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Journal of Chemical Neuroanatomy 96 (2019) 34–40

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Fornix deep brain stimulation induces reduction of hippocampal synaptophysin levels

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ARTICLE INFO

Keywords:
Deep brain stimulation
Fornix
Neuroplasticity
Synaptophysin

ABSTRACT

Fornix deep brain stimulation (DBS) has the ability to refurbish memory functions in animal models with experimental dementia. One of the possible underlying mechanisms is the acute increase of acetylcholine in the hippocampus. Another suggested hypothesis is neuroplasticity. Recent work in rats has shown that acute fornix DBS can modulate neurotrophic factors as well as synaptic plasticity markers on the short-term. Here, we want to test the hypothesis that acute fornix DBS can also lead to long-term effects on neuroplasticity. Rats received DBS at 100 Hz, 100 µA and 100 µs pulse width for 4 h with electrodes placed bilaterally in the fornix. Seven weeks after stimulation, rats were sacrificed. BDNF, p-CREB, SV2 and synaptophysin immunohistochemistry was performed for their brains. No differences were found in the number of BDNF, p-CREB or SV2 positive cells for fornix DBS rats when compared to sham. Surprisingly, the density of synaptophysin immunoreactive presynaptic boutons was significantly decreased in the CA1 and CA3 subregion of the hippocampus for DBS rats. Therefore, fornix DBS might induce long-term depression related mechanisms.

1. Introduction

Deep brain stimulation (DBS) is a technique that has been used for the treatment of more than 130,000 patients worldwide. It is an interventional minimal invasive neurosurgical procedure that is used to suppress or activate the dysfunctional brain circuits (Sironi, 2011). It involves a pulse generator which sends electrical pulses to the target brain circuits, electrodes which are placed in specific brain regions and an electrical extension to connect the electrode with the pulse generator (Hamani and Temel, 2012; Kringelbach et al., 2007).

DBS has been shown to be an effective treatment for some neurological and psychiatric disorders such as Parkinson's disease (Temel and Visser-Vandewalle, 2006), essential tremor (Benabid et al., 1991), dystonia (Holloway et al., 2006), obsessive compulsive disorder (Denys et al., 2010) and Tourette's syndrome (Ackermans et al., 2006). Promising single case studies and small clinical DBS trials have been carried out in some other neurological and psychiatric disorders such as depression (Bewernick et al., 2010), epilepsy (Andrade et al., 2006), drug addiction (Luigjes et al., 2012), headache (Leone, 2006), anorexia (Wu et al., 2013) and morbid obesity (Halpern et al., 2008). In recent years, DBS has also been explored in Alzheimer's disease (Laxton et al., 2010; Smith et al., 2012).

The idea of applying DBS in order to restore memory loss emerged serendipitously in 2008 when a patient suffering from morbid obesity started to recall autobiographical memories while being stimulated in the fornix/hypothalamus area (Hamani et al., 2008). Based on this case observation, two years later the same group performed a phase I trial on 6 Alzheimer patients with DBS electrodes implanted in the fornix (Laxton et al., 2010). Although the study was performed in an open-label fashion and without a sham surgery control group, the authors found that less severely affected patients responded to this therapy. After one month of stimulation the authors found that fornix DBS normalized cerebral brain glucose levels in the temporal and parietal cortex as observed in positron emission tomography (PET) scans (Laxton et al., 2010). In two patients with best clinical response to fornix DBS, the authors found evidence for neuroplastic changes. These changes included increased hippocampal volume which strongly correlated with volume change in the fornix and mammillary bodies indicating a circuit-wide effect of stimulation (Sankar et al., 2015). Moreover, recent work in rats has shown that acute fornix DBS can induce c-Fos activation in the hippocampus and also modulate neurotrophic factors such as brain derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF); as well as synaptic plasticity markers such as growth associated protein 43, α-synuclein and synaptophysin (Gondard et al., 2015).

Here, we want to investigate whether acute fornix DBS can also

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https://doi.org/10.1016/j.jchemneu.2018.12.001
Received 27 June 2018; Received in revised form 3 December 2018; Accepted 4 December 2018
Available online 05 December 2018
0891-0618/

have long-term effects on neuroplasticity. Previously, we have described that fornix DBS can improve long-term spatial memory independent of neurogenesis (Hescham et al., 2017). We now hypothesize that the long-term spatial memory effects of acute fornix DBS might be associated to other neuroplasticity mechanisms, for instance synaptic potentiation or changes in the enzymatic machinery of neurons and terminals. We have tested this hypothesis by performing immunohistochemistry of neuronal and synaptic plasticity markers such as phosphorylated cAMP response element-binding protein (p-CREB), BDNF, synaptic vesicle glycoprotein 2 (SV2) and synaptophysin. P-CREB is a transcription factor associated with long-term memory formation (Bourtchuladze et al., 1994). BDNF is a growth factor that works as a synaptic plasticity regulator and plays a key role in modulating long-term potentiation (LTP). SV2 is a glycosylated protein that functions as a storage and transporter for neurotransmitters in the presynaptic vesicles (Tao-Cheng, 2007) and synaptophysin is an integral membrane glycoprotein that is found in nearly all neurons in the CNS, particularly in the presynaptic vesicles (Masliah et al., 1989; Sze et al., 1997).

2. Materials and methods

2.1. Subjects

Seventeen Sprague–Dawley rats from Charles River (Sulzfeld, Germany) were used in this experiment. Their weight ranged between 280 and 300 g at the time of surgery. The room temperature of the colony room was preserved at 21 ± 1 °C. Rats were housed with two to three per cage in a reversed 12:12 h light-dark cycle and water and rat chow was available *ad libitum*. All animal procedures were carried out in accordance to the EU Directive 2010/63/EU for animal experiments.

2.2. Experimental groups

Two experimental rat groups were made: sham (n = 7) and fornix DBS (n = 10). The rats were randomly assigned to either one.

2.3. Surgical procedure

A detailed description, of the surgical procedure and the electrodes used for DBS, can be found in another paper (Tan et al., 2010). In summary, isoflurane was used for anesthetic purposes (IsoFlo®, Abbott Laboratories Ltd, Berkshire, Great Britain). Two bilateral burr holes were made for the implantation of the DBS electrodes at the level of the fornix. Electrodes are gold plated and composed of inner and outer parts. The inner wire consists of a platinum-iridium combination and functions as the negative contact and the outer electrode (stainless steel) as the positive contact. The maximum outer diameter of the electrode is approximately 250 µm with a tip diameter of approximately 50 µm (Technomed, Beek, the Netherlands). Using bregma as a reference point as stated in the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 2006), the coordinates are as follows: AP: -1.8 mm; ML: 1.3 mm; DV: -8.0 mm. A rodent stereotactic apparatus (Stoelting, Wood Dale, IL, USA, model 51653) was used for the implantation of the DBS electrodes. The permanent attachment of the construct was achieved by means of dental cement (Paladur, Heraeus Kulzer GmbH, Hanau, Germany). The same procedure was followed for sham rats.

2.4. Deep brain stimulation

A digital stimulator was used (DS8000, WPI, Berlin, Germany) to apply DBS for 4 h at100 Hz, 100 µA and 100 µs pulse width in awake, freely-moving rats. The different bilateral electrodes had separate stimulus isolators (DLS100, WPI, Berlin, Germany). No stimulation was performed for sham rats although they had cables attached to them. All rats were sacrificed 7 weeks after stimulation. Rats were also subjected to the Morris Water Maze before sacrifice; these results have been reported in a previous publication (Hescham et al., 2017).

2.5. Tissue collection

Pentobarbital overdose (Apotheek Faculteit Diergeneeskunde, Utrecht, The Netherlands) was used to sacrifice the rats 7 weeks after DBS. Transcardial perfusions with Tyrode buffer and Somogyi fixative containing picric acid, 4% paraformaldehyde, PBS and glutaraldehyde were performed. The brains were stored in a fresh fixative (similar to Somogyi but lacking glutaraldehyde) at 4 °C for 2 h. Brains were thereafter conveyed to 1% NaN3 at 4 °C for long-term storage.

For vibratome sectioning (Leica®, Wetzlar, Germany) brains were set in 10% gelatin from porcine skin (Sigma-Aldrich, Zwijndrecht, The Netherlands). Subsequently, the brains were cut into 30 µm slices in the frontal plane and instantaneously stored in 1% NaN3 at 4 °C.

2.6. Verification of electrode placements

Sections that contained electrode indentations from all rats were mounted on gelatin-coated glass slides and stained with standard hematoxylin and eosin. Bright field microscopy was used for inspection of the sections and verification of electrode placements.

2.7. Immunohistochemistry

Four series of coronal sections containing the hippocampus were processed for immunohistochemical staining using the following primary antibodies: rabbit-anti-p-CREB (1:200, Cell Signaling, Danvers, MA, USA), mouse-anti-synaptophysin (1:800; Jackson Immunolabs, West Grove, PA, USA), rabbit-anti-BDNF (1:400, Millipore-Chemicon, Burlington, MA, USA) and mouse-anti-SV2 antibody (1:8000, Developmental Studies Hybridoma Bank, Iowa, USA). We incubated sections with rabbit-anti-p-CREB for 3 nights at 4 °C and with mouse-anti-synaptophysin, rabbit-anti-BDNF and mouse-anti-SV2 for one night at 4 °C. Subsequently, brain sections were incubated with biotinylated donkey anti-rabbit (dilution 1:400) or donkey- anti- mouse (dilution 1:800; Jackson Immunolabs, West Grove, PA, USA) secondary antibody for one hour, followed by an ABC-step (avidin–biotin-peroxidase complex, diluted 1:800, Elite ABC-kit, Vestastatin, Burlingame, CA, USA). Sections were then incubated with DAB (3,30-diaminobenzidine tetrahydrochloride; synaptophysin and SV2 staining) together with nickel chloride intensification (p-CREB and BDNF staining). BDNF slides were also counterstained with hematoxylin. All slides were washed, dehydrated and coverslipped using Pertex (Histolab Products ab, Goteborg, Sweden).

2.8. Analysis of immunoreactivity

2.8.1. p-CREB and BDNF

The number of p-CREB and BDNF positive cells was counted using the stereological procedure, optical fractionator. Counts were done using a motorized stage and a stereological computer microscopy system (Stereo Investigator, Microbrightfield Bioscience, Williston, VT) (Jahanshahi et al., 2010). The granule cell layer of the dentate gyrus and the pyramidal cell layer of the CA1 and CA3 were defined as the regions of interest. All p-CREB and BDNF positive cells in an average of four sections, 300 µm apart, were counted with a 100x objective. The counting frame was set to 30 µm x 30 µm, while the grid size was 100 µm x 100 µm. The chosen brain sections extended from Bregma -3.1 mm to Bregma -4.3 mm. The total number of positive cells was estimated as a function of the number of cells counted and the sampling probability (Schmitz and Hof, 2000).

2.8.2. Estimates of the density of SV2 and synaptophysin-immunoreactive presynaptic boutons

The estimation of the density of SV2 and synaptophysin immunoreactive presynaptic boutons (SV2IPBs and SIPBs) followed the description of (Rutten et al., 2005). All measurements were performed on a single focal plane. Cell^{P} (Olympus soft imaging solutions) imaging software with an Olympus AX70 microscope was used for the detection of SV2IPBs and SIPBs with a 100x oil objective (Olympus UplanApo, NA = 1.35). In short, the area of interest was identified per image and thus, hippocampal neuropil was excluded from the analysis. To correct for microscope illumination irregularities, shading error correction was performed. The differential contrast enhancement filter (DCE) was used for the selective augmentation of weak differences in contrast. Detection threshold was maintained at the same level for all samples. Measurements were performed on five hippocampal sections per animal ranging from Bregma -3.1 to -4.3. In these sections, three randomly sampled areas of 5690 $\mu m^2 \pm 215\ \mu m^2$ for synaptophysin and 5890 $\mu m^2 \pm 17\ \mu m^2$ for SV2 within the stratum radiatum of CA1, stratum lucidum of CA3 and the stratum moleculare of the dentate gyrus were analyzed (Fig. 5). Analysis of immunoreactivity was made by calculating the mean grey value of SV2IPBs and SIPBs immunoreactivity within the region of interest divided by the number of all pixels. From these data, the SV2IPB or SIPB density per unit area (100 μm^2) was calculated in each area.

3. Statistical analyses

SPSS (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. Independent samples t-tests were used to analyze immunohistochemical findings. All p values < 0.05 were considered to be statistically significant.

4. Results

4.1. Verification of electrode placements

The DBS electrodes in sham and DBS animals were all placed in the vicinity of the fornix as can be seen in Fig. 1.

4.2. Analysis of immunoreactivity

P-CREB and BDNF are key mediators of activity-dependent processes in the brain that have a major impact on neuronal development and plasticity. To determine the effect of fornix DBS on the hippocampus, we have counted the number of p-CREB and BDNF positive cells in the CA1, CA3, and DG sub-region. There was no significant difference for the number of p-CREB positive cells between fornix DBS and sham rats in the CA1 (sham: 50356 ± 8059; DBS: 50364 ± 18530; t(12) = 0.0, n.s.), CA3 (sham: 21270 ± 3955; DBS: 23627 ± 4893; t(12) = 0.355, n.s.) and DG (sham: 241664 ± 22199; DBS: 230417 ± 19433; t(12) = 0.381, n.s.; Fig. 2). When looking at the BDNF positive cells, there was also no significant difference between fornix DBS and sham animals in the CA1 (sham: 97619 ± 11817; DBS: 95647 ± 10655; t(12) = 0.123, n.s.), CA3 (sham: 66838 ± 11754; DBS: 69341 ± 8274; t(12) = 0.18, n.s.) and DG (sham: 277830 ± 32972; DBS: 292135 ± 29530; t(12) = 0.322, n.s.; Fig. 3).

In order to evaluate the effects of fornix DBS on the synapse, we have estimated the density of SV2-immunoreactive presynaptic boutons (SV2IPB) and synaptophysin-immunoreactive presynaptic boutons (SIPB) per 100 μm^2 in the stratum radiatum of CA1, stratum lucidum of CA3 and stratum moleculare of the DG. We did not find any significant difference on the number of SV2IPBs in the stratum radiatum of CA1 (sham: 48.5 ± 0.9; DBS: 49.9 ± 1; t(162) = -1.022, n.s.), stratum lucidum of CA3 (sham: 40.7 ± 0.7; DBS: 39.7 ± 0.8; t(161) = 0.985, n.s.) and stratum moleculare of the DG (sham: 34.6 ± 0.8; DBS:

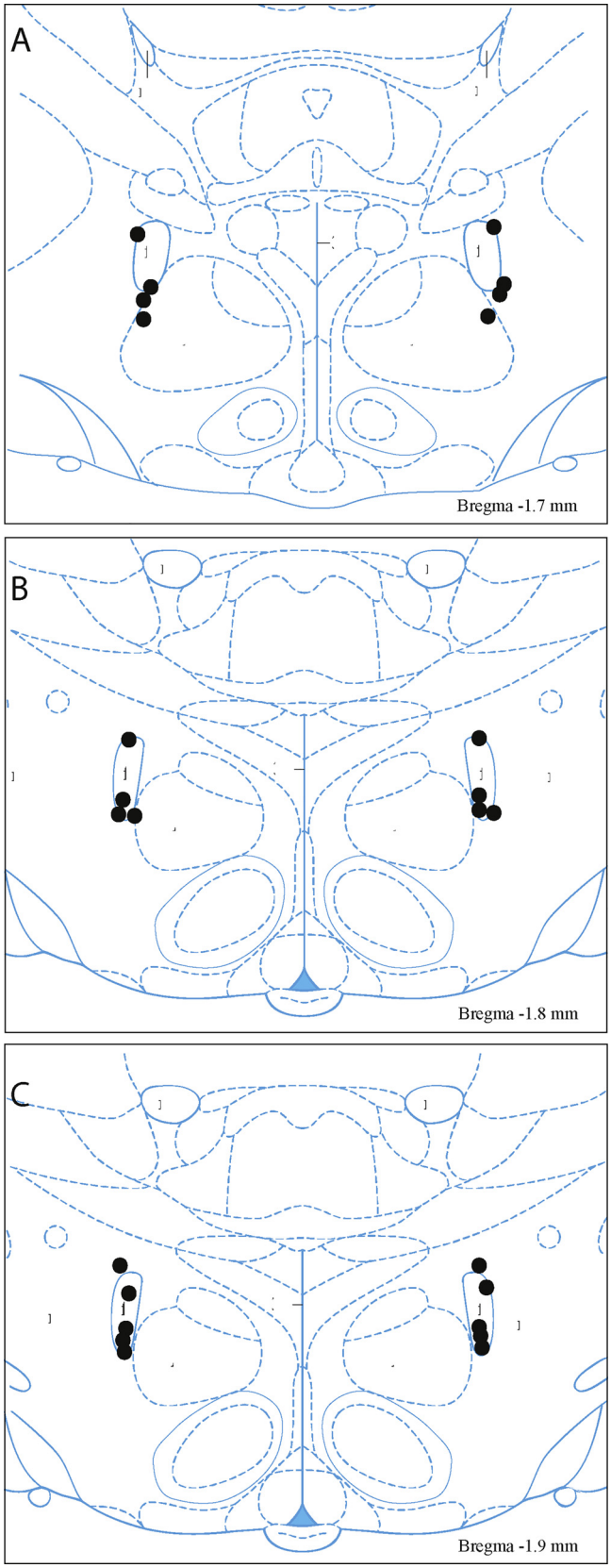

Fig. 1. Histological evaluation of electrode tips. Anatomical locations of bilateral stimulation sites close to the fornix at bregma level (A) -1.70 mm, (B) -1.80 mm and (C) -1.90 mm. Sites were localized by microscopic examination of histologically prepared tissue. Black points indicate the tip of the electrodes.

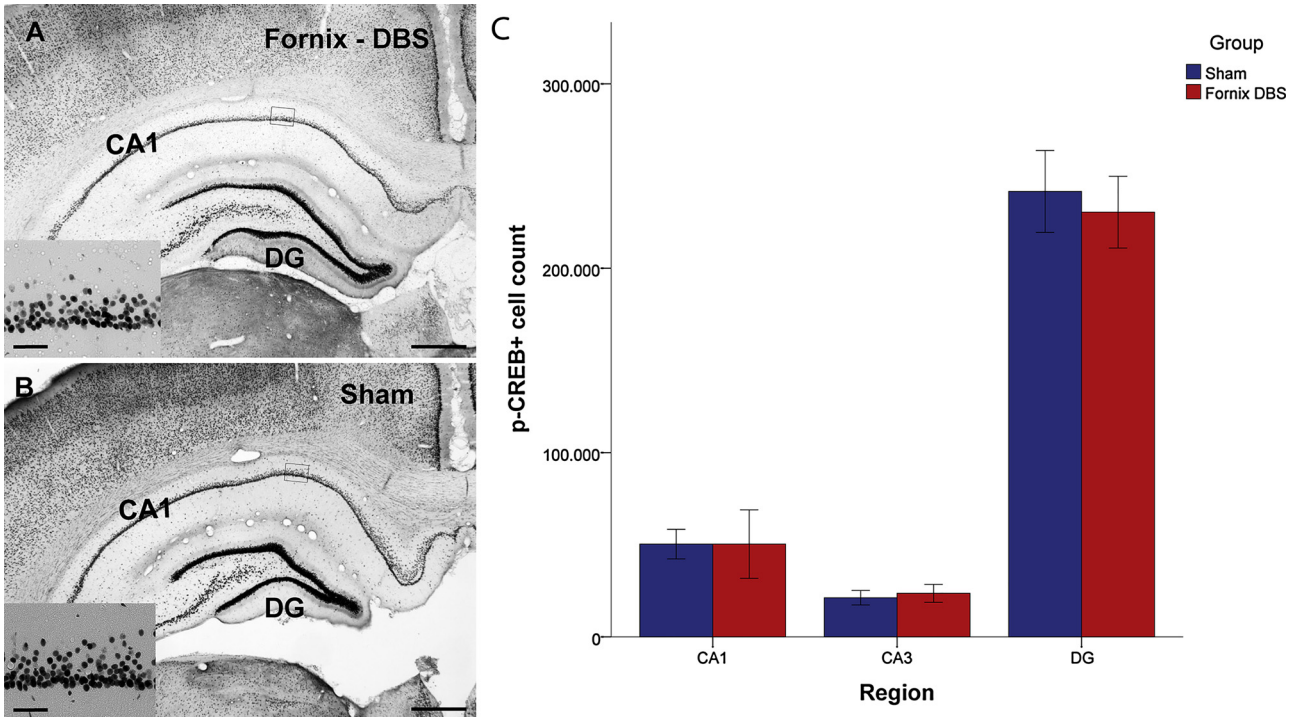

Fig. 2. (A–B) Representative low-power photomicrographs (scale bar = 500 μm) of coronal brain sections stained for p-CREB showing the hippocampus of fornix DBS (A) and sham animals (B). The high-power photomicrograph insets in the lower left corner show the pyramidal cell layer of CA1 (scale bar = 50 μm). (C) Mean number of p-CREB positive cells in the CA1, CA3 and dentate gyrus (DG). There was no significant difference between fornix DBS and sham rats for p-CREB positive cells in the different subregions of the hippocampus. Data is presented as mean ± SEM.

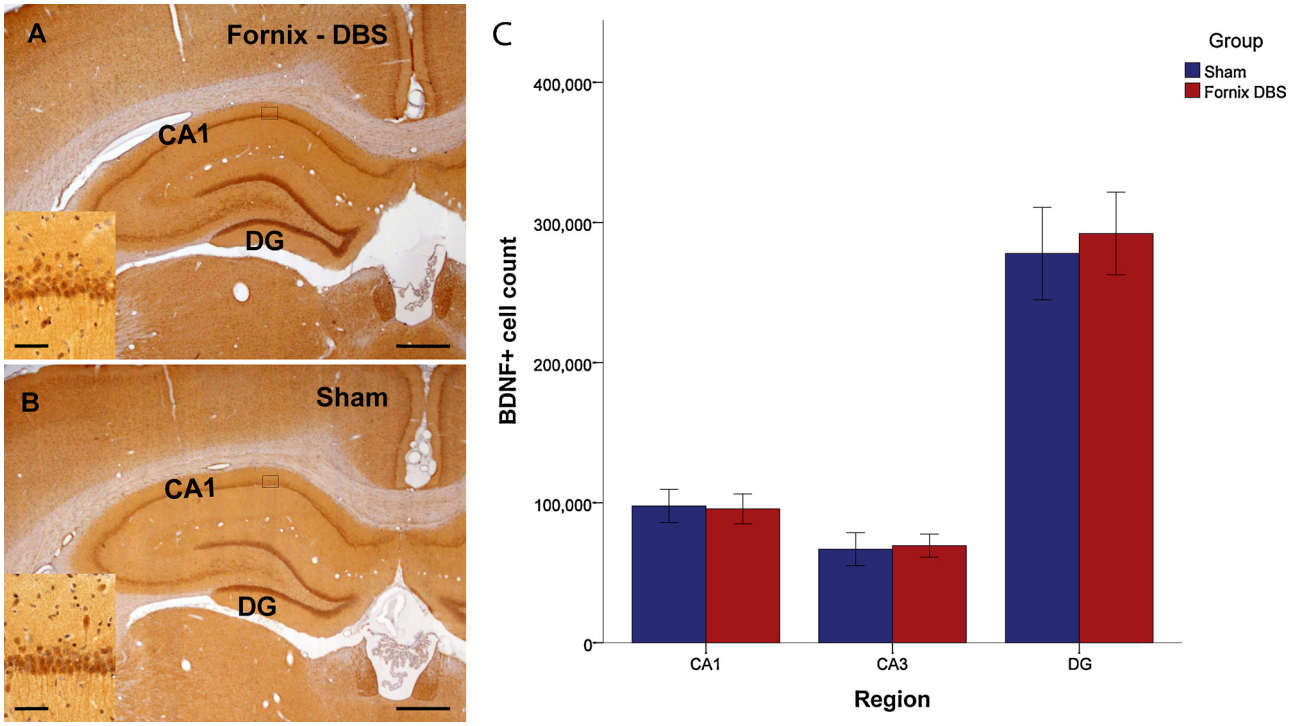

Fig. 3. (A–B) Representative low-power photomicrographs (scale bar = 500 μm) of coronal brain sections stained for BDNF and counterstained with Hematoxylin showing the hippocampus of fornix DBS (A) and sham animals (B). The high-power photomicrograph insets in the lower left corner show the pyramidal cell layer of CA1 (scale bar = 50 μm). (C) Mean number of BDNF positive cells double labeled with Hematoxylin in the CA1, CA3 and dentate gyrus (DG). There was no significant difference between fornix DBS and sham rats in the different subregions of the hippocampus. Data is presented as mean ± SEM.

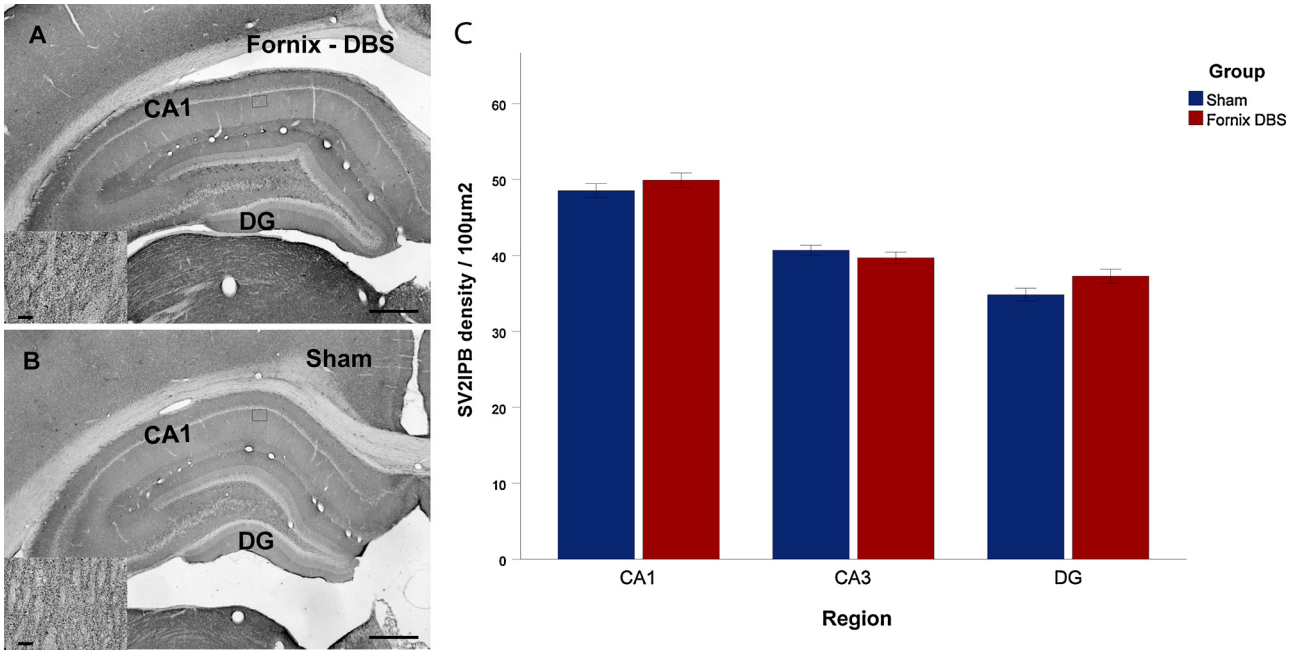

Fig. 4. (A–B) Representative low-power photomicrographs (scale bar = 500 µm) of coronal brain sections stained for SV2 showing the hippocampus of fornix DBS (A) and sham animals (B). The high-power photomicrograph insets in the lower left corner show the stratum lucidum of CA3 (scale bar = 50 µm). (C) Analysis of the SV2- immunoreactive presynaptic boutons (SV2IPBs) density per 100 µm^2 in the stratum moleculare of the DG, the stratum lucidum of the CA3 and the stratum radiatum of the CA1 revealed no significant difference between fornix DBS and sham rats. Data is presented as mean ± SEM.

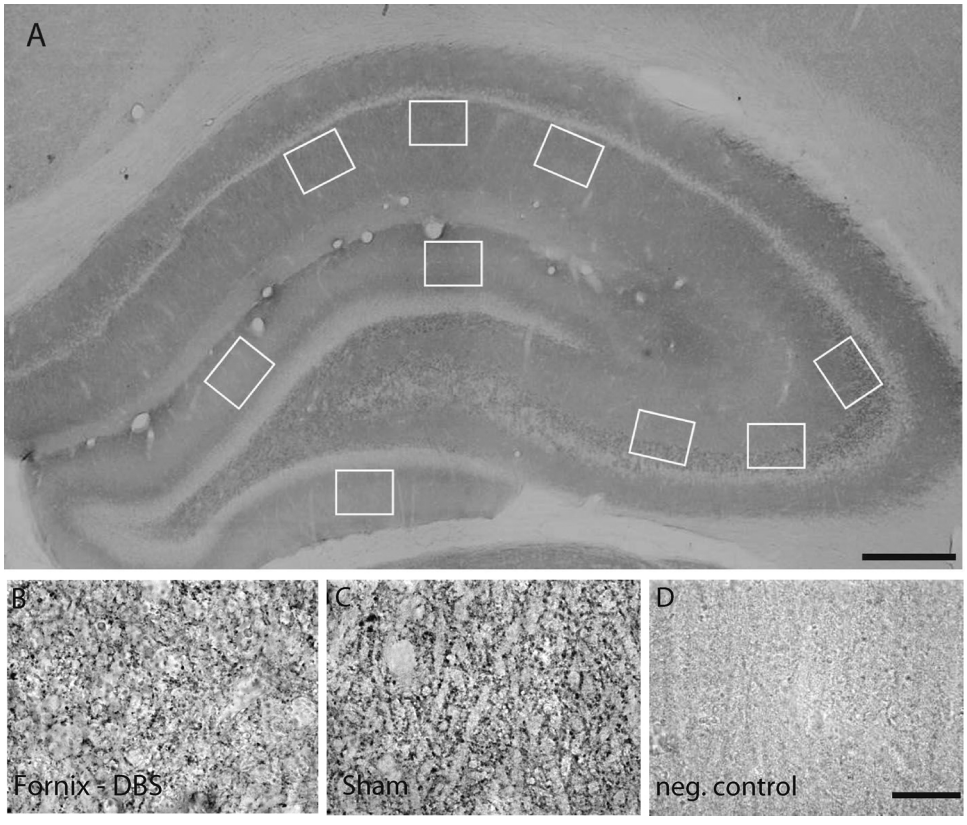

Fig. 5. Synaptophysin-immunoreactive presynaptic boutons in the hippocampus. A-C: representative images of the dorsal hippocampus at 10x (A) and the stratum lucidum of the CA3 subregion at 100x of a Fornix DBS rat (B) and Sham rat (C). In (D) we show the negative control. White rectangles in A represent sites at which high-magnification photomicrographs were taken for quantitative analysis of Synaptophysin-immunoreactive presynaptic boutons density (per section, 3 sites for the stratum moleculare, 3 for the stratum lucidum, and 3 for the stratum radiatum were analyzed). Scale bars represent 500 µm in A and 10 µm in B, C and D.

39.2 ± 0.9; t(161) = -1.949, n.s.; Fig. 4).

A significant decrease has been shown in the density of SIPBs per 100 µm^2 in rats that were exposed to fornix DBS compared to sham rats in the stratum radiatum of CA1 (sham: 53.2 ± 1.5; DBS: 48.3 ± 1.3; t(163) = 2.508, $p < 0.02$) and stratum lucidum of CA3 (sham: 39.3 ± 1; DBS: 33.6 ± 1; t(163) = 4.003, $p < 0.001$; Figs. 5 and 6).

There was no difference in the number of SIPBs between fornix DBS and sham in stratum moleculare of the DG (sham: 34.6 ± 1.3; DBS: 33.5 ± 1.3; t(163) = 0.597, n.s.).

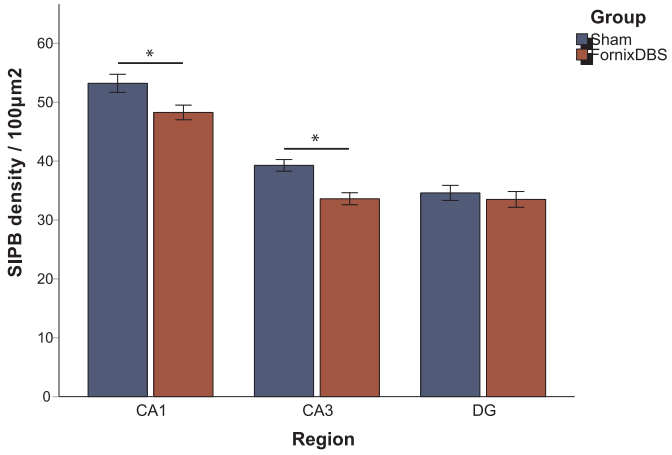

Fig. 6. Mean density of synaptophysin-immunoreactive presynaptic boutons (SIPBs) per 100 µm^2 in the stratum moleculare of the DG, the stratum lucidum of the CA3 and the stratum radiatum of the CA1. There is a significant decrease of the SIPBs density per 100 µm^2 in the stratum lucidum of the CA3 and the stratum radiatum of the CA1in rats that were exposed to DBS when compared to sham rats. Data is presented as mean ± SEM, * indicates $p < 0.05$.

5. Discussion

In this study we have tested the hypothesis that beneficial effects of fornix DBS on memory (reported in Hescham et al., (2017)) can be explained by enhancing histological parameters on neuronal and synaptic plasticity. We did not find any differences for the number p-CREB and BDNF cells in the different hippocampal sub-regions for fornix DBS rats when compared to sham. BDNF is an important neuronal growth factor in the brain that promotes neuronal maturation and neurogenesis by activating CREB and protein kinase A. Both BDNF and p-CREB are therefore key factors in the regulation of hippocampal neurogenesis and improved cognition (Ortega-Martínez, 2015). In fact, the presence of p-CREB has been found in many new-born immature neurons within the subgranular zone of the DG (Jagasia et al., 2009; Nakagawa et al., 2002a, b), the olfactory bulbs and the subventricular zone (Giachino et al., 2005; Herold et al., 2011). Moreover, it has been described that CREB expression in the DG persists for 3–21 days after cell generation and overlaps with doublecortin expression (Ortega-Martínez, 2015). The expression of p-CREB decreases with the emergence of mature neuronal markers such as calbindin and NeuN (Giachino et al., 2005; Herold et al., 2011). In our previous study, we have demonstrated improved cognition for fornix DBS rats when compared to sham 6.5 weeks after stimulation (Hescham et al., 2017). We found that this effect was independent of neurogenesis, since there was no difference in the number of double-labeled BrdU/NeuN cells between fornix DBS and sham animals in the subgranular zone of the DG (Hescham et al., 2017). Not detecting any significant difference in hippocampal BDNF and p-CREB positive cells between the two groups is therefore in line with our previous findings. As mentioned above, it might be possible, however, that the time frame of 7 weeks between stimulation and sacrifice was too long to detect any differences in p-CREB levels, since it was shown that p-CREB subsides in late maturation stages of neurons. Also, we have only investigated BDNF and p-CREB cells in the hippocampus in order to have a direct comparison to our previous results on BrdU/NeuN. It would be interesting to see whether fornix DBS might have an effect on the presence of BDNF and p-CREB in other neurogenic niches, such as the olfactory bulbs and the subventricular zone.

We also did not find a significant difference between fornix DBS and sham animals for the density of SV2IPBs in the hippocampus. SV2 is a transporter-like protein that is located in synaptic neurotransmitter-containing vesicles, binds synaptotagmin and is therefore involved in the regulated secretion of neurotransmitters from presynaptic neurons (Yao et al., 2010). In other words, SV2 modulates the expression, trafficking and stability of synaptotagmin, through which it regulates neurotransmitter release. Since we did not observe a difference in the number of hippocampal SV2IPBs between fornix DBS and sham animals despite improved cognition, we hypothesize that fornix DBS might only have immediate effects on neurotransmitter release, which cannot be detected at long-term.

Surprisingly, the density of SIPBs was significantly decreased in the CA1 and CA3 subfield of the hippocampus for fornix DBS rats when compared to sham. Synaptophysin and synaptotagmin are major integral membrane proteins localized to synaptic vesicles. As mentioned before, synaptotagmin plays a major role in neurotransmitter release (Yao et al., 2010). Synaptophysin, on the other hand, is thought to comprise part of the pore complex, which forms when the vesicle fuses with the presynaptic membrane. A decreased density of SIPBs is therefore indicative of reduced vesicle fusion at the presynapse. Although quantitative electron microscopy remains the only definitive means of determining synapse number, synaptophysin immunoreactivity provides a reliable marker of the synapse levels (Heinonen et al., 1995).

Recently, Gondard and colleagues have shown that fornix DBS significantly increases synaptophysin levels in the hippocampus (Gondard et al., 2015). In their experiment, however, rats were stimulated for 1 h and sacrificed at different time points (1 h, 2.5 h, 5 h, 25 h) after stimulation. These results therefore represent short-term effects of fornix DBS. Here, we have sacrificed animals 7 weeks after DBS and found decreased levels of synaptophysin in the hippocampus, despite improved cognition, indicating an activity-dependent reduction in synaptic strength or long-term depression (LTD) related mechanisms. Our findings appear to be contradictory to conventional knowledge in which the reduction of synapses affects the integrity of hippocampal connectivity and is highly associated to the magnitude of learning impairment (Burke and Barnes, 2010; Grillo et al., 2013).

LTD is considered to be the fourth common criteria of synaptic plasticity together with long term potentiation (LTP), sensitization and sprouting of axon terminals. Any aspect of prolonged depression in synaptic transmission or lowering of transmission efficacy is known as LTD (Barrionuevo et al., 1980). It has been shown that decreasing the synaptic efficacy or strength can play an important role in allowing the neuronal network to store information more effectively (Bear, 1999; Bermúdez-Rattoni, 2007; Mulkey et al., 1994). In particular, we have found decreased SIPBs in the stratum radiatum of CA1 and stratum lucidum of CA3. Studies using hippocampal slices have shown that acetylcholine can dramatically facilitate LTD (Kirkwood et al., 1999) and depotentiation (Huerta and Lisman, 1996) in these regions. We have found before, that fornix DBS induces an acute increase of hippocampal acetylcholine (Hescham et al., 2016). It might thus be possible, that acetylcholine can transiently facilitate hippocampal LTD following fornix DBS. This hypothesis, however, needs further investigation, because it remains questionable whether 4 h of stimulation and the associated transient acetylcholine release can indeed induce long-lasting effects (Picciotto et al., 2012). Since synaptophysin is localized at both types of excitatory and inhibitory neurons, electrophysiological recordings might provide clarity, whether mechanisms are mediated by LTP or LTD.

To summarize, we have shown previously that the improved water-maze performance of fornix DBS rats was independent of neurogenesis (Hescham et al., 2017). Based on our present findings, enhanced synaptic neuroplasticity is also not involved in the long-term memory effects following fornix DBS. It is possible that other neuroplasticity mechanisms might be involved, such as LTD. Further research is needed to reveal the exact pathways and mechanisms involved in this process.

Declarations of interest

None.

Ethical statement

All animal procedures were carried out in accordance to the EU Directive 2010/63/EU for animal experiments.

Acknowledgements

MA has been funded by King Khalid University, Saudi Arabia.

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