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Translocation and Phosphorylation of AMPA Receptors Following Transcranial Direct Current Stimulation in vivo

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TRANSLOCATION AND PHOSPHORYLATION OF AMPA RECEPTORS FOLLOWING TRANSCRANIAL DIRECT CURRENT STIMULATION IN VIVO

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

JUSTIN ANDREW STAFFORD

B.S., Miami University, 2014

2016

Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Justin Andrew Stafford ENTITLED Translocation and Phosphorylation of AMPA Receptors Following Transcranial Direct Current Stimulation \textit{in vivo} BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Stafford, Justin Andrew. M.S., Department of Neuroscience, Cell Biology, and Physiology. Wright State University, 2016. Translocation and Phosphorylation of AMPA Receptors Following Transcranial Direct Current Stimulation in vivo.

Transcranial direct current stimulation (tDCS) is the current technique used clinically to attenuate the effects of various neurological related deficits and augment functions such as memory and learning. The cellular and molecular mechanisms behind tDCS remain largely unknown and this study provides some of the first insights into the mechanisms behind tDCS. Direct current stimulation has been used to increase levels of long term potentiation (LTP) ex vivo suggesting that this stimulation has an effect on the LTP mechanisms of action. Subcellular protein extraction and fractionation methods were used to isolate synaptoneuroosomes from various brain regions to assess the effects of this treatment. One protein specifically targeted was the glutamatergic ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor because of the large role that this receptor plays in LTP in areas of the brain such as the hippocampus. The AMPA receptor translocates to perisynaptic locations and laterally diffuses to the post synaptic density (PSD) where proteins phosphorylate and retain the receptor to increase synaptic efficacy during LTP. Results from this study show that after rats are subjected to a single bout of tDCS in vivo, AMPA receptor is translocated from a
cytosolic to a synaptic region in the hippocampus and with significant phosphorylation in both hippocampal and hypothalamic regions. These results indicate that tDCS has a global effect on the brain and causes an LTP-like response in the hippocampus providing novel findings on the underlying processes of direct current stimulation.
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**Introduction**

*Purpose*

Long-term potentiation is an increase in synaptic efficacy that persists even after stimulation of the synapses ceases. Imagine multiple axons firing on a dendrite helping to relay a message. That signal is increased when there is a change in receptor activation or number, allowing that signal to be amplified in the postsynaptic neuron. This is done with the help of glutamate receptors called AMPARs, which can alter many cognitive abilities. This altered cognitive function along with an increase in synaptic efficacy can be accomplished when electrical stimulation is applied on the scalp or directly on the skull. The purpose of this study is to determine whether the mechanisms behind long term potentiation and electrical stimulation have any similar mechanisms of action.

*Transcranial Direct Current Stimulation*

Transcranial direct current stimulation (tDCS) is a non-invasive brain stimulation method that can affect neuronal excitability and modulate synaptic plasticity. Bindman et al. (1962) showed that direct current, when applied to the brain, alters neuronal electrical activity. Several years before that work, (Terzuolo, and Bullock, 1956) showed that applying low voltage gradients to active neurons can modulate their frequency. Since then the discovery and application of tDCS has been used as a
neuromodulatory tool leveraging change in electric field potentials to evoke long term potentiation (LTP)-like or long term depressive (LTD)-like effects. These effects of tDCS have been shown to modulate memory and learning, (Hilgenstock et al., 2016), (Ferrucci et al., 2013) and to help reduce pathological symptoms in diseases such as Parkinson’s, Alzheimer’s disease, depression, and aphasia presumably by increasing cortical excitability (Brasil-Neto, 2012), (Broeder et al., 2015). However, the exact mechanism as to how tDCS is increasing cortical excitability and causing the attenuation of these pathological symptoms is unclear (Broeder et al., 2015).

Transcranial direct current stimulation can either be anodal or cathodal, which can be determined based on the direct of applied current. Anodal tDCS results in increased neuronal excitability using an electrode montage that places the anode (stimulating) on the head and the cathodal (reference) electrode onto the body to complete the electrical circuit. Cathodal tDCS decreases neuronal excitability and uses the opposite set up as anodal, placing a cathodal electrode on the head and an anodal electrode onto the body (Nitsche et al., 2003). When tDCS stimulation is applied in vivo followed by LTP induction ex vivo there is significant enhancement of LTP, which was done by measuring changes in excitatory field potentials and their amplitudes using electrophysiological techniques (Rohan et al., 2015). This induced
LTP response, augmented by tDCS, occurs in a N-Methyl-D-Aspartate receptor (NMDA)-dependent fashion. The LTP response is diminished in both sham and stimulated samples when NMDAR antagonists are applied (Rohan et al., 2015). With the growing applications of tDCS, it is important to understand its underlying mechanisms that are involved in tDCS and the safety limits. There are still several parameters that must be defined in order to avoid harmful side effects, e.g. current density for brain lesions (Liebetanz et al., 2009).

*Long Term Potentiation (LTP)*

There are two types of LTP: NMDA-independent LTP and NMDA-dependent LTP. NMDA-dependent LTP is more commonly described in the literature (Bliss, and Collingridge, 2013), and is the type of LTP that will be focused on in this discussion. NMDA-independent LTP can be achieved using voltage gated calcium channels (VGCC's), and is shown to occur when these channels are activated and NMDA receptors have been blocked (Kessey, and Mogul, 1997). The present paper will focus on the involvement of NMDA-dependent LTP because the involvement of NMDA receptors is largely discussed in the literature of LTP as well as tDCS.

LTP is an increase in neuronal sensitivity to glutamate through glutamate-mediated ionotropic receptors due to the coincidental firing of pre- and post-synaptic neurons
altering the receptor content in the post-synaptic membrane (Luscher, and Malenka, 2012). In 2008 researchers postulated that the NMDA receptor (NMDAR) is also a voltage-dependent receptor that requires a change in the extracellular environment to expel a magnesium ion from the receptor to allow an influx of calcium into the postsynaptic neuron (Clarke, and Johnson, 2008). Glutamate must bind to NMDAR and the post synaptic neuron must be depolarized in order to expel the magnesium ion fully from NMDAR, making this receptor a coincidence receptor because both actions must occur (Luscher, and Malenka, 2012). The influx of calcium allows for the binding of calcium to calmodulin, forming a complex, that subsequently binds to calcium-calmodulin kinase II (CaMKII), leading to phosphorylation events in the post synaptic density (PSD) (Johansen et al., 2011). CaMKII is one of the most studied mediators in LTP because CaMKII is responsible for the phosphorylation of many different proteins that are required for LTP (e.g. AMPAR, CREB, and stargazin) (Lisman, Yasuda, Raghavachari, 2014). These events are the initial steps of early LTP (E-LTP), which is characterized as a change in receptor location and phosphorylation.

E-LTP is defined as a short term synaptic modification that lasts for about 1-2 hours, and it should also be noted that there is late LTP (L-LTP) that requires mRNA transcription and subsequent protein translation (Chai et al., 2015). Furthermore,
the next critical event that occurs after CaMKII phosphorylation is the persistence of CaMKII until the induction of other mechanisms can begin. Once that has occurred, CaMKII can continue to phosphorylate proteins as well as phosphorylate itself. The phosphorylation of an AMPAR subunit, GluA1, at the Ser831 and/or Ser845 epitopes is of particular importance because these sites help modulate the resulting post-synaptic modification. Therefore, the conductance probability of the GluA1 subunit is determined by the phosphorylation of Ser831 and Ser845 (Lee et al., 2003).

Finally, the retention of AMPAR receptors in the post synaptic membrane is required for E-LTP because the turnover rate of AMPARs in the membrane is normally transient involving many different proteins (Luscher, and Malenka, 2012). These proteins include PSD-95, transmembrane AMPA receptor proteins (TARPs), stargazin, and many others (Dakoji et al., 2003). Fluorescent imaging has shown that with inactivation of PSD-95, a PSD protein, there are decreased levels of AMPA receptors in the synaptic membrane (Yudowski et al., 2013). The phosphorylation of these proteins occurs within minutes after LTP and certain proteins, such a cAMP response-binding element (CREB), need to be phosphorylated in order to sustain LTP into its late phase (Racaniello et al., 2010).

Due to the phosphorylation of CREB, mRNA levels of downstream targets such as cFos, Egr-1 (immediate early genes) and brain-derived neurotrophic factor (BDNF)
are all significantly increased (Racaniello et al., 2010). These transcriptional events could be relevant for the translation of new proteins, increasing total protein levels in the post synaptic domain. The events of E-LTP must be established in order to have a better understanding of the immediate events that are occurring after LTP induction and, more importantly, to investigate whether these same changes are occurring following tDCS treatment. One of the most important effector molecules is the AMPA receptor because without the AMPA receptor a synapse is considered silent showing no excitatory postsynaptic potentials (EPSPs) at resting membrane potential (Isaac et al., 1995). Hence, these AMPA receptors are necessary to modulate the long term potentiation and should be a topic of discussion, along with other key modulators of LTP such as CaMKII and CREB.

*α*-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) Receptors

A key biomarker in the LTP response is the AMPA receptor because it is a fast acting protein that allows for an influx of calcium. This project focuses on the changes in the AMPA receptor following tDCS treatment to start to uncover the mechanism behind this treatment, and if this mechanism is similar to the changes seen during LTP. AMPA receptors are heterodimers that consist of four subunits (GluA1-GluA4) in a series of combinations. AMPA receptors are ionotropic glutamate receptors that are permeable to cations, namely sodium and calcium ions (Chater, and Goda, 2014).
Different heterodimeric combinations of AMPA subunits play unique roles in synaptic plasticity and it has been shown that GluA1 and GluA2 combinations play a major role in this plasticity (Heynen et al., 2000). Depending on the type of subunits that are incorporated into an AMPA receptor, there are changes in the receptor’s permeability and conductance. AMPA receptors are located in the post synaptic dendritic spine, in peri-synaptic locations, the synaptic membrane, or synaptic vesicles (Chater, and Goda, 2014). For the induction of E-LTP to occur there must be an alteration in the number or activation of AMPA receptors in the post synaptic membrane (Isaac et al., 1995). The mechanism by which this occurs is still debated but there are three major postulates for AMPA receptor insertion and activation: 1) increased levels of phosphorylation of AMPA receptors already in the membrane, 2) increased insertion of AMPA receptors into the synapse, 3) Insertion of AMPA receptors into silent synapses, which are designated as synapses that previously had no AMPA receptors. Evidence for all three mechanisms have been shown through previous research, not necessarily describing the alteration in AMPA receptors as one singular method but perhaps a combination of two or more (Yudowski et al., 2013), (Lee et al., 2003).

One mechanism that has been shown to be necessary for the activation of AMPA receptors is the phosphorylation of certain amino acids mediated by CaMKII on the
GluA1 subunit (Lisman et al., 2012) and (Lee et al., 2003). Not only is phosphorylation of GluA1 important in the E-LTP pathway, but retention of these AMPA receptors in the post synaptic membrane is also important, and is accomplished by the assistance of other proteins such as stargazin and PSD-95 (Lisman et al., 2012). The time course of AMPA activation in the post synaptic membrane is still a question largely debated. AMPA phosphorylation is shown to occur as early as 5 minutes following LTP induction, and last up until 60 minutes (Barria et al., 1997).

**Bringing It All Together**

Despite the growing popularity of tDCS with commercially available products, the molecular mechanisms underlying tDCS remain unknown. Our group has previously shown that tDCS causes an augmentation in LTP with only one prior tDSCS treatment (Rohan et al., 2015). Others have also shown the molecular mechanisms underlying LTP with the trafficking of AMPARs from cytosolic and/or perisynaptic locations to the synapse (Bassani et al., 2013) and it's phosphorylation (Lee et al., 2003), yet there has been no direct connection between tDCS and these molecular mechanisms. The central hypothesis of the current study is that anodal tDCS causes some of these molecular mechanisms involved in LTP, such as the phosphorylation and translocation of the AMPA receptor from the cytosol to the synapse. With the
understanding of tDCS on a molecular level, we can begin to understand the mechanisms by which it affects the brain and how it is modulating cognition.

**Materials and Methods**

*Animals*

The animals used for these experiments were adult, male, Sprague-Dawley rats (200-400 grams). Once the rats were received they were quarantined for up to 10 days. Rats were individually housed in shoebox style cages with water and food available *ad libitum*. Animals were handled daily (excluding weekends) by qualified technicians. Body weights recorded in order to ensure that all animals were gaining weight and were not subject to any stress.

*Surgery and Electrode Casing Implantation*

Body weight was recorded prior to surgery, with body weight measurement taken prior to electric stimulation to deduce if surgery effected the animal, then the animal was placed in an anesthesia induction chamber and given (5% isoflurane and 3% oxygen flow rate). After the animal was at the appropriate level of sedation, measured by lack of response during hind foot pinch test, the animal was removed from the induction chamber and had the chest hair and the hair from the tip of the nose to behind the ears shaved. Each subject was injected with approximately 50 μL
of lidocaine subcutaneously (SC) into the dorsal side of the rat’s head for a local anesthetic. Subsequently, 0.1-0.05 mg/kg of Buprenex was injected intramuscularly (IM) to help with post-operative analgesia. Finally, 5-8 mg/kg of Gentamicin (SC) was injected to help prevent bacterial infections. Before the animal was transferred to the surgical suite, the shaved portion of the head was lathered with betadine followed by 70% isopropyl alcohol for antibacterial and antiseptic purposes (this procedure was performed twice), leaving a final scrub of betadine on during the transfer to the surgical suite. Once the animal was placed on the surgical table, their nose was inserted into the nose cone with a 1-2% isoflurane flow rate and 3% oxygen flow rate with constant monitoring of the animal. Proper personal protection equipment (PPE) was always worn at all times while sterile gloves were changed if they were potentially compromised. Prior to the first incision, the excess Betadine solution was removed from the animal’s skull. A sagittal incision was made along the dorsal side of the animal’s head. Next, the connective tissue layering on the skull was cleaned by mechanically scraping the connective tissue with a scalpel. The electrode casing was then secured directly caudal to bregma along the sagittal suture (Fig. 1).
Figure 1. Diagram of electrode casing Placement. The square represents where the casing was placed during surgery.
The electrode casing (a 25-mm² square bottom casing) was secured to the skull with dental cement (vet bond) while simultaneously ensuring that none of the bond entered the casing, which would disrupt the electrode-skull conductance. The dental cement was placed around the bottom of electrode and partially under the open skin of the animal that was pulled back so that, once the skin was placed back over the skull, it will attach to the skull again to have complete closure of the surgical area preventing any infection or bleeding. Each subject was then returned to an incubator and wrapped in a towel to help with heating and allowing the animal to exert minimal energy for recovery. Each animal was allowed one week for recovery while body weight was monitored and the animal was visually inspected for electrode cap movement and/or bleeding.

**Acute Transcranial Electric Current Stimulation**

Each rat in these experiments was subjected to an acute, single round of electric current stimulation. Prior to the experiment, each subject was weighed and body weight was recorded. Experiments were carried out using a DC-stimulator (Neuro-Conn) box. Stimulator box was a direct current device that was programmed to deliver 250 micro amperes (µA) through the electrode for 30 minutes. First, the rat’s cranial electrode casing was examined for any movement ensuring it was secure on the rodent’s skull. The ventral side of the rodent on the chest in between both arms
was shaved, lathered with conductive gel, and fitted with a reference electrode secured with Pet Flex tape. Conductive gel was then inserted into the electrode casing mounted on the rodent’s skull to assist with transfer of current to the skull. The primary electrode was then screwed into the electrode casing and both electrodes are then tested for impedance before being attached to the stimulator box. The rat was then placed into a novel 45 x 40-cm open field environment with white walls on all sides. The electrode wiring was suspended above the rodent using a 15 mm washer held above the center of the open field using fishing wire. This allows for the rodent to move freely without becoming entangled in the wiring. Ethovision software monitored the rodent’s movements while technicians monitored the current, impedance, and the rodent itself ensuring that all devices were working properly. This experiment was carried out using two different sets of rats under the same parameters: (250µA, 30 minute stimulation) for half of the animals in each group while the other half was a sham group undergoing the same surgical and stimulation paradigm with the exception of not having any electrical stimulation. In summary, the cohort was given a single round of stimulation at 250 µA for 30 minutes or placed in the open field for 30 minutes with the identical set up and subjects were euthanized immediately after stimulation.
Euthanasia

All personnel performing the euthanasia via rapid decapitation were certified to perform the experiment. The guillotine used was sterilized prior to the first subject and after each additional subject. This method was performed due to the possible effects of anesthesia in altering cellular and molecular parameters, which could skew results.

Tissue Collection

Brains were processed on a cooled surface and immediately dissected into cerebellum, hippocampus, olfactory bulb + tract, hypothalamus, somatic sensory cortex, and the rest of the brain. Following the extraction of each brain region they were stored in 1.5 mL tubes and placed on dry ice for rapid freezing to prevent tissue degradation. Tissue samples were placed in an -80°C freezer until they were ready to be processed.

Experiment

Each animal was placed in the novel open field environment after being prepared as previously described. Sham group animals were placed in the novel open field environment for 30 minutes without stimulation while the stimulated group was placed in the novel open field environment with stimulation for 30 minutes as well.
Following the stimulation each animal was euthanized and tissue was immediately collected. The outline for the experiment is shown below (Fig. 2).
Experiment 1:

Figure 2. Experimental design and timeline of procedure. Animals are prepared, stimulated, euthanized, then tissue is collected for further analysis.


*Tissue Homogenization*

For this experiment, the left hippocampal and the hypothalamic samples were both selected for individual tissue homogenization of each and subsequent protein extraction. Before the experimental samples were tested, sample tissue was used to optimize the protein extraction method to isolate these proteins prior to performing a western blot. Syn-PER protein extraction method (ThermoFisher Scientific, catalog # 87793) was selected and optimized to ensure recovery of the cytosolic and synaptic fractions. Other reagents were added to the Syn-PER solution such as: protease inhibitor (catalog #P8340) to prevent protein degradation, phenylmethanesulfonyl fluoride (PMSF) (catalog # 329-98-6), and phosphatase inhibitor cocktail 2 (catalog # P5726) and 3 (catalog # P0044) to prevent de-phosphorylation of the proteins, all purchased from Sigma. These inhibitors were added at a 1:100 dilution just prior to use. Prior to homogenization, all samples were kept on ice to prevent thawing and degradation of the tissue. 200 µL of the Syn-PER solution was added to 2.0 mL round bottom centrifugation tubes and the tubes were subsequently weighed.

Following this procedure for the allotted number of samples, a spatula was used to remove the frozen tissue samples from their respective tubes and was placed in the centrifugation tubes with the Syn-PER solution. This mixture was then weighed
again to deduce the weight of the tissue. The Syn-PER solution must be added at a 10x dilution (volume/weight) to the tissue sample. The tissue weight, in milligrams, of each sample was multiplied by 10 and then subtracted from the 200 µL initial that was added to the centrifugation tubes to calculate the Syn-PER solution that needed to be added to achieve the final 10x dilution. Samples were homogenized into a 10x volume of Syn-PER buffer, and a 5-mm lead bead is added to the tubes and they are fitted with parafilm. The samples are then placed in the TissueLyser II (Qiagen) and set to shake at 20,000 Hz for 2 minutes to be homogenized. Incomplete tissue homogenization of a sample resulted in additional time in the TissueLyser II allowing for the tissue to dissolve into solution. A 50 µL aliquot was taken from the crude homogenized tissue for storage and a 10 µL aliquot was taken to be used in a bicinchoninic acid (BCA) assay for protein concentration analysis.

Following homogenization, the samples still in the same centrifugation tubes were placed in a table top centrifuge and spun at 1,200 x g for 10 minutes to separate the proteins from the other cellular components. A 10 µL aliquot was taken from the supernatant for BCA assay. The remaining supernatant is then transferred to a 1.5 mL centrifugation tube, now termed supernatant 1 (S1), that was spun on a table top centrifuge at 15,000 x g for 20 minutes. The S1 was then transferred into another 1.5 mL centrifugation tube that is termed supernatant 2 (S2), a 10 µL
aliquot was taken for BCA assay. The pellet that formed after S1 was centrifuged was termed pellet 2 (P2) and was reconstituted in 300 µL of Syn-PER (hypothalamic samples were reconstituted in 100 µL of Syn-PER), a 10 µL aliquot is taken for BCA assay. The P2 fraction (synaptoneurosomes) contains synaptic proteins such as the AMPA receptor and the S2 fraction is the cytosolic (soluble) fraction containing cytosolic proteins such as CaMKII. The centrifugation process is outlined below (Fig. 3).
Figure 3. Syn-PER protein extraction outline used for both hippocampal and hypothalamic samples.
**Bicinchoninic Acid Assay (BCA)**

Protein concentration for each sample (crude, S1, S2, and P2 fractions) was determined using the BCA protein assay kit (ThermoFisher Scientific, catalog #23225). The 10 µL aliquots taken from each fraction were diluted 10x by adding 90 µL of MilliQ water. A known solution with a concentration of 2 mg/mL was subject to a serial dilution creating known protein concentrations of 2 mg/mL, 1mg/mL, 0.5 mg/mL, 0.25 mg/mL, and 0.125 mg/mL as standards. 25 µL of each standard was added to a well in triplicate in a noncoated 96-well plate. 25 µL of each sample was also added in triplicate to a well in the 96-well plate. Once the plate was loaded with the diluted samples, 200 µL of the BCA reagent solution was added. Approximately 20 mL of the solution was needed to fill each well with 200 µL each. The reagent solution was made by adding 400 µL of reagent B to 19,600 µL of reagent A and vortexed to evenly distribute the two reagents within each other. Using a multi-channel pipette 200 µL of the mixture was added to each well. The top of the well-plate was sealed during transfer and unsealed prior to reading and entry into the analyzer.

Using the SpectraMax 190 (molecular Devices) with the SoftMax Pro software the plate was analyzed at 562 nanometers (nm). Two numbers of importance are given after analysis: the value, which is the absorbance of each well plate, and the result,
which is the diluted protein concentration. To find the accurate protein concentration for each sample two options can be taken 1) using the value or 2) using the result. During the analysis of protein concentration in this experiment option one was utilized. Using the mean value for the standard solutions create a graph with a linear equation and rearrange the equation to solve for the “X” value, which will be the diluted protein concentration while the “Y” value was the absorbance given and multiplying the “X” value by 10 since each fraction was diluted 10x. The following equation was rearranged as such:

\[ Y = mx + b \Rightarrow x = \left(\frac{y-b}{m}\right)\times10 \]

**Western Blot Antibody Optimization**

For each given primary antibody used in these experiments there is an optimal dilution, along with an optimal secondary antibody dilution for each primary. The four antibodies that are tested are tAMPAR (catalog # ab31232), pAMPAR (S831) (catalog # ab109464), and pAMPAR (S845) (catalog # ab76321), all purchased from Abcam, which have been previously discussed and Beta-actin from EMD Millipore (catalog # ABT264). A single sample’s synaptoneurososome fraction was utilized to reduce variability from sample to sample and, because this is a synaptic fraction, we expected to see the majority of these AMPA receptors in this fraction. Initially, we sought to optimize the appropriate concentration of primary antibody and
appropriate amount of protein to load in each lane of the gel. From here further optimization was needed to find the appropriate secondary antibody concentration.

**Western Blot Procedure**

All antibody optimization procedures and tDCS samples were carried out using the same general western blot protocol, only modified to accommodate the specific experiment, such as the cutting of the nitrocellulose membrane in the optimization portion. Samples that were prepared using the Syn-PER protein extraction method were prepared for western blots. Each sample was normalized to 1 µg/µL of protein to prepared sample. The sample was prepared with 50% 2x Laemmli blue buffer (for real time visualization of movement of the protein through the gel) with 5% by volume of the total Laemmli buffer volume being comprised of 2-mercaptoethanol (to reduce disulfide bonds and denature the tertiary structure of the protein allowing for movement through the gel). The appropriate amount of protein was then added for normalization and rest of the buffer solution was filled with MilliQ water to achieve the desired volume. All samples during preparation remained on ice until ready for use. When ready, the samples were heated to 95°C for 10 minutes to help facilitate the reduction of the disulfide bonds and break down the tertiary structure of the proteins, while preserving the total volume of the solution without causing the water to boil.
While the solution was heated, a Bio-Rad Mini Protean Tetra System powered by a Bio-Rad PowerPac Universal was set up. Pre-cast Bio-Rad Mini Protean TGX stain-free (4-20% gradient) gels were placed in either side of the western blot cassette. The inside of the cassette was filled with fresh (125 mM tris-HCl, 950 mM glycine, and 0.5% Sodium Dodecyl Sulfate) tank buffer that was diluted from a 5x tank buffer to 1x tank buffer. Spent tank buffer was used to fill the area outside of the cassette because an important factor in the fresh tank buffer was the SDS, due to its binding to the protein, changing the charge distribution on the proteins so that all proteins now have a similar charge, allowing for the applied current to move the proteins at different speeds based on size.

Once the samples have finished heating, they were loaded into the precast gel wells with the desired volume (15 wells per gel that can hold up to 15 µL in each well). The gel was then ran at 50V for 5 minutes followed by 150V for up to 60 minutes (each run time varies from approximately 45-60 minutes). Once the samples have been run through the gels, allowing for separation of proteins by size, the proteins must be transferred onto a nitrocellulose membrane. The gels, nitrocellulose membrane, and the transfer blot filter paper were soaked in transfer buffer (200 mL methanol, 5.82 grams of tris base, 2.93 grams of glycine, 3.75 mL of 10% SDS, and
filled to one liter with MilliQ water) for 5 minutes prior to transfer. The transfer system used was a Bio-Rad transblot SD semi-dry transfer system powered by a Bio-Rad PowerPac HC. After soaking in transfer buffer, the components were assembled on the transfer system with the Bio-Rad extra thick blot paper filter paper and placed on the transfer device, rolled out with a glass pipette tip to extrude any bubbles that may have formed in the paper, followed by laying the 0.45 µm nitrocellulose membranes on top (making sure not to touch the membranes with anything except forceps), with placement the gel on top of the membrane, ending with another extra thick blot paper filter paper on top of the gel that was rolled out once to extrude any bubbles from that piece. The setup is shown below (Fig. 4).
Figure 4. Western blot transfer set up used in the semi-wet transfer protocol.
Once the components were assembled the system was closed and run at 15V for 45 minutes. The system’s current was monitored for a few minutes following the start to ensure that the current went down and not up. Increase in current indicates a decrease in resistance, which causes damage to the apparatus and/or results in inadequate transfer of proteins to the nitrocellulose membrane. Once the proteins have been transferred, the membranes were washed in 0.1% Tween-20 Phosphate buffered saline (0.1% PBST) 3x for 5 minutes each wash. Following the three washes, the membranes were then placed in blocking buffer (5% reconstituted bovine serum albumin (BSA) PBST solution) to limit non-specific binding of antibodies. After the membranes were in blocking buffer for 1 hour, they were again washed in 0.1% PBST with 3 washes at 5 minutes each wash. The membranes were then ready to be probed with primary antibodies. The antibodies were diluted in the 5% BSA PBST solution to the desired antibody concentration and vortexed for proper distribution. Each membrane was placed in a blue MilliPore non-coated plastic box with 10 mL of the diluted primary antibody and were incubated at 4C in a refrigerator overnight.

The following day, after the primary antibody incubation, the membranes were washed 3x for 5 minutes each in 0.1% PBST. The membranes were then ready to be
probed with the secondary antibodies. Secondary antibodies were horseradish peroxidase (HRP) conjugated antibodies that were used in conjunction with enhanced chemiluminescence (ECL) for visual detection of antibody bound proteins. The secondary antibody used for all three AMPA receptor primary antibodies and the β-actin was an HRP-conjugated goat anti-rabbit antibody. Secondary antibody was diluted to 1:15K for all target primary antibodies, using 0.1% PBST and vortexed for proper distribution. The membranes were incubated in secondary antibody for 1 hour at room temperature. After the secondary antibody incubation, the membranes were washed 3x for 5 minutes each in 0.1% PBST. After these washes, the membranes should have primary antibody selectively bound to a specific protein’s antigenic sequence with secondary antibody bound to the primary (Fig. 5).
Figure 5. Representation of antibody binding sequence to a protein sequence. The primary antibody binds to a specific sequence of the protein, and the secondary antibody recognizes the foreign primary antibody and binds to it.
The final step was to develop the western blot using enhanced chemiluminescence. The western blot membrane was incubated in a peroxide and luminol solution, SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, catalog # 34095), which was combined in a 1:1 ratio (3-5mL of each solution) for 5 minutes with minimal exposure to light. While the solutions were relatively stable (up to 8 hours), prolonged exposure to light can cause some degradation of the compounds. After incubation, the membrane was wrapped in plastic wrap with aluminum foil to reduce light exposure. The membrane was then read in an ImageQuant RT ECL device (GE Healthcare) with varying light exposures. Subsequently, the membranes will be analyzed using ImageJ software to quantify the results given from the ImageQuant device.

ImageJ Analysis Software

ImageJ is an analysis software that allows for the quantification of western blot lanes. For quantification, a lane was measured by creating a box that encompasses the entire lane measured and an average pixel value was given to that space. Once this was done for all of the lanes and the background (to later subtract the background noise from the sample pixels), these values were converted into optical density values. A computer used for analysis was calibrated measuring a set number of pixel ranges with their corresponding known optical densities. Using this and a
Rodbard equation shown below, an equation that makes a best fit curve for the given data points, the optical density (O.D.), Y in this case, could be determined using the pixel value (X) and the A,B,C, and D values (pre-determined values given when using the equation). The O.D. was measured for each target sample and the positive beta-actin controls as well. Using this data, the relative O.D. of phosphorylation and total receptor level was quantified and analyzed, which will be discussed in the results section.

RodBard Equation

\[ y = c \times \left( \frac{(x-a)/(d-x))^1}{b} \right) \]

Following the quantification of optical density, the O.D. values were ran through different statistical analyses to determine different parameters from the experimental groups. SigmaPlot software was used for all graphing and analysis. For this experiment two-way ANOVAs and student t-tests were utilized to analyze the differences among cytosolic sham, cytosolic stimulated, synaptic sham, synaptic stimulated, and synaptic vs. cytosolic fractions and whether there was any significant difference between the groups. The key relationship analyzed was the significance between cytosolic sham and stimulated fractions, and synaptic sham and stimulated fractions.
Results

Antibody Optimization

Each antibody used in the analysis was optimized to achieve western blots that were in range of detection using the ImageQuant RT ECL device (table 1). Each antibody was ran using varying primary antibody concentrations paired with varying secondary antibody concentrations. This approach allowed for the determination of the range necessary for each antibody that also minimized nonspecific binding. Varying amount of protein was also tested to determine the optimal amount of protein that should be loaded into each well of the western blot gel. Each combination of protein and antibody concentration was subjected to different time points of exposure to the ECL device, which determined the most accurate reading time for the western blot. Before each sample was loaded into a gel for the western blot procedure, the concentration of each sample was diluted to 1 µg/µL of protein to ensure that the same amount of protein was loaded into each well.
### Table 1. Primary and secondary antibody concentrations used in western blots.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>S831 pAMPAR (host: rabbit) 1:20K</td>
<td>HRP conjugated Goat –anti – rabbit (1:15K)</td>
</tr>
<tr>
<td>S845 pAMPAR (host: rabbit) 1:1K</td>
<td>HRP conjugated Goat –anti – rabbit (1:15K)</td>
</tr>
<tr>
<td>C-term tAMPAR (host: rabbit) 1:20K</td>
<td>HRP conjugated Goat –anti – rabbit (1:15K)</td>
</tr>
<tr>
<td>B-actin (host: rabbit) 1:40K</td>
<td>HRP conjugated Goat –anti – rabbit (1:15K)</td>
</tr>
</tbody>
</table>
Experiment 1 analysis

To determine if the transcranial direct current stimulation had any effect on AMPA receptors in the hippocampus and hypothalamus, western blot gels were analyzed and quantified using ImageJ software and analyzed using SigmaPlot to look for significant translocation and phosphorylation of the receptor between sham and stimulated samples in the cytosolic and synaptic regions.

S845 pAMPAR Western Blot Analysis Hippocampus

S845 phosphorylation site western blot probe (Fig. 6) has a 100 kDa band marked to indicate the AMPA receptor (due to the approximate 100 kDa weight for the GluA1 subunit). The 40 kDa band is marked to indicate β-actin. Actin was used as a positive control to normalize each AMPA band’s optical density (O.D.) to the respective actin O.D. No significant phosphorylation was seen in the hippocampus at the S845 site of the GluA1 subunit with a synaptic sham fraction mean O.D. of 0.36 with SE +/- 0.03 and a synaptic stimulated mean O.D. of 0.36 with SE +/- 0.03 (Fig. 7), with an unpaired student’s t-test p-value of 0.94. Phosphorylation was also determined for the cytosolic fractions at the S845 site. The cytosolic fractions showed no significant change in phosphorylation with a cytosolic sham mean O.D. of 0.07 with SE +/- 0.03, a cytosolic stimulated mean O.D. of 0.06 with a SE +/- 0.02, and an unpaired student’s t-test p-value of p=0.86 (Fig. 8). Overall, there was no change in the phosphorylation state of the
S845 site on AMPA within the cytosolic sham or stimulated fractions. A two-way ANOVA shows that there is not a significant difference between sham and stimulated samples (p=0.99) or between the different fractions based on the stimulation (p=0.97). A significant difference between the phosphorylation in total cytosolic and total synaptic fractions was observed (no image) (p<0.001).
Figure 6. Hippocampal S845 phosphorylation site of GluA1 subunit of AMPAR and β-actin. This western blot is a representation of the S845 blot series, there are a total of 3 blots, each similar to Fig. 6. This blot was loaded with samples from both cytosolic and synaptic fractions from sham or tDCS stimulated hippocampi. There were a total of 16 samples with cytosolic and synaptic fractions for each one. On the left shows the cytosolic sham (n=8) and cytosolic stimulated (n=8) samples, while on the right is the synaptic sham (n=8) and synaptic stimulated (n=8) samples.
Figure 7. Hippocampal S845 phosphorylation site synaptic sham vs. stimulated.

Quantification of the synaptic sham (n=8) and synaptic stimulated (n=8) fractions. No significant differences were observed between the sham and stimulated samples (p=0.940).
Figure 8. Hippocampal S845 phosphorylation site cytosolic sham vs. stimulated samples.

Quantification of S845 site phosphorylation in the cytosolic sham (n=8) and stimulated (n=8) fractions. No significant difference was found between treatments (p=0.859).
**S831 pAMPAR Western Blot Analysis Hippocampus**

The S831 phosphorylation site was also probed for in hippocampal samples using both cytosolic and synaptic fractions under sham and stimulated conditions. S831 phosphorylation site western blot probe and β-actin (Fig. 9) shows 100 kDa band for the phosphorylated S831 AMPA receptor and the 40 kDa band for β-actin, the positive control. Quantification of the S831 phosphorylation site reveals no significant difference between the cytosolic sham (mean O.D. = 0.376) and the cytosolic stimulated sample (mean O.D. = 0.358) with p=0.574 (Fig. 10). Quantification at S831 in synaptic fractions show significantly more phosphorylation in the stimulated synaptic fractions (mean O.D. of 1.23 with SE +/- 0.038 ) than in the sham synaptic samples (O.D. of 1.02 with SE +/- 0.062) with an unpaired student’s t-test p-value of <0.05 (Fig. 11). Two-way ANOVA results show a significant difference (p=0.018) (F=6.303) between the fractions depending on what type of stimulation (sham or 250 µA) is present. The ANOVA also reveals that there is a significant difference (p<0.001) between the cytosolic and synaptic fraction phosphorylation as a whole (no image) (F=135.047).
Figure 9. Hippocampal S831 phosphorylation site on the GluA1 subunit of AMPA receptors and β-actin. This representative blot shows the cytosolic sham (n=8) and cytosolic stimulated (n=8) banding on the left and the synaptic sham (n=8) and synaptic stimulated (n=8) banding on the right.
Figure 10. Hippocampal S831 phosphorylation site in cytosolic sham (n=8) and stimulated (n=8) samples. No significant difference between cytosolic fractions was found (p=0.574).
Figure 11. Hippocampal S831 phosphorylation site synaptic fraction sham vs. stimulated.

Synaptic sham (n=8) and synaptic stimulated (n=8) samples. There was significant increase in phosphorylation of AMPAR at the S831 site following 30 minute - 250µA stimulation (p<0.05) in the stimulated synaptic fraction then in the synaptic sham fraction.
C-terminal AMPAR (tAMPAR) Western Blot Analysis Hippocampus

The last AMPA receptor marker that was studied in this experiment in the hippocampus was the GluA1 subunit of AMPA regardless of phosphorylation state. Western blot for tAMPA (Fig. 12) indicates banding for the AMPA receptor at the 100 kDa region and β-actin at the 40 kDa region. Quantification shows significantly more tAMPARs in the synaptic fraction (mean O.D. of 2.391 with SE +/- 0.161) then in the sham synaptic fraction (mean O.D. of 1.478 with SE +/- 0.0713) with a p-value of <0.001 (Fig. 13).

Cytosolic fractions show a significantly greater amount of tAMPARs in the sham samples (mean O.D. of 0.67 with SE +/- 0.10) then in the stimulated samples (mean O.D. of 0.36 with SE +/- 0.040) (Fig. 14) with a p-value of <0.05. A two-way ANOVA shows that there is a statistically significantly interaction between the fraction (cytosolic or synaptic) and stimulation (sham or 250 µA) with P<0.001 and F=34.399.
Figure 12. Hippocampal C-terminal probe of the GluA1 subunit of AMPARs and β-actin. The cytosolic stimulated (n=8) and cytosolic sham (n=8) samples are shown on the left while the synaptic stimulated (n=8) and cytosolic sham (n=8) samples appear on the right.
Figure 13. Hippocampal tAMPAR synaptic sham vs. stimulated samples; synaptic sham (n=8) and stimulated (n=8). There is a significant increase in AMPARs following 30 minute-250 µA stimulation (p<0.001) from synaptic sham to stimulated samples.
Figure 14. Hippocampal tAMPAR cytosolic sham vs. stimulated sample; synaptic sham (n=8) and stimulated (n=8). There is a significant increase in AMPARs following 30 minute – 250 µA stimulation (p<0.05) in the cytosolic sham vs. stimulated samples.
C-terminal AMPAR (tAMPA) Western Blot Analysis Hypothalamus

After quantification of the hippocampal samples was completed, hypothalamic samples were studied as a region of interest. These hypothalamus samples were probed for the same AMPA receptor targets as the hippocampus. Western blot for tAMPA (Fig. 15) indicates banding for the AMPA receptor at the 100 kDa region and β-actin at the 40 kDa region. Analysis yields no significant different between the synaptic sham (mean O.D. of 0.02 with SE +/- 0.09) and synaptic stimulated (mean O.D. of 0.11 with SE +/- 0.07) samples with a p-value of 0.22 (Fig. 16). The O.D. of the cytosolic sham (mean O.D. of -0.04 with SE +/- 0.04) and stimulated fractions (mean O.D. of -0.06 with SE +/- 0.06) with a p-value of 0.21. The cytosolic levels are not shown due to indiscernible levels between target and background. This indicates that levels of AMPA receptor in the cytosolic fraction is indistinguishable from background noise and taken as zero.
Figure 15. Hypothalamic C-terminal probe of the GluA1 subunit of AMPARs and β-actin.

The cytosolic stimulated (n=8) and cytosolic sham (n=8) samples are shown on the left while the synaptic stimulated (n=8) and cytosolic sham (n=8) samples appear on the right.
Figure 16. Hypothalamic tAMPAR synaptic sham vs. stimulated sample; synaptic sham (n=8) and stimulated (n=8). There is no significant increase in AMPARs following 30 minute – 250 µA stimulation in the synaptic sham vs. stimulated samples (p=0.22).
S831 pAMPAR Western Blot Analysis Hypothalamus

The S831 phosphorylation site was also studied in the hypothalamus to look for significant changes between non-stimulated and stimulated samples. Western blot for S831 phosphorylation site (Fig. 17) indicates banding for the AMPA receptor at the 100 kDa region and β-actin at the 40 kDa region. No significant difference between the cytosolic sham (mean O.D. of 0.04 with SE +/- 0.09) and stimulated (mean O.D. of 0.11 with SE +/- 0.07) samples (p=0.10) (Fig. 18). Quantification of the synaptic group for the phosphorylation of S831 in the hypothalamus yields a significant difference between the synaptic sham (mean O.D. of 0.68 with SE +/- 0.05) and the synaptic stimulated (mean O.D. of 0.92 with SE +/- 0.17) samples (p<0.004) (Fig. 19). A two-way ANOVA also shows that there is a significant difference between the cytosolic and synaptic fractions as a whole (p<0.001) (F=134.285).
Figure 17. Hypothalamic S831 phosphorylation site probe of the GluA1 subunit of AMPARs and β-actin. The cytosolic stimulated (n=8) and cytosolic sham (n=8) samples are shown on the left while the synaptic stimulated (n=8) and cytosolic sham (n=8) samples appear on the right.
Figure 18. Hypothalamic S831 cytosolic sham vs. stimulated sample; synaptic sham (n=8) and stimulated (n=8). There is no significant increase in AMPARs following 30 minute – 250 µA stimulation in the cytosolic sham vs. stimulated samples (p=0.10).
Figure 19. Hypothalamic S831 synaptic sham vs. stimulated sample; synaptic sham (n=8) and stimulated (n=8). Significant increase in AMPARs following 30 minute – 250 µA stimulation in the synaptic sham vs. stimulated samples (p<0.004).
S845 pAMPAR Western Blot Analysis Hypothalamus

The last analysis completed on the hypothalamus was to probe the S845 phosphorylation site on the AMPA receptor. Western blot for S831 phosphorylation site (Fig. 20) indicates banding for the AMPA receptor at the 100 kDa region and β-actin at the 40 kDa region. A two-way ANOVA analysis shows that there is no difference between the different stimulation groups (250 µA and sham) with a p-value of 0.37 or the stimulation group depending on what fraction it is in (cytosolic or synaptic) with a p-value of 0.67. There is a significant difference between the fractions when stimulation is not accounted for, with a p-value of <0.001. No significant difference was observed between the sham cytosolic fraction (mean O.D. of 0.04) and cytosolic stimulated group (mean O.D. of 0.07) with a p-value of 0.74 (Fig. 21). Also, no significant difference is seen comparing the synaptic sham group (mean O.D. of 0.35) and stimulated group (mean O.D. of 0.43) with a p-value of 0.35 (Fig. 22).
Figure 20. Hypothalamic S845 phosphorylation site probe of the GluA1 subunit of AMPARs and β-actin. The cytosolic stimulated (n=8) and cytosolic sham (n=8) samples are shown on the left while the synaptic stimulated (n=8) and cytosolic sham (n=8) samples appear on the right.
Figure 21. Hypothalamic S845 cytosolic sham vs. stimulated sample; cytosolic sham (n=8) and stimulated (n=8). No significant increase in AMPARs following 30 minute – 250 µA stimulation in the cytosolic sham vs. stimulated samples (p=0.74).
Figure 22. Hypothalamic S845 synaptic sham vs. stimulated sample; cytosolic sham (n=8) and stimulated (n=8). No significant increase in AMPARs following 30 minute – 250 µA stimulation in the synaptic sham vs. stimulated samples (p=0.35).
Discussion

Several major findings occurred during this study show AMPA receptor modification, which is a key process normally observed in classical LTP. This study shows that tDCS, an augmenter of LTP, causes an increase in phosphorylation and translocation of the AMPA receptor following stimulation. This study also shows that tDCS causes a global effect on the brain due to the increased phosphorylation seen in the hypothalamus. These results show that tDCS is causes wide spread molecular changes in the brain and, therefore warrants extensive future research.

Translocation of AMPARs in the Hippocampus

One of the first and major findings of this study was the translocation of AMPA receptors in the hippocampus. Based on the results as seen in Fig. 13 and 14, tDCS has resulted in a significant translocation of the AMPA receptor demonstrated by the increased AMPA receptor in the cytosolic sham samples and the synaptic stimulated samples when compared to their counter group. This gives some insight to the change in AMPA receptor location following the in vivo tDCS treatment. This stimulation causes the translocation of these receptors from the cytosolic area to the synaptic area. This is consistent with what is seen in “traditional” LTP with the vesicle insertion and lateral diffusion of AMPA receptors from the cytosol to the synapse (Bassani et al., 2013). NMDA-dependent LTP in the hippocampus is a topic
of great debate. Most of the mechanisms behind NMDA-dependent LTP that are currently under investigation have contributed to an evergrowing in-depth understanding of the process of memory and learning (Luscher, and Malenka, 2012).

GluA1 containing AMPA receptors are eventually trafficked from the cytosol to the synapse and inserted into the postsynaptic neuronal membrane. Then AMPARs move to the postsynaptic density where they are anchored and retained in the membrane for the maintenance of LTP (Luscher, and Malenka, 2012). Previous results from our laboratory have shown that a single bout of in vivo tDCS increased levels of LTP in the hippocampus when LTP is induced after stimulation ex vivo (Rohan et al., 2015). Under these same conditions (30 minute stimulation at 250 µA) this experiment shows that a single bout of tDCS has resulted in AMPA receptor translocation in vivo with no exogenous LTP induction. This truly novel finding gives more insight into the mechanism behind tDCS and demonstrates that tDCS treatment alone is causing neuronal changes in the hippocampus, which are reflective of the LTP process that occurs during memory and learning.
Phosphorylation of S831 and S845 on the GluA1 Subunit of AMPA

Another finding in this experiment was the significant phosphorylation of the S831 site on AMPA in the hippocampus. This phosphorylation site on AMPA is directly phosphorylated by CaMKII, a key mediator in the LTP process (Lisman et al., 2012). The phosphorylation site plays a role in synaptic plasticity in the hippocampus during LTP (Barria et al., 1997) and also plays a specific role with channel conductance and possible signaling for retention in the postsynaptic membrane (Lee et al., 2010). The results from this current study show that in the synaptic stimulated group there is a significant increase in phosphorylation at the S831 site. This increase in phosphorylation in vivo following stimulation, shown for the first time, gives even more insight into the effects of tDCS on the hippocampus. This study shows that all of these mechanisms- the translocation of AMPA receptors and their phosphorylation- are classic downstream effects of NMDA-dependent LTP.

The S845 site was not significantly altered during this experiment in the cytosolic or the synaptic stimulated groups, but this may be due to several factors. It is not yet defined whether or not the S845 phosphorylation site on AMPA is a target of CaMKII, it could be a target of another kinase (Lisman et al., 2012). Also, after the 30-minute stimulation, the tissue was collected immediately after (0 minute following stimulation) so the phosphorylation of S845 could be at different levels of
phosphorylation at this time compared to the S831 site. Multiple time points would need to be analyzed for further interpretation. Regardless, the fact that there is significant phosphorylation of the S831 site is another indication of this LTP-like response suggesting that tDCS is causing and is consistent with the time frame of phosphorylation events during LTP (Racaniello et al., 2010), (Barria et al., 1997). One disputed question among scientists involving LTP is the movement and phosphorylation of AMPARs, temporally and spatially. Information gathered from this study show significant phosphorylation of AMPA receptors in the synaptic fraction at this time point, even for the S845 site. Whether or not phosphorylation occurs before translocation or after can not be elucidated, but it is apparent that after 30 minutes of stimulation, there is a significant abundance of phosphorylated GluA1 containing AMPA receptors in this synaptic fraction, regardless of stimulation. Further experimentation needs to be conducted for further analysis.

*Phosphorylation of S831/S845 and Translocation of GluA1 Subunit of AMPARs in the Hypothalamus*

Similar to the hippocampus, significant phosphorylation was observed in this study at the S831 site in the hypothalamus and no significant phosphorylation in the S845 on the AMPA receptor. Results also show that there was not a significant change in the translocation of AMPA receptors in the cytosolic or synaptic samples in the
hypothalamus. Interestingly, the cytosolic samples appeared to have almost no quantifiable amount of AMPA receptors, independent of phosphorylation. There are several speculations behind the results seen in the hypothalamus. One reason could be that tDCS is causing a global effect on all neuronal tissue, because the electrode was placed over bregma on the skull and, because the hypothalamus is one of the most inferiorly located structures in the brain, the effect of tDCS is weakened due to possible diffusion and shunting. Another possibility is that the tDCS treatment is stressing the rodents and is causing an activation of the hypothalamus, and subsequent release of stress hormones such as ACTH and eventually corticosterone. However, hormone levels would need to be analyzed in both sham and stimulated samples before tDCS could be ruled as a potential stressor.

Conclusions

The experimentation behind this research project has yielded noteworthy findings in the area of brain stimulation. We have seen through the results presented in this experiment that with in vivo direct current stimulation with no exogenous induction of LTP causes a translocation and phosphorylation of AMPA receptors. The translocation and phosphorylation of AMPA receptors in the hippocampus due to tDCS is the same response that is seen in the hippocampus under normal LTP conditions in memory and learning. With this new insight into the molecular
mechanisms behind tDCS, the search for other key modulators that may be involved and effected by tDCS such as CaMKII, CREB, and BDNF can begin, which may determine if this treatment augments memory and learning by pairing it with a cognitive challenge. As previously shown, tDCS has effected the hypothalamus along with the hippocampus. This result beckons the question as to what other areas of the brain are being effected? Further analysis of other brain regions should be studied for a clearer picture of where tDCS is causing this excitatory response. This research starts to uncover the cellular and molecular mechanisms behind anodal tDCS and lays a framework for future research in this field.
References


