is typically required to stimulate, however, because there must be
a transparent optical path between the light source and the tissue to be stimulated. Optogenetic stimulation is possible without invasive surgery if long-wavelength optogenetic proteins are used (Miri et al., 2014), though only at superficial levels. Deeper than several millimeters, however, even long wavelength infrared light intensity is too low as a result of absorption by tissue. Since magnetic fields are not absorbed by tissue, the magnetic stimulation technique I propose in this thesis would not have these same challenges as optical techniques.

Advanced technologies make behavioral control of neurons possible in vivo. While these technologies have contributed advanced medical treatments for otherwise untreatable diseases, they suffer from being highly invasive. Electrical stimulation of the brain in vivo enabled the development of Deep Brain Stimulation (DBS) treatments for Parkinson’s, depression and many other neurological disorders (Miocinovic, Somayajula, Chitnis, & Vitek, 2013; Perlmutter & Mink, 2006). Installation of microelectrodes, however, requires high-risk brain surgery with potential for infection, severe inflammation and brain damage. Over time, furthermore, an insulating glial scar tissue often forms around the microelectrodes, preventing effective delivery of stimulation. Our magnetic field stimulation tool leads the way towards a potential replacement for microelectrode stimulation which is noninvasive, thereby completely negating the necessity of invasive and high-risk brain surgery.

In a 1979 Scientific American article, Francis Crick stressed the inestimable value of “a method by which all neurons of just one type could be inactivated, leaving the others more or less unaltered” (Crick, 1979). Optogenetics has to a large degree satisfied this need. To perform in vivo optical stimulation of the brain at deeper levels than several
millimeters, however, such as those depths required for stimulation to treat Parkinson’s disease, surgery is required. There are many side-effects due to this surgery from gliosis and infection to psychosis and depression (Burn & Tröster, 2004). The method I propose in this thesis would eliminate those side-effects directly caused by the surgery itself.

Magnetic fields are a minimally invasive option for two reasons. First, they do not require surgery, as most optical and electrical methods do. Second, magnetic fields interact weakly with mammalian tissue. In a review by Funk et al. (Funk, Monsees, & Ozkucur, 2009), the interaction of magnetic fields with cells and mammalian tissue is discussed in detail. They note that most mammalian biological tissue is diamagnetic and therefore has magnetic properties close to vacuum. It is not surprising, therefore, that studies of the effects of DC magnetic fields on humans is minimal. Chakeres and De Vocht, (Chakeres & De Vocht, 2005) report no short and long term effects of static magnetic fields by studying patients subject to exposure of magnetic fields as high as 8T in MRI machines. In the article, they conclude the following: “These studies did not demonstrate any clinically relevant adverse effects on neuro-cognitive testing or vital sign changes. One short-term memory, one sensory, and one cognitive–motor test demonstrated adverse effects, but the significance is not clear.” Therefore, although the magnetic stimulation technique I outline in this thesis theoretically will require a magnetic field as high as 250mT, it is unlikely that there will be adverse side-effects in mammalian studies.
1.2. Approaches to controlling the behavior of neurons

There are a variety of approaches to controlling the behavior of neurons. The first two methods that I will discuss are widely used and have been used for at least ten years: electrical and optical. I will also discuss two methods that are relatively new: ultrasonic and magnetic methods.

1.2.1. Electrical stimulation of neurons

Approaches that employ direct electrical stimulation of neurons have been of inestimable value to neuroscience. The 1991 Nobel Prize in Physiology or Medicine was awarded to Erwin Neher and Bert Sakmann “for their discoveries concerning the function of single ion channels in cells” (Nobel Media AB 2014, 2015). The technique that allowed Neher and Sakmann to make their discoveries is patch clamp electrophysiology, which they developed over the course of their work (Hamill, Marty, Neher, Sakmann, & Sigworth, 1981; Neher & Sakmann, 1976; Sigworth & Neher, 1980) and which is still the gold standard today for taking accurate, low noise recordings of single ion channel or whole cell activity (Bébarová, 2012).

Electrical stimulation with micropipette electrodes is capable of low signal-to-noise ratio (SNR) and high spatial and temporal resolution, but micropipette electrophysiology is limited because it cannot be easily multiplexed. More recently, capacitative stimulation via Multi-Electrode Arrays (Bakkum, Chao, & Potter, 2008; Dranias, Ju, Rajaram, & VanDongen, 2013; Potter, 2001) and direct current injection with nanowire arrays (Patolsky et al., 2006; Robinson et al., 2012) can facilitate research to investigate properties of a network of neurons and not just single cell units. In vivo,
microelectrodes are often used to perform experiments that investigate network activity via electrical recording and stimulation (Jog et al., 2002). Electrical stimulation methods are low noise and high resolution, but are unavoidably invasive, requiring either direct injection of ionic current into a cell or capacitative stimulation by modifying the electric field in the vicinity of the cell. Magnetic stimulation with the technique described herein is far less invasive and multiplexed than standard electrical stimulation techniques.

1.2.2. Optical stimulation of neurons

Channelrhodopsin-2 (ChR2) was cloned from the green algae *Chlamydomonas reinhardtii* in 2003 (Nagel et al., 2003). Nagel et al. expressed ChR2 in *Xenopus* oocytes and mammalian cells and demonstrated that ChR2 exhibits properties of a cationic membrane channel, directly gated by blue light. In 2005, Ed Boyden and Feng Zhang, graduate students working in the lab of Karl Deisseroth and in collaboration with Georg Nagel, published research on the successful expression of ChR2 in cultured mammalian hippocampal neurons. They were able to optically elicit action potentials with high temporal resolution (Boyden et al., 2005). In 2007, Han and Boyden transduced cultured mammalian neurons with the mammalian-optimized codon sequence of the light-driven chloride pump, Halorhodopsin (NpHR), from *Natronomas pharaonis* archaeabacterium (Han & Boyden, 2007). Boyden and Han were able to hyperpolarize neuronal membrane potential with millisecond precision simply by illuminated these cells with yellow light. Since NpHR is gated by yellow light, whereas ChR2 is gated by blue light, co-expressing both proteins in mammalian neurons allows for independent photostimulation and photoinhibition with millisecond precision. Many more light-gated channels, both naturally occurring and engineered variants, have been added to the photostimulation
toolkit (Chow et al., 2010; Gunaydin et al., 2010; Han et al., 2011; Hochbaum et al., 2014; Klapoetke et al., 2014; Miri et al., 2014). Although a red-shifted opsin like Jaws (Miri et al., 2014) enables transcranial optical inhibition of neurons at depths of 3mm in mice brain, optical stimulation methods are inherently limited in vivo by the opacity of tissue to light. Tissue is essentially transparent to magnetic fields, and so magnetic stimulation is a viable solution to this problem.

1.2.3. Ultrasonic stimulation of neurons

The first evidence of neuromodulation by ultrasound was given in 1958 by Fry and colleagues who demonstrated that it was possible to produce reversible lesions in the brain by focused ultrasound (Fry, Ades, & Fry, 1958). Gavrilov et al. demonstrated that it was also possible to excite activity in the peripheral nervous system in 1976 (Gavrilov et al., 1976). But for several decades after, little work was done to investigate ultrasonic neuromodulation because invasive surgery was required in order to stimulate through the skull. Recently there have been developed technological innovations for generation of focused ultrasound, and imaging methods for guiding delivery of stimulation (Foley, Vaezy, & Crum, 2007). Neuromodulation were originally achieved only with high intensity (>1W/cm²) focused ultrasound (HIFU), but at this high intensity, neuromodulation is caused by thermal effects which can cause irreversible damage to biological tissue by overheating or inertial cavitation of interstitial fluids (Dalecki, 2004).

In order to noninvasively modulate neural activity, therefore, Tyler et al. investigated pulsing ultrasound at lower frequency methods and were able to achieve
ultrasonically invoked action potentials in vitro (Tyler et al., 2008) and transcranially in vivo (Legon et al., 2014; Tufail et al., 2010; Tufail, Yoshihiro, Pati, Li, & Tyler, 2011).

Unfortunately, ultrasonic neuromodulation is nonspecific and spatial resolution is poor (~3mm) (Tufail et al., 2010). Specificity could potentially be enhanced by acoustic cavitation of microbubbles, but work in that area is only preliminary (O’Brien; Wu & Nyborg, 2008). Furthermore, the mechanism is not well understood (Tyler, 2011) and long term effects have not been explored.

1.2.4. Magnetic stimulation of neurons

Magnetic neuromodulation methods come in essentially two different varieties, since magnetic fields can potentially interact with both electric charges as well as magnetic dipoles. In the first variety, an applied, temporally varying magnetic flux $\Phi_m(t)$ induces a voltage or “electromotive force”, $\varepsilon$ which is described mathematically by Faraday’s Law of Induction (Equation 1.1)

$$\varepsilon = -\frac{d\Phi_B}{dt}$$

Equation 1.1 – Faraday’s Law of Induction

If a voltage is induced across the cell membrane, then physiologically relevant electrical signals can be transmitted to the cell, thereby facilitating control of cellular behavior. An example of a method of this first variety is transcranial magnetic stimulation (TMS), in which an alternating magnetic field induces electrical activity in the brain (Walsh, Walsh, Cowey, & Cowey, 2000). TMS has been performed for more
than thirty years (Barker, Jalinous, & Freeston, 1985), but the details of the mechanism of TMS are poorly understood. In vitro evidence does suggest, however, that transmembrane ionic currents are caused by a temporally varying magnetic field (Pashut et al., 2014).

In the second variety, a magnetic dipole \( \mathbf{m} \) in a spatially varying magnetic field \( \mathbf{B} \) experiences a force given by Equation 1.2

\[
\mathbf{F} = \nabla (\mathbf{m} \cdot \mathbf{B})
\]

**Equation 1.2 – Force on magnetic dipole in a magnetic field**

In this case, a force instead of a voltage is the direct result of the applied magnetic field. Methods of this variety require a secondary mechanism to translate this force into physiologically relevant signals like voltage or ionic current. Since tissue is so transparent to magnetic fields (Funk et al., 2009) this requirement can be utilized to target only those places in the tissue which have been genetically or otherwise modified to be capable of translating magnetically induced force into physiological signal. Lee et al. working in the Bozovic Lab performed this second variety of magnetic neuromodulation to stimulated peripheral neurosensory inner ear hair cells (Lee et al., 2014; Rowland, Roongthumskul, Lee, Cheon, & Bozovic, 2011). In this case, the mechanism translating the force into a physiological signal was the force-gated channel natively expressed by the inner ear hair cells they chose to work with. This work provides a method that can facilitate research to better understand and control the force gating mechanism of inner ear hair cells, but it is not a method that can be applied to any cells of the nervous system.
Furthermore, an “electromagnetic needle” was required in order to apply a high enough magnetic field gradient to force open the channels. Hughes et al. (Hughes, McBain, Dobson, & El Haj, 2008) transfected COS cells with the force-gated TREK1 channel and induced voltage changes across the membranes of cells by applying magnetic fields with a rare earth magnet. Outward rectifying K^+ currents of up to several picoamps were recorded by the whole cell patch clamp method. While innovative and useful for doing basic research to understand the mechanisms force-gating by TREK1, the method developed by Hughes et al. was unable to achieve currents of sufficient magnitude and consistency to induce physiologically relevant changes from rest state in most cells.

RF magnetic field heating of nanoparticles has also been used for modulation of cellular physiology. Although the magnetic fields used for RF heating are temporally varying at high frequencies, this type of magnetic neuromodulation falls into the second category of magnetic neurostimulation described earlier in this section, and Faradaic induction of electromotive currents are secondary (and often undesirable) effects.

Nanoparticles will be driven to oscillate with the RF magnetic field and at high frequencies, will dissipate enough power to produce substantial heat. because of energy required to flip a multi-domain ferromagnetic particle. The power dissipated as heat is given by integrating over the hysteresis B-H curve (Borrelli, Luderer, & Panzarino, 1984), as in equation 1.3.

\[ P = \int f (HdB) \]

**Equation 1.3 – Power dissipation in magnetothermal stimulation**
Heating nanoparticles with RF magnetic fields is clinically known as “hyperthermia” (Pankhurst, Connolly, Jones, & Dobson, 2003) and is a common treatment for cancer. Hyperthermia methods have been practiced for over fifty years to kill undesired tissue (Borrelli et al., 1984; Gilchrist et al., 1957; Salunkhe, Khot, & Pawar, 2014).

RF heating of magnetic nanoparticles has been used very effectively by the Polina Lab at MIT for wireless deep brain stimulation in mice (Chen, Romero, Christiansen, Mohr, & Anikeeva, 2015). The work being done in the lab of Polina Anikeeva to magnetically induce neuromodulation is promising because they not only can genetically target cells with selective expression of TRPV1 heat sensitive channel, but they also are able to multiplex the method by selectively heating different types of particles by stimulation at target frequencies (Christiansen, Senko, Chen, Romero, & Anikeeva, 2014).

A recent publication by Stanley et al. demonstrated regulation of glucose levels in mice genetically encoded with TRPV1 heat-sensitive channels and genetically encoded superparamagnetic ferritin nanoparticles. They give evidence to support their ability to control levels of insulin by RF magnetic stimulation. They go on to suggest that they can also activate TRPV1 by application of force with a static magnetic field. Unfortunately, however, it seems to be physically unlikely that the insulin increase is due to magnetic force on ferritin in a magnetic field gradient. Stanley et al. failed to provide a detailed description of the experimental conditions (magnitude of the field gradient), but we can make approximations. They suggest that a force of 0.2 pN is required to open the TRPV1 channel by directed application of force to the channel itself, since this is the force
needed to open other force-gated channels (Dobson, 2008; Hughes et al., 2008; Walker et al., 1999). It is likely that Stanley et al. use magnet fields and field gradients close to those used in the paper they cite in the description of the setup (Hughes et al., 2008). Hughes et al. measured the field gradient to be 5.5 T/m and the magnetic field to be 80 mT at a distance of 1.5 cm from the magnet. Ferritin has a magnetic moment at saturation magnetization of between $10^{-22}$-$10^{-20}$ J/T (Galvez et al., 2008; Martínez-Pérez et al., 2010; Wajnberg, El-Jaick, Linhares, & Esquivel, 2001). Although 80 mT is far below the field strength required to saturate magnetoferritin, we will still use the “best case” scenario and the higher estimate of saturation magnetic moment, $10^{-20}$ J/T. Such a ferritin molecule in a magnetic field of gradient 5.5 T/m will experience a force of approximately $5.5 \times 10^{-20}$ N. There would need to be on the order of $10^8$ ferritins per channel in order to achieve forces of the magnitude that Stanley et al. suggest are required (0.2 pN). It is important to note here that Stanley et al. design the experiment so that only a single ferritin binds to each TRPV1 channel. And so they claim to be acting upon the TRPV1 channel with a force that seems to be $10^8$ times larger than what is physically possible.

RF magnetothermal neuromodulation is promising, but there are numerous drawbacks. The Anikeeva group is able to elicit trains of action potentials in vivo, but only at very low temporal resolution since it is necessary to apply RF for 5-10 seconds before the spike train begins. RF magnetic neuromodulation is also potentially harmful to the tissue being stimulated, since it is necessary to achieve local temperatures of $>43^\circ$C, which is an appropriate temperature to oblate cancerous tissue by magnetic hyperthermia (Salunkhe et al., 2014). Finally, RF magnetic fields can interfere with electrophysiological recording and can induce stray Faradaic currents, as is purposefully
done with transcranial magnetic stimulation (Walsh et al., 2000). The magnetic neuromodulation described in this thesis is unique because of the mechanism of application of force and because unlikely most methods a uniform, static magnetic field is used.

1.2.5. Conclusion

The methods described above illustrate only a sample of the many available neuromodulation techniques that have been developed. Each technique has its own advantages and disadvantages, but all are important to the development of new ones. Building upon the current techniques, the method described in this thesis is similar to the magnetic neuromodulation employed by the Bozovic Lab (Lee et al., 2014) in that magnetic nanoparticles are used to act upon mechanosensory channels but our method is different in that it is genetically programmable and stimulation is with a uniform, static magnetic field. The method could also be said to be similar to that used by the Anikeeva lab (Christiansen et al., 2014) since both methods are genetically programmable, but we use static rather than RF magnetic fields and there is no risk of overheating the tissue. The genetically programmable element is discussed in the following section: we use the mechanosensitive Piezo1 ion channel (Coste et al., 2010) to transduce magnetically induced forces.

1.3. Piezo1 discovery, characteristics

Before the discovery of Piezo1 and Piezo2, few eukaryotic mechanosensitive ion channels had been identified and molecular mechanisms for mammalian
mechanotransduction were poorly understood (Chalfie, 2009; Tsunozaki & Bautista, 2009). This subsection will review relevant literature related to the discovery of Piezo1, its characteristics, and the motivation for using it in our experimental method.

1.3.1. Discovery of Piezo1

Piezo1 was discovered in 2010 by Coste et al. (Coste et al., 2010) working in the lab of Ardem Patapoutian. They sought to identify a protein responsible for mechanically activated currents similar to those previously recorded in primary cells (Coste, Crest, & Delmas, 2007). They assayed mouse and rat cell lines (Neuro2A, C2C12, NIH/3T3, Min-6, 50B11, F11 and PC12) by recording in whole cell mode while applying mechanical stimulus with a glass pipette. Out of two cell lines (Neuro2A and C2C12) that exhibited mechanically activated currents, those from Neuro2A cells were the most consistent and displayed faster kinetics. They performed DNA microarray screening to find proteins predicted to span the membrane at least twice and also proteins either known to be cation channels or of unknown function. They then performed short interfering RNA (siRNA) screens to knock down each candidate protein and found that knockdown of Fam38A (which they subsequently named “Piezo1”) produced the most pronounced decrease in mechanically activated currents. Transgenic expression of Piezo1 in HEK293T cells transferred mechanically sensitive currents with similar kinetics and current-voltage relationships to those found in Neuro2A cells. By these results, they showed conclusively that Piezo1 is necessary for mechanically activated currents, but did not conclusively report it as a mechanosensitive ion channel.
We have replicated the results of Coste et al. in our lab by recording from Neuro2A cells and stimulating using the method they describe.

1.3.2. Characteristics of Piezo1 ion channels

In 2012, the Patapoutian lab showed conclusively that Piezo1 is a pore-forming ion channel or is a subunit of a pore-forming ion channel (Coste et al., 2012). This led to increased interest in Piezo1 and to many studies of its characteristics (P. a Gottlieb & Sachs, 2012).

Piezo1 multichannel currents are well modeled by a 3-state model (closed, open, inactivated). The rate constant from closed to open state is known very small (less than 2 ms) and is pressure dependent but is difficult to measure precisely because it is so short relative to the kinetics of the mechanical stimulus (P. A. Gottlieb, Bae, & Sachs, 2012). The inactivation time constant is strongly voltage-dependent: 16 ms at -80 mV, 31 ms at -40 mV, 170 ms at +40 mV and 450 ms at +80 mV (P. A. Gottlieb et al., 2012).

Figure 1.1 Whole cell currents recorded from wild type Neuro2A cells. (A) Data from Coste et. Al, 2010 (B) Data acquired by author, 2014
Evidence suggests that Piezo1 forms homotetramers in the membrane. Coste et al. (Coste et al., 2012) reported that fluorescent bleaching of Piezo1-GFP fusion oligomers happened in 4 discrete steps, suggesting that Piezo1 forms tetramers in the membrane. They corroborated this by running the mouse Piezo1 protein on a native gel and staining with Coomassie blue, finding a prominent band at 1.2 MDa, the size of Piezo1 homotetramer. Using a super-resolution optical microscopy technique, Wang et al. (Wang et al., 2014) measured the distance between adjacent and diagonal subunits of the tetramer to be 36 ± 10 nm and 22 ± 5 nm, respectively.

Further evidence suggests that many Piezo1 proteins can form domains in the membrane. Gottlieb and Sachs recorded whole cell currents from cells expressing Piezo1. They demonstrated that repeated mechanical stimulation or treatment with cytoD, which breaks down actin, leads to loss of inactivation. Loss of inactivation seemed to occur simultaneously over all channels being recorded (~50-100 channels) suggesting that the channels were coupled together somehow, perhaps as a lipid raft (P. A. Gottlieb et al., 2012). This hypothesis of channel coupling via aggregation within the cell membrane is supported by our observations of immunostaining of the Piezo1 channel (see Chapter 2).

Piezo1 are reversibly blocked by Ruthenium Red and GsMTX, but only when applied to the extracellular side of the cell membrane (Bae, Sachs, & Gottlieb, 2011; Coste et al., 2012). Reversible blocking with gadolinium is also possible, but it has not been shown whether this blocking is specific to extracellular application (Coste et al., 2010).
1.3.3. Motivation for using Piezo1

With a variety of mechanosensitive channels available, it is natural to ask the question: why use Piezo1 as the mechanosensitive piece of the tool? Piezo1 is an attractive candidate for several reasons. It is the only mammalian mechanosensitive cation channel that does not appear to have alternative sensitivity modalities. TRP channels are heat- or cold-sensitive as well as mechanosensitive (Story & Gereau, 2006; Voets et al., 2004). Evidence suggests that Piezo1 is directly force-gated, requiring no other proteins, not even cytoskeleton, for activation (P. a Gottlieb & Sachs, 2012; Miyamoto et al., 2014)

Of importance for using this tool for neuroscience, Piezo1 is outward-rectifying, meaning that the currents it conducts depolarize the cell. Depolarizing currents can generate action potentials in neurons if sufficiently strong enough to depolarize the cell to threshold potential (usually about -30 mV). In contrast, TREK and TRAAK channels are mechanosensitive only, but are selective for K^+ ionic current and therefore inward rectifying (Brohawn, Su, & MacKinnon, 2014). Using a method similar to the one I discuss herein, TREK and TRAAK could potentially be modified to develop a magnetomechanical tool for inhibition of neuronal activity.

Piezo1 is also a good candidate ion channel because of its relevance in other research areas. Mutation of the Piezo1 protein is responsible for the hereditary disease xerocytosis (Bae, Gnanasambandam, Nicolai, Sachs, & Gottlieb, 2013; Zarychanski et al., 2012). Furthermore, Piezo1 plays a critical role in vascular endothelial cells of developing mice (Ranade et al., 2014). The insights we will gain about Piezo1 for
practical application in our technique will be useful to these other areas of research as well.

Finally, preliminary work performed in our lab (Section 2.1.2) validates the potential for Piezo1 to translate magnetically activated mechanical forces into action potentials in neurons.

1.4. Superparamagnetic nanoparticles

Superparamagnetic nanoparticles are used in our method to translate the applied uniform magnetic field into mechanical force on the Piezo1 protein. In this section, I will review the physics of superparamagnetism, the properties of superparamagnetic iron oxide nanoparticles (SPIONs) and some relevant literature.

1.4.1. Physics of superparamagnetism

In 1930, Frenkel and Doefman (Frenkel & Doefman, 1930) predicted in qualitative terms that sufficiently small ferromagnetic particles would consist of a single domain and may not display hysteresis observed in larger ferromagnetic material. A rigorous theoretical framework was derived by Louis Néel in 1949 (Néel, 1949) and William Brown in 1963 (Brown, 1963) which has come to be known as Néel relaxation theory or Néel-Brown theory. Superparamagnetism, phenomenon observed in single domain ferromagnetic and ferrimagnetic nanoparticles, can be explained by Néel-Brown relaxation theory.
A single-domain nanoparticle is one which is uniformly magnetized at any field (Bean & Livingston, 1959). The energy of a perfectly isotropic nanoparticle with magnetic moment, $\vec{\mu}$, in the presence of an applied field, $\vec{B}$ is given by Equation 1.4.

$$U = -\vec{\mu} \cdot \vec{B}$$

**Equation 1.4 – Energy of isotropic magnetic particle in a magnetic field**

We can calculate the fraction of magnetization aligned with the field for an assembly of such nanoparticles by averaging over a Boltzman distribution. From this we get the Langevin function, $L(x) = \coth x - 1/x$, where $x = \mu B / k T$. This gives rise to the familiar Langevin paramagnetism curve. The physics describing an ensemble of isotropic superparamagnetic nanoparticles is very similar to the physics of an isotropic paramagnetic solid.

However, a real nanoparticle is not magnetically anisotropic due to spin-orbit coupling interactions determined by crystal structure or physical shape. The phenomenon of superparamagnetism is observed when the magnetic anisotropy energy is significantly less than the thermal energy, and so the particle can be treated as being isotropic. For a uniaxially anisotropic nanoparticle, the energy will be given by $E_K = KV \sin^2 \theta$, (Bean & Livingston, 1959) where $V$ is the volume of the particle, $K$ is the anisotropy energy density and $\theta$ is the angle between the magnetization of the particle and its easy axis (or symmetry axis). The total energy of an anisotropic particle in a magnetic field is $E_K + U$ (Equation 1.5)
\[ E_{\text{total}} = KV \sin^2 \theta - \mu B \cos \theta \]

Equation 1.5 – Energy of magnetically anisotropic particle in magnetic field

For an ensemble of particles in zero external magnetic field, there will be two stable configurations, at \( \theta = 0 \) and \( \theta = \pi \), separated by an energy barrier \( KV \). In a nonzero magnetic field, however, the energies of the two stable configurations will shift, so that \( \theta = 0 \) becomes more energetically favorable than \( \theta = \pi \). The energy barrier will be correspondingly increased by \( \mu B \) for \( \theta = 0 \) and decreased by \( \mu B \) for \( \theta = \pi \).

![Diagram](image)

Figure 1.2 – Taken from (Bean & Livingston, 1959)

If an ensemble of uniaxially anisotropic particles is demagnetized from an initial state of being fully aligned with total magnetization \( M_s \), the fractional magnetization will decay as described by Equation 1.6 (Bean & Livingston, 1959), where \( t \) is the time from
the moment the field is turned off and \(\tau_N\) is the time constant of the decay, called the Néel relaxation time (Néel, 1949).

\[
M(t) = M_s \exp \left(-\frac{t}{\tau_N}\right)
\]

**Equation 1.6 – Fractional magnetization, \(M\), for an ensemble of particles**

For a given particle in an ensemble at temperature, \(T\), the probability of a transition between energy states separated by energy \(\Delta E\) is given by the Boltzmann factor \(\exp(-\Delta E/k_B T)\), and so the Néel relaxation time is given by Equation 1.7, where \(\tau_0\) is a time constant characteristic of the material (called the “attempt time”, typically on the order of \(10^{-9}\) or \(10^{-10}\) seconds).

\[
\tau_N = \tau_0 \exp\left(KV/k_B T\right)
\]

**Equation 1.7 – Néel relaxation time, \(\tau_N\)**

For longer Néel relaxation times, the magnetization of a particle will be more stable.

If the measurement time is much longer than the Néel relaxation time, then the magnetization will average out to zero during the measurement at zero external field. A particle is said to be “superparamagnetic” when the measurement time is much greater than the Neel relaxation time \((\tau_m \gg \tau_N)\). For a particle of a given volume and anisotropic energy density, \(K\), the temperature at which \(\tau_m = \tau_N\) is called the “blocking temperature”. This can be easily derived from Equation 1.7:
\[ T_N = \frac{KV}{k_B \ln \left( \frac{t_m}{t_0} \right)} \]

**Equation 1.8 – Blocking temperature, \( T_N \)**

It is important to note that the Néel relaxation time, and thus the stability, depends exponentially on the volume of the nanoparticle. For this reason, there is a sharp transition from the stable ferromagnetic regime to the superparamagnetic regime. In order to determine the size at which a particle becomes unstable, and enters the superparamagnetic regime, we can define, as others do (Bean & Livingston, 1959) “stability” to be at a Néel relaxation time of \( 10^2 \) seconds. This gives an energy barrier, \( KV \), of \( 25k_BT \). Blocking temperature is equal to room temperature for h.c.p cobalt particles of 4 nm radius, f.c.c. cobalt particles of radius 14 nm and iron particles of 12.5 nm radius. We use iron oxide particles of 2.5 – 5 nm radius, which are expected to exhibit superparamagnetic behavior at room temperature.
In this chapter I will describe the details of a novel magnetic neuromodulation method beginning with a discussion of the Piezo1 protein, followed by a section on superparamagnetic iron oxide nanoparticles (SPIONs) and concluding with a brief section describing how this system could be used in vivo. Briefly, an externally applied magnetic field modulates the interactions between multiple SPIONs attached to the subunits of the Piezo1 channel. Interactions between the SPIONs lead to tension forces on the Piezo1 channel. These forces are predicted to be sufficient to open the channel, leading to cationic currents.
2.1. Piezo1

2.1.1. Calculating the gating force of Piezo1

Hughes et al. tagged an extracellular domain of TREK1 with magnetic particles and pulled on the particles with the magnetic field gradient from a neodymium magnet (Hughes et al., 2008). They recorded currents activated by mechanical forces as low as 0.2 pN per particle. Walker used optical tweezers to activate force-gated Ca\(^{++}\) channels in cells derived from bone, using 7pN force (Walker et al., 1999). The force is exerted on the cell membrane and is not localized to a channel, and so we can suppose that the single-channel gating force will be lower than 7pN. Howard & Hudspeth measured the gating force for mechanosensitive channels (MSCs) of hair cells to be between 0.2-0.4 pN by using a fiber-optical cable to exert force on hair cells to be in this range (Howard & Hudspeth, 1988).

We can make approximations using measurements of the Young’s modulus of elasticity of HEK cell membrane (Kamgoué, Ohayon, & Tracqui, 2007) and measurements made of gating pressure of Piezo1 ion channel (P. A. Gottlieb et al., 2012). In the paper announcing the discovery of Piezo1 and Piezo2, Coste et al. (Coste et al., 2010) measured the mechanically induced current and plot it against pressure applied by pipet in cell-attached mode. The pressure at which a single channel in the patch is 50% likely to be open is -28 ± 1.8 mmHg. We can use the definition of the Young’s modulus to calculate the force generated by pressure application to the cell membrane. This force is given by Equation 2.1, where E is the Young’s modulus, \(A_0\) is the area upon which the
force is applied, $\Delta L$ is the change in the circumference of the cell, $L_0$ is the initial circumference of the cell.

$$F = \frac{EA_0\Delta L}{L_0}$$

**Equation 2.1 – Force on elastic cell membrane due to deformation**

For a patch radius of 1 $\mu$m, Suchyna et al. measure a patch deflection of 0.15 $\mu$m for negative pressure application of 30 mmHg (Suchyna, Markin, & Sachs, 2009). Experimental values of Young’s modulus of the cell membrane are in the range of 30-120 pA (Kamgoué et al., 2007). Assuming a cell radius of 10 $\mu$m, we calculate a $F_{50}$ force of 0.225-0.9 pN. Not surprisingly, this is close to single channel gating force measurements of many MSCs (Dobson, 2008).

These measurements of $P_{50}$, made in cell attached configuration, are measuring between 50-100 channels (P. A. Gottlieb et al., 2012), so this is an approximation for the gating force on a population of channels, not the single-channel gating force. Dobson suggests that activation of a mechanosensitive channel directly requires less force than activating indirectly through stretch forces in the membrane (Dobson, 2008), so we expect that the gating force of a single Piezo1 channel is lower than the value calculated above. Our experimental tool could allow us to make more exact measurements of the gating force of a single Piezo1 channel, and this technique could also be used to characterize this property of other mechanosensitive channels.
2.1.2. Transgenic expression of Piezo1 in neurons

To test the feasibility of using Piezo1 to manipulate the behavior of neurons, we needed to ensure that Piezo1 could be transgenically expressed in neurons. Neurons cultured from primary rat hippocampus were transfected with Piezo1 cDNA using the BrainBits transfection online protocol and reagents. 5 days after transfection, neurons were mechanically stimulated using a fire-polished glass pipet while recording in current clamp and voltage clamp mode in whole cell configuration (Coste et al., 2010). Stimulation with pulses of 1 second duration induced strong (~500 pA) currents in voltage clamp and robust action potential waveforms in current clamp. These mechanically activated currents exhibit inactivation with time constant of 5.7 ms. The action potentials induced by mechanical stimulation exhibit abnormally long decay constants from peak voltage. This is most likely due to the long decay constant of Piezo1, which is approximately 250 ms at +40 mV membrane potential and 450 ms at +80 mV membrane potential.
2.1.3. Engineering Piezo1 mutants for binding superparamagnetic particles

In order to exert a direct force on Piezo1 it was necessary to mutate the protein by inserting tags where the superparamagnetic particles could bind. We selected candidate
insertion sites based on 2D modelling of Piezo1 (Figure 2.3A). We generated 15 Piezo1 mutant plasmid constructs, each with a single c-Myc-tag insertion at one of the candidate locations. The cells used to test Piezo1 mutants were HEK293, because they are easier to culture than neurons and, unlike Neuro2A, do not natively express any mechanosensitive ion channels. They are capable of transgenically expressing WT Piezo1 via transfection, as is evident from mechanocurrents shown in figure.

Figure 2.2 – Mechanosensitive currents recorded from HEK293 cells transfected with WT Piezo1. (A) Mechanically gated currents recorded from HEK293 cells transgenically expressing WT Piezo1 “3 um” and “4 um” scale bars indicate displacement of stimulation pipet from starting location (B) Technique used to stimulate. Stimulation pipet on left, recording pipet on right
We tested the accessibility of these insertion sites in HEK293 cells transfected with these plasmids by performing immunofluorescence assays (Figure 2.3B). We tested the effect of insertion mutations on protein function by performing electrophysiological mechanosensitivity assays (Figure 2.3C).

Figure 2.3A is the 2D structure of Piezo1 based on hydrophobic analysis of amino acid domains using Phobius software (http://phobius.sbc.su.se/instructions.html). Regions in blue were predicted to be extracellular by both Phobius and Topcons, a similar software. The residues in red are the locations where c-Myc-tag was inserted.

Several of the c-Myc-tag insertions were found to be accessible. We assayed accessibility by transfecting HEK293 cells with the plasmids and then incubating at 4°C (to minimize endocytosis) with c-Myc antibody clone 9B11 (Cell Signalling) conjugated to AlexaFluor 647. After incubation with antibody, cells were washed and fixed and either analyzed by flow cytometry or by confocal microscopy (Figure 2.4). c-Myc-tags located at insertion sites ECD2, ECD5, ECD8 and ECD9i4 were found to be the most accessible. These assays strongly suggest that ECD2, 5, 8, 9i4 are extracellular domains of Piezo1, however they give insufficient evidence to make the claim that the other sites are intracellular (ECD1, 3, 4, 6, 7i1, 7i2, 9i1, 9i2 and 9i3). It is very possible that these sites are simply inaccessible to anti-Myc due to protein folding.
Figure 2.3 – Model of Piezo1 generated based on hydrophobicity. (A) Predicted topology of Piezo1 based on the Phobius prediction program. The regions also predicted to be extracellular with Topcons are in blue. The red colored residues are the locations where c-Myc-tag are inserted i.e. where no significant folded structure could be predicted. (B) Accessibility of the c-Myc-tags assessed by flow cytometry. The values are in % of transfected cells that where labelled by the 9B11 antibody. n=3 for each construct. Average ± sd (C) Inactivation time constants from traces obtained with Piezo-1 and P1ezo-1 with c-Myc tags. The insert shows a representative trace (here ECD3). Error bars are the standard deviations., n is the number of cell tested. ECD: extracellular domain.

Most of the mutants preserved mechanosensitivity. We assayed electrophysiological response to mechanical stimulation using the method described by Coste et al. (Coste et al., 2010). Briefly, cells were HEK293 transfected with Piezo1 mutants and monitored by voltage clamp electrophysiology in whole cell configuration. Whole cell membrane current was recorded while the cell membrane was deformed using a fire-polished micropipette driven by a micromanipulator. Mechanosensitivity of Piezo1
was conserved in all of the mutants except for three in ECD9 (ECD9i1, ECD9i2, ECD9i3). These results are summarized in Figure 2.3.

We used confocal microscopy and flow cytometry to determine which insertion sites are more accessible than others. The most accessible sites proved to be ECD2, ECD8 and ECD9i4. Data from these sites as well as ECD3 and ECD5, two less accessible sites, are shown in Figure 2.4. Several sites that were predicted to be extracellular by hydrophobicity analysis using Phobius (see Figure 2.3) were unlabeled by extracellular c-Myc antibody in our immunoassays.

![Confocal images and summary of flow cytometry data indicating that ECD2, ECD8 and ECD9i4 on Piezo1 are extracellular and accessible. Note that the punctate membrane staining patterns we observe agree with the hypothesis of channel coupling described by Gottlieb et al. (P. A. Gottlieb et al., 2012)](image)

This does not, however, mean that these sites are intracellular because they could be inaccessible for other reasons such as protein folding configurations of Piezo1 such that
these sites are not exposed. For the purposes of the magneto-mechanical neuromodulation method described in this thesis, these details of protein structure are unimportant, but for fundamental biological studies of Piezo1, these details are certainly of interest.

2.2. Superparamagnetic iron oxide particles (SPIONs)

2.2.1. Forces calculations for SPIONs

In this section, I consider the interactions between superparamagnetic iron oxide nanoparticles (SPIONs) bound to the Piezo1 protein. Fe$_3$O$_4$ nanoparticles theoretically exhibit superparamagnetic behavior at any diameter less than about 30nm (Krishnan, 2010), but due to fabrication defects, superparamagnetic properties are typically not manifested above 17 nm (Kolhatkar, Jamison, Litvinov, Willson, & Lee, 2013). In a review article, Kolhatkar et al. provide a summary of data from other papers, which I will rely on heavily (Kolhatkar et al., 2013). Importantly, the saturation magnetization are size-dependent. I will consider SPIONs of 5 nm and 10 nm diameter and assume values of magnetization saturation of 75 emu/g, and 60 emu/g, respectively (Caruntu, Caruntu, & O’Connor, 2007; Guardia, Labarta, & Batlle, 2011; Kolhatkar et al., 2013). Since 5nm and 10nm particles have masses of 0.33 x 10$^{-18}$ and 2.6 x 10$^{-18}$ g, single particle magnetic moments will be 2.5 x 10$^{-17}$ and 16 x 10$^{-17}$ emu (2.5 x 10$^{-20}$ and 16 x 10$^{-20}$ A*m$^2$). I will be assuming that the particles are at saturation magnetization. This is a reasonable assumption because the fields that our solenoid can generate (>200 mT) are sufficient for the ensemble to reach saturation magnetization (Caruntu et al., 2007).
The interactions between SPIONs bound to Piezo1 are of particular interest. Recall from Section 1.4.2 that Piezo1 is thought to form tetramers in the membrane, and Wang et al. (Wang et al., 2014) measured the distance between adjacent and diagonal subunits of the Piezo1 tetramer to be 36 ± 10 nm and 22 ± 5 nm, respectively. It is straightforward then, to calculate the forces between particles bound to the Piezo1 subunits of a single channel.

The force on a single nanoparticle in the presence of the magnetic field produced by a second nanoparticle is straightforward to estimate if we approximate each nanoparticle as a single magnetic dipole, with magnetic moment, $\vec{m}$. The magnetic field due to a single dipole is given by Equation 2.2

\[
\vec{B} = -\frac{\mu_0}{4\pi} \nabla \left( \frac{\vec{m} \cdot \vec{r}}{r^3} \right)
\]

**Equation 2.2 Magnetic field from a single dipole**

Choosing our polar coordinates so that $\vec{m} = m\hat{y}$, $\vec{r} = r\cos\theta\hat{x} + r\sin\theta\hat{y}$

\[
\vec{B} = -\frac{\mu_0}{4\pi} \nabla \left( \frac{m \sin \theta}{r^2} \right)
\]

\[
= -\frac{\mu_0}{4\pi} \left( \hat{\rho} \frac{\partial}{\partial \rho} + \hat{\theta} \frac{1}{\rho} \frac{\partial}{\partial \theta} \right) \left( \frac{m \sin \theta}{r^2} \right)
\]

\[
= \frac{\mu_0 m}{4\pi r^3} \left( 2 \sin \theta \hat{\rho} - \cos \theta \hat{\theta} \right)
\]

The force on a second magnetic dipole at $\vec{r}$ with magnetic moment parallel to the first will be:
\[ \vec{F} = \vec{\nabla}(\vec{m} \cdot \vec{B}) \]

\[ = \vec{\nabla} \frac{\mu_0 m^2}{4\pi r^3} (2 \sin^2 \theta - \cos^2 \theta) \]

This can be expanded to give the following equation:

\[ \vec{F} = \frac{\mu_0 m^2}{4\pi r^4} \left[ -3(2 \sin^2 \theta - \cos^2 \theta)\hat{r} + 6 \sin \theta \cos \theta \hat{\theta} \right] \]

**Equation 2.3 – Force between two magnetic dipoles**

We can also find the energy:

\[ E = \int_{\infty,\theta}^{r,\theta} \int \vec{F} \cdot d\vec{r} \]

This is expanded to give:

\[ E = \frac{\mu_0 m^2}{4\pi r^4} (\cos^2 \theta - 5 \sin^2 \theta) \]

**Equation 2.4 – Energy between two magnetic dipoles**

The interaction force and energy of a two particle system is plotted in Figure 2.5, with a single nanoparticle fixed at the origin and a second particle located at \( \vec{r} \) for both plots. The most stable configuration is with both magnetic moments aligned and therefore attracting one another. Two 5 nm particles can get no closer than 5 nm apart – at this
distance, the force between the two particles is 0.54 pN. This is in range of the forces required to open Piezo1 (0.2-0.9 pN), which I calculated in Section 2.1.1.

Figure 2.5 Interactions between two 5 nm nanoparticles attached to Piezo1
A) Force on second identical particle in the field of the first, B) Energy of the two particle system for one particle at origin, second particle at $\vec{r}$

Figure 2.6 – Diagram of Piezo1 tetramer, with distances as measured by (Wang et al., 2014)
A summary of various configurations of particles present or absent on the Piezo1 tetramer at locations indicated by Figure 2.6 is given in Table 2.1. Two 5 nm particles located on adjacent subunits of the Piezo1 tetramer ($\Delta r = 22 \pm 5$ nm) will interact with a force of 0.35-2.2 fN, and two 5 nm particles located on diagonal subunits of the tetramer ($\Delta r = 36 \pm 10$ nm) will interact with a force of 0.041-0.40 fN. It is also helpful to consider the energy of the system for each configuration. It is expected that some configurations may be less energetically favorable (positive energy values with respect to infinite separation), and so it is less likely for particles to be bound in that configuration. Since the values for configuration energy are on the order of $10^{-23}$ J, much less than room temperature molecular energy ($10^{-21}$ J), configuration energy is unlikely to play a role. These values are also given in Table 2.1.

<table>
<thead>
<tr>
<th>Loc. 1</th>
<th>Loc. 2</th>
<th>Loc. 3</th>
<th>Loc. 4</th>
<th>Force (fN)</th>
<th>Energy($\times 10^{-23}$ J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sqrt{\ }$</td>
<td>$\sqrt{\ }$</td>
<td>0</td>
<td>0</td>
<td>0.35 to 2.5</td>
<td>0.32 to 1.3</td>
</tr>
<tr>
<td>$\sqrt{\ }$</td>
<td>$\sqrt{\ }$</td>
<td>$\sqrt{\ }$</td>
<td>0</td>
<td>0.35 to 2.5</td>
<td>-1.3 to -5.1</td>
</tr>
<tr>
<td>$\sqrt{\ }$</td>
<td>$\sqrt{\ }$</td>
<td>$\sqrt{\ }$</td>
<td>$\sqrt{\ }$</td>
<td>0.38 to 2.5</td>
<td>-1.4 to -5.8</td>
</tr>
<tr>
<td>$\sqrt{\ }$</td>
<td>0</td>
<td>$\sqrt{\ }$</td>
<td>$\sqrt{\ }$</td>
<td>0.73 to 4.7</td>
<td>-1.7 to -7.1</td>
</tr>
<tr>
<td>$\sqrt{\ }$</td>
<td>0</td>
<td>$\sqrt{\ }$</td>
<td>0</td>
<td>0.71 to 4.5</td>
<td>-1.6 to -6.4</td>
</tr>
<tr>
<td>$\sqrt{\ }$</td>
<td>0</td>
<td>0</td>
<td>$\sqrt{\ }$</td>
<td>0.047 to 0.46</td>
<td>-0.13 to -0.71</td>
</tr>
</tbody>
</table>

Table 2.1 Table of force and energy on particle at “Location 1” for different configurations of particles present (“$\sqrt{\ }$”) or absent (“0”) on Piezo1

In order to exert sufficient force on the Piezo1 channel without the SPIONs touching one another, we decided to use a short linker to attach the SPIONs to the Piezo1
channel. In the next section I discuss the possibilities we considered and the rationale behind choosing bungarotoxin (BGTX) as a linker.

2.2.2. Conjugating SPIONs to bungarotoxin protein

We chose to use bungarotoxin (BGTX) to attach the SPIONs to the Piezo1 channel because of the small size. Figure 2.7 depicts components of the various components used in our method, in a scaled drawing.

Figure 2.7 – Scaled drawing of components of this method. Blue circles: 5 and 10 nm nanoparticles, Black circles: Piezo1 single proteins, subunits of tetramer

We first explored the use of a c-Myc tag and antibodies to c-Myc. For this reason the characterization of the accessibility of different binding sites on Piezo1 was performed using c-Myc and anti-Myc immunofluorescence staining. Once we had determined which sites were most accessible (ECD2, 8, 9) we transitioned to a smaller
linker between the SPIONs and the channel. Two ligand pairs were considered: nanobodies (Wang et al., 2014) and bungarotoxin (Changeux, Kasai, & Lee, 1970).
Nanobodies are non-toxic and have high affinity (picomolar to nanomolar $K_D$ values) for their antigen, however, nanobodies against small peptides are not currently available and so a large protein like GFP would need to be also part of the linker, which is not desirable. Therefore we chose bungarotoxin, which is an AChR antagonist and therefore highly toxic to living animals, but is sufficient for proof of principle in vitro experiments. For in vivo experiments, it is likely that we will transition to using nanobodies against anti-myc or against a specific extracellular domain of the Piezo1 protein.

SPIONs functionalized with carboxylic acid groups were conjugated to bungarotoxin by carboxyl crosslinking with primary amine groups on bungarotoxin protein. This process is detailed in Figure 2.8

Conjugated nanoparticle product was characterized by gel electrophoresis, which indicates successful conjugation of all of the SPIONs (see brightfield image of gel in Figure 2.9). The conjugated SPIONs are expected to move slower than the unconjugated SPIONs, which is what we see in the gel results. Furthermore, we see no SPIONs in the supernatant of the conjugation reaction.
2.2.3. Potential issues we may encounter

It has been demonstrated that static magnetic fields of 400 mT strength decrease the opening probability of the bacterial mechanosensitive channel, MscL (Petrov & Martinac, 2007). Petrov and Martinac posit that this is due to properties of the membrane, and not the MscL protein itself, so it is possible that static magnetic fields may reduce channel opening probability of Piezo1 also. This can be easily tested. If this is a factor, it is possible that the reduced channel opening probability will be offset by the forces exerted by the SPIONs.

The magnetic field stimulus may interact with the electrophysiological recording equipment. Static magnetic fields are likely to interact much less than alternating fields used in other magnetic neuromodulation methods (Christiansen et al., 2014). At the times
when the magnet is turned on or off, there will be currents induced in any unshielded conductive material. If this poses serious issues with patch clamp recording, then Ca sensitive fluorescent dyes or genetically encoded voltage sensing proteins like GCaMP can be used.

The electromagnet itself may mechanically deform when turned on and off. The silicon steel core will become strongly magnetized when current is passed through the coil, and at the gap there is a magnetic field of 500mT.

### 2.3. Towards in vivo applications of this method

As an *in vitro* tool, magneto-mechanical neuromodulation could facilitate previously infeasible experiments, surpassing current methods (discussed in Chapter 1) by being less invasive. Noninvasiveness is an even more important characteristic of a good neuromodulation tool *in vivo*. To make use of this tool *in vitro*, all that is needed is the DNA in plasmid form so that primary cells or a cell line can be transiently transfected. This same approach is not possible *in vivo*, and instead viral transduction is more typical. The cDNA (~8000 bp) for our Piezo1 mutants could be inserted into a viral construct downstream of a cell-type specific promoter such as parvalbumin (basket cell interneurons), calcium/calmodulin kinase IIα (pyramidal neurons), Thy1 (Purkinje cells) or glial fibrillary astrocytic protein (astrocytes) (Campsall, Mazerolle, De Repentiny, Kothary, & Wallace, 2002; Hippenmeyer et al., 2005; Mayford et al., 1996; Rogan & Roth, 2011; Sweger, Casper, Scearce-Levie, Conklin, & McCarthy, 2007). Wild-type mice, for example, could then be infected with the viral construct by injection into a particular region of the brain. Alternatively, mouse lines expressing our Piezo1
mutants under specific promoters could be established using the Cre-LoxP system. As an alternative to promoters, Guillaume Duret, a post-doc in the Robinson Lab, has experience with using BACs and flanking the transgene of interest with long sequences upstream and downstream of the genomic DNA of proteins that differentiate cells, thereby limiting expression of the transgene only to those cells which can naturally express the differentiation protein.

Specific promoters will be intended to sensitize specific populations of cells or brain regions to magnetic fields. However, promoter-driven expression of transgenes is often not uniquely specific to one cell type or brain region and such expression is exhibits temporal dependence (Campsall et al., 2002). Therefore it may prove to be necessary to target specific brain regions by precise injection of SPION conjugates to those regions. For this reason, it is in fact advantageous that cells in these mouse lines that express the mutant Piezo1 proteins would remain insensitive to magnetic fields until SPION conjugates are introduced.

Two objections may be raised with regard to the claim that our system is minimally invasive. First, it can be argued that the injection of SPIONs into the brain is invasive. While this is true, it is significantly less invasive than implanted electrodes or fiber-optic cables, which must remain implanted during stimulation (see Chapter 1). Second, bungarotoxin-SPION conjugates are extremely invasive since bungarotoxin is a Nicotinic acetylcholine receptor (nACHR) antagonist. For an in vivo system, bungarotoxin would not be a viable option for tagging SPIONs to the Piezo1 protein. Instead, nanobodies (Rothbauer et al., 2006; Wang et al., 2014) against the Piezo1 protein or against c-Myc would need to be generated.
Chapter 3

Methods

3.1. Cell culture

3.1.1. HEK293 and Neuro-2a Cell Lines

HEK293 cells (ATCC CRL-1572) were maintained in DMEM high glucose (Life Technologies 11965) with 10% fetal bovine serum (Life Technologies 26400). Neuro-2a cells (ATCC CCL-131) were maintained in EMEM (ATCC 30-2003) with 10% FBS (Life Technologies 26400). Cells were grown up to confluency in T75 flasks and passaged approximately every 1-2 weeks.

3.1.2. Primary Rat Hippocampal Cell Culture

Neurons were cultured on an astrocyte feeder layer as described in various protocols, with minor modifications (Albuquerque, Joseph, Choudhury, & MacDermott, 2009; Brewer, Torricelli, Evege, & Price, 1993). Both cell types were derived from
hippocampal tissue from E18 Sprague Dawley rats that was purchased from BrainBits, LLC (SKU: hp). Astrocyte cultures were established by growing cells from dissociated hippocampal tissue in NbAstro medium (BrainBits, SKU: NbASTRO). Astrocytes were harvested with Tryple (Life Technologies, 12605) and plated on PDL-coated 12 mm coverslips at a density of 5,000 cells/mm². E18 hippocampal neurons (BrainBits, LLC) were plated on astrocyte substrate at a density of 10,000 cells/mm² in NbActiv1 medium supplemented with 3% fetal bovine serum (Life Technologies 26400). 50% of the medium was replaced with fresh medium every 3-4 days. Experiments described herein were performed at DIV 7-14.

3.2. Molecular Biology

3.2.1. Protein engineering

Q5 Site-Directed Mutagenesis Kit (New England BioLabs) and corresponding protocol was used for insertion of mutations at the various ECD# sites. DH5α bacteria (NEB) were transformed with plasmids for mutagenesis and plasmid preparations. DNA sequencing was performed by SeqWright. DNA analysis

Plasmid map of WT Piezo1 is shown in Figure 3.1
3.2.2. Transfection

Transfections were performed on cell cultures that were 50-70% confluent using Lipofectamine-2000 reagent (Life Technologies, 11668) with Opti-Mem transfection medium (Life Technologies, 31985) and corresponding protocol. 24 hours after transfection, medium was exchanged for maintenance medium (see Section 3.1 Cell culture). Cells were then used for immunofluorescence assays or electrophysiological experiments.
3.3. Whole cell patch clamp electrophysiology

Whole cell patch clamp electrophysiology was performed on HEK293 cells using extracellular solutions and pipet solutions used by Gottlieb and Sachs for their whole cell patch clamp experiments (P. A. Gottlieb et al., 2012). Micropipettes were pulled to have a resistance of 2.5-5 MΩ. For electrophysiology measurements of rat hippocampal neurons, extracellular solution contained NaCl (119 mM), KCl (5 mM), HEPES (20 mM), CaCl₂ (2 mM), MgCl₂ (2 mM), glucose (30 mM) and glycine (0.001 mM). Extracellular solution had pH of 7.35 and osmolarity of 330 mOsm (adjusted with sucrose). Micropipettes were backfilled with a pipet solution containing potassium gluconate (130 mM), KCl (10 mM), MgCl₂ (5 mM), EGTA (0.6 mM), HEPES (5 mM), CaCl₂ (0.06 mM), Mg-ATP (2 mM), GTP (0.2 mM), leupeptine (0.2 mM), phosphocreatine (20 mM) and creatine phosphokinase (50 U/ml). Recordings were performed at room temperature using Multiclamp 700B (Molecular Devices) headstages. Current clamp recordings were performed with a holding current of 0 pA. All acquisitions were performed using the pCLAMP 10 software (Molecular Devices) and analysis was performed using Clampfit 10 (Molecular Devices) and Matlab (Mathworks).

The planned setup of electrophysiology recording and magnetic stimulation is depicted in Figure 3.2, which was created in Google Sketchup.
3.4. Immunofluorescence Assays

3.4.1. Adherent cell staining

Immunofluorescence assays testing extracellular accessibility were performed as follows. 48 hours after transfection, cells were re-suspended using Accutase (Life Technologies, A11105) and re-plated on 12mm PDL-coated coverslips. 12mm PDL-coated coverslips with cells transfected with Piezo1 mutant or WT plasmids were washed three times with room temperature DMEM and then incubated in blocking solution made of 0.5% BSA in DMEM for 1 hour at 4°C. Antibody staining solution was made up of
Myc-Tag Mouse antibody, clone 9B11 conjugated to AlexaFluor 647 (Cell Signaling Technologies 2233S), diluted 1:200 in 0.5% BSA in DMEM. After 1 hour block, the blocking solution was removed, antibody staining solution was directly added, and cells were incubated in this for 30 min at 4°C. After this incubation, cells were washed 3 x with room temperature DMEM and then fixed with 4% formaldehyde solution (16% formaldehyde solution from Thermo Scientific, Prod# 28908, diluted 1:4 in PBS) for 15 minutes at room temperature. After fixation, cells were washed 3 x in PBS and then mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes P36935).

3.4.2. Flow Cytometry

Samples were prepared using Cell Signalling online protocol: [http://www.cellsignal.com/common/content/content.jsp?id=flow](http://www.cellsignal.com/common/content/content.jsp?id=flow). 48 hours after transfection, cells were detached by Accutase treatment. Cells were then centrifuged and re-suspended in 2-3 ml of blocking solution (0.5% BSA in PBS). Cells were then re-suspended in 100 ul of antibody staining solution and incubated for 2 hour on ice. Antibody staining solution was made up of Myc-Tag Mouse antibody, clone 9B11 conjugated to AlexaFluor 647 (Cell Signaling Technologies 2233S), diluted 1:200 in 0.5% BSA in DMEM. After antibody staining, cells were washed once with blocking solution and then once with 0.75 ml of PBS. Then, 0.25 ml of 16% formaldehyde was added to achieve a final concentration of 4% and cells were incubated in fixative solution for 10 minutes at 37°C. After fixation, cells were chilled on ice until being used for flow cytometry measurements.
Flow cytometry measurements were performed using a BD FACSCanto II flow cytometer with 488nm and 633nm lasers for excitation of GFP and AlexaFluor 647, respectively. This equipment is maintained by the Rice Shared Equipment Authority.

### 3.5. Confocal Microscopy

Confocal microscopy was performed using a Nikon A1-Rsi confocal microscope with CFI Plan-Apochromat VC 60x/1.4 oil objective or a LSM 710 confocal microscope with Plan-Apochromat 63x/1.4 oil objective. This equipment is maintained by the Rice Shared Equipment Authority.

### 3.6. Conjugation of SPIONs to bungarotoxin

ICK conjugation kit (Ocean Nanotech, ICK05-005) was used to conjugate 5 nm nanoparticles to α-bungarotoxin (Molecular Probes, B-1601). ICK kit protocol was followed, and separation by ultracentrifugation was done (90,000g for 1 hr) instead of magnetic separation using Ocean Nanotech SuperMag Separator.

### 3.7. Design of electromagnet

Electromagnet was designed in collaboration with Chris Yeckel at Stangenes Industries, Inc. Diagrams of the electromagnet, created using Google Sketchup are shown in Figure 3.3. In order to achieve a field of 228 mT in the gap of the coil, where the sample sits, the coil must be driven at 36 A (with about 14 V), requiring about 500W.
According to estimates by Stangenes, the coil can be run for a maximum of 30 seconds before overheating and risking melting the magnet wire insulation.

Figure 3.3 – 3D rendering of electromagnet. Created in Google Sketchup

3.8. MATLAB

Matlab (Mathworks) was used for analysis of electrophysiology data and for calculations of magnetic forces.
References


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