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Modulation of the Sodium/Potassium ATPase Function and Expression by Transcranial Direct Current Stimulation of the Right Sensorimotor Cortex in Mice

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MODULATION OF THE SODIUM/POTASSIUM ATPASE FUNCTION AND EXPRESSION
BY TRANSCRANIAL DIRECT CURRENT STIMULATION OF THE RIGHT
SENSORIMOTOR CORTEX IN MICE

By

SALIM BENDAOUD

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2017
This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

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Abstract

MODULATION OF THE SODIUM/POTASSIUM ATPase FUNCTION AND EXPRESSION BY TRANSCRANIAL DIRECT CURRENT STIMULATION OF THE RIGHT SENSORIMOTOR CORTEX IN MICE

By
Salim Bendaoud

Advisor: Professor Zaghloul Ahmed, PhD

Direct current stimulation is a technique used in various therapeutic applications. Despite this broad utilization, its biological mechanisms in the target tissues have not been elucidated yet. Studies in the neuroscience field have shown that DC stimulation affects neurons’ physiology. The way DC influences the neurons’ function is not understood as well. Converging conclusions have proven that DC stimulation provokes changes of the axonal excitability. Nevertheless, most studies related to the application of direct current on the nervous organs failed to look at the possibility that it could influence the factors that control the neurons’ excitability. For example, up to this time, sodium channels, crucial elements in the generation of membrane potentials, have not been investigated. Therefore, to understand the neurobiological aspects underpinning the action of DC stimulation on nervous structures, we looked at the molecular changes that may happen following the application of transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice. Our study essentially focused on \( \text{Na}^+\text{K}^+\text{ATPase} \) given its role in the generation and the control of the nerve excitability. We particularly examined patterns of expression of the catalytic \( \alpha_1 \) and \( \beta \) subunits following transcranial direct current stimulation because of their contribution to the pump function. Alternately, we investigated other molecules...
implicated in the regulation of the Na\textsuperscript{+}K\textsuperscript{+} ATPase activity. We also inspected whether electrophysiological changes could occur after DC stimulation of the cortical region of interest. Data from two electrophysiological experiments confirmed the overall conclusions reported in the scientific literature. The first one originating from Dr. Ahmed’s unpublished work done in our lab on the mice’s sciatic nerves confirmed that anodal and cathodal direct current stimulation oppositely influences axonal excitability. In this experiment design, two chambers, lateral and central, made of petroleum jelly, enclosed the sciatic nerve. DC electrode was permanently placed in the central chamber to deliver 10 µA of either anodal or cathodal stimulation for 2 minutes. Another electrode was alternately positioned in the lateral or the central chamber to deliver test stimulations during and after anodal or cathodal DC stimulation. Preliminary findings showed that whether test-stimulation originated from the lateral or the central chamber, anodal DC produced prolonged and increased axonal excitability embodied by the elevated number of compound action potentials (CAP). On the other hand, cathodal DC decreased the number of CAP over a period of 25 minutes after the end of the stimulation. Additional findings revealed that a-DC consistently elicited increased and prolonged axonal excitability in the presence of ouabain, a steroid inhibitor of the Na\textsuperscript{+}K\textsuperscript{+} ATPase activity, when it was alternately added to the lateral or the central chamber. In contrast, the addition of ouabain in either chamber, reduced, and in some instances reversed, the prolonged hyperpolarization that usually c-DC stimulation induces. The presence of ouabain particularly in the central chamber showed an initial exaggeration of c-DC hyperpolarization followed by a persistent inhibition over 15 minutes. This result suggests that immediate and long-lasting effects of c-DC are independent from each other. Furthermore, the observation of these findings in the presence of an inhibitor of Na\textsuperscript{+}K\textsuperscript{+} ATPase alludes to the possibility that DC long-lasting effects involved changes of the
pump activity or expression. The second electrophysiological experiment involved the transcranial stimulation of the right sensorimotor cortex of CD-1 mice for 20 minutes with either 200 µA anodal or cathodal DC. Then, the recording of sensory evoked potentials (SEP) after test-stimulations of different frequencies were initiated from the left sciatic nerve. Our findings demonstrated that anodal and cathodal DC elicited SEPs with opposite amplitudes. The difference was not yet significant. SEPs with high and low amplitudes were respectively associated with c-TDCS and a-TDCS. These findings may reflect the occurrence of immediate synaptic responses. On the other hand, high amplitude SEPs following c-TDCS could be the consequence of the hyperpolarization of pre-synaptic neurons, which might have led to the disinhibition of post-synaptic neurons.

Collectively, these electrophysiological studies, besides confirming the effects of anodal and cathodal DC on axonal excitability, also highlighted the role that Na⁺K⁺ATPase may assume in the initiation of the cell excitability changes after DC stimulation is applied to the nervous organs. Moreover, these findings provided us with the necessary background to undertake the investigation of molecular changes in the neurons that could happen following TDCS of the sensorimotor cortex.

Knowing the importance of Na⁺K⁺ATPase in the elicitation of membrane potentials, we hypothesized that DC effects on neurons should involve changes targeting this integral protein. We assumed that these modifications could target Na⁺K⁺ATPase synthesis rate or its gene expression. They could affect its activity as well. We investigated Na⁺K⁺ATPase subunit expression using three experimental paradigms: single direct stimulation of the exposed right sensorimotor cortex, single and repeated transcranial stimulation of the same cortical region. Our results unequivocally showed that c-DCS in all these experimental protocols consistently
induced a significant increase of Na\(^{+}\)K\(^{-}\) ATPase \(\alpha_1\) expression. Likewise, the expression of Na\(^{+}\)K\(^{+}\) ATPase \(\alpha_3\) subunit, exclusively found in neurons, was significantly elevated except with single transcranial direct stimulation. Concurrently, our results showed that a-DCS stimulation of the sensorimotor cortex globally reduces Na\(^{+}\)K\(^{+}\) ATPase \(\alpha_1\) and \(\alpha_3\) expression, but not always significantly. These findings agree with the notion that both subunits are needed for the function of neurons; hence their overall similar response to DC stimulation.

To see whether changes in the Na\(^{+}\)K\(^{+}\) ATPase \(\alpha_1\) protein expression were parallel to modifications of Na\(^{+}\)K\(^{+}\) ATPase \(\alpha_1\) gene expression, we investigated levels of mRNA after single DC stimulation of the exposed right sensorimotor. Our findings demonstrated that Na\(^{+}\)K\(^{+}\) ATPase \(\alpha_1\) gene expression was significantly increased with cathodal DC stimulation. Therefore, the increase of Na\(^{+}\)K\(^{+}\) ATPase \(\alpha_1\) protein expression was concurrent to the rise of Na\(^{+}\)K\(^{+}\) ATPase \(\alpha_1\) gene expression. These conclusions are in line with the limited studies that have shown DC use can influence gene expression.

The increase of Na\(^{+}\)K\(^{+}\) ATPase \(\alpha_1\) protein expression following cathodal DC stimulation prompted us to look for the origin of the factor that could have influenced the rise of the pump expression. We evoked two possibilities to explain this increase. The first one was that DC stimulation could directly affect the expression of Na\(^{+}\)K\(^{+}\) ATPase \(\alpha_1\) gene. The second one was that DC could increase the catalytic activity of the enzyme, which would have required the synthesis of additional pumps. Comparison of tissues exposed to 1mM lidocaine, needed to block the Na\(^{+}\)K\(^{+}\) ATPase pump activity, with non-lidocaine tissues, both stimulated by single cathodal direct stimulation, showed mixed results. The expression of Na\(^{+}\)K\(^{+}\) ATPase \(\alpha_1\) protein was alternately elevated, not significantly yet, in non-lidocaine stimulated tissues and in lidocaine non-stimulated tissues. We also found that lidocaine stimulated tissues had a decreased
Na⁺K⁺ ATPase α1 expression compared to non-lidocaine stimulated tissues. These findings led us to cautiously postulate that the enhancement of Na⁺K⁺ ATPase α1 protein expression could be triggered by different stimuli directly and through changes of the pump activity.

Since Na⁺K⁺ ATPase pump activity is directly inhibited through the phosphorylation of several serine residues by protein kinases PKA and PKC, and indirectly by dopamine and cAMP-regulated phosphoprotein (phospho-Darpp-32), we examined the effect of DC on the control of the pump function. We first looked at the expression of Na⁺K⁺ ATPase α1 phosphorylated at serine 943, a conserved residue across various species, by PKA. Our findings revealed that single a-DCS steadily and significantly increases the Na⁺K⁺ ATPase α1 phospho-serine 943 protein expression whether in the exposed or covered right sensorimotor cortex. These outcomes indicated that a-DCS directly inhibits the activity of the pump inserted in the membrane as the phosphorylation at serine 943 is not accompanied with an internalization of the pump. Our results also revealed that single cathodal transcranial direct current stimulation significantly decreases Na⁺K⁺ ATPase α1 phospho-serine 943 expression. The action of PKA on serine 943 is also associated with concomitant phosphorylation of Darpp-32, which inhibits protein phosphatase 1 (PP1). However, our findings did not show that a-DCS simultaneously increases the expression of Na⁺K⁺ ATPase α1 phospho-serine 943 and Darpp-32. We only observed this dual increase with only repeated a-TDCS. In addition, we found out that the expression of phospho Darpp-32 was significantly decreased and increased by single a-TDCS and c-TDCS respectively. This inconsistent result suggests that a-DCS and c-DCS could alternately activate protein kinases or protein phosphatases in each experimental protocol.

PKC phosphorylation of the Na⁺K⁺ ATPase at serine 23 leads to the endocytosis of the pump causing the inhibition of its activity. Our findings explicitly displayed a consistent effect of a-
DCS on the expression of $\text{Na}^+\text{K}^+$ ATPase $\alpha1$ phospho-serine 23. There was significant increase in the expression of the pump phosphorylated at this site in all three experimental paradigms. We also witnessed a significant decrease of the pump elicited by single c-TDCS. These results agreed with the rise of $\text{Na}^+\text{K}^+$ ATPase $\alpha1$ phospho-serine 943 expression produced by a-DCS. PKA phosphorylation at serine 943 is considered a permissive action for PKC phosphorylation at serine 23. The similar impact that a-DCS had on the pump expression phosphorylated at these two residues supports the notion that these two kinases interact with one another to inhibit $\text{Na}^+\text{K}^+$ ATPase activity.

$\text{Na}^+\text{K}^+$ ATPase $\beta$ subunit contributes to the adequate functionality of the pump by transporting $\alpha$ subunit to the plasma membrane. Our findings revealed that either single a-DCS or a-TDCS elicited an increase of $\text{Na}^+\text{K}^+$ ATPase $\beta2$ expression. Conversely, our results showed that both c-DCS and c-TDCS produced a decrease of $\text{Na}^+\text{K}^+$ ATPase $\beta2$ expression. These results were opposite to those related to the expression of $\text{Na}^+\text{K}^+$ ATPase $\alpha1$ expression. They suggest that DCS has a different impact on each of the $\text{Na}^+\text{K}^+$ ATPase subunits to balance their expression.

The present study demonstrated that transcranial direct current stimulation of the sensorimotor cortex brings about changes that are not limited to modifications of the axonal excitability. More importantly, the present study revealed that direct current stimulation of the nervous tissue also affects molecular targets in the neurons. These molecules could be those that play an overt role in the production and maintenance of neurons’ membrane potentials like the $\text{Na}^+\text{K}^+$ ATPase or those that regulate the pump activity like phospho-Darpp-32. Furthermore, our findings confirmed that DC stimulation of the right sensorimotor cortex is also accompanied with changes of the axonal excitability.

The present study showed that transcranial direct current stimulation affects the expression of
Na⁺ K⁺ ATPase protein and gene. They also revealed that the regulation of the pump activity is also influenced by either anodal or cathodal direct current. Furthermore, our results suggested that DC stimulation may exert its effects through modifications of cellular components involved in the elicitation of the membrane potential. Ultimately, this study was an attempt to identify cellular molecules that DC targets to exert its effect.
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To all other CSI faculty and administrative personnel, my sincere thanks for making this college a place where I intellectually flourished.

To my wife, children, and the rest of my family, without your support and patience, the completion of this project would not have been possible.

Finally, I dedicate this dissertation to the memory of my parents and my sister Salima who passed away while pursuing a residency in obstetrics/gynecology.
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Repeted Anodal Transcranial Direct Stimulation and Mice Behavior.

**Discussion**

**Conclusion**

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### ABBREVIATIONS

- **a-DC**: anodal direct current
- **a-DCS**: anodal direct current stimulation
- **anodal-sDC**: anodal-sciatic direct current stimulation
- **ANOVA**: analysis of variance
- **a-TDCS**: anodal transcranial direct current stimulation
- **ATP 1A1**: ATPase, Na+/K+ transporting, alpha 1 polypeptide
- **CAP**: compound action potentials
- **cathodal-sDC**: cathodal-sciatic direct current stimulation
- **c-DC**: cathodal direct current stimulation
- **c-DCS**: cathodal direct current stimulation
- **c-TDCS**: cathodal direct current stimulation
- **Darpp-32**: dopamine and c-AMP regulated phosphoprotein-32
- **DC**: direct current
- **EPSP**: excitatory post-synaptic potentials
- **LS**: lidocaine-stimulated
- **LNS**: lidocaine-non-stimulated
- **LSD**: least significant difference
- **MEP**: motor evoked potentials
- **Na\(^+\)K\(^+\) ATPase**: sodium/potassium ATPase
- **nCAP**: number of compound action potentials
- **NLNS**: non-lidocaine-non-stimulated
- **NLS**: non-lidocaine-stimulated
- **PP1**: phosphatase protein 1
- **PP2B**: phosphatase protein 2B
- **SEP**: sensory evoked potentials
- **Tbp**: tata box protein
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INTRODUCTION

Direct current stimulation is a non-invasive technique that is largely used in humans and animals. Giovanni Aldini, an Italian physicist and nephew of Galvani, introduced it for the first time in 1804 as a therapeutic means to treat patients suffering of melancholia, a mood disorder (Parent, 2004). Following the seminal and pioneering experiments done on humans (Priori, Berardelli, Rona, Accornero, & Manfredi, 1998), which have paved the way for the safe utilization of weak pulses, clinical applications of transcranial direct current stimulation have been used to improve cognitive functions, manage psychiatric disorders, treat neurological and brain degenerative conditions, and correct motor impairments resulting from stroke accidents.

For instance, the application of anodal transcranial direct current stimulation over the dorsolateral prefrontal cortex improved the working memory of healthy individuals (Fregni et al., 2005). In the same way, transcranial direct current stimulation of the fronto-cortical region during the sleeping period helped individuals perform better in declarative memory tests (Marshall, Mölle, Hallschmid, & Born, 2004). Recent inclusion of transcranial direct current stimulation techniques in the psychiatric domain yielded promising results (Tortella et al., 2015). The use of these stimulation methods in the treatment of depression displayed an efficiency equal to that of classical therapy (Loo et al., 2012). The generalization of transcranial direct current stimulation to the treatment of neurological conditions revealed optimistic results, too.

Furthermore, the reduction of motor symptoms associated with Parkinson’s disease has been confirmed consistently in different studies following the application of anodal transcranial direct current stimulation (Benninger et al., 2010; Fregni et al., 2006). Also, the introduction of transcranial weak pulses in the treatment of neurodegenerative conditions proved to be a successful therapeutic means. Individuals diagnosed with Alzheimer disease, for instance,
bettered their cognitive functions following three sessions of 30 minutes of anodal transcranial direct current stimulation over the temporo-parietal region (Ferrucci et al., 2008). Additionally, transcranial direct current stimulation has been commonly employed to rehabilitate patients’ motor injuries following the occurrence of stroke. Results of clinical trials showed that cathodal transcranial direct current stimulation applied over the unaffected motor cortex of individuals suffering of hand motor disturbances subsequent to subacute stroke produced tangible correction of those impairments (D.Y. Kim et al., 2010). Post stroke hemiplegic patients responded more positively to transcranial direct current stimulation sessions than to functional treatment, according to a recent study (Cha, Ji, Kim, & Chang, 2014). Alongside the utilization of transcranial direct current stimulation on humans, numerous experiments have been also done on animals. Findings of these studies confirmed the therapeutic effects evaluated in various clinical settings (S. J. Kim et al., 2010). Additionally, these outcomes originating from research on animals contributed to the refinement of direct current techniques on humans (Liebetanz et al., 2009). Despite the accumulation of substantial data showing the benefit of direct current stimulation in the treatment of different conditions affecting humans, mechanisms underpinning the effectiveness of this therapeutic technique remain completely unknown. Therefore, more research is required to reveal the neurobiological effect of direct current stimulation on the target tissues.

Sodium potassium ATPase pump in the brain and in other organs belongs to the P-ATPase group. Enzymes of this category are labeled so because they are phosphorylated temporarily at an aspartate residue during the catabolic process (Ohtsubo, Noguchi, Takeda, Morohashi, & Kawamura, 1990). Each P-ATPase pump hydrolyzes one molecule of ATP to actively transport directly or indirectly relevant cations (Ca\textsuperscript{2+}, H\textsuperscript{+}, Na\textsuperscript{+}, K\textsuperscript{+}, and others) against an electrochemical
gradient (Bublitz, Poulsen, Morth, & Nissen, 2010). In neurons, the pump constantly maintains an electrochemical gradient between the intracellular and extracellular spaces by releasing three \( \text{Na}^+ \) outside the cell and introducing two \( \text{K}^+ \) in the cytoplasm. This electrochemical difference across the plasma membrane contributes to the establishment of the resting and active membrane potential (Suhail, 2010). The same gradient is required in neurons and other cells to provide them with the necessary energy to power secondary active transports, which allow them to take into the cytoplasm essentials nutrients (Lingrel, 1992). Intracellular sodium concentrations and cytosol acidity also depend indirectly on the \( \text{Na}^+ \text{K}^+ \)-ATPase pump (Therein, Goldshleger, Karlish, & Blostein, 1997). Consistently, many neurotransmitter transporters use antiport co-transport systems (Isaksen & Lykke-Hartmann, 2016). For example, glycine, an inhibitor neurotransmitter in the central nervous system, is co-transported by GlyT2, which uses sodium and chlorine ions. The sodium ionic gradient necessary for this transport rests on the \( \text{Na}^+ \text{K}^+ \)-ATPase pump activity (López-Corcuera et al., 1998). Consequently, the conveyance of various neurotransmitters like, dopamine, norepinephrine, GABA, and serotonin through the cell membrane might be compromised, as this type of carrier mediated transport is reliant on the \( \text{Na}^+ \text{K}^+ \)-ATPase activity (Kristensen et al., 2011). Taken together, these assessments show the importance for the neurons’ function to maintain an electrochemical gradient, which the \( \text{Na}^+ \text{K}^+ \)-ATPase pump generates. Therefore, any genetic mutation that affects the enzyme’s molecular conformation or any impediment that alters partial or complete activity of the pump will disrupt the ionic gradient across the plasma membrane and furthermore the cell’s function. In the event the electrochemical gradient is no longer preserved, neurons would have difficulty in responding to sensory stimuli, as this physiological faculty is related to the flow of sodium and potassium currents through specific transmembrane voltage channels. (Isaksen & Lykke-Hartmann, 2016).
Interfering with Na\(^+\)K\(^+\)-ATPase enzyme activity could also have other serious effects on the cell’s biological tasks. Co-transporter mechanisms like the Na\(^+\)/Ca\(^+\) exchanger will fail to keep low cytoplasmic calcium concentrations raising the possibility of activating calcium dependent gated channels and triggering the release of unwanted neurotransmitters (de Lores Arnaiz & Ordieres, 2014). Decrease of the cytosolic pH and increase of the cell volume are additional complications that may appear following the loss of the electrochemical gradient (DiPolo & Beaugé, 2006; Therien & Blostein, 2000). On the other hand, inadequate activity of the Na\(^+\) K\(^+\)-ATPase has been linked to pathologies and behavioral disorders beyond the simple impairment of the cell function. The normal physiology of the nervous system cells entails that an equilibrium between the ions flowing passively or actively across the plasma membrane must be constantly present (Goodman, Hall, Avery, & Lockery, 1998; Wright, 2004). The Na\(^+\) K\(^+\)-ATPase pump activity is therefore decisive in maintaining these currents well balanced (Hodgkin & Huxley, 1952). The loss of the ionic equilibrium in neurons has been associated with various conditions affecting the nervous system. In line with these statements, the modification of the Na\(^+\) K\(^+\)-ATPase activity was linked to frequent seizures (Vaillend, Mason, Cuttle, & Alger, 2002). Furthermore, mutation of Na\(^+\) K\(^+\)-ATPase alpha 3 subunit gene was found to trigger epileptic activity in mice and humans (Clapcote et al., 2009). Recent findings showed that amyloid beta peptide (A\(\beta\)) plaques, which are characteristically accumulated in the extracellular matrix of people affected with Alzheimer’s disease, contribute to the increase of sodium and potassium ions concentrations in the cytoplasm of astrocytes because of the low expression of the Na\(^+\) K\(^+\)-ATPase pump (Graham et al., 2015). This will have affected the ability of astrocytes to uptake glutamate neurotransmitter released in the synapses (Vitvitsky, Garg, Keep, Albin, & Banerjee, 2012). Rapid Onset Dystonia Parkinsonian (RDP), Alternating Hemiplegia of
Childhood (AHC), Cerebellar Ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss (CAPOS) are three neurological conditions associated with Na⁺K⁺-ATPase alpha 3 subunit gene mutation, which is likely to interfere with sodium and potassium currents (Isaksen & Lykke-Hartmann, 2016). The role of Na⁺K⁺-ATPase alpha 3 subunit in the control of behavior has been well highlighted in Myshkin mice known to have an inactivating mutation of this isoform. The occurrence of abnormal behavior could be associated with the failure of the pump to feed the Na⁺/Ca⁺ exchanger. It is very credible that a mutation of the pump’s alpha3 subunit gene will induce an elevation of intracellular calcium concentration, triggering frequent signaling pathways dependent on this ion. In support of this assertion, maniac behavior, which is similar to that of the Bipolar disorder, was seen in Myshkin mice, showing that a dysfunction of Na⁺K⁺-ATPase alpha 3 isoform could be associated with uncharacteristic conducts (Kirshenbaum et al., 2011). Additional atypical behavior was also identified in mice with mutation of the Na⁺K⁺-ATPase alpha 2 subunit gene, which causes Familial Hemiplegic Migraine Type 2 (Lingrel, Williams, Vorhees, & Moseley, 2007). Concomitant to these disorders, dysfunctions affecting the pump function proved to have an impact on cognitive functions. For example, mice carrying Na⁺K⁺-ATPase alpha 3 subunit mutation performed poorly in tests of spatial learning and memory (Holm et al., 2016). Similarly, impaired cognitive performances were diagnosed in patients with Rapid Onset Dystonia Parkinsonian (RDP), a condition that is related to a mutation of Na⁺K⁺-ATPase alpha 3 subunit. (Cook et al., 2014).

Together, these results highlight the pivotal role of Na⁺K⁺-ATPase in the generation and maintenance of neurons’ resting membrane potential. (Scheiner-Bobis, 2002; Takeuchi, Reyes, Artigas, & Gadsby, 2008). They also reveal the importance of maintaining the enzyme in an intact functional state to avoid certain depressive, neuro-degenerative, and behavioral disorders.
Furthermore, these reports infer that any change in the neurons’ function is most likely to involve Na\(^+\) K\(^+\) -ATPase pump. In parallel to these affirmations, transcranial direct current stimulation has been found to affect neurons’ membrane voltage (Di Lazzaro et al., 2013; Priori et al., 1998) without triggering an action potential (Nitsche et al., 2008). In general, anodal direct current stimulation increases the axonal excitability whereas cathodal direct current stimulation reduces it (Nitsche & Paulus, 2000). It is plausible that changes of neurons’ excitability after stimulations with different transcranial direct currents could arise from modifications of the Na\(^+\) K\(^+\) -ATPase structure or activity.

Therefore, we hypothesize that transcranial direct current stimulation effect on neurons’ axonal excitability is driven whether by adjustments of the function, regulation or expression of the Na\(^+\) K\(^+\) -ATPase.
MATERIAL AND METHODS

Animals

CD-1 mice were bred and reared within the following conditions: 12:12 light dark cycle with access to food and water ad libitum. Authorization allowing experiments on the animals was given by the College of Staten Island’s Institutional Animal Care and Use Committee (IACUC) according to NIH guidelines.

Preparation of mice for single direct current stimulation over the exposed right sensorimotor cortex

Mice of one month age were anesthetized with ketamine/xylazine (100/10 mg/kg) solution through intra peritoneal injection at the dose of 100 µl per10 g of animal weight. Animals were then stabilized on a stereotaxic apparatus. Using a dental round burr (Union Broach Co., Switzerland) mounted on a dental handpiece, a circular craniotomy over the right sensorimotor cortex area extending 1.5 mm rostral and caudal to bregma in the sagittal plan and 3mm laterally to bregma was performed to expose the underlying mice brain with its intact dura mater. Right sensorimotor cortex cortices of anesthetized sham animals were covered with a sterile cotton imbibed with saline solution (NaCl 0.9 %) for 20 minutes. A circular electrode (thickness 5 µm, diameter, 3.5 mm) connected to a stimulator was placed over the right sensorimotor cortices of experimental animals to deliver for a period of 20 minutes whether an anodal or a cathodal direct current of 0.2 mA intensity (I). The reference electrode was placed on the mouse’s tail. Current density (J) value based on the current intensity and the electrode area was equal to 10.2 Am⁻². Two hours following the end of the application of saline solution in sham mice and the end of direct current stimulation in experimental mice, animals were euthanized using an intraperitoneal injection of a lethal dose of ketamine-xylazine (100/10 mg/kg) anesthetic solution.
Preparation of mice for single and repeated transcranial direct current stimulation of the right sensorimotor cortex

Animals were anesthetized using an intra peritoneal injection of ketamine/xylazine (100/10 mg/kg). Through a sagittal incision, the skull was exposed, and the underlying tissues were removed. A plastic hub, to which DC simulating electrode will be tightened, was stabilized over the right sensorimotor cortex area, extending 1.5 mm rostral and caudal to bregma in the sagittal plan and 3 mm laterally to bregma, with a dental resin. To stimulate the animal, the electrode was tightened through a luer to the plastic hub filled with 0.9% NaCl saline solution (Figure 1, A). The reference electrode was placed in the mouse’s tail. Anodal or cathodal direct current of 0.2 mA intensity was applied for 20 minutes each day for 5 consecutive days. Animals were euthanized with an injection of a lethal dose of ketamine-xylazine (100/10 mg/kg) solution 24 hours after the end of the last stimulation session.

Figure 1: Preparation of CD-1 mouse for transcranial direct current stimulation: A) Cartoon showing the different parts needed for direct current transcranial stimulation of the right sensorimotor cortex. B) Picture showing the fixation of the hub (electrode) by dental resin over the skull area covering the right sensorimotor cortex.

RNA isolation and cDNA synthesis

At the end of a single 20 minutes session of either anodal or cathodal direct current stimulation of the exposed right sensorimotor region, cortices of CD-1 mice were collected 2 hours and left
at – 80 °C. Total RNA was extracted from frozen brain tissues using TRIZOL® (Life Technologies, USA). To rid the extracts from any possible presence of genomic DNA, they were further submitted to an additional treatment with Aurum Total RNA mini kit (Bio-Rad, USA) in accordance with the manufacturer’s instructions. Optical density measurements of the samples were done at 260 nm and 280 nm using Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA) to assess the RNA purity and concentration of each sample. cDNA was obtained by combining 4 µl of iScript Reverse Transcription Supermix for RT-qPCR kit (Bio-Rad, USA) with appropriate volume of RNA template and nuclease-free water in a mixture of 20 µl volume. Total RNA (1 µg per 20 µl RT reaction) was reverse transcribed in a thermal cycler (Primus 25, Cole-Parmer USA) using the following parameters: 5 minutes priming at 25 °C, 30 minutes reverse transcription, and 5 minutes reverse transcription inactivation at 85 °C. The resulting cDNA had a concentration of 50 ng/µl, which was further diluted to 10 ng/µl before use.

**Real-time quantitative RT-PCR**

A total volume of 10 µl per reaction containing 2 µl of cDNA mixed with 0.5 µl of 20X PrimePCR assay (Bio-Rad, USA), 5 µl of 2X SsoAdvanced universal SYBR® Green (Bio-Rad, USA), and a variable volume of nuclease-free water was prepared for each gene. Triplicates of the sample of interest or sham were run in the real-time quantitative PCR apparatus CFX384 Real Time System (Bio-Rad, USA) using the following parameters: 2 minutes activation step at 95 °C, then 40 cycles, each one requiring 5 seconds of denaturation at 95 °C, and 30 seconds of annealing at 60 °C. Mean of Ct values of the gene of interest (alpha 1 Na⁺ K⁺ ATPase) and the reference gene, TATA box binding protein (Tbp), from experimental and sham samples were used to calculate the relative expression of mRNA.
Primers, for probing transcripts of Alpha1 Sodium Potassium ATPase, gene of interest, and TATA Box Binding Protein, reference gene, in the right sensorimotor cortex of CD-1 mice were ordered from (Bio-Rad, USA).

Gene Name: ATPase, Na+/K+ transporting, alpha 1 polypeptide, Gene Symbol: Atp1a1
Unique Assay ID: qMmuCID0017752

Gene Name: TATA box binding protein, Gene Symbol: Tbp
Unique Assay ID: qMmuCID0040542

**Preparation of mice for checking whether the increase of the Na⁺ K⁺ ATPase expression is activity-dependent**

Mice were anesthetized and prepared following the immediate effect of direct current stimulation on the Na⁺ K⁺ ATPase expression protocol in open skulls. In two groups of mice, a sterile gauze imbibed with 1mM lidocaine was applied over the exposed right sensorimotor cortex for 5 minutes. One of the lidocaine treated groups, known as lidocaine stimulated (LS) group, was subjected to 20 minutes of only cathodal direct current stimulation. The second one referred to as lidocaine non-stimulated (LNS) group did not receive any stimulation. A third group of mice, identified as non-lidocaine stimulated (NLS) group, underwent also only a single cathodal direct current stimulation for 20 minutes. In the fourth batch of mice, named non-lidocaine non-stimulated (NLNS) group, a sterile gauze imbibed with sterile saline solution (NaCl 0.9%) was applied for a duration of 20 minutes. The intensity of the current was equal to 0.8 mA. Two hours after the end of 20 minutes of saline solution application or cathodal direct current stimulation, mice groups were euthanized, their right sensorimotor cortices collected and placed in the freezer at – 80 °C.

**Preparation of mice for assessing the effects of direct current stimulation on the sciatic nerve**

Study of sciatic nerve excitability was done using Ahmed-unpublished data. Mice were
anesthetized with an intraperitoneal injection of ketamine/xylazine (90/10 mg/kg). To have access to the sciatic nerve, an incision was made through the skin of the left hind limb of mice stabilized on a stereotaxic apparatus. To deliver direct current stimulation and evaluate its effect on the sciatic nerve excitability, one single DC electrode, made of stainless steel plate (thickness, 5 µm; width, 7 mm; length, 15 mm), was placed under the sciatic nerve. Sciatic nerve was fixed to a rubber silicone, whose shape fits the exposed region, to prevent any interference with body tissues and surrounding area. Two chambers of Ringer solution, central and lateral were created by pouring at the periphery of the exposed area petroleum jelly mixed with silicone oil. DC stimulating electrode was placed in the central chamber and DC ground electrode was positioned on the abdominal skin (Figure 2). Other electrodes were additionally used: a concentric bipolar for stimulating the sciatic nerve and a hook shaped one for recording its potentials. Stimulating reference and ground electrodes were respectively attached to the skin of the left paw and of the right abdominal region. Two electrophysiology protocols were used to stimulate and record sciatic nerve responses. Lateral test stimulation where the stimulating electrode was placed in the lateral chamber away from DC electrode. Central test stimulation where the stimulating electrode was inserted in the central chamber. Ouabain 450 µM, known to inhibit the catalytic activity of the Na⁺ K⁺ ATPase pump, was added alternately in lateral and central chambers during each protocol. Sciatic nerves were exposed to 10 µA DC for a period of 2 minutes. Test stimulations were done before, during, and several times after DC application.
Figure 2: Preparation of CD-1 mouse for direct current stimulation of the sciatic nerve. Sciatic nerve is represented in yellow. Central and lateral chambers are circumscribed with the blue color. In this experimental representation, test-stimulation electrode is placed lateral to DC electrode.

Preparation of mice for assessing the effects of single transcranial direct current stimulation of the right sensorimotor cortex on the sensory evoked potentials amplitude

CD-1 mice were anesthetized with an intraperitoneal injection of xylazine/ketamine (90/10 mg/kg). Animals were later stabilized in a mouse stereotaxic device. An incision through the skin covering the left hind limb was made to expose the sciatic nerve. A nerve cuff electrode was placed around it to allow test stimulations. The recording electrode was directly implanted on the right sensorimotor cortex through a perforation of the skull. Ground and reference electrodes associated respectively with cuff and recording electrodes were placed on the wall of the platform covered with aluminum. A plastic hub, destined to receive DC stimulating electrode, was stabilized with a dental resin over the right sensorimotor cortex area extending 1.5 mm rostral and caudal to bregma in the sagittal plan and 3mm laterally to bregma. Reference electrode was attached to the left ear of the animal. Alternately, anodal and cathodal DCS of 0.2 mA was applied for 20 minutes over the right sensorimotor cortex. Sensory evoked potentials triggered by 1 pulse, 17 pulses (50 Hz), and 17 pulses (333 Hz) were recorded before and after DC application.
Western blotting

Exposed brains that have been stimulated directly, and those that have received a single or repetitive transcranial stimulation were processed according to the following protocol. Brains removed from animals’ skulls were first put in sterile Petri dishes placed on wet ice. Right sensorimotor cortices were then dissected, weighted, and frozen on dry ice within 5 minutes following the sacrifice of animals. Next, using (Abcam Co., USA) protocol, brain cortices were first manually homogenized in a solution containing the following compounds: 1X protease inhibitor, 1X phosphatase inhibitor, 5mM EDTA, 1mM PMSF, and RIPA buffer. Furthermore, partially homogenized samples were sonicated, and centrifuged at 12,000 g for 20 minutes in a centrifuge apparatus (Beckman, USA) to extract membrane-bound and soluble proteins. Supernatant proteins concentrations were established using Bradford protein assay (Bio-Rad) and a microplate spectrophotometer (MPM 6-Bio-Rad). Proteins were first run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After separation, proteins were transferred to a PVDF membrane (Bio-Rad) in an electrophoresis chamber containing Tris-buffered saline with 20% methanol. Membranes were initially incubated overnight at 4 °C with one of the following antibodies: mouse monoclonal anti-sodium potassium ATPase alpha 1 (1:5,000 dilution-Santa Cruz Biotechnology, USA), goat polyclonal anti-sodium potassium ATPase alpha 1 phospho serine 943 (1:400 dilution-Santa Cruz Biotechnology, USA), rabbit polyclonal anti-alpha 1 phospho serine 23 sodium potassium ATPase (1:1,000 dilution-Abcam, USA), mouse monoclonal anti-alpha 3 sodium potassium ATPase (1:2,500-Santa Cruz Biotechnology, USA), rabbit monoclonal anti-sodium potassium ATPase beta 2 (1:45,000 dilution-Abcam, USA), rabbit polyclonal anti-Darpp-32 (1:1,000 dilution-Santa Cruz Biotechnology, USA), goat polyclonal anti-phospho Darpp-32 (1:500 dilution-Santa Cruz Biotechnology, USA).
Biotechnology), and mouse monoclonal anti-beta actin (1:5,000 dilution-Santa Cruz Biotechnology, USA). After that, membranes were incubated at room temperature with one of the following secondary antibodies: HRP conjugated goat anti-mouse (1: 5,000 dilution-Santa Cruz Biotechnology, USA), goat anti-rabbit (1: 5,000 dilution-Santa Cruz Biotechnology, USA) or donkey anti-goat (1: 5,000 dilution-Santa Cruz Biotechnology, USA). Detection of immune-reactive proteins of interest was performed using an HRP enhanced chemiluminescence (WB-100) kit (Boston BioProducts, USA). Measurements of the proteins bands’ intensity was done using a densometer. Using Image J software (NIH, USA), the relative density of each protein band of interest was determined by normalizing it to the density of β-actin band.

Open field activity

The open-field test is used to assess rodents’ anxiety, hyperactivity, and motor abilities. The field where animals’ behavior was tracked is a rectangular arena measuring 80x60x15cm. A video hanging 1.5 m above it recorded mice locomotor activity. Before the recording started, mice were placed in the center zone of the arena. The recording time was set for 10 minutes. Mice were kept in their home cages for 15 minutes in a neighboring room to facilitate acclimation before the beginning of the experiment. After the end of 10-minute recording, mice were brought back to their cages. A thorough cleaning of the arena with 70% ethanol was performed between each animal testing. Using a software called Anymaze®, parameters like, animal motion speed, and time spent immobile in central zone were analyzed according to a protocol adapted from El Idrissi, et al., 2008.

Statistical Analysis

Statistical analysis was done using Graphpad prism (GraphPad Software, Inc., USA). Data were
analyzed using t-test, single or multifactorial ANOVA, and expressed as means ± standard error (SE). Post-hoc analyses were conducted for unequal n. Significance was set at a confidence of 95% and results were statistically significant if \( p < 0.05 \).
RESULTS

COMPARISON OF Na⁺K⁺ ATPase ALPHA 3 PROTEIN EXPRESSION IN SHAM AND CONTROL CD-1 MICE

Cathodal direct current stimulation has the same effect on Na⁺K⁺ ATPase α3 protein expression in sham and control CD-1 mice

Before assessing the expression of any protein of interest in any animal’s right sensorimotor cortex, we verified if there was a difference in the expression of Na⁺K⁺ ATPase alpha 3 between sham and control mice. After the end of the craniotomy, a period of 20 minutes corresponding to the time of the stimulation was first observed followed by 2 hours waiting period before control and sham mice were euthanized. The analysis of western blots of tissues originating from sham and control mice (Figure 3, A), all of them treated with a mouse anti-Na⁺K⁺ ATPase alpha 3 primary antibodies (1: 2,500 dilution), did not show any significant difference in the levels of expression of Na⁺K⁺ ATPase alpha 3 (Figure 3, B).

Figure 3: Cathodal direct current stimulation does not induce any significant change of Na⁺K⁺ ATPase α3 protein expression in right motor cortices of control and sham CD-1 mice.

A) Western blots showing the expression of Na⁺K⁺ ATPase alpha 3 protein in samples collected from the right motor cortices of control and the open motor cortices of sham mice. Actin was used as an internal control. B) Unpaired t-test graph shows no significant difference between control and sham animals Na⁺K⁺ ATPase α3 expression: (t = 0.2237, df = 6); P = 0.83. Bars represent means of the densitometry ratios of Na⁺K⁺ ATPase alpha 3 to actin normalized to the mean of the densitometry ratio of the sham samples. 6 sham and 2 control mice were used.

EFFECT OF DIRECT CURRENT STIMULATION ON Na⁺K⁺ ATPase ALPHA 1 PROTEIN EXPRESSION
Single cathodal direct current stimulation increases $\text{Na}^+\text{K}^+$ ATPase alpha 1 protein expression in the exposed right sensorimotor cortex of CD-1 mice

One of our research objectives was to see whether direct current stimulation affects $\text{Na}^+\text{K}^+$ ATPase protein expression. Synthesis, insertion or removal of $\text{Na}^+\text{K}^+$ ATPase proteins from the plasma membrane is considered a long-term regulation of the pump’s function (McDonough & Farley, 1993) because these processes take time before they occur (Therien & Blostein, 2000). Sodium/potassium pump generates currents responsible of the membrane potential (Horisberger, 2004), and DCS was found to affect neurons’ membrane voltage (Nitsche & Paulus, 2000). Given this relation, we wanted to see if changes of cells’ excitability during DCS are related to modifications of the enzyme expression. The $\text{Na}^+\text{K}^+$ ATPase pump has many subunits: alpha, beta, and gamma, all of them found in different isoforms (Sweedner, 1991). We focused our work on alpha 1 subunit because it is the site of the pump catalytic function (Pedemonte & Kaplan, 1990), and it is ubiquitous in all brain cells (Lingrel et al., 2007). Using western blots experiments (Figure 4, A), we found that cathode stimulated samples have a significant expression of $\text{Na}^+\text{K}^+$ ATPase alpha 1 compared to anode stimulated, **$p=0.008$, and sham tissues, *$p=0.03$, (Figure 4, B).

![Figure 4](image)

**Figure 4:** Single cathodal direct current stimulation significantly increases $\text{Na}^+\text{K}^+$ ATPase alpha 1 protein expression in CD1-mice exposed right motor cortices. A) Western blots showing the expression of $\text{Na}^+\text{K}^+$ ATPase alpha 1 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows significant main effect between the groups: $F (2, 10) = 5.9; P = 0.01$. Optical density in cathode stimulated animals was significantly higher than in sham and anode stimulated animals;
Fisher’s LSD test: *p = 0.03 from sham; **p = 0.008 from anode. Bars represent means of the densitometry ratios of Na\(^+\)K\(^+\) ATPase alpha 1 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

**Single anodal and cathodal transcranial direct current stimulation of the right sensorimotor cortex over an intact skull of CD-1 mice have opposite effects on Na\(^+\)K\(^+\) ATPase alpha 1 protein expression**

Significant findings related to the expression of the Na\(^+\)K\(^+\) ATPase protein in the exposed right sensorimotor cortex samples led us to investigate whether transcranial direct current stimulation would generate the same findings. Our results showed that the effect of the cathodal transcranial direct current stimulation over the right sensorimotor cortex of mice was also associated with a significant increase of the Na\(^+\)K\(^+\) ATPase alpha 1 expression in comparison to anode treated, ***p = 0.0001, and sham mice, *p = 0.022, (Figure 5, A & B). This outcome confirmed the trend we have already witnessed in mice subjected to cathodal direct stimulation over the exposed right sensorimotor cortex. We also found that the expression of Na\(^+\)K\(^+\) ATPase alpha 1 protein after anodal transcranial direct current stimulation was significantly smaller, *p= 0.028, (Figure 5, B) than in sham samples. These two outcomes are in line with our hypothesis, which suggests that cathodal and anodal direct current stimulation increases and decreases respectively the Na\(^+\)K\(^+\) ATPase alpha 1 protein expression.

**Figure 5:** Single anodal and cathodal transcranial direct current stimulation of the right sensorimotor cortex over an intact skull of CD-1 mice significantly and respectively decreases and increases Na\(^+\)K\(^+\) ATPase alpha 1 protein expression. A) Western blots showing the expression of Na\(^+\)K\(^+\) ATPase alpha 1 protein in samples collected from sham,
anodal and stimulated mice. Actin was used as an internal control. **B)** One way ANOVA graph reveals significant main effect between groups: $F(2, 10) = 18.3; P = 0.0005$. Optical density in cathode stimulated animals was significantly higher than in sham and anode stimulated animals; Fisher’s LSD test: *p = 0.022 from sham; ***p = 0.0001 from anode. Optical density in anode stimulated mice was significantly lower than in sham animals: *p = 0.028. Bars represent means of the densitometry ratios of $\text{Na}^+\text{K}^+$ ATPase alpha 1 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

**Daily 20 minutes of cathodal transcranial direct current stimulation of the right sensorimotor cortex through an intact skull over a period of 5 days increases $\text{Na}^+\text{K}^+$ ATPase alpha 1 protein expression**

According to previous studies, using a single transcranial direct current stimulation triggers a change of the underneath cortex excitability that could persist for more than 90 minutes after the end of the stimulation (Nitsche & Paulus, 2000). Additional studies revealed that repeated transcranial direct current stimulation of the motor cortex of individuals with no health problem, done over a period of 5 days, efficiently influences the cortex excitability (Alonzo, Brassil, Taylor, Martin, & Loo, 2011). Given these findings, we tested whether daily 20 minutes of transcranial direct stimulation would have also an impact on the $\text{Na}^+\text{K}^+$ ATPase $\alpha$1 protein expression as we assessed it already with a single session of anodal and cathodal stimulation.

Western blots analysis of the experimental samples reveals that repetitive cathodal transcranial direct current stimulation has similar influence on the expression of $\text{Na}^+\text{K}^+$ ATPase $\alpha$1 protein (Figure 6, A). We found that tissues treated with cathodal direct current show significant enhancement of the protein in comparison to anode stimulated, ***p = 0.0004, and sham, **p = 0.008 samples, (Figure 6, B). The expression of $\text{Na}^+\text{K}^+$ ATPase $\alpha$1 in anode treated animals showed also a decreased pattern compared to sham mice. However, the difference did not rise to a significant level. Despite its moderation, this result proved that anodal transcranial direct current stimulation had an opposite effect on $\text{Na}^+\text{K}^+$ ATPase $\alpha$1 protein expression compared to that of the cathodal stimulation thus supporting our hypothesis. Taken together, these results,
particularly those seen with cathodal stimulation, did not contradict those observed with the immediate effect of cathodal stimulation on $\text{Na}^+\text{K}^+\text{ATPase} \alpha 1$ expression in open and closed skulls.

**Figure 6:** Daily 20 minutes cathodal transcranial direct current stimulation of the right sensorimotor cortex through an intact skull over a period of 5 days induces a significant increase of $\text{Na}^+\text{K}^+\text{ATPase} \alpha 1$ protein expression. **A)** Western blots showing the expression of $\text{Na}^+\text{K}^+\text{ATPase} \alpha 1$ protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. **B)** One way ANOVA graph shows significant main effect between groups: $F (2, 10) = 16.43; p = 0.0004$. Optical density in cathode treated mice was significantly higher than in sham and anode stimulated animals; Tukey’s test: **$p = 0.008$ from sham; ***$p = 0.0004$ from anode. Bars represent means of the densitometry ratios of $\text{Na}^+\text{K}^+\text{ATPase} \alpha 1$ to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

**EFFECT OF DIRECT CURRENT STIMULATION ON THE EXPRESSION OF $\text{Na}^+\text{K}^+\text{ATPase ALPHA 1 (ATP 1A1) GENE}$**

Single cathodal direct current stimulation of the exposed right sensorimotor cortex of CD-1 mice increases $\text{Na}^+\text{K}^+\text{ATPase} \alpha 1$ relative mRNA transcripts

We reserved a part of our study to the exploration of a possible link between DC stimulation and gene expression. Our previous results revealed changes in the expression of $\text{Na}^+\text{K}^+\text{ATPase} \alpha 1$ protein following the application of c-DC in different experimental settings. However, we could not affirm whether the increase of the pump expression reflected an activation of $\text{Na}^+\text{K}^+\text{ATPase} \alpha 1$ gene. We found out that most studies aimed at identifying the mechanisms of direct current stimulation effects were approached mainly through the analysis of pharmacological or electrophysiological effects. Scarce were the investigations devoted to unveiling its molecular mechanisms. One of them showed that daily and concomitant application
of anodal and cathodal transcranial stimulation respectively to the ipsilateral and contralateral cortices of rats with induced stroke decreases the levels of hemichannel pannexin 1 mRNA, a cell membrane protein found in non-selective channels (Jiang et al., 2012a). This result proved that transcranial direct current stimulation impacts the gene expression of proteins located in the plasma membrane. Recent data suggested that the mechanisms sustaining the action of transcranial direct current stimulation require gene expression and synthesis of newly formed proteins (Cirillo et al., 2016). Consequently, we inspected whether Na\(^+\)K\(^+\) ATPase alpha 1 relative mRNA transcription was affected following the respective anodal and cathodal direct current stimulation of the exposed right sensorimotor cortex. Real time quantitative polymerase chain reaction (RT-qPCR) experiments showed that Na\(^+\)K\(^+\) ATPase alpha 1 relative mRNA transcripts in the exposed sensorimotor were increased with c-DC. These findings proved that the rise of the pump’s protein expression after the application of cathodal current over the exposed sensorimotor was also accompanied by an augmentation of the expression of Na\(^+\)K\(^+\) ATPase alpha 1 relative mRNA transcripts, (Figure 7, A & B). We found a significant increase of Na\(^+\)K\(^+\) ATPase alpha 1 relative mRNA transcripts in cathode treated mice compared to the transcripts in anodal treated, **p= 0.0022, and in sham animals, **p= 0.0017 (Figure 7, B). Therefore, our results supported early findings that DC stimulation influences the expression of genes, too.

**Figure 7:** Cathodal direct current stimulation of the exposed right sensorimotor cortex generates a significant increase of Na\(^+\)K\(^+\) ATPase alpha 1 relative mRNA transcripts. A) melt curve, amplification, and melt peak showing RT-qPCR run of samples collected from right
motor cortices of sham and experimental animals subjected to 20 minute of anodal or cathodal direct current stimulation through an open and closed skull respectively. B) One way ANOVA graph in animals stimulated through open skulls displays significant main effect between the groups: F (2,10) = 16.43; p= 0.0004. Na⁺ K⁺ ATPase alpha 1 mRNA relative quantity in cathode treated mice was significantly higher than in sham and anode stimulated animals; Tukey’s test: **p= 0.0017 from sham; **p= 0.0022 from anode. 3 sham, 7 cathode treated and 8 anode treated mice.

IS THE INCREASE OF NA⁺ K⁺ ATPase ALPHA 1 PROTEIN EXPRESSION, FOLLOWING DIRECT CURRENT STIMULATION, DIRECTLY OR ACTIVITY-DEPENDENT TRIGGERED?

Expression of Na⁺ K⁺ ATPase alpha 1 protein is alternately increased and decreased, but not significantly, when its activity is respectively on and off

Consistent results showed that cathodal direct current stimulation in either paradigm, single or repeated, influences Na⁺ K⁺ ATPase alpha 1 protein expression. How this impact is brought about? Does direct current stimulation exert its effect directly on the gene expression? Does direct current stimulation increase the expression of Na⁺ K⁺ ATPase alpha 1 pump because its activity is increased? Thus, more pumps are required? To find out, we studied four groups in the experiment: non-lidocaine non-stimulated (NLNS), non-lidocaine stimulated (NLS), lidocaine non-stimulated (LNS), and lidocaine stimulated (LS). We did not see any significant difference in the expression of Na⁺ K⁺ ATPase alpha 1 protein between any pair, (Figure 8, B). There were some trends in the expression of the pump showing slight contrast in the Na⁺ K⁺ ATPase alpha 1 expression between many groups. For instance, within the category where the activity was on, non-lidocaine stimulated (NLS) subgroup has higher expression of Na⁺ K⁺ ATPase alpha 1 protein compared to non-lidocaine non-stimulated (NLNS) (Figure 8, B). This result is reminiscent of the difference seen between cathodal stimulated and sham tissues in the previous analyses as non-lidocaine non-stimulated (NLNS) batch could be considered as sham control (Figure 1, A& B). Despite its non-statistical significance, this result confirmed that cathodal direct current stimulation has an increasing effect on Na⁺ K⁺ ATPase alpha 1 protein expression.
There was not also any notable difference between lidocaine non-stimulated (LNS) and lidocaine stimulated (LS) samples sets, both had their pump activity off (Figure 8, B). Our results suggest that Na⁺K⁺ ATPase alpha 1 pump activity contributes to the increase of its own expression. The difference of the expression of Na⁺K⁺ ATPase alpha 1 between non- lidocaine stimulated (NLS) and lidocaine stimulated (LS) subgroups was not significant either. In the former set (NLS), the pump activity was on and its expression went up. In the latter group (LS), the activity of Na⁺K⁺ ATPase alpha 1 was off and its expression decreased. This pattern of expression showed that simultaneous stimulation and blockage of the pump’s activity tends to reduce its expression (Figure 8, B). However, we also found non-significant increase of Na⁺K⁺ ATPase alpha 1 protein expression in lidocaine non-stimulated (LNS) samples where the pump’s activity was off. This assessment proves that the simple fact of blocking the pump’s activity can lead to its increased expression, too. Collectively, these results could not lead to a clear conclusion regarding the effect of the activity on the overall expression of the pump based only on the comparison of NLS and LS groups’ Na⁺K⁺ ATPase alpha 1 expression.

Figure 8: No significant difference in the expression of Na⁺K⁺ ATPase alpha 1 protein is found between non-lidocaine and lidocaine treated tissues after stimulation of the exposed right sensorimotor cortex of CD-1 mice by single cathodal direct stimulation. A) Western blots showing the expression of Na⁺K⁺ ATPase alpha 1 protein in samples collected from non-lidocaine non-stimulated (NLNS), non-lidocaine stimulated (NLS), lidocaine non-stimulated (LNS), and lidocaine stimulated (LS) samples. Actin was used as an internal control. B) Two-
way ANOVA graph shows no significant interaction between the groups: F (1-16) = 1.4, p = 0.25; no significant effect of cathodal direct current stimulation: F (1-16) = 0.14, p = 0.7 or significant effect of lidocaine application over the open right motor cortex: F (1-16) = 0.00008, p = 0.99 on the expression of alpha 1 Na$^+$K$^+$ ATPase. The graph still shows non-significant difference in the expression of alpha 1 Na$^+$K$^+$ ATPase between non-lidocaine non-stimulated (NLNS) and non-lidocaine stimulated (NLS) samples. It also reveals no significant difference between (NLS) and lidocaine stimulated (LS). The graph reveals that the blockage of Na$^+$K$^+$ ATPase alpha 1 pump activity in (LNS) samples increases its expression. Bars represent means of the densitometry ratios of Na$^+$K$^+$ ATPase alpha 1 to actin, which have been normalized to the mean of the densitometry ratio of the sham samples. 5 mice for each group were used.

**EFFECT OF DIRECT CURRENT STIMULATION ON Na$^+$K$^+$ ATPase ALPHA 3 PROTEIN EXPRESSION**

Single cathodal direct current stimulation of the exposed right sensorimotor cortex of CD-1 mice significantly increases Na$^+$K$^+$ ATPase alpha 3 protein expression

We extended our research to the study of Na$^+$K$^+$ ATPase alpha 3 isoform expression because it is exclusively found in neurons (Moseley et al., 2007). Brains’ neurons have also alpha 1 and 2 isoforms present in their plasma membranes (Juhaszova & Blaustein, 1997). Furthermore, studies showed that both alpha 1 and 3 contribute to the basic function of neurons (Blanco & Mercer, 1998; Dobretsov & Stimers, 2005). A recent study found out that whenever there is an increase of intracellular concentrations of sodium, it would be mainly Na$^+$K$^+$ ATPase alpha 3 that contributes to the expulsion of this cation (Azarias et al., 2013). These findings indicate the importance of alpha 3 subunits in the establishment of the membrane potential. We therefore examined how direct current stimulation could influence Na$^+$K$^+$ ATPase alpha 3 isoform protein expression. Western blots results (Figure 9, A) showed that cathodal direct current stimulation causes a significant increase of Na$^+$K$^+$ ATPase alpha 3 protein, **p = 0.006, in comparison to the expression of the same protein in tissues submitted to anodal current was applied (Figure 9, B). Nevertheless, the level of the expression of Na$^+$K$^+$ ATPase alpha 3 protein in anode treated tissues was still lower than in sham samples. These results are like those seen with the effect of cathodal direct current stimulation on Na$^+$K$^+$ ATPase alpha 1 protein expression.
**Figure 9:** Single cathodal direct current stimulation of the exposed right sensorimotor cortex of CD-1 mice induces a significant increase of Na\(^+\) K\(^+\) ATPase α3 protein expression. 

A) Western blots showing the expression of Na\(^+\) K\(^+\) ATPase alpha 3 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows significant main effect between groups: F (2, 10) = 6.043; P=0.02. Optical density in cathode stimulated animals was significantly higher than in anode stimulated animals; Fisher’s LSD test: **p = 0.006 from anode.

Bars represent means of the densitometry ratios of Na\(^+\) K\(^+\) ATPase alpha 3 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

**Single cathodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice through an intact skull is not associated with any significant change of Na\(^+\) K\(^+\) ATPase α3 protein expression**

An important objective of our research was to verify the consistency of direct current stimulation effect on different molecular targets. Would it be possible that cathodal transcranial direct stimulation of the right sensorimotor cortex through an intact skull could cause an increase of Na\(^+\) K\(^+\) ATPase alpha 3 protein expression? Western blots examination of samples subjected to cathodal transcranial direct current stimulation showed that Na\(^+\) K\(^+\) ATPase alpha 3 protein expression was not significantly increased, p = 0.10, compared to that seen in anode treated tissues, (Figure 10, A). Overall, patterns of Na\(^+\) K\(^+\) ATPase alpha 3 protein expression in anode and cathode treated tissues still followed the trend that we have already seen with the effect of direct current stimulation of the exposed right sensorimotor cortex. Regardless of its non-significant statistical value, (Figure 10, B). This outcome did not oppose our hypothesis. It
simply confirmed the increasing effect that cathodal direct current stimulation has on $\text{Na}^+\text{K}^+\text{ATPase}$ alpha 3 expression.

![Figure 10: Single cathodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice through an intact skull has no effect on $\text{Na}^+\text{K}^+\text{ATPase}$ $\alpha$3 protein expression.](image)

A) Western blots showing the expression of $\text{Na}^+\text{K}^+\text{ATPase}$ alpha 3 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows no significant main effect between groups: $F(2, 10) = 2.89; P = 0.10$. Optical density in cathode stimulated animals was still higher than in sham and anode stimulated animals. Bars represent means of the densitometry ratios of $\text{Na}^+\text{K}^+\text{ATPase}$ alpha 3 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

**Daily 20 minutes cathodal transcranial direct current stimulation of the right sensorimotor cortex through an intact skull over a period of 5 days significantly increases $\text{Na}^+\text{K}^+\text{ATPase}$ $\alpha$3 protein expression**

We ultimately evaluated $\text{Na}^+\text{K}^+\text{ATPase}$ alpha 3 protein expression in the right sensorimotor cortex of CD-1 mice that underwent daily 20 minutes of transcranial direct current stimulation for 5 consecutive days. The observed results (Figure 11, A) were reminiscent of what we have already assessed in tissues submitted to direct current stimulation of the exposed sensorimotor cortex. More specifically, the expression of $\text{Na}^+\text{K}^+\text{ATPase}$ alpha 3 protein was significantly higher, $****p = 0.0001$, in tissues submitted to repeated cathodal transcranial direct current stimulation than in those subjected to anodal stimulation and in sham, $****p = 0.0001$, (Figure 11, B). We should note that unlike the non-significant reduction of $\text{Na}^+\text{K}^+\text{ATPase}$ produced by a-DCS of the exposed sensorimotor (Figure 9, B), the expression of $\text{Na}^+\text{K}^+\text{ATPase}$ alpha 3
protein in anode treated mice in this experiment was not lower than that seen in sham animals.

**Figure 11:** Na\(^+\)K\(^+\) ATPase α3 protein expression is significantly increased in tissues treated with daily 20 minutes cathodal transcranial direct current stimulation over a period of 5 days than in anode treated and sham samples. A) Western blots showing the expression of Na\(^+\)K\(^+\) ATPase alpha 3 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows significant main effect between groups: F (2, 10) = 51.01; P = 0.0001. Optical density in cathode treated mice was significantly higher than in sham and anode stimulated animals; Tukey’s test: ****p= 0.0001 from sham; ****p= 0.0001 from anode. Bars represent means of the densitometry ratios of alpha 3 Na\(^+\)K\(^+\)ATPase to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

**EFFECT OF DIRECT CURRENT STIMULATION ON Na\(^+\)K\(^+\)ATPase ALPHA 1 PHOSPHO-SERINE 943 PROTEIN EXPRESSION**

Single anodal direct current stimulation of the exposed right sensorimotor cortex of CD-1 mice significantly increases Na\(^+\)K\(^+\) ATPase α1 phospho-serine 943 protein expression

Changes affecting the Na\(^+\)K\(^+\) ATPase protein expression constitute an example of long term regulation of the pump’s function (McDonough & Farley, 1993). Other forms of the enzyme’s regulation include the control of the activity of the inserted pump in the plasma membrane (Blanco, Berberian, & Beauge, 1990; Ewart & Klip, 1995). These types of control are considered as short-term regulation of the pump activity (Wu, Chen, Chi, & Liu, 2007). When prompted, the control of the pump’s function occurs in a matter of minutes or hours. As a result, the Na\(^+\)K\(^+\) ATPase pump’s turnover rate, more specifically the ions transport, may increase or decrease. A well-known short-term regulation of the enzyme is the one that phosphorylates some of its serine/threonine residues through protein kinases: PKA and PKC. Following the attachment of a
phosphate group at one these residues, the pump’s activity may increase or decrease (Bibert, Roy, Schauer, Horisberger, & Geering, 2008; Cornelius & Logvinenko, 1996). Among these sites, serine 943 is the target of protein kinase A (PKA) (Beguin et al., 1994). The effect of PKA on the pump is the inhibition of its activity; a phenomenon seen in Na\(^+\)K\(^+\) ATPase pumps of various tissues (Nairn, Cheng, Ho, Greengard, & Aperia, 1997). Additionally, the phosphorylation of serine 943 residue is not necessarily accompanied with an internalization of the pump (Fisone et al., 1994) confirming that certain forms of control are exerted locally within the enzyme. Given this background about the pump activity, we wondered whether the immediate effect of direct current stimulation would influence short-term regulation of the pump. The change in the expression and the identification of pumps with phosphorylation sites at serine 943, after the end of direct current stimulation, could be an indication of the level of the activity in these enzymes. Serine 943 residue, the target of PKA, is conserved across Na\(^+\)K\(^+\) ATPase isoforms and species, (Blanco & Mercer, 1998; Poulsen, Nissen, Mouritsen, & Khandela, 2012). We focused on the ubiquitous alpha 1 isoform using an anti-Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 943 antibody to detect the presence of phosphorylated pumps in the exposed right sensorimotor cortices of CD-1 mice after the application of anodal or cathodal direct current stimulation respectively. The analysis of western blots (Figure 12, A) results showed a significant increase of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 943 protein expression in anode treated animals compared to cathode treated animals, **p = 0.0074, (Figure 12, B). It is important to mention that this result exhibited a reversed trend of what we have been seeing in the previous experimental protocols where cathodal direct current usually was found to cause an increase of both Na\(^+\)K\(^+\) ATPase alpha 1 and alpha 3.
Single anodal direct current stimulation of the exposed sensorimotor cortex of CD-1 mice significantly increases Na\(^+\)K\(^+\) ATPase α1 phospho-serine 943 protein expression. A) Western blots showing the expression of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 943 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows significant main effect between groups: F(2, 9) = 8.49; P = 0.008. Optical density in anode stimulated mice was significantly higher than in cathode stimulated animals; Tukey’s test: **p = 0.0074 from cathode. Bars represent mean of the densitometry ratios of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 943 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

Single anodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice through an intact skull significantly increases Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 943 protein expression. Single cathodal stimulation significantly decreases it.

We tested the immediate effect of anodal and cathodal transcranial direct current stimulation on the expression of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 943 in the right motor cortex of CD-1 mice as well. The result of the western blots (Figure 13, A) demonstrated that anodal current greatly influenced the expression of this subunit, ***p = 0.0002, in comparison to cathodal treated samples (Figure 13, B). Concurrently, our analysis revealed that cathode treated tissues have a significant decrease, *p = 0.04, in contrast to the expression of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 943 in sham samples, (Figure 13, B). This was a new trend. This outcome showed that single c-TDCS affects the pump differently when it shifts from one conformational state to another one. Here also, single anodal transcranial direct current stimulation reversed the pattern of protein expression that we have been witnessing while studying other target proteins. Interestingly, this result was consistent with single stimulation of the exposed cortex.
Figure 13: Single anodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice through an intact skull causes a significant rise of Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 protein expression. In contrast, single cathodal transcranial direct current stimulation significantly decreases Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 protein expression. Western blots showing the expression of Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows significant main effect between groups: F (2, 11) = 18.24; P = 0.0003. Optical density in anode stimulated mice was significantly higher than in cathode stimulated animals; Tukey’s test: ***p = 0.0002 from cathode. Optical density in cathode stimulated animals was significantly lower than in sham mice; Tukey’s test: *p = 0.04 from sham. Bars represent means of the densitometry ratios of Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

Repeated anodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice does not substantially change Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 protein expression

The analysis of the delayed effect of 20 minutes of daily anodal and cathodal transcranial direct current stimulation on Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 expression was important to us as we wanted to confirm the consistency of the results obtained with single direct and transcranial stimulation of sensorimotor cortices. Western blots of Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 did not reveal any significant increase of the phosphorylated pump in tissues that underwent repeated a-TDCS, F (2, 10) = 1.3, p = 0.31, (Figure 14, A). However, we saw a pattern of Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 protein expression in anodal and cathodal treated samples that was the replication of the trend observed with single direct and transcranial direct current stimulation (Figure 14, B). Despite their non-significance, these outcomes
indicated that repeated a-TDCS affects Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 943 expression in a way that was opposite to repeated a-TDCS effect on \(\alpha 1\) and \(\alpha 3\) Na\(^+\)K\(^+\) ATPase protein expressions.

**Figure 14:** Daily 20 minutes anodal or cathodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice over a period of 5 days does not elicit any significant change of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 943 protein expression. A) Western blots showing the expression of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 943 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows no significant main effect between groups: \(F (2, 10) = 1.3; p = 0.31\). Optical density in anode treated mice was still higher than in sham and cathode stimulated animals. Bars represent means of the densitometry ratios of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 943 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

**EFFECT OF DIRECT CURRENT STIMULATION ON REGULAR DOPAMINE AND C-AMP REGULATED PHOSPHOPROTEIN (DARPP-32) PROTEIN EXPRESSION**

Daily 20 minutes anodal or cathodal transcranial direct current stimulation of the right sensorimotor cortex through an intact skull over a period of 5 days has no impact on regular dopamine and c-AMP regulated phosphoprotein (DARPP-32) protein expression

Protein kinase A (PKA) phosphorylates directly the sodium potassium pump at serine 943 and concomitantly dopamine and cAMP-regulated phosphoprotein (DARPP-32), an inhibitor of protein phosphatase 1 (PP1), which is known to oppose the action of protein kinases in the pump (Bibb et al., 1999; Fryckstedt, Meister, & Aperia, 1992; Hemmings, Greengard, Tung, & Cohen, 1984). The result of this double action of PKA on these two targets is the inhibition of Na\(^+\)K\(^+\) ATPase activity (Therien & Blostein, 2000). It was therefore relevant to our research to examine
Darpp-32 protein as any change in its expression could be an indication of the state of the pump activity following the application of direct current stimulation. Before making any extrapolation regarding the effect of direct current stimulation on this protein, we decided to investigate first the levels of non-phosphorylated or regular dopamine and cAMP regulated phosphoprotein (DARPP-32) protein expression. We chose to do it using repeated daily transcranial direct stimulation protocol because we planned to compare regular Darpp-32 to Phospho-Darpp-32. We were not sure that one single direct current stimulation would be enough to reveal the expression of Phospho-Darpp-32 in sufficient amounts. Western blots results (Figure 15, A) showed that sham, anodal and cathodal treated tissues express approximately the sample levels of DARPP-32 protein, $F (2, 10) = 0.08; p = 0.9$, (Figure 15, B). These outcomes proved that daily repeated transcranial stimulation with either current polarity does not affect normal DARRP-32. We anticipated these results because regular DARPP-32 has no impact on the pump’s activity.

**Figure 15:** No significant difference in the expression of regular Darpp-32 protein is seen in sham, anode and cathode treated tissues with daily 20 minutes of transcranial direct current stimulation over a period of 5 days. 

A) Western blots showing the expression of Darpp-32 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows no significant main effect between the groups: $F (2, 10) = 0.08; P = 0.9$. Optical density is relatively the same in sham, anode and cathode stimulated animals. Bars represent means of the densitometry ratios of Darpp-32 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.
EFFECT OF DIRECT CURRENT STIMULATION ON PHOSPHO-DARPP-32 DOPAMINE AND C-AMP REGULATED PHOSPHOPROTEIN (DARPP-32) PROTEIN EXPRESSION

Daily 20 minutes anodal transcranial direct current stimulation of the right sensorimotor cortex through an intact skull over a period of 5 days significantly increases phospho-dopamine and c-AMP regulated phosphoprotein (Phospho-DARPP-32) protein expression.

We probed phospho-Darpp-32 protein expression in the right sensorimotor cortical tissues submitted to daily 20 minutes of anodal or cathodal transcranial direct current stimulation over a period of 5 days. This examination was important because any change of phospho-Darpp-32 expression compared to that of regular Darpp-32 would be an indication of the impact that delayed transcranial stimulation could have on the short-term regulation of the pump activity.

The analysis of phosphorylated Darpp-32 western blots (Figure 16, A), unlike those of regular Darpp-32, revealed a significant increase of its expression in anode treated tissues contrasted to its expression in cathodal stimulated samples, *p= 0.04, (Figure 16, B). These findings were of two folds. The first one was that repeated transcranial direct stimulation effect is not the same in regular darpp-32 and in phospho-Darpp-32. The second one was that this experimental protocol replicated the trend, although it was not significant, seen with the expression of Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 protein in anodal treated animals (Figure 14, B). At the onset, it may seem irrelevant to compare phospho-Darpp-32 to Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 protein. However, both are phosphorylated by PKA when the inhibition of the Na⁺ K⁺ ATPase activity is desired; hence the importance to check their phosphorylated state. Our results showed that there is a tendency of a-DC stimulation to phosphorylate both PKA targets.
Figure 16: Daily 20 minutes of anodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice over a period of 5 days through an intact skull generates a significant rise of phospho-dopamine and c-AMP regulated phosphoprotein (Phospho-DARPP-32). A) Western blots showing the expression of phospho-Darpp-32 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph reveals a significant main effect between the groups: F (2, 10) = 4.24; P = 0.04. Optical density in anode stimulated animals was significantly higher than in cathode stimulated animals; Tukey’s test: *p= 0.04 from cathode. Bars represent means of the densitometry ratios of phospho-Darpp-32 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

**Single anodal or cathodal direct current stimulation of the exposed right sensorimotor cortex of CD-1 mice is not associated with any significant change of phospho-Darpp-32 protein expression**

To check the consistency of the results obtained with repeated a-TDCS, it was necessary to evaluate levels of phospho-Darpp-32 protein expression in single DC stimulation experimental paradigms. Western blots of sham, anode and cathode treated tissues in the exposed right sensorimotor cortex (Figure 17, A) did not reveal any significant change regarding the expression of phospho-Darpp-32, F (2, 10) = 0.81; p = 0.47, (Figure 17, B). Nevertheless, we found that both anodal and cathodal treated tissues have a level of phospho-Darpp-32 protein expression that was moderately lower than in sham tissues. These results showed that direct current stimulation has no apparent effect on the regulation of the sodium potassium pump activity.
Figure 17: No significant difference in the expression of Phospho-Darpp-32 protein is seen in sham, anode and cathode treated tissues with single 20 minutes of direct current stimulation of the exposed right sensorimotor cortex. A) Western blots showing the expression of phospho-Darpp-32 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows no significant main effect between the groups: F (2, 10) = 0.81; p = 0.47. Optical density is relatively the same in sham, anode and cathode stimulated animals. Bars represent means of the densitometry ratios of phospho-Darpp-32 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

Single anodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice through an intact skull significantly decreases phospho-Darpp-32 protein expression

Non-significant findings of phospho-Darpp-32 protein expression observed with DC stimulation of the exposed right sensorimotor cortex prompted us to study the effect of TDCS on the phosphorylated phosphoprotein inhibitor (Darpp-32) expression. Using uncorrected Fisher’s least significant difference (LSD) statistical test, we found out that phospho-Darpp-32 protein expression in anode treated tissues was significantly smaller, (Figure 18, A) than that witnessed in both sham, p = 0.03, and cathode stimulated samples, 0.04, (Figure 18, B). These results were at odds with the ones obtained with repeated TDCS where the expression of phospho-Darpp-32 protein was significantly higher in anode treated tissues compared to the expression in cathode stimulated samples (Figure 16, B). Simultaneously, when we compared them to phospho-Darpp-32 protein expression in the exposed motor cortex, we found that anodal current stimulation has also a non-significant reducing effect on phospho-Darpp-32 expression (Figure 17, B). These
two results displayed consistency of the effect of a-DCS on phospho-Darpp-32 protein expression in open and closed right sensorimotor cortices.

Figure 18: Single anodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice through an intact skull significantly decreases phospho-Darpp-32 protein expression in comparison to that seen in cathode treated and sham tissues. A) Western blots showing the expression of phospho-Darpp-32 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows significant main effect between the groups: F (2, 11) = 3.73; p = 0.05. Optical density is significantly reduced in anode treated animals than in sham and in cathode stimulated mice; Fisher’s LSD test: *p = 0.03 from sham, *p = 0.04 from cathode. Bars represent means of the densitometry ratios of phospho-Darpp-32 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

EFFECT OF DIRECT CURRENT STIMULATION ON Na\(^+\)K\(^+\)ATPase ALPHA 1 PHOSPHO-SERINE 23

Single anodal direct current stimulation of the exposed right sensorimotor cortex of CD-1 mice increases Na\(^+\)K\(^+\)ATPase alpha 1 phospho-serine 23 protein expression

Data demonstrates that PKA direct phosphorylation of alpha 1 Na\(^+\)K\(^+\)ATPase at serine 943 is not ultimate. By attaching a phosphate at this location of the catalytic subunit, PKA appears to facilitate the phosphorylation of PKC at another residue: serine 23 (Cheng, Höög, Nairn, Greengard, & Aperia, 1997). Dopaminergic circuits trigger PKC phosphorylation at this site leading to the endocytosis of the pump (Chibalin et al., 1999). Taken together, these results show that dopamine-activated PKC and PKA pathways, and Darpp-32 phosphorylation result in the inhibition of the pump activity. Nonetheless, these activating proteins are not always triggered through dopaminergic pathways. For example, Darpp-32 action can be initiated by other means.
than the neural circuits activated by dopamine (Snyder et al., 1992). The whole process of attaching a phosphate group at serine 23 is an example of short-term regulation of the pump’s function. Because of these interactions, we studied the levels of Na⁺K⁺ ATPase alpha 1 phospho-serine 23 to determine whether direct current stimulation of the exposed right motor cortex of CD-1 mice would also have an influence on the short-term regulation of the pump’s activity.

Western blots results (Figure 19, A) revealed that anodal direct current direct stimulation of the exposed right sensorimotor cortex significantly increased Na⁺K⁺ ATPase alpha 1 phospho-serine 23 protein compared to the expression in sham tissues, ***p = 0.0005, and in cathode treated samples, ***p= 0.0006, (Figure 19, B).

Figure 19: Single anodal direct stimulation of the exposed right sensorimotor cortex is accompanied with a significant increase of Na⁺K⁺ ATPase α1 phospho-serine 23 protein expression in contrast to that seen in cathode treated and sham tissues. A) Western blots showing the expression of Na⁺K⁺ ATPase alpha 1 phospho-serine 23 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows significant main effect between the groups: F (2, 10) = 18.16; P = 0.0005. Optical density in anode stimulated animals was significantly higher than in sham and cathode stimulated animals; Fisher’s LSD test: ***p = 0.0004 from sham and ***p = 0.0006 from cathode. Bars represent means of the densitometry ratios of Na⁺K⁺ ATPase alpha 1 phospho-serine 23 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

Single anodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice through an intact skull significantly increases Na⁺K⁺ ATPase α1 phospho-serine 23 protein expression. Cathodal stimulation significantly decreases it.

Does single anodal transcranial direct current stimulation of the right sensorimotor cortex through an intact skull generate also similar effect on Na⁺K⁺ ATPase alpha 1 phospho-serine 23
protein expression? To answer this question, we subjected CD-1 mice right sensorimotor cortical regions to either anodal or cathodal transcranial direct current. Western blots analysis (Figure 20, A) showed that Na⁺K⁺ATPase alpha 1 phospho-serine 23 protein expression in anode treated tissues was highly expressed compared to the expression in cathode stimulated tissues, p = 0.0002, (Figure 20, B). Concurrently, single cathodal transcranial direct stimulation significantly reduced the expression of Na⁺K⁺ATPase alpha 1 phospho-serine 23 protein compared to the expression in sham, p = 0.009, (Figure 20, B). It is evident that stimulations of the right sensorimotor cortex with opposite polar currents stimulations induced antagonist effects on Na⁺K⁺ATPase alpha 1 phospho-serine 23 protein. Reexamining the action of PKA on Na⁺K⁺ATPase alpha 1 phospho-serine 943 (Figure 13, B) and its facilitating role of PKC action on Na⁺K⁺ATPase alpha 1 serine 23 residue, we found a concordance between the levels of expression of the pump at these two different residues under the immediate effect of anodal transcranial direct current stimulation. Our findings do not contradict the notion that both PKA and PKC are “mobilized” during the regulation of the membrane’s pump activity.

Figure 20: Single anodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice through an intact skull significantly increases Na⁺K⁺ATPase α1 phospho-serine 23 protein expression. Cathodal stimulation significantly decreases it. A) Western blots showing the expression of Na⁺K⁺ATPase alpha 1 phospho-serine 23 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows significant main effect between the groups: F (2, 11) = 15.86; P = 0.0006. Optical density in anode stimulated animals was significantly higher than in cathode stimulated mice; Fisher’s LSD test: ***p = 0.0002 from cathode. Optical density in cathode treated animals was significantly lower than in sham mice; Fisher’s LSD test: **p = 0.009 from...
sham. Bars represent means of the densitometry ratios of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 23 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

Daily 20 minutes of anodal transcranial direct current stimulation of the right sensorimotor cortex through an intact skull over a period of 5 days significantly increases Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 23 protein expression. Cathodal stimulation significantly decreases it.

A part of our research plan was to check if repeated transcranial direct current stimulation would have also an effect on the short-term regulation of the pump activity that could persist 24 hours beyond the last stimulation of the right sensorimotor cortices. Western blots analysis (Figure 21, A) showed that delayed effect of a-TDCS influences the expression of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 23 protein in the same way a-DCS did on the expression of the same protein in the open and closed sensorimotor cortices. More specifically, delayed effect of a-TDCS was associated with a significant increase of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 23 protein expression, \(p = 0.0005\), when contrasted to the expression of the same pump in cathode treated samples (Figure 21, B). There was also a significant difference in the expression of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 23 protein between anode treated and sham tissues, \(p = 0.01\), (Figure 21, B). All in all, Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 23 protein expression was a replication of the trend viewed with the immediate effect of both single direct current stimulation, (Figure 19 & 20, B). We should note though that repeated c-TDCS, unlike single c-TDCS paradigm (Figure 20, B), did not cause a significant decrease of the Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 23 protein expression in comparison to that seen in sham samples.
Figure 21: Daily 20 minutes anodal transcranial direct current stimulation of the right sensorimotor cortex through an intact skull over a period of 5 days significantly increases Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 23 protein expression in comparison to the expression seen in cathode treated and sham tissues. A) Western blots showing the expression of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 23 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows significant main effect between the groups: F (2, 10) = 17.08; P = 0.006. Optical density in anode treated mice was significantly higher than in sham and cathode treated animals; Tuckey's test: *p= 0.01, ***p= 0.0005 respectively. Bars represent means of the densitometry ratios of Na\(^+\)K\(^+\) ATPase alpha 1 phospho-serine 23 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

**EFFECT OF DIRECT CURRENT STIMULATION ON Na\(^+\)K\(^+\) ATPASE BETA 2 PROTEIN EXPRESSION**

Single anodal direct current stimulation of the exposed right sensorimotor cortex of CD-1 mice significantly increases Na\(^+\)K\(^+\) ATPase beta 2 protein expression. Single cathodal stimulation significantly decreases it.

Parallel to the study of Na\(^+\)K\(^+\) ATPase alpha 1 subunit, we addressed the effect of direct current stimulation on beta Na\(^+\)K\(^+\) ATPase subunit. The contribution of this subunit to the overall function of the pump is important to be disregarded. Studies have proven that any alteration in this glycoprotein’s structure would impair the entire pump activity (Jaunin et al., 1993). For instance, beta subunit’s participation in the delivery of alpha 1 subunit to the plasma membrane is imperative (Käthi Geering, 2008; Tokhtaeva, Sachs, & Vagin, 2009). Beta subunit acts also like a chaperone protein as it first assembles with alpha subunit in the endoplasmic reticulum prior to their insertion in the plasma membrane (Higashi, Kawamura, & Nogushi, 1990). Study of beta Na\(^+\)K\(^+\) ATPase subunit was necessary for us to fully assess the effect of direct current on the
pump’s function. We wanted to see whether long-term regulation of the pump’s activity concerns also other subunits than alpha 1. We chose to examine Na\(^+\) K\(^+\) ATPase β2 protein expression as it is one of the two isoforms expressed in the brain tissue (Koksoy, 2002). Western blots results (Figure 22, A) revealed that anodal direct current stimulation induced a significant increase of Na\(^+\) K\(^+\) ATPase β2 protein expression, p= 0.0005, (Figure 22, B) compared to cathodal direct. Additionally, the expression of Na\(^+\) K\(^+\) ATPase β2 protein after the application of c-DCS was significantly lower compared to that seen in sham tissues, p = 0.03, (Figure 22, B). We already found that direct current stimulation of the right sensorimotor cortex influences long-term regulation of the pump activity through changes of the alpha 1 subunit expression. It was not surprising to see that DC stimulation also impacts beta 2 subunit as the function of the pump requires both subunits. Furthermore, these two results demonstrated that the immediate effect of direct current stimulation did not affect only the catalytic subunit.

**Figure 22: Single anodal direct current stimulation of the exposed right sensorimotor cortex of CD-1 mice significantly increases Na\(^+\)K\(^+\) ATPase β2 protein expression. Single cathodal stimulation significantly decreases it.**

A) Western blots showing the expression of Na\(^+\) K\(^+\) ATPase β2 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows optical density in anode stimulated mice was significantly higher than in cathode stimulated animals; Tukey’s test: ***p= 0.0005 from cathode. Optical density in cathode stimulated animals was significantly lower than in sham mice. Tukey’s test: *p= 0.04 from sham. Bars represent means of the densitometry ratios of Na\(^+\) K\(^+\) ATPase β2 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.
Single anodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice through an intact skull increases Na\(^+\) K\(^+\) ATPase β2 protein expression. Single cathodal stimulation significantly decreases it.

We tested the expression of Na\(^+\) K\(^+\) ATPase β2 protein under the immediate effect of transcranial direct current stimulation experimental protocol as well. Optical density analysis (Figure 23, A) of tissues obtained from the right sensorimotor cortices revealed the same pattern seen with single direct current stimulation of the exposed right sensorimotor cortex (Figure 22, A). We confirmed that anodal compared to cathodal transcranial direct current stimulation has a significant increasing effect on Na\(^+\) K\(^+\) ATPase β2 protein expression, p = 0.001, (Figure 23, B). Similarly, we found out that single c-TDCS replicated the decreasing effect of DC stimulation on Na\(^+\) K\(^+\) ATPase β2 protein expression in comparison to that seen in sham tissues, p = 0.02, (Figure 23, B).

**Figure 23**: Single anodal transcranial direct current stimulation of the right sensorimotor cortex over an intact skull causes a significant increase of Na\(^+\) K\(^+\) ATPase β2 protein expression. Single cathodal transcranial direct current stimulation significantly decreases it contrary to the expression seen in sham tissues. A) Western blots showing the expression of Na\(^+\) K\(^+\) ATPase β2 protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows significant main effect between the groups: F (2, 11) = 9.415; P= 0.004. Optical density in anode stimulated mice was significantly higher than in cathode stimulated animals; Fisher’s LSD test: **p = 0.001 from cathode. Optical density in cathode stimulated animals Fisher’s LSD test: *p = 0.02 from sham. Bars represent means of the densitometry ratios of Na\(^+\) K\(^+\) ATPase β2 to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.
Daily 20 minutes anodal transcranial direct current stimulation of the right sensorimotor cortex through an intact skull over a period of 5 days is not associated with any significant change of Na\(^+\) K\(^+\) ATPase β2

The effect of daily 20 minutes anodal or cathodal transcranial direct stimulation of the right sensorimotor cortex over a period of 5 days was not accompanied with any significant difference of Na\(^+\) K\(^+\) ATPase β2 protein expression. These outcomes were contrary to those consistently seen with the effect of single 20 minutes direct current and transcranial direct current stimulation of exposed or closed sensorimotor cortices respectively. Tissues’ optical density evaluation revealed a change of Na\(^+\) K\(^+\) ATPase β2 protein expression pattern (Figure 24, A). Noticeably, repeated c-TDCS still showed a rise, not significant yet, of beta 2 subunit expression. It was only in this paradigm that a trend of this kind was seen. Single c-DCS of either the exposed or the covered right sensorimotor cortex produced a reversed pattern of beta 2 subunit expression, (Figure 22 and 23, B).

**Figure 24: Daily 20 minutes anodal or cathodal transcranial direct current stimulation of the right sensorimotor cortex over a period of 5 days through an intact skull does not induce any significant change of Na\(^+\) K\(^+\) ATPase β2 protein expression.** A) Western blots showing the expression of beta 2 Na\(^+\) K\(^+\) ATPase protein in samples collected from sham, anodal and stimulated mice. Actin was used as an internal control. B) One way ANOVA graph shows no significant main effect between the groups: F (2, 10) = 1.44; P=0.28. Bars represent means of the densitometry ratios of beta 2 Na\(^+\) K\(^+\) ATPase to actin normalized to the mean of the densitometry ratio of the sham samples. 3 sham and 5 experimental mice for each category were used.

**EFFECT OF DIRECT CURRENT STIMULATION ON LOCOMOTOR ACTIVITY AND ANXIETY OF CD-1 MICE**

Single cathodal transcranial direct current stimulation is not associated with any notable change of CD-1 mice locomotor activity or anxiety
Transcranial direct current stimulation of the right motor cortex has already shown that molecular changes occur. One of our research objectives was to examine if behavioral modifications may also follow upon the application of direct current stimulation. We tested the behavior of CD-1 mice in the open field. Tracking mice traveled distance and time spent in the central zone before and after cathodal transcranial direct stimulation will give us an idea about the impact of this treatment on the locomotor activity and the level of anxiety respectively. The evaluation of our results does not reveal any significant change in the traveled distance by mice before and after cathodal direct current stimulation. Also, no contrast between the traveled distances of sham mice with that of either pre-cathode stimulated or post-cathode stimulated animals exists (Figure 25, A, B, & C). A slight decrease is still seen with pre-stimulated mice compared to sham and post-cathode stimulated animals (Figure 25, D). Similarly, our results do not show any significant change in the time spent by animals in central zone before or after cathodal transcranial direct stimulation (Figure 25, E). Nevertheless, pre-stimulated mice spent more time than both sham and post-cathode stimulated mice.

**Figure 25**: Single cathodal transcranial direct current stimulation has no effect on CD-1 mice locomotor activity or anxiety. A, B, C: track plot tracing of mice in the open field arena showing traveled distance and crossing of the central zone from which time spent in can be
inferred path of mice: A) In sham B) In CD-1 mice before stimulation. C) In CD-1 mice after stimulation. D) One way ANOVA graph of total distance traveled by mice shows no main effect between the groups; Tuckey’s test: F (2, 7) = 1.27, P = 0.33. E) One way ANOVA graph of time spent by animals in central zone shows no main effect between the groups; Tuckey’s test: F (2, 7) = 2.28, P = 0.17. 2 sham and 4 experimental mice were used. Data represent mean ± SEM.

EFFECTS OF DIRECT CURRENT STIMULATION ON THE SCIATIC NERVE EXCITABILITY

Direct current stimulation of the sciatic nerve confirms changes of the axonal excitability and hints to the involvement of the Na⁺K⁺ATPase in these modifications

To shed the light on the immediate and long-lasting effects elicited by DC stimulation, a study was conducted in our lab (Ahmed-unpublished work) to see the immediate and long-lasting effects of DC stimulation on the sciatic nerve. The analysis of (Ahmed-unpublished data) confirms the role that either anode or cathode subthreshold current has on the changes of axonal excitability. The data also hints to a possible involvement of the sodium potassium ATPase as a factor in the persistence of long-lasting effects. In the absence of ouabain, (Figure 26, A) when test stimulation was initiated from the lateral chamber, anodal-sDC enhanced the number of nCAP during and after 15 minutes following the offset of the stimulation. Conversely, cathodal-sDC provoked a depression of nerve excitability, reflected by a decrease of nCAP, lasting over 25 minutes. Adding ouabain to the lateral chamber (Figure 26, B) caused a decrease of nCAP during sDC with both anodal and cathodal current. Nevertheless, anodal sDC significantly increased nerve excitability following current offset that continued for 25 minutes. After the end of sDC, cathodal current displayed a short inhibition followed by a rise of nCAP toward baseline levels. When ouabain was added to the central chamber (Figure 26, C) anodal sDC elicited prolonged nerve excitability less intense than the one seen in only Ringer solution or ouabain in lateral chamber. Cathodal sDC induced short time inhibition that lasted 5 minutes before nCAP returned to baseline levels.

In the absence of ouabain (Figure 26, D), when test stimulation was initiated from the central
chamber, during sDC, anodal current decreased whereas cathodal increased nCAP thus reversing the direction of nerve excitability. After the offset, anodal sDC increased nCAP over a period of 25 minutes whereas cathodal decreased them over the same period. Addition of ouabain to the lateral chamber (Figure 26, E), anodal sDC generated moderate and shorter increase of nerve excitability; cathodal produced increased and sustained nCAP. When ouabain was added to the central chamber (Figure 26, F), during sDC, anodal and cathodal respectively decreased and increased nCAP. After the offset of sDC, ouabain reduced anodal sDC nerve excitability augmentation, while blocking cathodal sDC inhibition, which were seen with only Ringer solution.

Figure 26. Long-lasting effects of subthreshold direct current stimulation in the sciatic nerve. In these experiments, sciatic nerves were exposed to 10 µA DC for 2 minutes. Two
chambers of Ringer solution were created: one covering DC electrode and the other covering the lateral test-stimulation area. It should be noted here that Ringer solution increased the nerve threshold to DC stimulation. On the top of the figure are insets showing the experimental arrangement. **A)** When test-stimulation was lateral to DC electrode, anodal DCS caused enhancement of nerve excitability that lasted for 15 minutes, and cathodal DCS depressed nerve excitability for at least 25 minutes following current offset. **B)** When ouabain was added to the lateral chamber, during DCS, both anodal and cathodal DCS induced inhibition. However, following DCS, anodal current produced long-lasting enhancement up to 25 minutes, *p < 0.001 from baseline, and cathodal current provoked short long-lasting inhibition, *p < 0.001 from baseline. **C)** When ouabain was added to the central chamber, anodal DCS caused long-term enhancement of the nerve excitability, *p < 0.001 from baseline, which was less pronounced than Ringer solution and when ouabain was added to the lateral chamber. Cathodal DCS caused short period (5 min) of inhibition before nerve excitability reverted to baseline level. **D)** When test-stimulation was in the central chamber, the direction of nerve excitability was reversed during DCS. After current offset, anodal current increased the nerve excitability whereas cathodal decreased it. **E)** When ouabain was added to the lateral chamber, both anodal and cathodal current provoked an inhibition during DC application. After the end of DCS, anodal current caused smaller increase, while cathodal current induced stronger and longer enhancement of the nerve excitability, *p < 0.001 from baseline. **F)** When ouabain was added to the central chamber, it blocked cathodal induced inhibition, and it reduced anodal enhancement, *p < 0.002 from baseline. Data represent mean ± SEM. 5 mice for each category were used.

**EFFECTS OF SINGLE TRANSCRANIAL DIRECT CURRENT STIMULATION OF THE RIGHT SENSORIMOTOR CORTEX ON SENSORY EVOKED POTENTIALS**

Single cathodal transcranial direct current stimulation increases the amplitude of sensory evoked potentials. Anodal stimulation decreases it

Studies have shown that transcranial direct current stimulation affects nerve excitability. This effect has been assessed through electrophysiological data (Antal, Kincses, Nitsche, Bartfai, & Paulus, 2004), motor evoked potentials (Rivera-Urbina et al., 2015), visual evoked potentials (Antal et al., 2004; Ding et al., 2016). Parallel to these investigations, the study of sensory evoked potentials (SEP) has been also studied in concurrence with transcranial direct current stimulation (Mori et al., 2012). To analyze other aspects of TDCS on the right sensorimotor cortex, we also checked the amplitude of sensory evoked potentials (SEP) triggered by the stimulation of the sciatic nerve before and after DC stimulation of 0.2 mA. We found out that after cathodal-DCS of the right sensorimotor cortex, the trend of SEPs in response to sciatic
nerve stimulation was the same. Whether the nerve was stimulated with 1 pulse, 17 pulses (50 Hz) or 17 pulses (333 Hz), SEP amplitudes were consistently greater with c-DCS than with a-DCS and control. Close analysis of the data shows that stimulation with 17 pulses (50 Hz) appears to produce the greatest amplitude of SEPs after c-DCS of the right cortical area (Figure 27, C). With this stimulatory type, the difference of SEP amplitude between anodal and cathodal current was also the neatest. In opposition, SEP amplitudes provoked by 17 pulses (333 Hz) were the smallest (Figure 27, E). Amplitudes of SEPs following single sciatic nerve stimulus pulse were intermediate between those produced by 1 pulse and 17 pulses (333 Hz), (Figure 27, D). These findings show the regularity of the effect of c-TDCS on the activity of sensory cortical neurons. Furthermore, the existence of an opposite impact of a-TDCS on SEPs has been also a verifiable fact with all types of sciatic nerve stimulations.

Figure 27: Single cathodal transcranial direct current stimulation enhances sensory evoked potentials (SEP). Effect of 20 min of tDCS on sensory evoked potentials (SEP) in awake mice. A) Implant of tDCS, recording, and stimulating electrodes. B) Representative traces of SEP 60 min following tDCS. D) Summary plot showing SEP in response to single sciatic nerve stimulus
pulse. SEP amplitudes after the end of c-TDCS are moderately higher than those produced by a-TDCS and sham. C) Summary plot showing SEP in response to 17 pulses (50 Hz) of stimulus to the sciatic nerve. SEP amplitudes associated with c-TDCS are neatly higher than those elicited by a-TDCS and sham. E) Summary plot showing SEP in response to 17 pulses (333 Hz) to the sciatic nerve. SEP amplitudes following c-TDCS are also higher than control and sham. Three animals were used in each group.

**Repeated Anodal Transcranial Direct Stimulation and Mice Behavior**

While proceeding with repeated anodal transcranial direct current stimulation of the right sensorimotor cortex experiment, we have seen in three mice a seizure-like behavior starting after the second day. For reminder, this protocol included 5 sessions of 20 minutes of either anodal or cathodal transcranial direct current stimulation of 0.2 mA intensity. The seizure-like behavior was seen in both the left forelimb and hindlimb. It was still more evident and intense in the left hindlimb. Two of the animals displayed this behavior during two separate sessions while the stimulation was going on. This behavior in all the mice was visible after a minimum of 10 minutes. Also, one mouse showed this conduct starting the second day after 14 minutes of stimulation and repeatedly in the following sessions. For example, in the fourth session, the same animal exhibited this behavior during the stimulation, at the offset of the current, and 30 minutes after the end of the stimulation. We didn’t see any immediate or delayed pathological effect following the end of the stimulation. Likewise, we have not seen this conduct in mice that underwent a single transcranial direct stimulation or those with the exposed sensorimotor cortex.
DISCUSSION:

Most studies aimed at elucidating the mechanisms of DC application have been focused on the changes affecting axonal excitability. During the initial experiments of DC stimulation, researchers did not look at the modifications that could affect proteins and second messengers involved in the initiation and the control of the so-called membrane potentials. Yet, some of them have already suggested that sodium (Nitsche et al., 2003) and calcium (Islam, Aftabuddin, Moriwaki, Hattori, & Hori, 1995) channels should be studied subsequent to DC application to the organs of the nervous system. The reason being these ions, particularly the sodium ones, play a role in the generation of membrane potentials. For instance, despite the proof that TDCS produces late LTP-like plasticity in the human brain (Monte-Silva et al., 2013), which is sustained by AMPA and NMDA receptors, the expression of these two proteins was never verified. Few studies in the field have been devoted to the examination of the relationship between DC stimulation and certain proteins like growth factors of cartilage and bone (Aaron, Boyan, Ciombor, Schwartz, & Simon, 2004), hemichannel pannexin 1 (Jiang et al., 2012), and BDNF (Wenjin et al., 2011). Our study was the first to address the effects of DC stimulation on the molecular “behavior” of the Na\(^+\)K\(^+\) ATPase pump, a protein involved in the establishment of the cell membrane potential. In conjunction with this primary investigation, our research focused on other proteins, which contribute to the overall function of this pump.

COMPARISON OF ALPHA 3 Na\(^+\)K\(^+\)ATPase PROTEIN EXPRESSION IN SHAM AND CONTROL CD-1 MICE

We started our research looking at the difference that may exist in the expression of Na\(^+\)K\(^+\) ATPase α3 in the right sensorimotor cortex of sham and control (intact) mice. This initial testing was necessary to draw accurate conclusions at the term of our research. For instance, the use of placebo control versus sham control in clinical studies has been questioned before making
acceptable conclusions. Each one of these controls has its advantages and limitations. In scientific investigations where surgical procedures are needed, the adoption of control sham as a comparator is not only more rigorous, but it is most likely to reduce inaccuracies (Sutherland, 2007). Preparation of sham animals in our lab was done with great care because procedures may injure cortical structures and impact the expression of the pump. A study relevant to our investigation showed that brain injuries were associated with decreased levels of Na\(^+\) K\(^+\) ATPase enzymes (Seddik, Habib, & Shamy, 1991). Our results showed Na\(^+\) K\(^+\) ATPase α3 subunit equally expressed in control and sham mice tissues. They also prove that surgical interventions exposing sensorimotor cortices with their preserved dura mater do not influence the expression of Na\(^+\) K\(^+\) ATPase α3 protein. Furthermore, they were a clearance for us to use sham animals as reliable comparators in our experimental studies.

**EFFECT OF DIRECT CURRENT STIMULATION ON Na\(^+\) K\(^+\) ATPase ALPHA 1 PROTEIN EXPRESSION**

Single cathodal direct current stimulation (c-DCS) of the exposed right sensorimotor cortex, and single or repeated cathodal transcranial direct current stimulation of the same cortical region of CD-1 mice increases Na\(^+\) K\(^+\) ATPase α1 protein expression

Our choice to investigate the expression of Na\(^+\) K\(^+\) ATPase α1 subunit in this particular part of the brain was justified in large part by the fact that DCS mainly affects cortical neurons’ excitability (Cambiaghi et al., 2010). Our studies showed that cathodal DCS of the right sensorimotor cortex modifies Na\(^+\) K\(^+\) ATPase α1 subunit expression. This trend has been verified with single cathodal direct current stimulation of the right exposed sensorimotor cortex. It has been also confirmed with single or repeated cathodal transcranial direct current stimulation of the same cortical region. In all experimental paradigms, evident differences in the expression of this subunit were observed between cathode, anode treated, and sham tissues. We found that Na\(^+\) K\(^+\) ATPase α1 subunit expression was highly increased in cathode stimulated tissues compared to
anode treated and sham samples. Compelling data concluded that c-TCDS decreases neurons’ excitability (Nitsche et al., 2004). Numerous publications exhibited the role that Na⁺K⁺ATPase pump activity plays in the modification of the membrane potential. For instance, activation of the enzyme reduced the burst frequency (Picton, 2017). In contrast, temporary blockage of Na⁺K⁺ATPase activity caused hyper-excitability of neurons (Vaillend et al., 2002). Similarly, mutation (I810N), which impedes the activity of normally synthesized Na⁺K⁺ATPase alpha 3 subunit was also associated with hyper-excitability of neurons in the hippocampus (Clapcote et al., 2009).

Taken together, these findings proved a strong link between disruptions of the pump activity and the state of neurons’ excitability. Yet, the increase of Na⁺K⁺ATPase α1 protein expression seen in our experiments could not be solely reduced to an issue of the pump activity. We did not simultaneously assess the catalytic activity and the expression of Na⁺K⁺ATPase alpha 1. It was not therefore possible to link the increase or decrease of the pump expression to a respective rise or decline of the enzyme activity. We could only speculate about the increase of Na⁺K⁺ATPase alpha 1 expression as we were the first in the field to examine the relationship between DCS and the pump’s expression. We hypothesized that the rise of this protein is due to the hyperpolarizing effect of the cathodal current application on the neurons’ excitability. It is plausible that induced and decreased membrane voltage might be perceived by these cells as a signal that their pumps are not efficiently working. It is also possible to impute the disruption of the enzyme activity to an insufficient number of functional pumps. Previous studies backed this suggestion. They established a direct link between increased activity and synthesis of Na⁺K⁺ATPase pumps during the first 4 weeks following the birth of rats (Kocak, Oner, & Oztas, 2002). From another stance, the rise of Na⁺K⁺ATPase protein expression could be viewed as the manifestation of a homeostatic response to the disrupting hyperpolarization caused by the c-DCS in each
experimental protocol. This result disclosed a new dimensional effect of DC stimulation, which is to provoke changes at the molecular levels.

To conclude, cathodal direct current stimulation’s effect on the increase of Na$^+$ K$^+$ ATPase expression has been a consistent fact in our three experimental paradigms. It demonstrated that besides inducing changes in membrane potentials, DC stimulation also affects proteins’ expression. For the first time, our findings proved that DCS of brain tissue is not an action exclusively circumscribed to changes in membrane voltage. Proteins that participate in the elicitation of the membrane potential like Na$^+$ K$^+$ ATPase are also susceptible to its effect.

**Single anodal transcranial direct stimulation significantly decreases Na$^+$ K$^+$ ATPase alpha 1 protein expression**

Anodal direct current is known to increase neurons’ excitability (Kidgell et al., 2013). This is a reverse of the effect of cathodal direct current. We therefore anticipated to see inverse effects of anodal direct current on the Na$^+$ K$^+$ ATPase alpha 1 protein expression. The analysis of our results supported our expectation. They revealed that anodal stimulations, regardless of the experimental setting in which they have been used, caused a decrease of Na$^+$ K$^+$ ATPase alpha 1 protein expression compared to the expression seen in cathode treated and sham tissues. Here also, we theorized that increased excitability of neurons would be countered by a decrease of the number of Na$^+$ K$^+$ ATPase pumps. If a change of the axonal excitability toward a positive value is not physiologically sought, it is conceivable that the cell will oppose it by affecting the structures that control the membrane voltage. We assumed that neurons must counteract the increasing excitability as there is a risk of depolarization, causing an action potential induction. Unwanted spikes may lead to downstream excessive calcium entry followed by exaggerated neurotransmitter release that could be toxic to cell survival (de Lores Arnaiz & Ordieres, 2014). Because of all these possibilities, a decrease of Na$^+$ K$^+$ ATPase could be considered as a mere
homeostatic response to the disruptions causing an elevation of the cell membrane potential. However, Na\textsuperscript{+}K\textsuperscript{+} ATPase alpha 1 expression in anode treated tissues was significantly decreased compared to cathode stimulated samples. It was not when contrasted to sham samples. Single anodal transcranial direct current stimulation was the exception in that regard. A close look at Na\textsuperscript{+}K\textsuperscript{+} ATPase alpha 1 protein expression following this stimulation showed not only a significant decrease compared to the expression in cathode treated tissues, but also a significant reduction in contrast to the expression in sham samples. The existence of the difference between single direct anodal direct stimulation (a-DCS) of the exposed sensorimotor cortex and single transcranial direct current stimulation (a-TDCS) of the same region might be attributed to the way currents flow over an exposed and a protected cortex (Brunoni et al., 2013). As for the discrepancy between single and repeated anodal transcranial direct current stimulation, one possible explanation is the fact that a brain that has been stimulated overnight may not react like the one that received one unique stimulation (Filmer, Dux, & Mattingley, 2014).

In any event, the absence of a constant significant difference of Na\textsuperscript{+}K\textsuperscript{+} ATPase alpha 1 protein expression between anode treated and sham tissues across different experimental patterns does not change anything to the fact that overall different direct currents elicit opposite effects on the expression of the pump. The notable reduction of Na\textsuperscript{+}K\textsuperscript{+} ATPase expression occasioned by anodal compared to that prompted by cathodal direct current stimulation is of two folds value. The first is that stimulated tissues were responsive to the effect of anodal current like they were with cathodal current, but in a reversed direction. The second one is that the decrease of the pump expression after a-DC proved indirectly that the increased expression of Na\textsuperscript{+}K\textsuperscript{+} ATPase after the application of cathodal stimulation could not be the consequence of random circumstances. It clearly affirms that direct current stimulation influences protein expression.
Effect of Direct Current Stimulation on the Expression of Na⁺ K⁺ ATPase Alpha 1 (ATP 1A1) Gene

Cathodal direct current stimulation of the exposed right sensorimotor cortex of CD-1 mice increases Na⁺ K⁺ ATPase alpha 1 relative mRNA transcripts

Western blots have already confirmed the increase of Na⁺ K⁺ ATPase alpha 1 protein expression with every type of cathodal direct current stimulation. Yet, there was still a question that we needed to answer to explain the origin of the Na⁺ K⁺ ATPase protein rise. Did the increase of the enzyme reflect de novo produced proteins or did it simply highlight the presence of cytoplasmic pools of already formed pumps? We have previously mentioned that only few studies have addressed direct current stimulation and gene expression (Aaron et al., 2004; Jiang et al., 2012b; Wenjin et al., 2011). However, there were precedents in the field where electrical stimulation was found to affect gene expression. As an example, chronic depolarization induced by K⁺ ions and electrical stimulation both prompted the expression of tyrosine hydroxylase. The blockage of N-type calcium channels allowed the genetic expression of this enzyme by K⁺ ions influx, but prevented it by electrical stimulation. This result proved that this particular gene is triggered by electrical stimulation through calcium channels (Brosenitsch & Katz, 2001). A recent study showed that electrical stimulation brings about BDNF expression in the spinal cord through calcium and ERK pathway (Wenjin et al., 2011). Together, these findings confirmed the sensitivity of genes to the action of DC. In our research, we examined the gene expression of Na⁺ K⁺ ATPase alpha 1 isoform, ATP 1A1, because it is ubiquitously found in all neurons. We studied the relationship between DC stimulation and gene expression after stimulating only the exposed right sensorimotor. Our protocol was set to allow the occurrence of transcription within reasonable time. Mice were euthanized 2 hours after the end of the stimulation protocol. Studies showed that this period of time was enough to produce substantial mRNAs (Janicki et al., 2004; Shav-Tal et al., 2004; Sheinberger & Shav-Tal, 2013). Analysis of RT-PCR of samples from
exposed right motor sensorimotor cortices showed that cathodal direct current stimulation (c-DCS) yielded a significant increase of ATP 1A1 mRNA transcripts compared to both a-DCS and sham. This outcome supported prior findings that electrical fields’ weak currents potentially affect gene expression and indirectly the activity of certain plasma membrane proteins (Huang, Chen, Yen, Chen, & Young, 2011). Relevantly, this result showed that the increase of Na\(^+\)K\(^+\) ATPase alpha 1 protein expression after cathodal direct current stimulation of the exposed sensorimotor cortex was not necessarily the consequence of pre-made pumps. It is worth mentioning that ATP 1A1 mRNA transcripts produced by a-DCS were increased compared to those seen in sham. This trend was not in line with the low expression of Na\(^+\)K\(^+\) ATPase alpha 1 protein after anodal-DCS of the exposed sensorimotor cortex. To account for this discrepancy, we think that post-translational factors could have played a role in the generation of this outcome. It is known that proteins’ expression can be modified following the intervention of those so-called post-translational factors (Chiarugi & Buricchi, 2007; Mahtani et al., 2001). It is therefore plausible that during a-DCS, post-translational factors were also activated, further decreasing the level of Na\(^+\)K\(^+\) ATPase alpha 1 protein expression.

The present study revealed another effect of DC at the molecular level. Linking these new outcomes to the previous ones will possibly help us clarify how DC exerts its influence on the neurons’ functional molecules. Moreover, the significant difference of ATP 1A1 mRNA transcripts expression found between cathode and anode treated animals, and between cathode and sham animals would not have any scientific value if we did not find similar difference in the Na\(^+\)K\(^+\) ATPase alpha 1 protein expression following stimulation of the right sensorimotor cortex with current of different polarity. New recommendations (MIQE) for optimum results in real time PCR experiments require the validation of the reference genes that are well suited for a
given tissue of interest (Kozera & Rapacz, 2013). We used the Tata box protein (Tbp) as a reference gene (Bio-Rad, USA). We determined the concentration of our transcripts using relative calculation, a method that computes the number of transcripts of interest in relation to a known number of reference transcripts (Huggett et al., 2013). Our RT-qPCR findings showed that the expression of ATP 1A1 gene and the expression of Na\textsuperscript{+}K\textsuperscript{+} ATPase alpha 1 protein following the stimulation of the right exposed sensorimotor cortex are not contradictory.

**IS THE INCREASE OF Na\textsuperscript{+} K\textsuperscript{+} ATPase ALPHA 1 PROTEIN EXPRESSION, FOLLOWING DIRECT CURRENT STIMULATION, DIRECTLY OR ACTIVITY-DEPENDENT TRIGGERED?**

The expression of Na\textsuperscript{+} K\textsuperscript{+} ATPase alpha 1 protein is alternately increased and decreased, but not significantly, when its activity is respectively on and off.

The increase of Na\textsuperscript{+} K\textsuperscript{+} ATPase alpha 1 protein expression with cathodal direct stimulation led us to try identifying what process could engender this rise. In other words, we had to find out whether it is directly the current or indirectly the increased activity that triggers the formation of new pumps. The Na\textsuperscript{+} K\textsuperscript{+} ATPase pump’s turnover rate, more specifically its ability to transport ions, depends on many factors (Bibert et al., 2008; Cornelius & Logvinenko, 1996). Most importantly, elevated cytosolic Na\textsuperscript{+} ions were found to trigger an acute activity of the pump. Therefore, any decrease of these ions’ concentration would affect the function of the Na\textsuperscript{+} K\textsuperscript{+} ATPase enzyme (Rayson & Guptall, 1985). Lidocaine, the most popular anesthetic, indirectly blocks the function of the pump. Its main targets are sodium voltage gated channels responsible for the generation of action potential (Cummins, 2007; Sheets & Hanck, 2003). By blocking the influx of Na\textsuperscript{+}, the pump becomes inactive (Kutchai & Geddis, 2001). The use of lidocaine in our experiments did not allow us to easily answer the question that we formulated to identify the factor that caused the increase of the Na\textsuperscript{+} K\textsuperscript{+} ATPase expression. The obtained results were not significant. They did not show any clear cutoff between Na\textsuperscript{+} K\textsuperscript{+} ATPase protein expression due
to direct current stimulation and those due to the interference with the pump’s activity. Nevertheless, their trends do not contradict what we have already compiled as data. For instance, we found that lidocaine-non-stimulated (LNS) tissues, a group whose activity was off, showed the highest level of Na\(^+\)K\(^+\) ATPase protein expression. In appearance, this result inferred that inhibiting the pump’s activity leads to the increase of its expression. This suggestion is plausible when comparing non-lidocaine non-stimulated (NLNS) with lidocaine non-stimulated (LNS) subgroups. This latter set showed the lowest Na\(^+\)K\(^+\) ATPase protein expression. Based on this comparison, we can cautiously assume that the blockage of the activity could play role in the control of the pump expression. There are two reasons to be careful in drawing a conclusion regarding the relation between activity and expression of the pump. The first one rests on the fact that the results are not significant. The second one lies on the fact that lidocaine itself was reported to influence gene expression, at least, in the fibroblasts of oral mucosa (Friederich & Schmitz, 2002). The increase of Na\(^+\)K\(^+\) ATPase protein in the non-lidocaine stimulated (NLS) subset compared to the (NLNS) subgroup could be viewed as a support to what we had already assessed in the early experiments where c-DC increased the Na\(^+\)K\(^+\) ATPase expression in all experimental paradigms. This result did not rule out that direct current stimulation could reliably induce gene expression. Furthermore, it indirectly confirms RT-qPCR results, which showed increased gene expression under cathodal stimulation. The decrease of Na\(^+\)K\(^+\) ATPase protein in the lidocaine stimulated (LS) batch compared to that seen in the (NLS) subgroup suggested that the hyperpolarizing c-DCS effect (Nitsche & Paulus, 2000) on the pump’s expression could not be fully exerted if the pump is not fully functional.

In conclusion, the lack of significant results obliges us to speculate only regarding the link between pump activity and Na\(^+\)K\(^+\) ATPase protein expression. There was a trend that seemed to
impute to the blockage of the activity a role in increasing the levels of the pump. As for the capacity of direct current to elicit Na\(^+\)K\(^+\) ATPase protein expression, it was not evidently proven in these experiments, but the mitigated results we obtained infer that possibility.

**EFFECT OF DIRECT CURRENT STIMULATION ON Na\(^+\)K\(^+\) ATPase ALPHA 3 PROTEIN EXPRESSION**

Both single cathodal direct current stimulation (c-DCS) of the exposed right sensorimotor cortex, and repeated cathodal transcranial direct current of the same cortical region of CD-1 mice increase Na\(^+\)K\(^+\) ATPase alpha 3 protein expression

Our investigation about the effects of DC on the sensorimotor cortex could not avoid studying Na\(^+\)K\(^+\) ATPase alpha 3 subunit. This experimental inquiry was necessary as this subunit is exclusively present in neurons (Juhaszova & Blaustein, 1997). Studies showed that Na\(^+\)K\(^+\) ATPase alpha 3 subunit in partnership with alpha 1 subunit contribute to the optimum function of neurons (Dobretsov & Stimers, 2005). For example, following a surge of Na\(^+\) ions in the cytosol, Na\(^+\)K\(^+\) ATPase alpha 3 subunit was reported to swiftly reestablish normal concentration of these cations (Zahler, Zhang, Manor, & Boron, 1997). From a health standpoint, behavioral disorders (Kirshenbaum et al., 2011), and cognitive deficits (Holm et al., 2016) have been linked to Na\(^+\)K\(^+\) ATPase alpha 3 subunit mutations. Our results showed that treatment of the exposed right sensorimotor cortex with a single c-DCS was enough to cause a significant increase of the Na\(^+\)K\(^+\) ATPase alpha 3 protein expression in contrast to the expression of this subunit following a-DCS. Yet, we did not see a significant difference in the expression of this subunit between cathode treated and sham mice. We saw comparable results when sensorimotor cortical regions of CD-1 mice were subjected to repeated cathodal transcranial direct current stimulation. Unlike the previous analysis, the expression of Na\(^+\)K\(^+\) ATPase alpha 3 protein, under cathodal current, was significantly higher than both anode treated and sham mice.

These results illustrated that the impact that cathodal direct current stimulation has on the
expression of \( \text{Na}^+ \text{K}^+ \text{ATPase} \) alpha 3 was roughly the same as it had on alpha 1 subunit. Both isoforms’ expression was increased after direct stimulation of the exposed sensorimotor cortex and repeated transcranial of the same brain area. The importance of these results rests on the consistency of cathodal current influence on these two isoforms. Had we obtained opposite effects with each one of them, we would have had legitimate reasons to question the constant impact that cathodal stimulation had on \( \text{Na}^+ \text{K}^+ \text{ATPase} \) alpha 1 subunit expression in all experimental settings.

In conclusion, these results supported the idea that in neurons, alpha 1 and 3 subunits perform similar functions as they were similarly affected by the same current stimulation.

**Single anodal transcranial direct current stimulation (a-TDCS) does not significantly influence \( \text{Na}^+ \text{K}^+ \text{ATPase} \) alpha 3 protein expression**

In opposition to the results acquired with single a-TDCS, the effect of the same current on \( \text{Na}^+ \text{K}^+ \text{ATPase} \) alpha 3 subunit was not replicated, at least significantly. We have already invoked possible reasons to explain the difference between single direct (exposed cortex) and transcranial stimulation when we discussed the \( \text{Na}^+ \text{K}^+ \text{ATPase} \) alpha 1 gene expression. Those explanations remain also credible in this study. Yet, in the context of \( \text{Na}^+ \text{K}^+ \text{ATPase} \) alpha 3 protein expression, things are slightly different than in alpha 1 subunit gene expression analysis. Despite the lack of significant difference, the analysis of single TDCS effect on \( \text{Na}^+ \text{K}^+ \text{ATPase} \) alpha 3 protein expression revealed a trend that was like the pattern observed in repeated TDCS. We still saw that cathode treated animals have a high expression of alpha 3 subunit compared to anode stimulated mice.

We postulated that because \( \text{Na}^+ \text{K}^+ \text{ATPase} \) alpha 1 and 3 subunits accomplish similar functions to maintain neurons’ membrane potentials, they were found to react in a similar way to the application of a given direct current stimulation in all experimental paradigms.
EFFECT OF DIRECT CURRENT STIMULATION ON Na⁺K⁺ATPase ALPHA 1 PHOSPHO-SERINE 943 PROTEIN EXPRESSION

Direct stimulation of the exposed right sensorimotor cortex and transcranial stimulation of the same brain region of CD-1 significantly increased Na⁺K⁺ATPase α1 phospho-serine 943 protein expression

We examined the possibility that direct current stimulation might have on the short term-regulation of the pump’s activity. The catalytic subunit of Na⁺K⁺ATPase hosts several sites that can be phosphorylated (Feschenko & Sweadner, 1994). For instance, a phosphate group is attached to an aspartate residue to permit the regular catalytic activity of the membrane enzyme (Ohtsubo et al., 1990). Other residues are also phosphorylated to modulate the activity of the pump. Protein kinase A (PKA) preferentially attaches a phosphate at serine 943 residue within the catalytic subunit (Cheng, Fisone, et al., 1997; Cheng, Höög, et al., 1997).

Our study showed that single stimulation of the exposed right sensorimotor cortex with anodal direct current provokes a significant rise of Na⁺K⁺ATPase α1 phospho-serine 943 protein expression in contrast to that induced by cathodal current. Similarly, we found out that single transcranial direct current stimulation causes the same effect on the alpha subunit phosphorylated at serine 943.

As we mentioned above, PKA phosphorylation of the pump at this site has been proven to inhibit its function. The identification of this phosphorylated protein did not allow us to evaluate quantitatively the state of the pump activity. It only let us indirectly infer the quality of potentially inactive pumps. It was a result that should be taken at its face value. Therefore, we can rightly theorize that anodal direct current contributes to the inhibition of the pump activity.

We can also argue that the pump activity blockage was a reaction to the depolarizing power of anodal current (Nitsche et al., 2008). It seems unlikely that subthreshold depolarization due to a-DC could be associated with a continuing functional pump, which could lead to increased axonal
excitability. It is the enzyme, through its catalytic function, that favors the depolarization to happen. Therefore, temporarily blocking the pump’s activity will counterbalance the influx of additional ions that a-DC causes.

Repeated anodal transcranial direct current stimulation of the right sensorimotor cortex of CD-1 mice does not substantially change \( \text{Na}^+ \text{K}^+ \text{ATPase alpha 1 phospho-serine 943 protein expression} \)

Our study also tackled the effect that repeated direct current stimulation might have on the expression of \( \text{Na}^+ \text{K}^+ \text{ATPase alpha 1 phospho-serine 943 protein expression} \). We could not confirm the previous significant results in which anodal direct current was associated with an increase of the phosphorylated protein expression. Nonetheless, we couldn’t dismiss their suggestive value. Even though they were not significant, the displayed pattern of \( \text{Na}^+ \text{K}^+ \text{ATPase alpha 1 phospho-serine 943 protein expression} \) confirmed the trend seen with both single direct and transcranial. Stimulation with a-TDCS still engendered the highest expression of \( \text{Na}^+ \text{K}^+ \text{ATPase alpha 1 phospho-serine 943 protein} \). Failure to confirm a significant outcome with repeated TDCS might be attributed to the fact that phosphorylation of the protein at serine 943 is a transient occurrence that will not sustain longevity. In this experimental paradigm, mice were euthanized 24 hours after the last stimulation, before tissues were prepared. Finally, besides influencing proteins’ expression, DCS also appears to influence the short-term regulation of the pump activity. In addition, these results showed that neurons can respond to DCS with a change in the expression of target proteins or with a change of the pump activity.

**EFFECT OF DIRECT CURRENT STIMULATION ON REGULAR DOPAMINE AND C-AMP REGULATED PHOSPHOPROTEIN (DARPP-32) PROTEIN EXPRESSION**

Our study also examined the impact that direct current stimulation may have on the factors implicated in the regulation of the pump activity. Consequently, we focused on dopamine and c-
AMP regulated phosphoprotein (Darpp-32), a protein phosphatase 1 inhibitor (Fisone, Snyder, Aperia, & Greengard, 1998). Data showed that upon activation of dopamine 1 receptor (DA1), PKA phosphorylates the pump at serine 943 residue and Darpp-32 in the cytosol to block the activity of Na⁺ K⁺ ATPase enzyme (Fienberg et al., 1998; Therien & Bost, 2000). Based on this information, before assessing the presence of phosphorylated Darpp-32, we began by evaluating the levels of regular Darpp-32. When making comparisons, this initial action will help us rule out the possibility that increased phospho-Darpp-32 expression is due to an existing elevated cytosolic pool of this protein. To compare regular Darpp-32 and phospho-Darpp-32 expressions, we only used repeated-TDCS. Our assumption was that this type of stimulation would be better at generating more phospho-Darpp-32. Our results revealed no difference in the expression of regular Darpp-32 between anode and cathode treated mice. We expected this outcome as regular Darpp-32 has no implication in modulating the pump activity.

EFFECT OF DIRECT CURRENT STIMULATION ON PHOSPHO DOPAMINE AND CAMP REGULATED PHOSPHOPROTEIN (PHOSPHO-DARPP-32) PROTEIN EXPRESSION

Repeated anodal transcranial direct stimulation of the right sensorimotor cortex significantly increases phospho-Darpp-32 protein expression

Studying phospho-Darpp-32 or the inhibitor of protein phosphatase PP1 was pertinent to identify a possible relationship between direct current stimulation and short-term regulation of the pump function. Still, possible variations in the expression of phospho-Darpp-32 after DC application would not account for any direct or accurate evaluation of the pump function. They would only let us indirectly appreciate the state of Na⁺ K⁺ ATPase activity. Reports have shown that protein kinase A (PKA) phosphorylates Darpp-32 at threonine 34 residue prompting it to inhibit phosphatase protein 1 (PP1) (Hemmings et al., 1984). Because the action of PKA is dopamine-dependent, downstream dopaminergic signaling pathways are triggered (Walaas, Hemmings,
Greengard, & Nairn, 2011). The review of Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 protein expression led us to postulate that DCS affects the pump activity. Given the functional association between the phosphorylation of the pump at serine 943 residue and Darpp-32 at threonine 34, modification of phospho-Darpp-32 expression could strengthen our conviction that DCS intervenes in the control of the pump activity. We found out that repeated a-TDCS significantly influenced the expression of phospho-Darpp-32 in contrast to c-TDCS. This result demonstrated that DCS acts also on molecular targets that regulate the pump activity. We could not directly prove that DCS influences the pump function as we were only looking at the expression of another phosphorylated protein. Yet, this result supported the notion that a-TDCS acts on phospho-Darpp-32 and Na⁺ K⁺ ATPase alpha 1 phospho-serine 943 in the same way to control the pump activity. We have already reported that phosphorylation of Na⁺ K⁺ ATPase alpha 1 at serine 943 and Darpp-32 contribute to the pump inhibition (Beguin et al., 1994; Bertorello, Aperia, Walaas, Nairn, & Greengard, 1991). Yet, repeated a-TDCS did not yield a significant increase of Na⁺ K⁺ ATPase alpha 1 phospho-serine 943. However, it still showed the same trend of influence that both single direct and transcranial stimulation had on the same protein. Moreover, increased phospho-Darpp-32 expression following repeated a-TDCS showed the consistency of the effect that this current polarity has on the pump activity modulation.

**Single stimulation of the exposed right sensorimotor cortex with either anodal or cathodal direct current has no impact on phospho-Darpp-32 expression**

We did not observe any effect of either single anodal or cathodal direct current stimulation of the exposed right motor on the expression of phospho-Darpp-32. Overall, the absence of any statistical difference prevented us from drawing any meaningful conclusion. Still, we noticed that the expression of phospho-Darpp-32 was approximately the same in anode and cathode treated mice. Interestingly, in both experimental animals, the expression of phospho-Darpp-32 was
reduced compared to the one seen in sham mice. One possible explanation is that the exposed right sensorimotor cortex could have acted as an injured brain. It is reported that traumatic brain wound causes a decrease of phospho-Darpp-32 (Bales, Yan, Ma, Li, Samarasingle, & Dixon, 2012). Concurring reports also showed that injured brains have cAMP-PKA signaling pathways downregulated (Atkinsa et al., 2008). Two other probable reasons could justify the approximate equality of phospho-Darpp-32 expression in anode and cathode treated animals. The first one is that the direct current effect could have been overwhelmed by other signaling pathways. Protein phosphatase 1 (PP1) could not have been inhibited by either current. This enzyme controls several downstream targets, among them transcription factor (CREB), calcium/calmodulin kinase II, and more interestingly voltage gated channels like sodium and calcium channels (Fernandez, Schiappa, Girault, & Le Novere, 2006). Influx of Na\(^+\) ions through their specific channels would be a stimulating, not an inhibitory factor, of the pump activity. The second reason is that mice in this paradigm were anesthetized to prepare them for stimulation protocols. It is possible that at the time of the stimulation, the effect anesthesia was not eliminated. Some anesthetics like lidocaine were found to increase the activity of the Na\(^+\) K\(^+\) ATPase pump in the gingiva (Villarruel, Orman, & Borda, 2014).

**Single anodal and cathodal transcranial direct current stimulation of the right sensorimotor cortex produce significant and antagonistic effects on phospho-Darpp-32 expression**

Single anodal and cathodal transcranial direct current stimulation produced opposite results than in any other experimental design. Strikingly, the expression of phospho-Darpp-32 was significantly higher with c-TDCS than with a-TDCS. Furthermore, phospho-Darpp-32 expression in anode treated animals was less than the one seen in sham mice. At the onset, these results seem to be at odds with those observed in the preceding experimental settings. A review
of them showed that reduced expression of phospho-Darpp-32 was indeed a constant trend even though in the absence of a significant difference. The exception was only found with repeated a-TDCS where the expression of phospho-Darpp-32 was elevated. However, to explain the increase of phospho-Darpp-32 under the influence of cathodal current during single transcranial stimulation, we need to remember the characteristics of this current. Cathodal current was found to lower membrane potentials (Cambiaghi et al., 2010). The present study concluded that it increased the expression of both alpha 1 and 3 isoforms. We don’t have any proof that newly formed pumps are systematically inserted in the plasma membrane. Yet, we can argue that the increase of phospho-Darpp-32 expression provoked by cathodal current could be a reaction by neurons to control the activity of their pumps: old and newly synthesized. It is plausible that the increase of the pump expression had to be halted by an inhibition of its activity. Conversely, the decrease of phospho-Darpp-32 expression seen with anodal current fits well with the idea that depolarization induced by the same current requires full function of the pump. On the other hand, Darpp-32 has at least two threonine residues where it can be phosphorylated (Fernandez et al., 2006; Snyder et al., 1992). These include threonine 34, which PKA targets, and threonine 75. Phosphorylation of Darpp-32 at threonine 75 inhibits PKA itself and prevents further inhibition of the pump’s function (Bibb et al., 1999). It is not impossible that anodal direct current could have initiated this signaling pathway to reduce the pump activity. Ultimately, DC stimulation with either polarity could not elicit the same effect on various targets. For example, if a-TDCS caused PKA phosphorylation and later the inhibition of PP 1, it is not excluded that it could have also activated PP2B, which is known to counter the effects of PKA by dephosphorylating its targets (Blanco, Gladis, & Mercer, 1998) and increasing the pump affinity for sodium ions (Aperia, Ibarra, Svensson, Klee, & Greengard, 1992).
Effect of direct current stimulation on the expression of phospho-Darpp-32 was not consistent enough to make an acceptable conclusion. Our results highlighted the impact that direct current of different polarity could have on triggering molecular changes. Despite these conflicting findings, the decrease and increase of phospho-Darpp-32 expression is an overwhelming confirmation of the opposite effects that a-DC and c-DC have on their molecular targets.

**EFFECT OF DIRECT CURRENT STIMULATION ON Na⁺ K⁺ ATPase ALPHA 1 PHOSPHO-SERINE 23**

Anodal direct current causes significant rise of Na⁺ K⁺ ATPase alpha 1 phospho-serine 23 protein expression in every stimulatory experimental paradigm of the right sensorimotor cortex

The regulation of the pump activity requires phosphorylation processes. The present study included those that directly affect the catalytic turnover of the pump and those that modulate it. PKA-dependent direct phosphorylation at serine 943 and phospho-Darpp-32 are respective examples of those so-called processes. We already referred to the coordination between PKA and PKC leading to the inhibition of the pump activity (Pinto-do-O, Chibalin, Katz, Soares-da-Silva, & Bertorello, 1997). We have also mentioned that direct phosphorylation of Na⁺ K⁺ ATPase alpha 1 at serine 943 residue causes the inhibition of the pump (Bertorello et al., 1991; Fisone et al., 1994). According to many reports, this phosphorylation is needed to facilitate the modulation of the pump activity by PKC (Cheng et al., 1997). Other scientific accounts indicated that PKC inhibition of the pump activity leads to the internalization of alpha and beta subunits (Chibalin, Katz, Berggren, & Bertorello, 1997; Chibalin et al., 1998). Remarkably, phosphorylation of Na⁺ K⁺ ATPase alpha 1 at serine 23 and its subsequent endocytosis was seen so far only in rats. Furthermore, the conservation of serine 23 residue across species has not been confirmed yet (Poulsen, Morth, Egebjerg, & Nissen, 2010). However, given the closeness between rats and mice species, we still examined the phosphorylation of alpha 1 subunit at this site. The study of
phosphorylation at serine 23 was another way to evaluate the link between direct current stimulation and short-term regulation of the pump activity. Like the preceding means, it is considered as an indirect assessment of the pump function. We found out that Na⁺K⁺ATPase alpha 1 phospho serine 23 was expressed in mice, and its expression varied correspondingly with the type of the current polarity.

The present study showed that single anodal direct current stimulation of the exposed right sensorimotor cortex generates a significant increase of Na⁺K⁺ATPase alpha 1 phospho serine 23 expression in contrast to cathode treated and sham mice. Distinctively, in this experimental design, the difference in the expression Na⁺K⁺ATPase alpha 1 phospho serine 23 between anode and cathode or between anode and sham was highly significant compared to the expression of any other molecular target. We could not assess any significant difference in the expression of Na⁺K⁺ATPase phospho-serine 23 between cathode treated and sham mice.

Single a-TDCS produced a significant increase of Na⁺K⁺ATPase alpha 1 phospho serine 23 protein expression compared to c-TDCS. Contrary to the stimulation of the exposed sensorimotor cortex, there was also a significant difference between cathode treated and sham mice. The expression of Na⁺K⁺ATPase alpha 1 phospho serine 23 after c-TDCS was significantly decreased. This finding was seen only in this experimental pattern.

Repeated-TDCS elicited patterns of Na⁺K⁺ATPase alpha 1 phospho serine 23 protein expression like those seen in single stimulation of the exposed sensorimotor cortex. Like the results seen in this later experimental paradigm, we found that repeated a-TDCS produced a significant increase of Na⁺K⁺ATPase alpha 1 phospho serine 23 expression compared to the one seen in cathode treated mice. The expression of Na⁺K⁺ATPase alpha 1 phospho serine 23 following a-DC was also significantly higher than that observed in sham mice.
Our findings displayed a significant increase of Na\(^+\)K\(^+\) ATPase alpha 1 phospho serine 23 protein expression by a-DC. This increase was a constant fact in all experimental patterns. Furthermore, the effect of anodal current on Na\(^+\)K\(^+\) ATPase alpha 1 phospho serine 23 expression did not antagonize the effect of the same current on Na\(^+\)K\(^+\) ATPase alpha 1 phospho serine 943 protein expression. This is a central point because phosphorylation at this residue is the mark of PKA, which is known to affect directly the pump activity, and further facilitate the action of PKC. Similarly, the expression of Na\(^+\)K\(^+\) ATPase phospho serine 23 is a hallmark of PKC action. Both kinases have been found to inhibit the pump catalytic function. Most importantly, our results did not show PKA and PKC with opposing effects on the regulation of the pump activity. In fact, the analysis of their respective impact on Na\(^+\)K\(^+\) ATPase alpha 1 phospho serine 23 expression supported the idea that both work together to achieve one goal, which is to inhibit the pump activity. In three various experimental approaches, our study showed that only anodal direct current stimulation allows PKA and PKC to attain this goal. To conclude, we believe that our findings have a significant scope. They proved that PKA and PKC under anodal stimulation do not affect the pump activity differently. We can arguably view the concordance of their respective action as another proof that both kinases collaborate to induce the same effect, namely the pump inhibition. They also strengthen the assertion that anodal direct current stimulation has a consistent influence on the short-term regulation of the enzyme.

**EFFECT OF DIRECT CURRENT STIMULATION ON Na\(^+\)K\(^+\) ATPASE BETA 2 PROTEIN EXPRESSION**

*Single anodal and cathodal stimulation of the right sensorimotor cortex respectively increases and decreases Na\(^+\)K\(^+\) ATPase β2 subunit expression*

After confirmation that DC influences Na\(^+\)K\(^+\) ATPase alpha subunits, we hypothesized that stimulation of the right sensorimotor cortex would also impact beta subunits. It is important to
mention that synthesis of Na\(^+\) K\(^+\) ATPase beta subunits (1, 2, and 3) was found to exceed those of alpha subunits (McDonough & Farley, 1993). Beta 1 and 3 subunits were also reported to influence the rate of the pump function (Jaisser, Jaunin, Geering, Rossier, & Horisberger, 1994). Studies showed that protection from cellular degradation, transportation, and optimization of the catalytic alpha subunit is dependent on beta subunits (Geering, Meyer, Paccolat, Kraehenbuhl, & Rossier, 1985; Käthi Geering, 2008; Jaisser et al., 1994). Taken together, these findings revealed that the beta subunit plays a role in the regulation of the sodium potassium pump activity.

Changes in the expression of this subunit are also considered as examples of long-term control of the Na\(^+\) K\(^+\) ATPase activity (Koksoy, 2002).

Our study showed that the beta 2 subunit, which is also expressed in the brain (Lingrel, 1992), displays a sensitivity to the action of direct current stimulation. We found that single direct stimulation of the exposed right sensorimotor with either anodal or cathodal direct current influences the expression of Na\(^+\) K\(^+\) ATPase beta 2 subunit. More specifically, a-DC prompted a significant increase of beta 2 protein expression in contrast to c-DC. Simultaneously, we saw the expression of this subunit significantly reduced in cathode treated mice compared to sham animals.

In parallel to these results, single a-TDCS significantly increased beta 2 subunit expression in contrast to that induced by single c-TDCS. Likewise, the expression of beta 2 subunit in cathode stimulated mice was significantly reduced in comparison to that seen in sham mice.

The comparison of beta 2 subunit and alpha 1 subunit expressions assessed in every experimental paradigm revealed an antagonistic effect of DC stimulation. In other words, anodal and cathodal current respectively decreased and increased alpha 1 subunit expression. Yet, anodal and cathodal current respectively increased and decreased beta 2 subunit expression. As Na\(^+\) K\(^+\)
ATPase pump function requires both subunits for its full function, we assumed that these findings suggested compensatory responses of neurons to DC influence. Homeostatic reaction is meant to maintain the cell’s function. For instance, if anodal current were to decrease both alpha and beta subunits, it would certainly impact the pump’s ability to work properly. Therefore, any alteration of any kind that may affect the neuron’s subunits must be corrected. In the aforementioned discussion, we have already suggested that DC stimulation does not necessarily induce the same effect on all molecular targets. Correspondingly, it is probable that neurons respond to DC effects whether with an increasing expression of a given subunit or with a decreasing expression of another subunit.

These findings showed that both direct and transcranial stimulation of the right sensorimotor cortex have similar effects on the expression of Na⁺K⁺ATPase beta 2 subunit. They also revealed that changes affecting the protein expression are not solely limited to the catalytic subunit of the pump.

Repeated transcranial direct stimulation of the right sensorimotor cortex does not influence Na⁺K⁺ATPase β 2 protein expression

We have pointed out that overall DC effect on Na⁺K⁺ATPase beta 2 subunit is the opposite of DC influence on Na⁺K⁺ATPase alpha 1 subunit. Yet, repeated a-TDCS induced a trend of beta 2 subunit expression that was not like the result seen with single DCs of the exposed right sensorimotor cortex or single transcranial direct current stimulation of the same cortical region. In these paradigms, anodal current caused a significant rise of beta 2 expression. However, repeated a-TDCS slightly elicited a decrease of beta 2 subunit. Contrary to these outcomes, repeated a-TDCS was associated with a decrease of Na⁺K⁺ATPase beta 2 subunit expression. Unlike the consistent increase of Na⁺K⁺ATPase alpha 1 subunit expression triggered by c-DCS in all the previous experimental paradigms, the increase of Na⁺K⁺ATPase beta 2 subunit
expression was seen with only repeated c-TDCS.

The absence of any significant difference in the expression of $\text{Na}^+\text{K}^+$ ATPase beta 1 subunit, following repeated stimulatory protocol, lets us minimize the scope of these apparent contradictory results. Recurrence of stimulation sessions and the time of their occurrence (Sale, Ridding, & Nordstrom, 2007) could have changed the physiological state of the cortex, thus eliciting different reactions from day to day (Filmer et al., 2014). It is possible that physical constraints have prevented the procurement of results like the ones seen with single stimulatory protocols. For instance, direct current flow varies according to the anatomy of the underlying structures and their deepness. The current is more efficient under the electrode and at the bottom of the sulci than toward the reference and at the surface of the cortex (Miranda, Mekonnen, Salvador, & Ruffini, 2013). Eventually, repeated stimulation could have caused the activation of signaling pathways that have inhibitory effects on transcriptional or translational factors.

**EFFECT OF DIRECT CURRENT STIMULATION ON LOCOMOTOR ACTIVITY AND ANXIETY OF CD-1 MICE**

Single cathodal transcranial direct current stimulation is not associated with any notable change of CD-1 mice locomotor activity or anxiety

Besides investigating potential molecular changes associated with DC stimulation, we also examined in the open field test two behaviors, locomotor activity and anxiety, before and after mice were subjected to single cathodal direct current stimulation. Our choice of cathodal current was justified in part because it was the only one that has consistently produced an increase of $\text{Na}^+\text{K}^+$ ATPase protein expression. Open field results showed no significant difference in the traveled distance, a parameter used to evaluate mice locomotor activity, between cathode pre-stimulated and cathode post-stimulated animals. There was also no significant difference between sham and cathode post-stimulated mice. Nonetheless, our results still displayed a slight increase of locomotor activity by post-stimulated compared to pre-stimulated animals. In the
same way, there was no significant difference in the time spent in central zone, a factor used to quantify animals’ anxiety, between pre-stimulated and post-stimulated mice. There was no significant difference between pre-stimulated or post-stimulated animals and sham mice as well. Yet, the time spent in central zone was shorter in post-stimulated than in pre-stimulated animals, suggesting that the former mice were more anxious. Despite their statistical insignificance, these findings seem to infer that c-DCS increases locomotor activity and anxiety. The results could be due to the fact that cathodal current is associated with a hyperpolarization of the neurons (Nitsche & Paulus, 2000) and an inhibition of the neurotransmission by glutamate. These effects should enhance GABAergic circuits (Ding et al., 2016; Filmer et al., 2014). Furthermore, animals with disrupted GABA systems have demonstrated increased locomotor activity and anxiety (Curia, Papouin, Seguela, & Avoli, 2009; D’Hulst et al., 2006; El Idrissi et al., 2005). Collectively, the limitation of our data did not allow us to come up with conclusion regarding the effect of cathodal direct current stimulation on mice’s behavior. Nevertheless, the observed results did not exclude the possibility that direct current might influence other functions besides membrane potential or protein expression. For instance, in humans, transcranial direct current stimulation has been shown to influence cognitive functions.

**EFFECTS OF SUBTHRESHOLD DIRECT CURRENTS ON THE SCIATIC NERVE**

Findings of the study of DCS effects on the sciatic nerve raised four questions. First, how do weak currents elicit an increase or a decrease of the nerve excitability? Second, are there factors governing long-term after effects of DCS? Third, is there any interaction between axonal effects and synaptic driven effects of DCS? Finally, how can these findings be used in the application of DC to the brain and spinal cord? The answer to the first question was given by the results of this study, which along with other reports, implied that acute changes of the axonal excitability
reflect variations in the axonal threshold (Del Castillo & Katz, 1954; Eccles, Kostyuk, & Schmidt, 1962). The response to the second question rested on the identification of at least one factor, Na\(^+\)K\(^+\) ATPase, in the control of distant effects. The addition of ouabain to the central chamber prevented the occurrence of long-lasting inhibition or distant effects at 10, 15, 20, and 25 mn. Ouabain also intensified the immediate effect of c-DCS. Furthermore, these findings showed that immediate and long lasting effects of DCS are independent from each other.

Ouabain, at the concentration used in this experiment, was expected to depolarize the membrane (Kagiava, Aligizaki, Katikou, Nikolaidis, & Theophilidis, 2012). The fact that it stopped the long-lasting inhibition of DC stimulation suggests that cathodal current hyperpolarizing nature was possibly due to changes of sodium potassium ATPase number or activity. Clinically, these findings make c-DCS a good candidate to replace medications, like Digoxin, aimed to regulate Na\(^+\)K\(^+\) ATPase. As for the third and fourth questions, there were no direct answers to them.

Nevertheless, the observed results were in line with the effects of DCS on spinal cord (Ahmed, 2011; Ahmed & Wieraszko, 2012). Stimulation of the spinal cord with c-DCS for 3 minutes provoked long-lasting depression of nerve excitability (Ahmed, 2011). Conversely, a-DCS of the spinal cord during 3 minutes induced an enhancement of spinal excitability (Ahmed, 2011).

There is a similarity between these findings and those of the present study. This suggests that the effects of DCS on more complicated regions of the nervous system must include non-synaptic components.

Collectively, these findings supported the idea that the immediate effect of DC on neurons’ excitability is exerted through changes of membrane threshold. The current study confirmed precedent findings that have shown stimulation with DC modifies cortical neurons’ excitability. Most importantly, this study revealed that Na\(^+\)K\(^+\) ATPase plays a role in the after-effects of DC.
Ultimately, findings of this investigation highlighted the role of cathodal-DCS, which can be harnessed to treat certain medical conditions.

**EFFECTS OF SINGLE TRANSCRANIAL DIRECT CURRENT STIMULATION OF THE RIGHT SENSORIMOTOR CORTEX ON SENSORY EVOKED POTENTIALS**

**Cathodal transcranial direct current stimulation effect is associated with an increase of sensory evoked potentials**

Responses to transcranial direct current stimulation in the brain cortex are not the same. For instance, motor evoked potentials (MEP) have been found to be increased and decreased by a-TDCS and c-TDCS respectively (Nitsche & Paulus, 2000). Visual evoked potentials (VEP) have also been found to be reduced after stimulation of the visual cortex (Ding et al., 2016). The use of c-TDCS over the human somatosensory cortex produced SEP with decreased amplitudes (Vaseghi, Zoghi, & Jaberzadeh, 2015). However, recent findings revealed that cervical cathodal trans-spinal direct current stimulation enhances somatic sensory responses (SEP), but less than responses elicited in the motor cortex (Song, Truong, Bikson, & Martin, 2015). A study on the effect of spinal transcutaneous direct current stimulation on SEP in humans revealed that anodal current reduces the amplitude of SEPs elicited by stimulation of the tibial posterior nerve (Cogiamanian, Vergari, Pulecchi, Marceglia, & Priori, 2008). Taken together, these conclusions about SEP are in line with what we obtained in our study. Our findings steadily showed that c-TDCS and a-TDCS respectively trigger an elevation and a decrease of SEP amplitude. Yet, we cautiously abstained from affirming the existence of significant difference between anodal and cathodal DCS because of the reduced number of mice used in this experiment. This was a limitation that should be corrected in future studies. Observed high amplitudes of SEPs generated by c-TDCS in the right sensorimotor cortex could be due to the hyperpolarization that this current induces. This will make it difficult for neurons to reach the threshold before triggering an
action potential. However, once the cells reach this threshold, they produce an action potential with a high amplitude peak. Another possible explanation is that a-TDCS increases spontaneous activity of presynaptic neurons, leading to a decrease of excitatory post synaptic potentials (EPSPs). This option should not be ruled out because studies have already shown that presynaptic hyperpolarizing currents increase the amplitude of EPSPs (Eccles et al., 1962; Hubbard & Willis, 1962). A third probability to explain the trend of SEPs recorded in the right sensorimotor is that c-TDCS may hyperpolarize interneurons to disinhibit their actions on post synaptic neurons.

To conclude, this electrophysiology study reinforced the view that DC stimulation can modify the processing of the information originating from different parts of the body. It may be used to reduce neuronal adaptation, thus increasing the perception of sensory stimuli.

**Repeated Anodal Transcranial Direct Stimulation and Mice Behavior**

The observation of seizure-like behavior in some mice that had undergone repeated transcranial direct current stimulation calls for a cautious interpretation. The fact that this pattern was seen more than once suggests that a biological effect is triggered in certain experimental conditions, namely repeated a-TDCS protocol. The analysis of our findings seems to exclude a fortuitous manifestation. On the other hand, the limited number of mice that have shown this behavior, and the variation of its occurrence in the three mice led us to disregard a biological cause as a unique explanation. Rather, we assumed that technical manipulations could be also a possible source of this conduct. During the preparation of the hub, which serves as an electrode, we used dental resin. This latter is prepared by mixing a liquid and a powder to obtain a moderate viscous consistency. To glue the hub over the skull, we poured around its base the dental resin. It is possible that some liquid spread over the area within the hub (electrode). With the subsequent hardening of the resin, the area of the hub thus of the electrode would be reduced. This would
increase the current density and expose the underlying sensorimotor cortex to an intense stimulation and induce seizure-like behavior in the left limbs of the mice.
CONCLUSION

Our current study revealed that direct stimulation is not strictly confined to changes affecting the axonal excitability. Yet, a part of our research has also confirmed that DC elicits also in the right sensorimotor cortex modifications of the membranes’ thresholds. The originality of our study rested on the revelation that DC stimulation triggers changes at the molecular level. Our findings showed that cathodal direct current stimulation indisputably provoked a significant increase of expression of both the ubiquitous Na⁺K⁺ ATPase alpha 1 and the neuron-specific Na⁺K⁺ ATPase alpha 3. They similarly exhibited a decreased trend of the enzyme expression associated with anodal-DC. In addition, the increase of Na⁺K⁺ ATPase alpha 1 protein expression after c-DC stimulation was also backed by an increase of the pump’s gene expression following the application of the same current. We intended to identify the factor that could have increased Na⁺K⁺ ATPase expression after stimulating the right sensorimotor cortex with c-DC in each experimental design. However, our study failed to identify the source. Changes of Na⁺K⁺ ATPase α1 expression patterns due to direct activation of the gene or by interference with the pump activity were both seen after c-DCS of the exposed right sensorimotor. In another approach, our results indirectly indicated that DC stimulation affects the short-term activity of Na⁺K⁺ ATPase. They showed that anodal DC in contrast to cathodal DC significantly increases Na⁺K⁺ ATPase-phospho serine 23 and Na⁺K⁺ ATPase-phospho serine 943 expressions. They also inferred that a-TDCS inhibits the activity of the pump. Furthermore, DC stimulation was found to affect the expression of Na⁺K⁺ ATPase β2 subunit as well. Our investigation demonstrated that both single a-DCS of the exposed right sensorimotor and single a-TDCS produce a significant increase of Na⁺K⁺ ATPase β2 subunit. These findings showed that DC stimulation may also affect beta subunit expression. This could have an impact on the long-term
regulation of the pump activity because of the important role of this subunit in the conveyance of alpha 1 subunit to the plasma membrane and the function of the entire pump.

Together, these findings confer to DC stimulation a new dimension that is to influence the Na$^+$ K$^+$ ATPase pump at the functional, genetic, and protein expression levels. The present study showed that DC can indistinctively target various molecules that are functionally involved in neurons’ excitability. These findings may help understand how the immediate mechanism of DC stimulation is exerted. They also constitute a justification for the exploration of other cellular structures like sodium and calcium channels, which control non-synaptic and synaptic function of the neurons.
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