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Tissue Response to Deep Brain Stimulation and Microlesion: A Comparative Study

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Abstract

Objectives—Deep Brain stimulation (DBS) is used for a variety of movement disorders, including Parkinson's disease. There are several theories regarding the biology and mechanisms of action of DBS. Previously, we observed an upregulation of neural progenitor cell proliferation in post-mortem tissue suggesting that DBS can influence cellular plasticity in regions beyond the site of stimulation. We wanted to support these observations and investigate the relationship if any, between DBS, neural progenitor cells and microglia.

Methods—We employed naïve rats in this study for DBS electrode implantation, stimulation and microlesions. We used immunohistochemistry techniques for labeling microglial and progenitor cells, and fluorescence microscopy for viewing and quantification of labeled cells.

Results—We present data that demonstrates a reciprocal relationship of microglia and neural precursor cells in the presence of acute high frequency stimulation. In our hands, stimulated animals demonstrate significantly lower numbers of activated microglia (P=0.026) when compared to microlesion and sham animals. The subthalamic region surrounding the DBS stimulating electrode reveals a significant increase in the number of neural precursor cells expressing cell cycle markers, plasticity and precursor cell markers (Ki67; P=0.0013, MCM2; P=0.0002).

Interpretation—We conclude that in this animal model, acute DBS results in modest local progenitor cell proliferation and influenced the total number of activated microglia. This could be of clinical significance in patients with PD, as it is thought to progress via neuroinflammatory processes involving microglia, cytokines and the complement system. Further studies are required

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to comprehend the behavior of microglia in different activation states and their ability to regulate adult neurogenesis under physiological and pathological conditions.

Keywords

Deep brain stimulation; Neural Precursor cell; Parkinson's disease; Plasticity; Microglia

Introduction

Deep brain stimulation (DBS) is becoming increasingly prevalent for addressing medication refractory symptoms in some neurodegenerative and neuropsychiatric disorders. It consists of electrode implantation into specific parts of the brain and delivery of local current to influence a neural network [1],[2]. Current targets for patients with Parkinson's disease (PD) are subthalamic nucleus (STN) globus pallidus internus (GPi) and ventral intermediate nucleus of the thalamus (VIM). Although clinical efficacy and usage of DBS have been investigated by many studies, the biology and mechanism(s) of action, specifically in the context of microglial cells and the neural stem cell compartment are not understood. Our preliminary results from human post-mortem studies of PD-DBS revealed up-regulation of neural progenitor cells (NPC) in the electrical stimulation field [3]. Despite demonstration that certain physiological stimuli, environments and physical activity can stimulate neural precursor cell genesis [4] [5] [6], it is unclear what cellular and molecular components facilitate the necessary neuropermissive environment. It has been recently described that it is possible to increase cell proliferation in the substantia nigra of 6-OHDA microlesioned rats under conditions of enriched environments (physical activity) [7], [8]. However, this proliferative response alone cannot explain the efficacy of DBS in all disease contexts.

Microglia normally exist in the central nervous system (CNS) in a quiescent, resting or 'ramified' state with a round cell body and thin processes, constantly monitoring the physiological environment [9]. In the event of an injury, microglia proliferate rapidly and undergo significant morphological alterations [10] [11] [12] [13] [14]. Initially, microglial cell bodies enlarge, their processes become thicker and begin to retract [15]. Microglia appear amoeboid as they progress toward becoming more phagocytic and pleiomorphic [16] [17]. Inflammatory processes in the CNS involving activated microglia [18] are believed to play a role in neuronal cell death in PD[19] [20] [21] [22]. Initial microglial reaction is reparative, however uncontrolled, it can lead to a release of pro-inflammatory and toxic factors [23]. Anti-inflammatory drugs and radiation [24]have been shown to repress microglial activation and exert neuroprotective effects in the CNS following injury [24] [25] [26].

Microglia have become candidates for modulation of neurogenesis in the injured and healthy brain. Up-regulation of microglia correlates to the production of neurotoxic factors, and inversely to neurogenesis[27]. There is evidence to show that local environmental cues, (including microglia and secreted proteins) influence the functionality of NPCs. In a recent study by Mosher et al, 2012 [27], it was reported that microglia were preferentially more densely populated in the neurogenic niches, and were present in close proximity with NPCs. Therefore, endogenous microglia and NPCs are well-posed to interact. A better

comprehension of the type of cell communication in the neurogenic niche and the ability of NPCs to modulate their environment indicated that NPCs maybe regulated by microglia, and in turn were capable of regulating microglia.

In this study, we established a rodent model to investigate the effect of a microlesion versus acute DBS on the interaction of microglia and NPCs in the context of electrical stimulation. We hypothesized that high frequency electrical stimulation (HFS) would activate molecular mechanisms decreasing the detrimental effects of activated microglia, perhaps through an upregulation of NPC proliferation [28], [29].

Material and Methods

All abbreviations used in the materials and methods are elaborated in Table 1.

Experimental animals

The University of Florida's Institutional Animal Care and Use Committee (IACUC) approved the study, and all procedures were in accordance with IACUC guidelines. Adult, male Sprague-Dawley rats (Harlan Labs, 200–250g) were used in these studies. In the final analysis, there were 5 rats in the stimulation group, 4 rats in the sham group, 5 rats in the microlesion group and 5 rats in the stimulation+BrdU group. Animals were housed with access to food and water ad libitum in a room, which was maintained at a constant temperature (20–22°C) on a 12h light-dark cycle.

Surgical Procedures, Electrical stimulation of the STN and BrdU administration

Rats were either unilaterally microlesioned, or implanted with stimulating electrodes. Animals were anesthetized using 5% isoflurane-O_2 mixture. Animals' heads were fixed to a stereotactic frame (Stoehling Instruments). Bregma was carefully delineated and marked. Bipolar stainless steel electrodes (Plastics One) (0.008inch diameter; approximately 200um) were implanted unilaterally in the subthalamic nucleus in the following coordinates relative to bregma (AP −3.8mm and ML+2.5mm, DV −7.6 from dura), according to Paxinos and Watson (2005). In the stimulation group as well as the sham group, the electrode was secured using screws and dental acrylic. In the microlesion group, animals were not implanted with electrodes. The microlesion or microinjury was generated unilaterally using a sterile Hamilton needle (33 gauge, approximately 200um) at the above stereotactic coordinates and withdrawn gently. Following surgeries, all animals were allowed to recover for 2 weeks. At the conclusion of experiments, the accuracy of electrode placement was confirmed histologically by standard Hemotoxylin & Eosin (H&E) staining. The placement of the DBS lead was considered accurately targeted in the STN if the electrode tip could be confirmed by examination of post-mortem sections.

High frequency electrical stimulation was carried out after a two-week recovery period at the following parameters: 130Hz frequency, 50us pulse width, at an intensity of 50uA for 1h daily, for two weeks post recovery. The parameters have been described in the literature and were selected to be therapeutic but below the threshold intensity for contralateral forepaw dyskinesia [30]. For HFS, animals were fixed to the stereotaxic frame and anesthetized. Stimulation was conducted using a World Precision Instruments Inc. stimulator, for one-hour

daily, for two weeks post recovery. Microlesion and sham animals were also anesthetized, but not stimulated.

Bromodeoxyuridine (BrdU) (Sigma) was dissolved in saline and sterile filtered. Animals (n=5) received a total of 50 mg/kg of BrdU intraperitoneally on the last three days of the stimulation paradigm.

Sacrifice

At the conclusion of the two-week stimulation period and following the last BrdU injections, the animals were deeply anesthetized (pentobarbital, 60mg/kg) and transcardially perfused with phosphate buffered saline (100mL PBS), followed by 4% paraformaldehyde (100mL PFA). The electrodes were carefully removed at this time, attempting to keep the tissue damage to a minimum, and the brains were removed from the skull and postfixed overnight in PFA and subsequently placed in 30% sucrose solution overnight in preparation for cryopreservation. Brains were then frozen in Optimal Cutting Temperature (OCT) compound and stored at −80°C until analysis.

Immunohistochemistry, microscopy and cell counting

Frozen brains from all the groups were cut on a cryostat at 5μm and mounted on SuperFrost Plus slides (Fisher).

Sections were immunostained with the following primary antibodies: MCM2 (Cell Signaling, 1:500), Ki67 (Novo Castra, 1:1000), Iba1 (Millipore, 1:500). Following block in serum for 1h, tissue was incubated in each of the antibodies overnight, at 4°C. Tween (0.1%) was added to the Tris buffer (0.1M) during incubation to permeabilize cell membranes. Following primary incubations, sections were incubated in secondary antibodies (Alexa Fluor 488, or 594) for 1h. Slides were coverslipped using a Vectashield (Vector Labs) containing DAPI. A total of 6 sections of interest were analyzed per antibody using a Nikon fluorescent microscope. Only cells whose nuclei (stained with DAPI) were unambiguously associated with the given marker were scored.

For sections pre-labeled with BrdU, slides were first incubated in HCl (1N) for 30min to break open the DNA in order to achieve a nuclear stain. The sections were then washed using borate buffer (0.1M) three times. Following this, the sections were blocked in serum and treated with the antibody BrdU (Abcam, 1:100) as described above.

Microscopy and Statistical analysis

Slides were viewed on a Nikon fluorescent microscope, equipped with a camera. The boundaries of the subthalamic nucleus were delineated according to the Paxinos and Watson Rat Atlas (~840um thick). The caudal third ventricle (C3V) was defined as that tissue slice containing both the dorsal hippocampus (∼3.8 to 4.5 mm from bregma) and the third ventricle, following the Paxinos and Watson Rat Atlas. The total number of immunoreactive (IR) cells were estimated by using a 10 × 10 mm grid, superimposed on images taken at 20× magnification, using Adobe Photoshop®. Profile counts were done on every 25th section containing IR cells by an observer blinded to the animal treatment groups and to the identity

of the sections (HK), and a total of six sections were counted per group. The contralateral side of the brain was used as control without treatment. Cell counts were obtained in a total of six sections on the contralateral (control) side as described above. For statistical analyses, Kruskal-Wallis, a non-parametric test was used.

Results

Microglial phenotypes around the site of electrode/microlesion

In the region of the implanted stimulation electrodes in the subthalamic nucleus, microglial morphology was studied using Iba1 immunolabeling. While both subtypes of microglia can be characterized by cytoplasmic staining, ramified microglia were clearly distinguishable from amoeboid microglia (Fig 1a, 1c). The ramified cells have a smaller cell body when compared to the amoeboid cell type, which have a denser cell body. Furthermore, the ramified cells typically possess thick, radially projecting processes when compared to the activated cells, which have few to no processes.

Activated microglia were identified around the site of the stimulation electrode and were readily recognized by their typical amoeboid morphology, with round cell bodies, with few to no ramifications or processes. This type of a morphological change from quiescent/ramified morphology to the amoeboid/macrophage-type is typically seen in microglia after infection, traumatic injury and/or neurodegenerative disease. As we moved away from the site of stimulation (1mm or more), the number of activated microglia dramatically decreased and there was a predominance of ramified or normal microglia (Fig. 1a, 1b, 1c). Quantitative analysis showed that in sham and microlesion-only animals, there was an increase in the density of activated microglia, when compared to quiescent microglia (Fig. 1d). The number of Iba1 positive amoeboid cells was significantly greater in the sham and microlesion only groups ($P=0.0026$) when compared to the stimulated group.

Cell proliferation around the site of electrical stimulation in DBS animals

BrdU—BrdU was administered to label dividing cells after the stimulation paradigm in a subset of animals after applying STN-DBS at similar parameters to those used in patients (130 Hz, 2.5 V, 60 usec) for 1h everyday under a state of general anesthesia. These animals were subsequently sacrificed the last day after the BrdU injection paradigm. Animals that received HFS-DBS showed an increase in BrdU-positive cells in the region immediately surrounding the site of electrical stimulation (Fig. 2a) and in an increase in the 3rd ventricle region (Fig 2b), confirming our previous observations from post-mortem human tissue[3]. The maximum number of labeled cells was observed 5 to 7 days post stimulation.

Quantitative analysis of proliferation positive cells in post-DBS animals

MCM2—For purposes of quantifying changes in proliferative cells in DBS and non-DBS animals, an experienced analyst (HK) who was blinded to the experimental groups evaluated the number of MCM2 positive cells around the site of the lead tip in stimulated, sham, as well as microlesion animals when compared to the contralateral (untreated) side (Fig. 3a, b, c). MCM2 was significantly increased ($P=0.0002$) in sections from HFS-DBS animals, indicating NPC proliferation as a result of electrical stimulation (Fig. 3d).

Correlation between amoeboid microglia density and progenitor cell proliferation in STN

Ki67 and Iba1—In order to further confirm our findings and correlate the microglial phenotype to progenitor cell proliferation, we quantified proliferative cells (Iba1 positive; green, Ki67 positive; red) in the region immediately surrounding the site of electrical stimulation, sham or microlesion (Fig. 4a, 4b, 4c). The number of Ki67 positive cells was fewer in the sham and microlesion groups when compared to the stimulated group (P=0.0013) (Fig. 4d). Conversely, the number of activated microglial cells was greater in the sham and microlesion groups when compared to the stimulated group (from Fig. 1d).

Discussion

In the CNS, resident microglia comprise approximately 5–20% of glia. In their resting state, microglia are typically ramified and their fine processes intertwine into their surrounding cells. As “sentinels” of the brain, microglia are able to switch from resting to an activated state upon injury. The resulting morphological change prepares them for phagocytosis, however recent evidence indicates that microglia may also be involved in regulation of neurogenesis as well as in migration [32] [33]. In the present study, we sought to delineate changes in microglial phenotypes in the presence and absence of high frequency electrical stimulation, and in the context of progenitor cell proliferation. Our findings show the presence of amoeboid and ramified microglia in the STN of stimulated and also in sham and microlesion animals. A significant increase in the density of amoeboid microglia was observed in the STN of the sham and microlesion only animals, and the presence of more progenitor cells in stimulated animals. These findings support the recent observation from human studies of the DBS Brain Tissue Network that there is an upregulation of progenitor cells around the DBS electrode, and around the third ventricle. Furthermore, our observations correlate with the recent demonstration in human postmortem PD tissue that there is an elevation of activated microglia in the STN when compared to HF-DBS tissue [31]. This study by Pienaar et al presents the novel hypothesis that DBS might influence the neuroinflammatory response in PD patients [31]. Pienaar et al demonstrated that in human postmortem subjects, the number of activated microglia was reduced in the STN of the STN-DBS cohort when compared to non PD DBS cases. Our study lends support to this finding. It has been previously reported that new glial cells can be generated in a rat lesion model (6-OHDA) as a result of increased cellular plasticity in an enriched environment [34]. Further, Steiner, B. et al (2008) showed that a unilateral ablative STN lesion can induce transient changes in the plasticity of the substantia nigra, by inducing the increase in proliferative cells, a proportion of which were identified as microglia [35]. Another study in a rat model of stroke revealed that electrical stimulation of the relevant region (cortex) suppressed microglial activation, whereas in control animals there was rampant microglial activation as was confirmed by Iba1 staining [36]. These findings further strengthen our current observations.

Our study shows that high frequency stimulation at clinically relevant parameters drives progenitor proliferation around the site of electrical stimulation and also suppresses microglial activation. While the activation state of microglia is likely to be regulated by numerous molecules, there is significant evidence accumulating that depending on their state

of activation, microglia can either be supportive or detrimental for adult neurogenesis in the healthy, as well as the injured brain. Recent imaging studies have demonstrated that patients with idiopathic PD have an increase in neuroinflammation in the basal ganglia, striatum, and frontal and temporal cortical regions. This has been corroborated in postmortem tissue, where activated microglial cells are present surrounding dopaminergic neurons, which are impaired in the SN, suggesting neuroinflammation. It is hypothesized from these results that microglial activation likely occurs at an early stage of the disease either before (or in parallel with) the important loss of dopaminergic neurons [37, 38]. There is now a preponderance of evidence in the literature suggesting that microglia, depending on their state of activation can either be beneficial or detrimental to adult neurogenesis, in both the diseased/injured and healthy brain [39]. Therefore, it is relevant to tease out the differences in activation states of microglia and the regulation of adult neurogenesis in physiological as well as in pathological conditions. It is possible that the functional role of microglia in neurogenesis depends on the location of the microglial population, and hence the protein expression of these cells but this remains unknown [39]. Early studies have provided evidence that inflammation and microglial activation can be detrimental for adult neurogenesis, however recent experimental evidence shows their involvement to be complex and not necessarily detrimental [39]. Whether the role and function of microglia in neurogenesis is dependent on differences in environmental cues and whether microglial function determines neurogenic potential are important future questions, which still need to be addressed. One must be cautious and distinguish between neurogenesis in the niche regions when compared to other parts of the adult CNS such as the SN or STN, where cellular plasticity, but not neurogenesis occurs. Further, our results have to be viewed in the context of the acute stimulation paradigm, which is significantly different than the constant stimulation that patients with PD DBS undergo. There is new enthusiasm in the field that various types of electrical stimulation (electroconvulsive therapy, transcranial magnetic stimulation (TMS), deep-brain stimulation (DBS), vagal nerve stimulation, epidural stimulation, and transcranial direct current stimulation (tDCS) are effective for a variety of disorders. The reason for the success of this relatively new method of treatment has been attributed to the following potential mechanisms: alteration of cortical excitability [40], modulation of the brain inflammatory response [31], permeability of the blood–brain barrier [41], brain perfusion and neuronal apoptosis [42, 43], and enhancement of neural plasticity [44]. Neural plasticity could include synapse formation, dendritic architecture, and also neurogenesis [45] [46]. Exogenous electrical fields have been shown to influence directional migration and differentiation of NPCs [47] [48] in vitro, and it is likely that these results are similar in in vivo systems, but the results and mechanisms are yet to be elucidated. While our observations support the concept of neural stimulation inducing changes in local cellular plasticity by driving NPC proliferation, the BrdU incorporating compartment could not be further characterized, likely because of the developmental stage of the cells (antigen markers of neural cells are not yet expressed). Further cell culture studies and time course models will likely resolve this problem. Furthermore, determining whether signaling via the Wnt/ß-catenin pathway is responsible for the NPC proliferation was beyond the scope of this study.

In summary, we present a rat model that further supports observations from human subjects and expands into a comparison of electrical stimulation and microlesion therapy. We present

animal data that reveal a reciprocal relationship of microglia and neural precursor cells (NPC) in the presence of acute high frequency stimulation (HFS). These observations add to our understanding of the biological changes that co-exist during DBS and microlesion therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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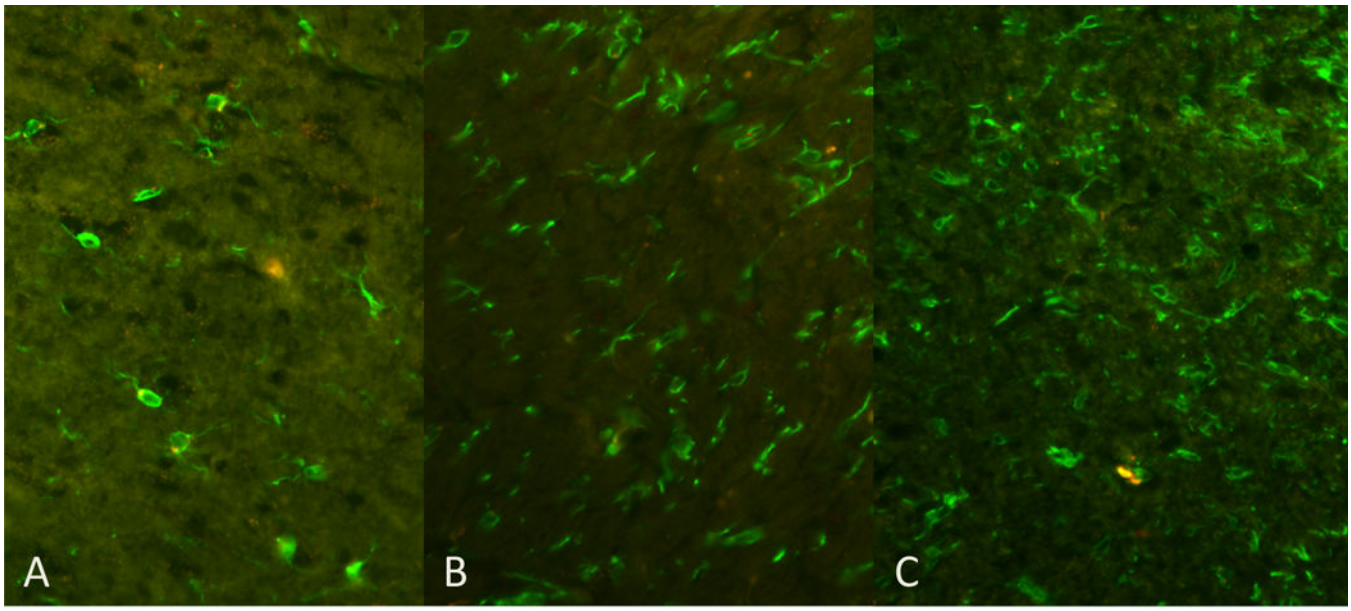

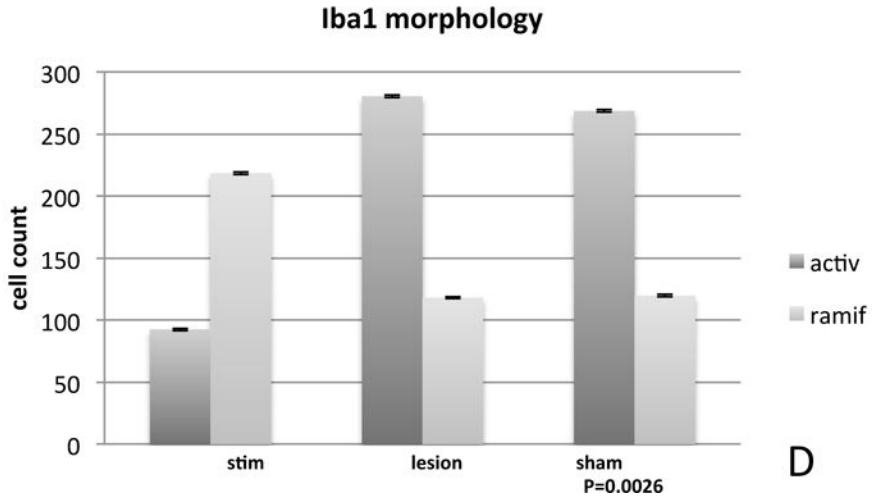

Figure 1.

Morphology of microglia: Figure 1a shows the normal or “ramified/quiescent” state of microglia, with branching processes. Figure 1b shows a mildly activated state of microglia, where the processes are beginning to retract. Figure 1c shows activated or “amoeboid” microglia, where processes are almost completely retracted and the cell bodies are round and macrophage-like. Figure 1d Morphometric quantification of the number of Iba1 positive cells in the STN of HFS animals compared to microlesion and sham animals and is representative of the quantitative difference (P=0.0026) between normal and activated microglia in animals that underwent HFS-DBS and sham animals as well as animals that underwent microlesioning.

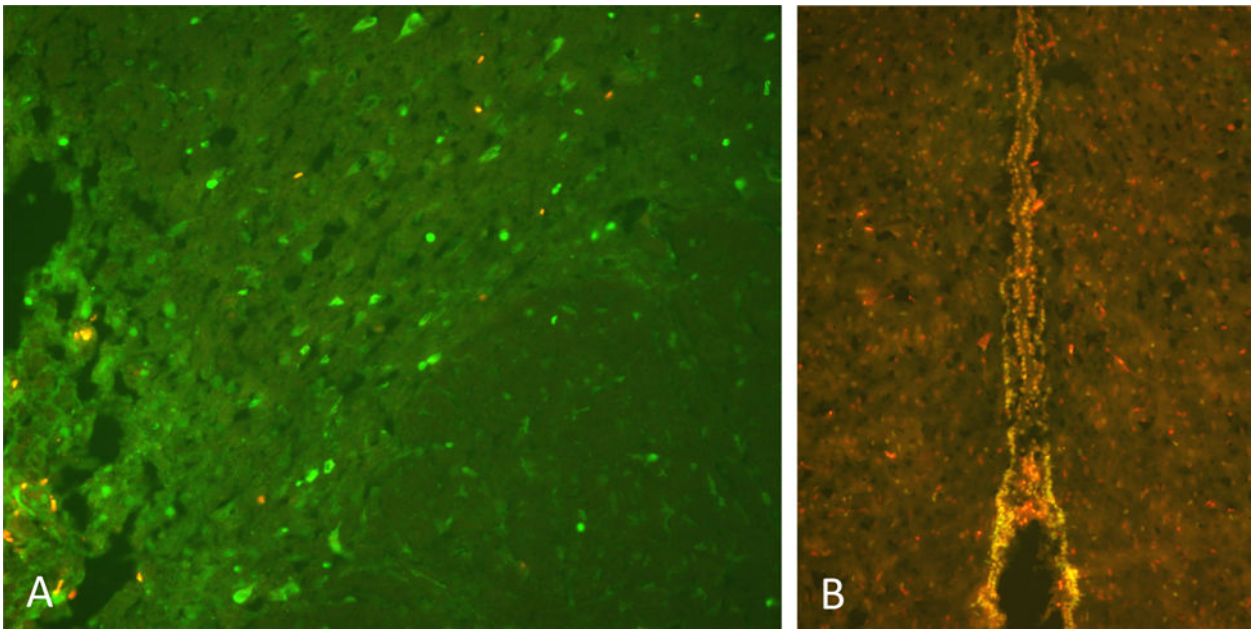

Figure 2.

a): BrdU labeling of proliferative cells immediately surrounding the site of HFS-DBS in the STN. b) Colabeling of Sox2 (green) and BrdU (red) positive cells around the third ventricle in STN-HFS animals.

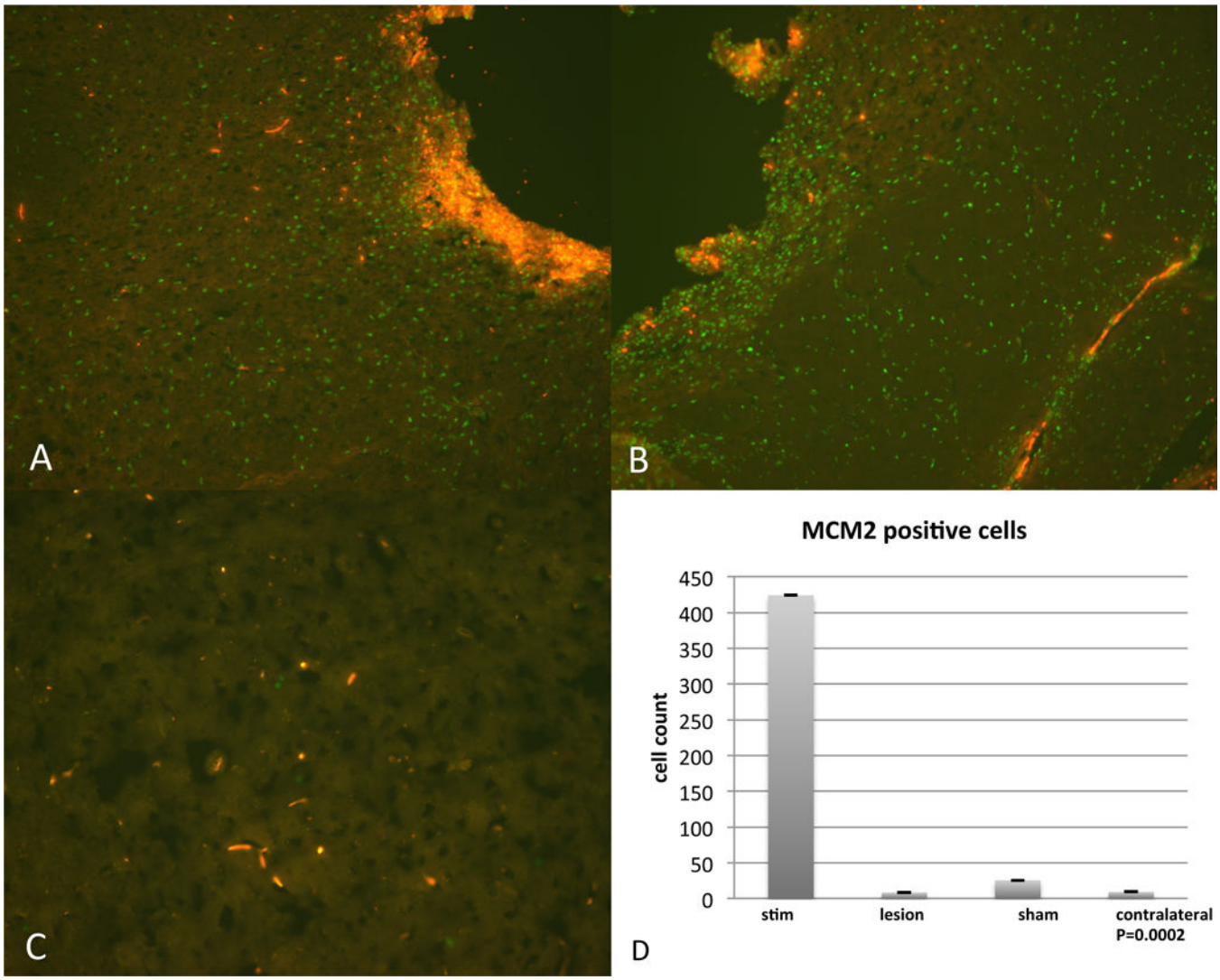

Figure 3.

a, b): MCM2 positive (green) cells immediately surrounding electrode tip in STN-HFS animals. [orange is auto-fluorescent blood]

c): Contralateral side to HFS-STN demonstrating almost no MCM2 positive (green) cells in the STN.

d): Graph showing quantification of the number of MCM2 positive cells in the STN of HFS animals compared to microlesion, and sham animals. Also shown is STN in contralateral side of an HFS animal. Values in graph represent mean number of MCM2 positive cells per group of animals, P=0.0002.

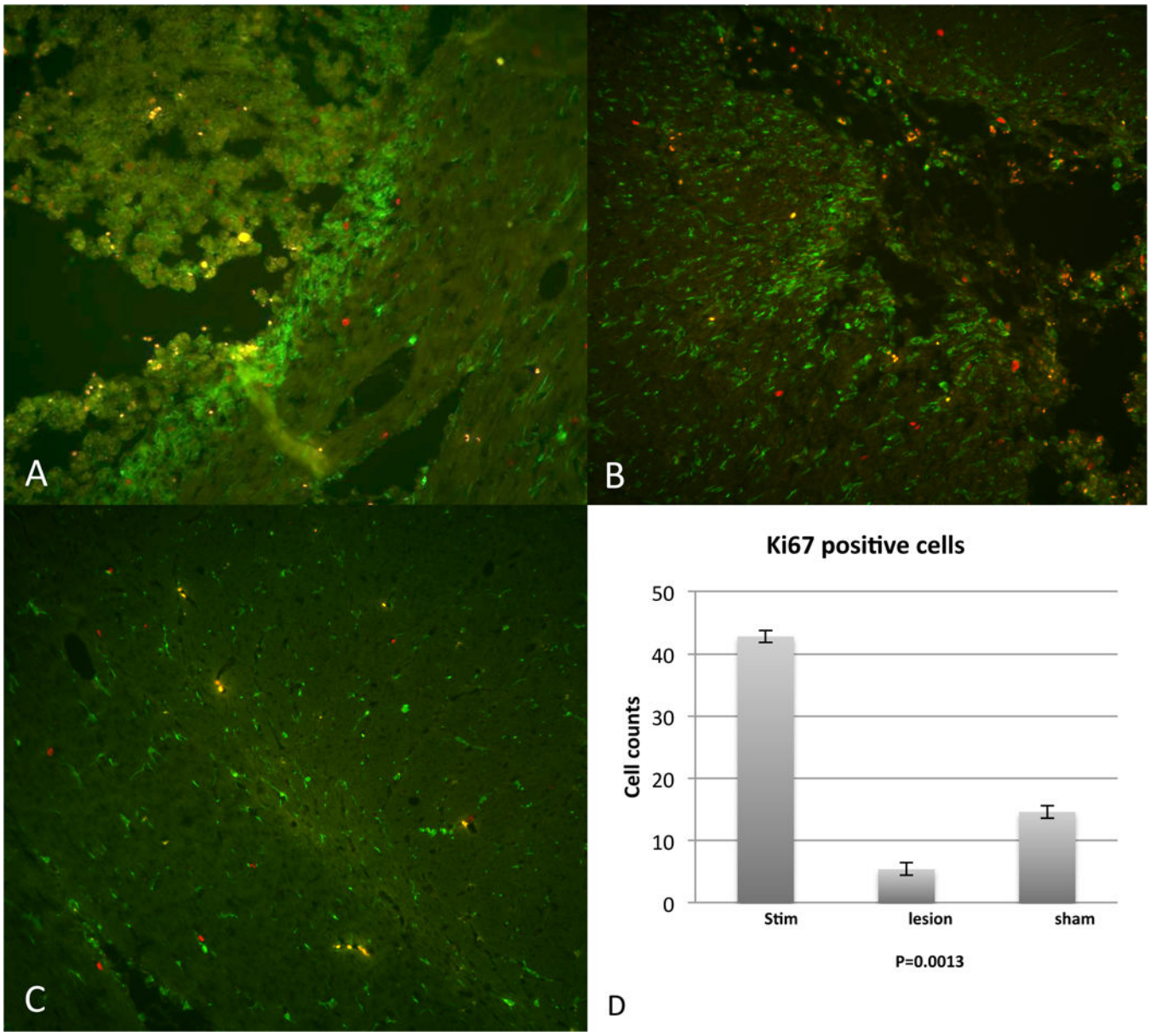

Figure 4.

a), b): Ki67 (red) and Iba1 (green) labeled cells in 2 representative HF-STN animals. Iba1 positive cells immediately surrounding the electrode site demonstrate amoeboid morphology, whereas immediately adjacent to the site of stimulation, cells demonstrate normal distribution and ramified morphology.

c): Bottom panel shows normal, ramified Iba1 microglia in the contralateral STN from HFS animals.

d): Graph representing the quantification of Ki67 positive cells in the STN in stimulated animals versus microlesion and sham animals, clearly demonstrating a significant increase in Ki67 positive cells in the stimulated group alone, P=0.0013.

Table 1

Abbreviation	Scientific name	Use/Definition
PBS	Phosphate Buffered Saline	Buffer solution
PFA	Paraformaldehyde	Used to
BrdU	Bromodeoxyuridine	Synthetic nucleoside, analog of thymidine used in the detection of proliferating cells in living tissues
OCT	Optimal Cutting Temperature compound	This product is a formulation of water-soluble glycols and resins and provides optimal conditions for cryostat sectioning at temperatures of −10°C (14°F) and below.
DAPI	(4′,6-diamidino-2-phenylindole)	Fluorescent label that binds to DNA, used to cell staining and visualization via fluorescent microscopy.
MCM2	Minichromosome maintenance complex	Part of a set of highly conserved proteins involved in the initiation of eukaryotic genome replication.
Ki67	Also known as MKi67	Nuclear protein associated with cell proliferation, and with rRNA transcription. Is active during all active phases of cell cycle.
Iba1	Ionized calcium-binding adapter molecule 1	A calcium binding protein, specifically expressed in microglial cells.