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# Effects of High Frequency Electrical Stimulation of the Infralimbic Cortex on Cellular Activity in the Rodent Brain

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An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

Effects of High Frequency Electrical Stimulation of the Infralimbic Cortex on Cellular Activity in the Rodent Brain

By Ashwin G. Ramayya

Deep brain stimulation (DBS) of the subgenual cingulate (Cg25) white matter (WM) is a novel surgical therapy which has recently been used to treat severely depressed, treatment-resistant individuals. While it has shown significant therapeutic effects, the mechanism by which DBS achieves clinical benefits is not completely understood. Functional neuroimaging studies have shown that Cg25WM DBS leads to a suppression of metabolic activity in the Cg25, which is a known mediator of negative mood. In this study, we aimed to gain some understanding of how this occurs by studying the effects of acute high frequency stimulation (HFS) on cellular activity in the rodent brain. We targeted the infralimbic cortex (IL), the rodent homologue of Cg25, and quantified cellular activity by measuring the density of cells expressing c-fos, an Immediate Early Gene (IEG) which is expressed in the nucleus of active cells. We compared cellular activity in stimulated and non-stimulated rats locally in the IL, in the monosynaptically connected basomedial amygdala (BMA) and the polysynaptically connected hippocampal dentate gyrus (DG). We found there to be a greater cellular activity in all three regions of the stimulated brain, suggesting that Cg25WM DBS has far-reaching acute effects on cellular activity. The increased cellular activity in the IL was found to be statistically significant; however, there were not enough subjects to conclusively determine the significance of increased cellular activity in the BMA and the DG.

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## Introduction

Major depression is a devastating psychiatric condition that affects the mental, physical and social health of millions of the people world-wide. Depressed individuals typically dwell in low-mood states and are unable to experience pleasure in previously enjoyable activities. These individuals also often experience other impairments such as fatigue, insomnia, loss of appetite, social withdrawal, and many have thoughts of suicide (American Psychiatric Association 2000). As the most common psychiatric disorder (Wang 2003), depression is the leading source of disability in adults in North America under the age of 50 (WHO 2003).

Major depression is a complicated condition and it is extremely difficult, if not impossible, to point to a single cause. In order to overcome this challenge, a biopsychosocial model of depression has been suggested where biological, psychological and social factors all play a role in causing depression (Department of Health and Human Services 1999). Depending on the case, a preexisting genetic vulnerability (Caspi et al. 2003), a traumatic life event (Heim et al. 2003), or chronic substance abuse (Fergusson et al. 2009), can be the driving factor in causing major depression. These complexities combined with converging lines of evidence from clinical, biochemical, neuroimaging and postmortem studies suggest that depression is likely to be a systems-level disorder affecting many regions of the brain, not just a single brain region or neurotransmitter system (Mayberg et al. 2005, 1997, Nemeroff 2002, Nestler et al. 2002, Vaidya and Durman 2001).

An emerging view in the field holds that depression arises out of a systemic irregularity in communication between limbic brain regions (which process motivational, affective, and emotional behavior) and cortical brain regions (which process sensory, motor, and cognitive behaviors) (Drevets 1999, Mayberg et al. 1997, 1999, 2003). The subgenual cingulate (Cg25) appears to be at the center of this cortico-limbic network as many functional neuroimaging studies suggest that it plays a critical role in regulating negative mood states (Mayberg et al. 1999, Seminowitz et al. 2004). Functional neuroimaging studies either measure the change in glucose metabolism (Positron Emission Tomography, PET), or blood perfusion (functional magnetic resonance imaging, fMRI) in specific brain regions over time (Weiller et al. 2006). Cg25's strong connections to various cortical and limbic regions (such as nucleus accumbens, amygdala, hypothalamus, and orbitofrontal cortex) have been implicated in underlying core behaviors altered in depressed patients (such as sleep, appetite, learning, motivation etc) (H. Johansen-Berg 2008, Barbas et al. 2003, Jugens and Muller-Preuss 1977, Carmichael and Price 1996, Vogt and Pandya 1987, Freedman et al. 2000). Also, the fact that most anti-depressant therapies have been shown to reduce Cg 25 activity supports the idea that Cg25 plays a critical role in depression (Dougherty et al. 2003, Mayberg et al. 2000, Goldapple et al. 2004, Mottaghy et al. 2002, and Nobler et al. 2001).

In many cases, the cortico-limbic irregularity in depressed individuals can be corrected through a variety of compensatory manipulations that re-establish the normal mood state (Vaidya and Duman 2001, Hyman and Nestler 1996, and Mayberg 2003). For example, many depressed individuals have low-levels of monoamine neurotransmitters (such as dopamine, serotonin and norepinephrine) which are necessary for healthy

communication between various cortico-limbic circuits. In these cases, pharmaceutical agents that increase the levels of one or more of these monoamine neurotransmitters have shown strong anti-depressant effects (Shah et al. 1999, Nutt et al. 2008, Hirschfield 2000). In other cases, various forms of evidenced-based psychotherapy have also proven to have effective anti-depressant effects (Roth et al. 2005). However, in up to 20% of depressed individuals, these standard interventions prove to be ineffective and trial-and-error combinations of various medications and electroconvulsive therapy are used as a last resort (Kennedy and Lam, 2003, Sackeim et al. 2001). The individuals who remain severely depressed despite these aggressive treatment strategies are considered treatment-refractory and are candidates for novel, experimental surgical therapies such as deep brain stimulation (DBS) (Mayberg et al. 2005, McNeely 2008).

DBS is a surgical technique that involves chronically implanting electrodes in specific brain regions and electrically stimulating them in order to modulate the activity of the targeted regions and their associated network(s). Pioneering work done in Parkinson's disease has shown that chronic high-frequency electrical stimulation of pathologically hyperactive brain regions leads to significant clinical benefits (Benabid, 2003, Lang and Lozano 1998). Pilot studies based on this principle has shown high-frequency DBS of the subgenual cingulate white matter (Cg25WM) to have significant anti-depressant effects in some severely depressed, treatment-refractory patients (Mayberg et al. 2005, Lozano 2008). Not only did these patients show sustained behavioral improvements after sustained high-frequency electrical stimulation, but PET studies conducted on these patients 3 and 6 months after the procedure showed significant metabolic changes in cortical and limbic networks that correlated with anti-

depressant benefits (Mayberg et al. 2005). Many of these changes were similar to those seen in patients receiving anti-depressant medication (reduced local activity in Cg25, and reduced remote activity in the hypothalamus, the brainstem and the prefrontal cortex), while other changes were similar to those seen in patients receiving cognitive psychotherapy (decreased activity in the medial frontal/orbital frontal cortices, and increased activity in the dorsal cingulate cortex) (Mayberg et al. 2000, 2005, Goldapple et al. 2004). Unique to Cg25WM DBS, however, is a sudden improvement in mood seen in the operating room. It has been hypothesized that this could be due to an acute deactivation of Cg25, which as discussed earlier, is a hypothesized mediator of negative mood (Mayberg et al. 2005). Also, pilot studies are currently being done to clinically evaluate DBS of the nucleus accumbens (NaCC) and the ventral capsule (Malone et al. 2009, Schlaepfer et al. 2008).

While PET suggest that Cg25WM DBS leads to a long-term suppression of metabolic activity in Cg25 (Mayberg et al. 2005), the short-term and long-term effect on cellular activity is unclear (Davis et al. 1997, McInteyre et al. 2004). One major view in the field holds that Cg25WM DBS could be suppressing local activity of neuronal cell bodies and their efferents (either by activating inhibitory afferents to the region, or by causing a depolarizing blockade). Another major view suggests that Cg25WM DBS activates Cg25 cell bodies and their efferents (and thus effecting regions down-stream to Cg25). A third view holds that Cg25 DBS inhibits Cg25 cell bodies, but activates efferents from Cg25. (Gabbott 1997, Lozano 2002, McIntyre et al. 2004, Mayberg et al. 2005, Vitek 2002). A recent study explored this controversial subject in the context of Parkinson's disease by using cutting-edge optogenetic approaches (which involve both

optical and genetic manipulation of neural circuits) to deconstruct neural circuitry in a Parkinsonian rat model. They elegantly demonstrated that therapeutic effects in Parkinsonian rats could only be achieved by activating white-matter afferents to the pathologically hyperactive subthalamic nucleus (STN) (Gradinaru et al. 2009). When they selectively inhibited STN neurons, they saw no therapeutic effects. These results suggest that STN DBS exerts its therapeutic effects by antidromically activating inhibitory white-matter afferents to pathologically hyperactive STN, rather than inhibiting STN neurons directly. Antidromic effects occur when electrical impulses from the electrode travel from the axon terminus "backwards" towards the cell body and activate the neuron. While these findings further complicate an already complex matter, they suggest that remote regions play a critical role in the therapeutic effects seen with DBS. In this study, we aim to investigate the mechanism of Cg25WM DBS and effects on depression by studying the effects of acute high-frequency electrical stimulation (HFS) on cellular activity in a rat model of the human brain.

The infralimbic cortex (IL) is the functional and anatomical homologue of Cg25 in rodents. First, a study of cortex metabolism in a rodent model of depression showed that the pathological IL has similar metabolic properties as the pathological Cg25 in severely depressed humans (Shumake 2000). Additionally, studies of anatomical connectivity in rodents (Vertes 2004, Conde 2005, Hurley et al. 1991), and non-human primates (Carmichael and Price 1994, Vogt and Pandya 1987, Barbas 2003, and Freedman 2000) have shown the IL to have similar connectivity to Cg25 in humans (Gutman et al. 2008, Johansen-Berg et al. 2007). Specifically, both regions show strong reciprocal connections with various prefrontal and cingulate cortical regions, and various

subcortical regions such as the hypothalamus, and the amygdala. Of particular relevance to Cg25WM DBS is the strong connectivity between the IL and the amygdala. The IL sends strong efferents to the basomedial nucleus of the amygdala (BMA) and receives inhibitory input from the basolateral amygdala (which is located adjacent to the BMA) (Perez-Jaranay et al. 1991). While there is much to understand about the nature of these connections, there appears to exist an inhibitory feedback loop between the IL and the amygdala, which could suppresses IL activity in the long-term (Madsen et al. 2008). The dentate gyrus (DG) of the hippocampus is another area that is relevant to Cg25WM DBS. While the DG plays a critical role in anti-depressant activity, it is not directly connected to the IL (Vertez 2004, Hurley et al. 1991, Conde et al. 1995). Specifically, it is one of the two sites in the brain where neurogenesis, or the generation of new neurons, takes place. It is hypothesized that neurogenesis is the critical process altered by anti-depressants that leads to sustained behavioral improvements in depressed patients who respond to treatment (Kempermann et al. 2003). Additionally, a study of rodent hippocampal microcircuitry suggests that DG activity decreases in the depressed state and increases with anti-depressant interventions (Airan et al. 2007).

The manner by which Cg25WM DBS acutely affects Cg25 and the various regions associated with Cg25 and depression is not known. We hope to gain insight on this question by examining the effect that acute IL HFS has on cellular activity in the IL, in the monosynaptically connected BMA, and the polysynaptically connected DG in the rodent brain. We measure cellular activity by quantifying the number of cells expressing c-fos, an immediate early gene (IEG) which is expressed in neurons in response to direct

stimulation by growth factors, neurotransmitters or electrical stimulation (Sagar et al. 1988, Kovacks et al. 2008).

## Materials and Methods

### *Experimental Animals*

We used adult Sprague-Dawley Rats (180-200g) housed with ad libitum access to food and water in a room maintained at constant room temperature (20-22$^{o}$C) on a 12 Hour: 12 Hour light-dark cycle. We received Institutional Animal Care and Use Committee (IACUC) approval for this study.

### *Surgery*

After being anaesthetized with inhaled isoflourane (1-4%; adjusted as needed), the animals' heads were fixed to a stereotaxic instrument to anatomically localize the rat infralimbic cortex. A craniotomy was made and a concentric bipolar electrode (Plastics One) was lowered into the relevant cortex of each animal using previously determined stereotaxic coordinates (2.7 mm anterior to bregma, 0.5 mm lateral, 4.6 mm ventral to pia) (Paxinos & Watson, 1986). The electrode tip was dipped in a phosphate buffered 1:500 DAPI solution (Hoestch) to aid in visualization of the electrode. The animals were then stimulated for 1 hour at the following parameters: 2.5 V, 90 microsecond pulse width, 130 Hz (HFS). These parameters have previously been used to induce remote immediate early gene (IEG) expression in the rodent brain (Toda et al. 2008). Sham + Electrode animals were also anesthetized and had the electrode implanted; however, they were not stimulated. Sham – Electrode animals were anesthetized and craniotomies were made,

but the electrode was not lowered into the cortex. After the procedures, the electrodes were removed, the surgical planes were closed and the animals were allowed to recover.

*Experimental Design*

Before surgery, the rats (n=10) were placed in either the HFS group (n=4), the Sham + Electrode group (n=4) or the Sham – Electrode group (n=2). Animals in the HFS group were electrically stimulated for 1 hour; animals in the Sham+ Electrode group had an electrode lowered into the brain, but were not stimulated; and animals in the Sham – Electrode group were prepared for electrode placement, but did not have an electrode lowered into the IL. All rats were perfused with saline 0.9%, followed by 4% paraformaldehyde 3 hours from the end of stimulation in order to analyze the acute cellular effects of IL HFS. Increased remote IEG expression following HFS has been shown to occur 3 hours after stimulation (Toda et al. 2008).

*Tissue Collection and Immunohistochemistry*

The animals were then deeply anesthetized with euthasol (50 mg/Kg administered intraperitoneally) and subsequently perfused with saline 0.9%, followed by 4% paraformaldehyde. Brains were then removed from the skull and post-fixed overnight in a phosphate buffered 30% sucrose solution. Free-floating 50 micro-meter sections were cut on a cryostat, and collected on a cryoprotectant and immunostained with mouse anti-NeuN (1:500, Sigma) and rabbit anti-c-fos (1:500, ABCAM). Secondary antibodies were used at a 1:500 concentration (Jackson ImmunoResearch Laboratories).

*Image Quantification*

Immunofluorescent images were captured using an immunofluorescent camera attached to a Nikon Eclipse E400 microscope and a computer. The exposure settings were kept constant across IL sections (gain 5.6, exposure 135 ms), BMA sections (gain 5.6, exposure 178 ms) and DG sections (gain 5.6, exposure 97 ms) from each subject. The contrast in each image was set based on the histogram for each image. Cells were counted within a region of fixed area (125664 pixel$^2$) in morphologically similar IL, BMA, and DG sections from each subject as identified by the technician (see figure 1). All four subjects from each group had 2 or more usable sections from the IL, however, due to poor perfusion; only 3 subjects from each group had 2 or more usable sections from the BLA and DG. Specific cell counts and contrast settings relative to the histogram were recorded for each subject.

*Statistical Analysis*

Unpaired, two-tailed student t-tests were used in all comparisons. There were two analyses done based on differential organization of the data. In the "By Section" analysis, cell counts were compared across stimulated and non-stimulated sections of tissue from all subjects. In the "By Subject" analysis, the mean cell count was obtained for each subject (by averaging cell counts from each section) and compared. The "By Subject" analysis is more robust because it is more sensitive to variance between individual subjects and has been used in the past (Deurveilher et al. 2004).

## Results

*Electrode Location*

Our first goal was to determine whether or not our surgical technique was robust enough to consistently lower the electrode into the IL. In order to aid with visualization of the electrode track, the electrode was dipped in a 1:500 phosphate-buffered solution of DAPI, which is immunofluorescent. We were able to visualize the electrode correctly placed in the IL using this method (Figure 2).

*C-fos Antibody Binding*

In order to determine the specificity of our rabbit c-fos antibody, we compared sections stained with both the c-fos primary anti-body and secondary rabbit antibody with sections stained only with secondary rabbit antibody. The sections without the c-fos primary antibody showed no nuclear staining (Figure 3d). Also, we stained sections with both the rabbit c-fos primary antibody and the mouse NeuN primary antibody to determine whether c-fos was being expressed in the nucleus of mature neurons (which express NeuN). We found there to be substantial co-localization of these antibodies (Figure 3 a,b,c).

*Sham-Electrode Subjects*

In order to determine the effect of placing an inactive electrode in the IL, we examined the baseline c-fos expression in the IL in two subjects. These animals were anesthetized, but had no electrode in the IL (Sham-Electrode group). While we did not quantify the results from this group (due to the small number of subjects), there was no dramatic difference in expression patterns in the IL, BMA, and DG of sham-electrode subjects compared to the sham+electrode subjects (Figure 4).

*IL C-fos Activity*

We counted cells within a region of fixed area (125664 pixels$^2$) in at least two morphologically similar sections of the IL from each subject in order to determine the effects of acute IL stimulation on local cellular activity. We found there to be a higher density of cells expressing c-fos in the IL sections from HFS subjects compared to sections from Sham+Electrode subjects (Figure 4). Cell counting revealed a mean of 47 (+/-3.9) cells in the IL of HFS subjects (n=4) compared to a mean of 20 (+/-2.8) cells in the IL of Sham+Electrode subjects (n=4). Both the "By Section" t-test ($p<0.001$) and "By Subject" t-test ($p=0.004$) showed these results to be statistically significant (Figure 5, Table 1).

Additionally, within HFS subjects, we compared IL sections from the stimulated side of the brain (where we could visualize an electrode track) with sections from the non-stimulated side of the brain (where we could not visualize an electrode track). We did not find a statistically significant ($p=0.94$) difference between mean cell counts in the stimulated hemisphere (mean=46+/-4.6) and the non-stimulated hemisphere (mean=47+/-6.2) (Figure 6).

*Basomedial Amygdala (BMA) C-fos Activity*

We also bilaterally counted cells in at least two morphologically similar sections of the BMA from each subject in order to determine the effect of acute IL stimulation on monosynaptically connected brain regions. Due to poor perfusion in two of the subjects, we only had 3 HFS subjects and 3 Sham+Electrode subjects with 2 or more usable BMA sections. Again, we found there to be a higher density of cells expressing c-fos in the BMA of HFS subjects (mean=32+/-4.4) compared to Sham+Electrode subjects

(mean=20+/-2.3) (Figure 4). The "By Section" t-test revealed that these values were statistically significant (p=0.03), however, the "By Subject" t-test did not show the result to be significant (p=0.39) (Figure 5, Table 1).

*Hippocampal dentate gyrus (DG) c-fos activity*

We also bilaterally counted cells in at least two morphologically similar sections of the DG from each subject in order to determine the effect of acute IL stimulation on limbic regions polysynaptically connected to the IL. Again, due to poor perfusion in two of the subjects, we only had 3 HFS subjects and 3 Sham+Electrode subjects with 3 or more usable DG sections. We found there to be a higher density of cells expressing c-fos in the of HFS subjects (mean=42+/-7.9) compared to Sham+electrode (mean=20+/-3.8) subjects (Figure 4). Again, the "By Section" t-test revealed that these values were statistically significant (p=0.02), however, the "By Subject" t-test did not show the result to be significant (p=0.278).

**Discussion**

In this study, we sought to determine the effect that acute IL HFS has on cellular activity in 1) the IL, 2) the monosynaptically connected basomedial amygdala (BMA), and 3) the polysynaptically connected hippocampal dentate gyrus (DG). We found that there was a higher density of cells expressing c-fos in the IL (mean=47+/-3.9, n=4), BMA (mean=32+/-4.4, n=3), and DG (mean=42+/-7.9, n=3) of stimulated rats compared to the IL (mean=20+/-2.8, n=4), BMA (mean=20+/-2.3, n=3), and DG (mean=20+/-3.8, n=3) of Sham+Electrode rats (Figure 4, 5, Table 1). A "By Section" student t-test, which compared cell counts across tissue sections showed results from the IL ($p<0.001$), BMA

(p=0.03), and DG (p=0.02) to all be statistically significant. A "By Subject" student t-test, which compared mean cell counts from each individual subject showed for the IL (p=0.004) but not the BMA (p=0.39) or the DG (p=0.28) (Table 1). Additionally, of the rats receiving HFS, there was no significant difference (p=0. 94) between cellular activity in IL sections ipsilateral to the electrode (mean=46+/-4.6) and IL sections contralateral to the IL (mean=47+/-6.2) (Figure 6).

We aimed to gain insight into the mechanism by which deep brain stimulation (DBS) of the subgenual cingulate white matter (Cg25WM) exerts its therapeutic effects. While functional neuroimaging studies have revealed that Cg25WM DBS leads to long-term metabolic suppression of the subgenual cingulate (Cg25) in severely depressed, treatment-refractory patients, the mechanism of action is still unclear. It has been proposed that this metabolic deactivation of Cg25, which is a known mediator of the negative-mood state, leads to both acute improvements in mood and long-term behavioral improvements in many severely depressed individuals (Mayberg et al. 2005). There are three major hypotheses in the field about the mechanism by which Cg25WM DBS suppresses activity in the Cg25. One holds that it locally inhibits neuronal cell bodies and efferents in the region (through a depolarization blockade, or through activation of inhibitory afferents to the region), while the other holds that DBS activates neuronal cell bodies and efferents from Cg25, and the third suggests that DBS inhibits Cg25 cell bodies but activates their efferents (Gabott 1997, Lozano 2002, McIntyre et al. 2004, Gadinaru and Mogri 2009, Mayberg et al. 2005, Vitek 2002). Based on a recent study of how DBS affects Parkinsonian circuitry, regions that are remotely located from the electrode are likely to be involved (Gadinaru and Mogri 2009). In the current study, we

aimed to gain an understanding of the acute effects that DBS has on neural circuitry associated with depression in the rodent brain. We hope that this will shed some light on the acute mechanisms by which Cg25WM DBS exerts its therapeutic effects.

In order to investigate the acute changes in cellular metabolism observed during chronic Cg25WM DBS, we studied the effects of acute IL HFS on c-fos expression in the IL, the BMA and the DG. We confirmed that our surgical technique was robust by visualizing the electrode track with DAPI immunofluoresence (Figure 2). Also, the parameters we used for high frequency stimulation (one-hour HFS; 2.5 V, 90 microsecond pulse width, 130 Hz) have previously been used to simulate DBS in the rodent brain (Toda et al. 2008). We hope that our study of acute IL HFS will shed some light on the acute effects of chronic Cg25WM DBS, which has therapeutic effects in severely depressed individuals.

*Sham-Electrode findings*

We studied c-fos expression in the IL, BMA, and DG of anesthetized rats (Sham-Electrode group) in order to determine the effect of lowering an electrode into the IL. While we did not quantify the cellular expression level in this group, we did not identify any dramatic difference in the general c-fos expression pattern in the IL, BMA, and DG of subjects in the Sham-Electrode group compared to subjects in the Sham+Electrode group. It is an appropriate finding that the placement of the electrode does not substantially change cellular activity of the IL as Cg25WM DBS patients only report improvements in mood after electrical stimulation is initiated (Mayberg et al. 2005).

*Increased Cellular Expression in the IL after HFS*

We found a higher number of cells expressing c-fos in the IL of stimulated animals (mean=47, n=4) compared to control animals (mean=47+/-3.9, n=4). This was our most statistically robust finding as both our t-test analyses showed it to be significant ("By Section," $p<0.001$; "By Subject," $p=0.04$). This increased cellular activity following acute stimulation is very relevant to uncovering the mechanism by which Cg25WM DBS suppresses Cg25 activity. Most directly, these results contradict the idea that DBS suppresses Cg25 activity by acutely inhibiting neurons. In fact, it appears that Cg25WM DBS increases short-tem neuronal activity as shown by the increased c-fos expression. However, our findings do not rule out the possibility that DBS suppresses Cg25 activity in the long-term.

Additionally, in the HFS subjects, we did not find a significant difference ($p=0.94$) between cellular activity in the IL ipsilateral to the electrode (mean=46+/-4.6) and the IL contralateral to the electrode (mean=47+/-6.2) (Figure 6). There are two possible explanations for this finding. First, due to the proximity of the IL to the midline (and thus the contralateral IL), it is feasible that the electrical field from the electrode in the ipsilateral IL could have "spilled over" and activated cells in the contralateral IL. Alternatively, the electrical stimulation of the ipsilateral IL could have activated the contralateral IL through callosal pathways that connect the regions across hemispheres (Carr et al. 1998, Vertes 2004). In the future, this can be tested in the future by lesioning these callosal pathways and measuring cellular activity across hemispheres.

*Increased Cellular Expression in BMA after HFS*

We also found a higher number of cells expressing c-fos in the BMA of stimulated animals (mean=32+/-4.4, n=3) compared to Sham+Electrode animals (mean=20+/-2.3, n=3). While the "By Section" t-test showed this result to be significant (p=0.03), the "By Subject" analysis did not show statistical significance (p=0.39). While it is likely IL HFS leads to increased cellular activity in this region, more subjects are needed to stastically validate our finding. Specifically, more subjects will account for variance between subjects, which could have been caused by differences in quality of perfusion between subjects.

The effect of IL HFS on cellular activity in the amygdala is also very relevant to understanding the mechanism by which Cg25 DBS acts because of its strong interconnectivity with the IL (Vertes 2004, Conde et al. 1995, Mcdonald 2001 and Hurley et al. 1991). If confirmed to be statistically significant, our finding would support the hypothesis that Cg25WM DBS affects cellular activity in remote regions by activating efferents from Cg25 and/or antidromically activating afferents to Cg25 (Gradinaru et al. 2009, Vitek 2002). While our findings shed some light on the general mechanism of Cg25WM DBS, they could also contribute more specifically. There appears to exist a strong inhibitory feedback loop between the IL and the amygdala with strong excitatory efferents from IL to the amygdala (Milad et al. 2002) and strong inhibitory afferents to the IL from the amygdala (Perez-Jaranay et al. 1991). If confirmed to be significant, our findings could support previous work suggesting that this inhibitory feedback loop between the IL and the amygdala could lead to long-term suppression of IL activity (Madsen et al. 2008).

*Increased Cellular Expression in DG after HFS*

In order to determine the effect that acute IL HFS has on cellular activity of limbic regions that are polysynaptically connected to the IL, we studied cellular metabolic changes in the DG. We found there to be a higher number of cells expressing c-fos in the DG of HFS rats (mean=20+/-3.8, n=3) than in the DG of Sham+Electrode rats (mean=20+/-3.8, n=3). As with the BMA, the "By Section" analysis showed these results to be significant (p=0.02), but the "By Subject" analysis did not show statistical significance (p=0.28). Again, while it is likely that IL HFS leads to increased cellular activity in the DG, more subjects are required to account for variance seen between subjects.

The DG is polysynaptically connected to the IL through the amygdala (Kazuho et al. 2003) and the CA1-CA2 fields of the hippocampus (Vertez 2004, Hurley et al. 1991, Conde et al. 1995). Additionally, the DG is intricately involved in depression as it undergoes significant metabolic changes during depression and after anti-depressant activity (Mayberg et al. 2000, Sheline 2003). Specifically, the DG is one of two sites in the brain for neurogenesis (the creation of new cells) (Kempermann 2003). Many consider this process to play a critical role in depression. Also, a study of functional hippocampal microcircuitry showed that DG cellular activity decreases during the depressed state and increases in response to antidepressant interventions (Airan et al. 2007). Our findings, if statistically verified, would support this idea that DG cellular activity is critically involved in anti-depressant activity. More generally, it suggests that Cg25WM DBS can acutely affect the cellular activity of regions that are involved in depression, but are not directly connected to the IL.

*Additional Regions*

Our findings in the BMA and the DG suggest that Cg25WM DBS acutely affects other remote regions in the brain. One area of interest is the nucleus accumbens (NAcc), which is directly connected to Cg25 and intricately involved with reward and pleasure circuits in the brain (Ikemoto et al. 1999, Johansen-Berg et al. 2007, Gutman et al. 2008). Cg25WM DBS acute activation of the NAcc could be responsible for the acute improvement in mood seen in the OR during Cg25WM DBS. Alternatively, the amydala-IL inhibitory feedback loop discussed earlier could also be responsible for this sudden change in mood. Additionally, Cg25 has also been shown to have strong connections to the frontal pole, hypothalamus, the dorsal thalamus, and the dorsal brainstem, which have all been implicated in anti-depressant mechanisms (Johansen-Berg et al. 2007, Gutman et al. 2008). Cg25WM DBS could be influencing the cellular activity in these various cortical, limbic, and viceromotor regions through efferents to these regions and/or antidromically through afferents from these regions. Further work needs to in order to characterize these interactions both acutely and in the long-term.

**Limitations**

While this study produced some relevant findings, there were several limitations. First, the technique used to count cells was not completely objective as cell counting was done by a biased observer. Even though every effort was made to only count cells with nuclear c-fos staining, the technique may have been inaccurate. In order to resolve this issue in the future, images will be quantified by an unbiased observer who is unaware of the experimental design.

Another limitation of the study was the low number of subjects with usable BMA and DG sections. As discussed earlier, the statistical significance of our BMA and DG results cannot be verified without additional subjects.

Also, we were unsure of which side of the BLA and DG sections was ipsilateral to the electrode as sections might have been flipped over during transfer between wells. Even though bilateral counts were done in all sections, we were unable to do any meaningful analysis on the hemispheric extent of unilateral HFS. In the future, we will use a blade coated in DAPI to make an incision along the stimulated side of the brain.

Additionally, we are unsure of the extent to which the electric field directly affects the cellular regions in the rodent brain. Without a negative control, we cannot be certain that the electrical field generated from the IL HFS is not large enough to increase cellular activity in the BMA and DG.

Finally, the variation in the manner in which rats were sacrificed is a possible statistical confound, particularly for the cellular activity in the amygdala. Even though all rats were injected with euthasol, some rats struggled more and may have had a fear response that lasted longer. In the future, the rats will not be allowed to recover from anesthesia and they will be sacrificed with an overdose of isofluorane.

**Conclusion**

In conclusion, we report two key findings regarding acute IL HFS: 1) it significantly increases cellular activity locally in the IL, and 2) there is no significant difference in IL cellular activity ipsilateral and contralateral to the electrode. These findings reveal important information about the local effects of acute IL HFS and suggest

that Cg25WM DBS does not acutely suppress Cg25 activity by directly inhibiting local neurons. Additionally, we report data suggesting that acute IL HFS increases cellular activity in the BMA, which is monosynaptically connected to the IL, and the DG, which is polysynaptically connected to the IL. While additional subjects are required to validate the statistical significance of these results, the current data is promising. If statistically verified, these findings would suggest that Cg25WM DBS can acutely affect various monosynaptic and polysynaptic regions that are involved in depression.

**Future Directions**

The most immediate future direction is to evaluate the cellular effect of IL HFS on the nucleus accumbens (NaCC) to determine whether or not Cg25WM DBS might be activating reward circuits. This would shed light on the source of the acute improvement in mood seen in depressed individuals. Also, studies should be done to better understand the importance of the stimulation parameters, the time course of cellular activity, and the spatial extent of electrical stimulation. Variables that can be altered include the frequency, pulse width and voltage of stimulation, and the time between electrical stimulation and sacrifice of the rat. Additionally, studies can be undertaken to understand the cellular mechanisms underlying DBS of other regions such as the NaCC (Schlaepfer et al. 2008) and ventral capsule (Malone et al. 2009).

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Table 1: Statistical Analyses of Mean Cell Counts in IL, BMA, and DG of HFS and Sham+Electrode (Sham+E) subjects.

| Region of Interest | Mean Cell Count in HFS subjects (+/- SEM) | Mean Cell Count in Sham+E subjects (+/- SEM) | "By Section" t-test | "By Subject" t-test |
|---|---|---|---|---|
| IL | 47 (+/- 3.9) | 20 (+/-2.8) | N=16, 14 (p<0.001)** | N=4 (p=0.004)* |
| BMA | 32 (+/-4.4) | 20 (+/-2.3) | N=16, 18 (p=0.03)* | N=3 (p=0.39) |
| DG | 42 (+/- 7.9) | 20(+/-3.8) | N=17,15 (p=0.02)* | N=3 (p=0.278) |

*=p<0.05

**=p<0.01

***=p<0.001

Figure 1: Morphologically similar sections of the IL (A), BMA (B), and DG (C) were used for cell counting.

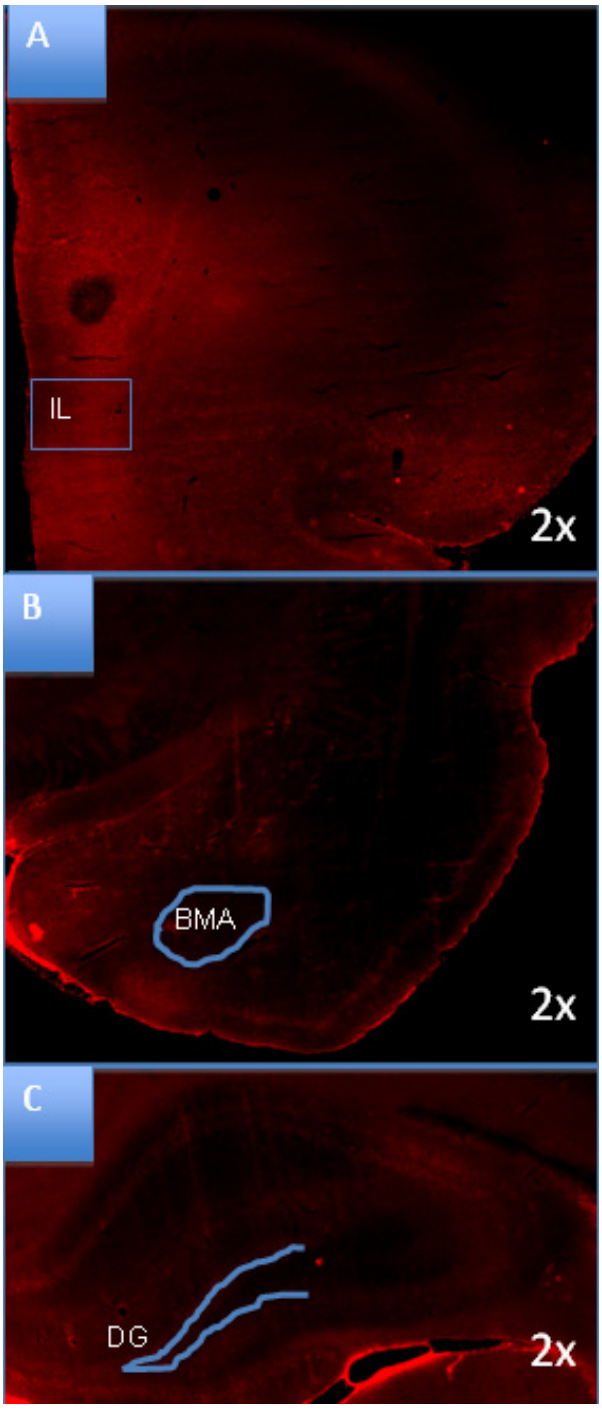


Figure 2: Electrode track in an IL section visualized with DAPI immunofluoresence (left). DAPI immunofluoresence was absent in an IL section without an inserted electrode (right).

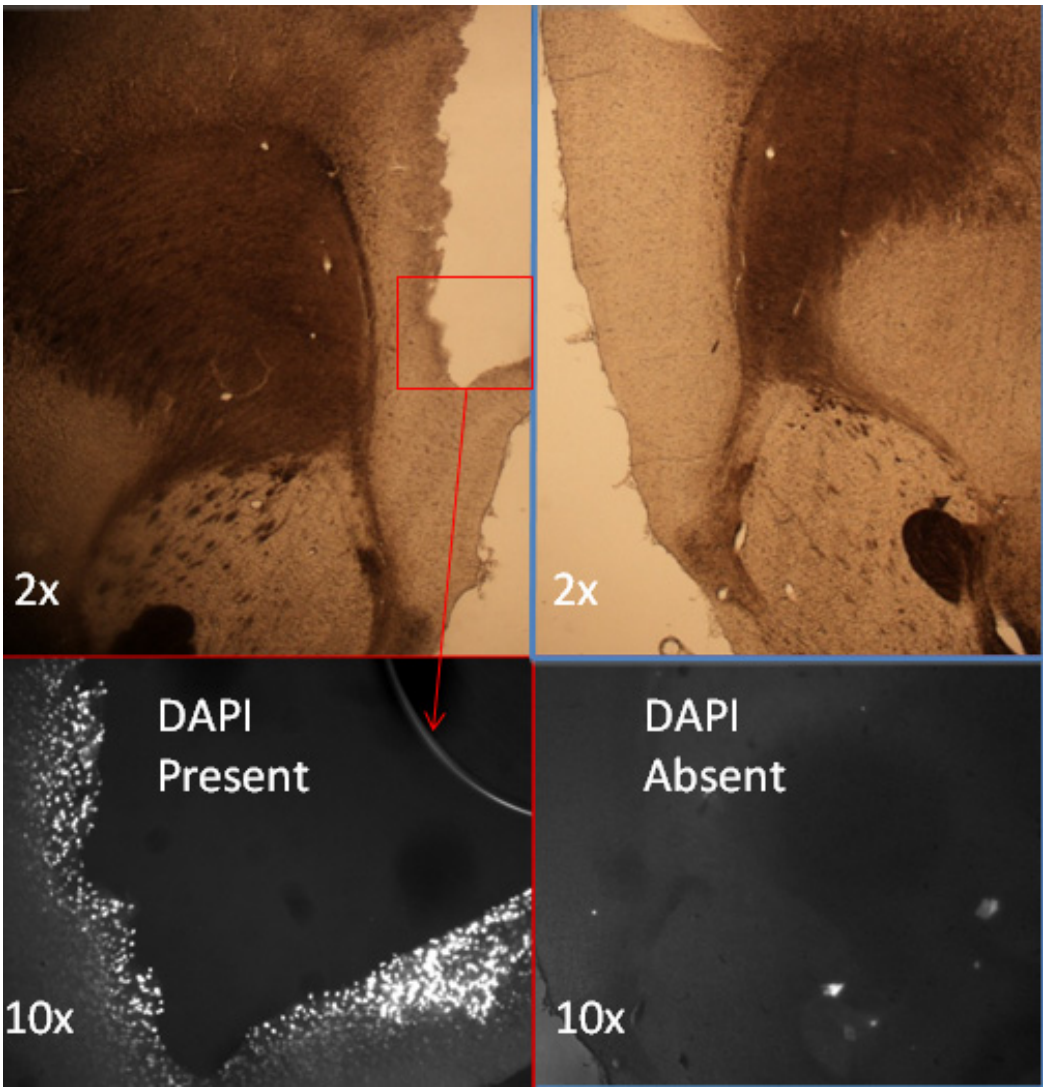
2x
2x
DAPI
Present
DAPI
Absent
10x
10x

Figure 3: Co-localization of NeuN (green) and c-fos (red) is present (A, B, C) indicating that c-fos is expressed in mature neurons. Also, absence of the c-fos primary antibody eliminates nuclear staining (D).

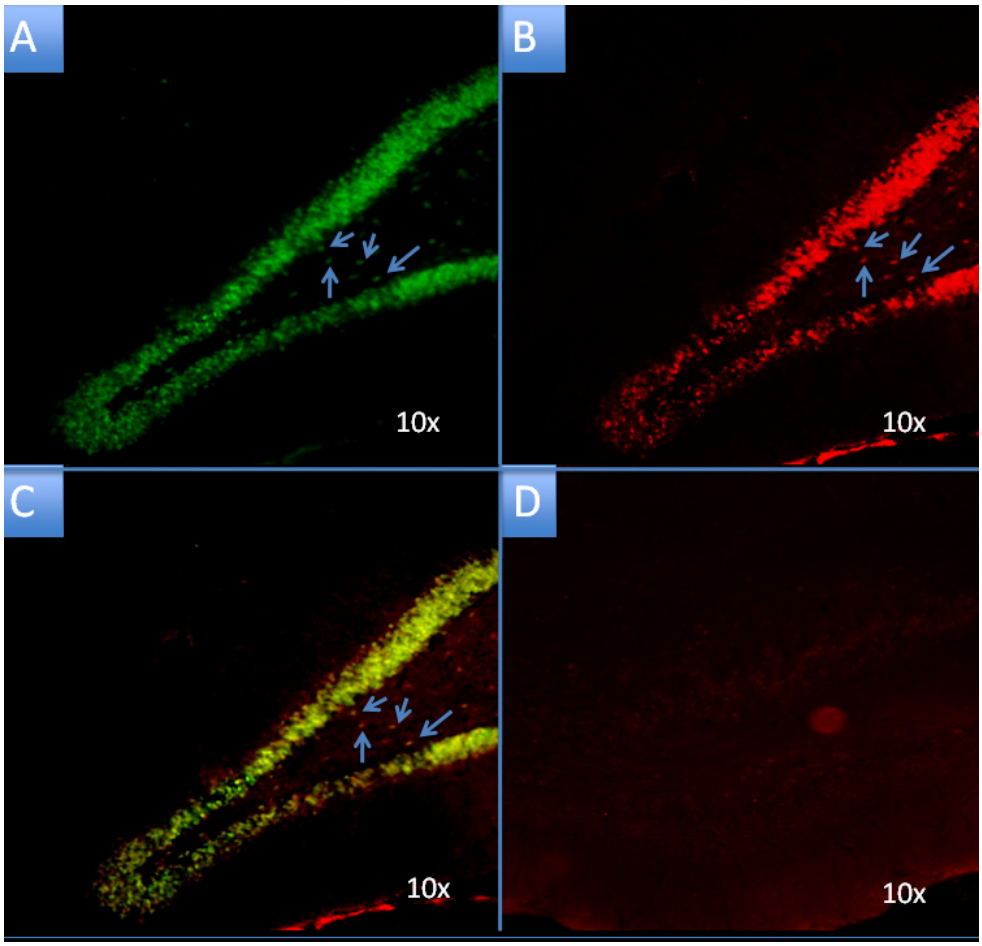

Figure 4: Representative c-fos expression in IL, BMA, DG sections from HFS (A,B,C), Sham+electrode (D,E,F), and Sham-electrode (G,H,I) subjects. HFS subjects showed increased c-fos expression in all three regions of interest.

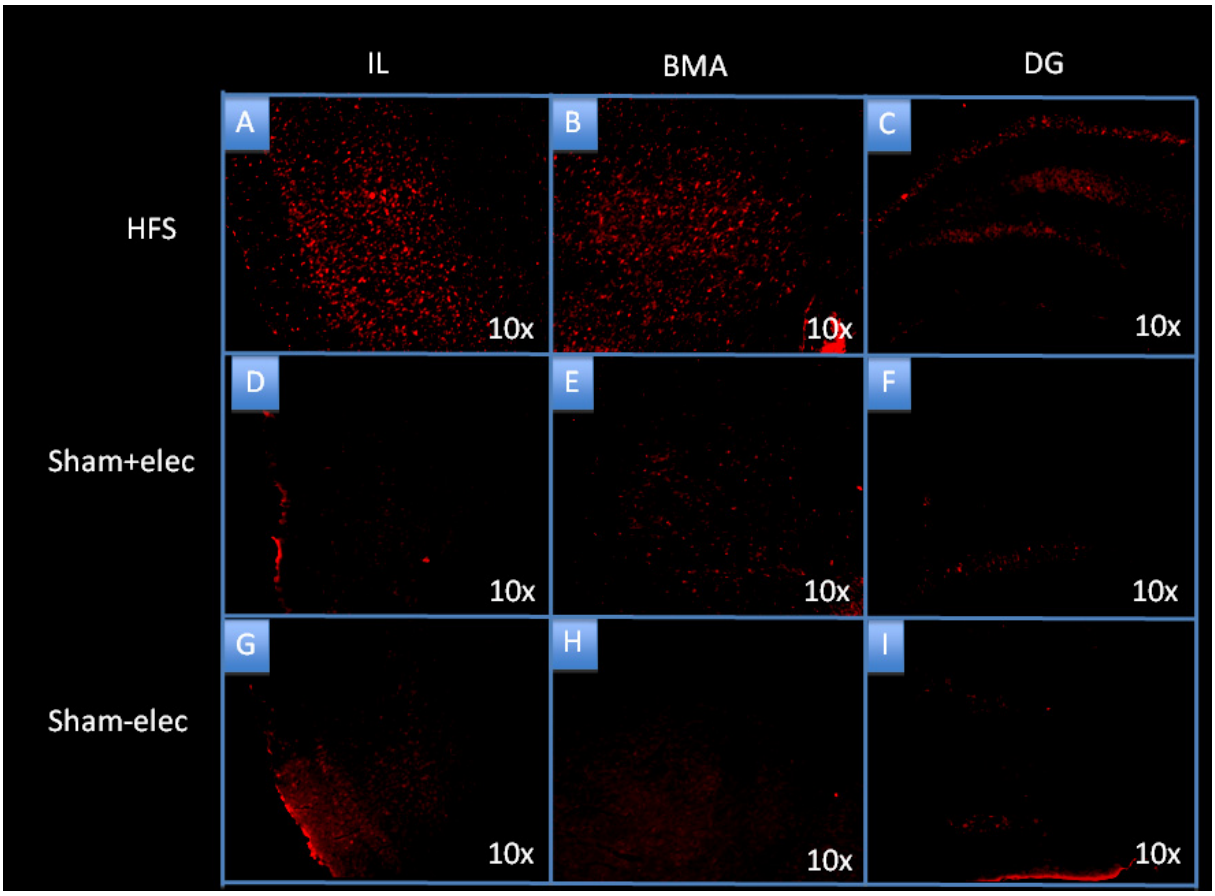

Figure 5: Mean Cell Counts from IL, BMA, and DG sections from HFS and SHAM+Electrode groups. "*" indicates statistical significance (at least $p<0.05$) as shown by the "By Section" t-test and "**" indicates significance shown by the both the "By Section" and "By Subject" t-test.

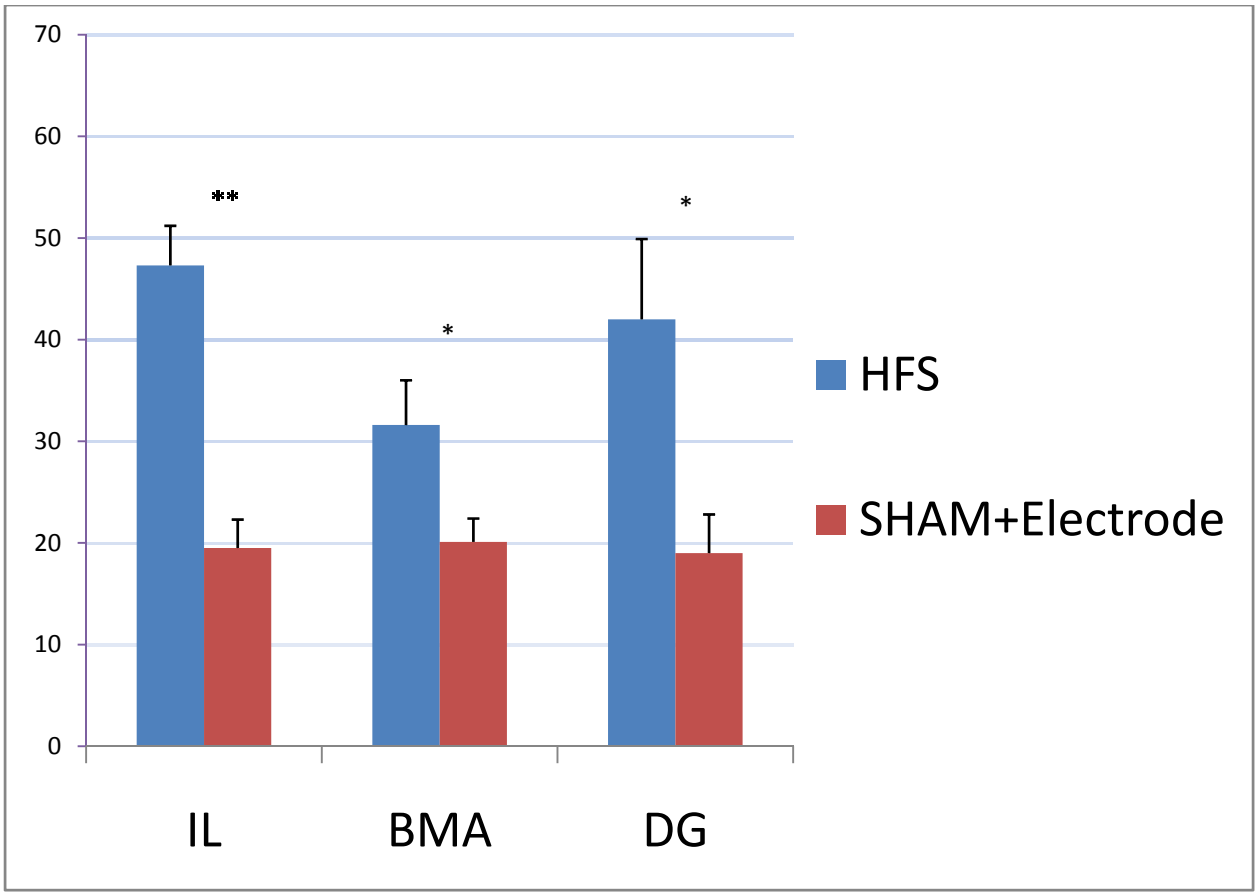

Figure 6: Mean Cell Counts from the stimulated IL and non-stimulated IL from HFS brains. No significant difference was found.