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ORIGINAL ARTICLE

Chronic fornix deep brain stimulation in a transgenic Alzheimer's rat model reduces amyloid burden, inflammation, and neuronal loss

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Abstract

Recent studies have suggested deep brain stimulation (DBS) as a promising therapy in patients with Alzheimer's disease (AD). Particularly, the stimulation of the forniceal area was found to slow down the cognitive decline of some AD patients, but the biochemical and anatomical modifications underlying these effects remain poorly understood. We evaluated the effects of chronic forniceal stimulation on amyloid burden, inflammation, and neuronal loss in a transgenic Alzheimer rat model TgF344-AD, as well as in age-matched control rats. 18-month-old rats were surgically implanted with electrodes in stereotactic conditions and connected to a portable microstimulator for chronic DBS in freely moving rats. The stimulation was continuous during 5 weeks and animals were immediately sacrificed for immunohistochemical analysis of pathological markers. Implanted, but non-stimulated rats were used as controls. We found that chronic forniceal DBS in the Tg-AD rat significantly reduces amyloid deposition in the hippocampus and cortex, decreases astrogliosis and microglial activation and lowers neuronal loss, as determined by NeuN staining. In control animals, the stimulation neither affects neuroinflammation nor neuronal count. In the Tg-F344-AD rat model, 5 weeks of forniceal DBS decreased amyloidosis, inflammatory responses, and neuronal loss in both cortex and hippocampus. These findings strongly suggest a neuroprotective effect of DBS and support the beneficial effects of targeting the fornix in Alzheimer's disease patients.

Keywords Alzheimer rat model · Alzheimer disease · Deep brain stimulation · Beta-amyloid plaques · Neuronal loss · Fornix · Chronic stimulation · Neuromodulation · Neuroprotection

Introduction

Alzheimer's disease (AD) is a slowly progressive disorder with insidious onset and progressive impairment of episodic memory leading to global cognitive decline including aphasia, apraxia, and agnosia. The pathological hallmarks of AD are progressive deposition of amyloid beta (Aβ) peptides forming amyloid plaques, neurofibrillary tangles constituted mainly by protein tau-associated hyperphophorylated microtubules, chronic neuroinflammation, neuronal injury, and synaptic loss (Blennow et al. 2006; McKhann et al. 2011). The amyloid cascade hypothesis suggests that deposition of Aβ triggers progressive neuronal dysfunction and death with a predilection for neuronal circuits dedicated to memory (Palop and Mucke 2010). The AD pathological features initially appear in the hippocampus, a main hub in memory circuits. As the disease progresses, additional regions of the brain become affected and brain atrophy occurs. Benefits

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from symptomatic medical treatments in AD are notoriously still insufficient (McKhann et al. 2011).

The fornix is mainly a projection tract connecting the hippocampus with the mammillary bodies (Lövblad et al. 2014) and is a major component of the circuit of Papez, which is involved in memory function. The key role of the fornix in memory function has been delineated in both animal and human, as forniceal lesions were found to trigger memory impairments (Tsivilis et al. 2008; Wilson et al. 2008; Vann et al. 2009; Browning et al. 2009).

Deep brain stimulation (DBS) involves the delivery of electrical current into specific areas of the brain through implanted electrodes and has emerged as a powerful technique to treat neurological and neuropsychiatric disorders from Parkinson's disease, to essential tremor, dystonia, obesity, depression, epilepsy, and obsessive compulsive disorder (Benabid et al. 1991; Krack et al. 2003; Deuschl et al. 2006; Mallet et al. 2008). Recently, two studies demonstrated that electrical stimulation of memory circuits can enhance memory in humans without cognitive deficit (Hamani et al. 2008; Suthana et al. 2012). First, in a study in epileptic patients explored by deep electrodes, visuo-spatial memorization was facilitated by electrical stimulation of the entorhinal cortex (Suthana et al. 2012). Second, improvement of verbal recollection was reported after deep brain stimulation (DBS) of the fornix in the hypothalamus in a patient treated for morbid obesity (Hamani et al. 2008). Further studies have suggested that DBS targeting the fornix could be useful to modulate memory circuits in patients with cognitive decline, to slow down this impairment. Pilot studies have evaluated the safety of chronic fornix DBS and suggested that DBS could stabilize or slow the memory decline of few patients with mild AD (Laxton et al. 2010; Fontaine et al. 2013). Moreover, bilateral hippocampal volume increased in the two patients exhibiting the best clinical response to fornix DBS compared to a matched group of AD patients without DBS (Sankar et al. 2015). More recently, a larger (42 patients) double-blinded phase II study confirmed the safety of fornix DBS in mild AD and suggested a cognitive benefit after 2 years of chronic stimulation for older patients with slowly progressing disease (Lozano et al. 2016; Ponce et al. 2016).

Until so far, only few studies have evaluated the biological effects of DBS on memory circuits in experimental animal models and none of them have investigated permanent chronic stimulation. Most of these animal studies used short duration DBS either in the fornix area, or in other Papez's circuit structures as the entorhinal cortex (EC) or the anterior nucleus of the thalamus (ANT) (Hescham et al. 2013; Gondard et al. 2015) in healthy rodents or in acute cognitive impairment models (injection of scopolamine or corticosteroids). In the present study, we investigated the influence of 5 weeks of continuous forniceal DBS in a rodent model of AD displaying the histological, anatomical, and behavioral alterations observed in AD-affected patients, as well as in age-matched control wild-type rats (Mann et al. 2017). Our study was designed to mimic the experimental conditions of AD patients treated by chronic fornix DBS. The effects on amyloid deposition, neuroinflammation, and neuronal loss were followed by immunohistochemical analyses.

Materials and methods

Animals

In this study, 18-month-old transgenic rats TgF344-AD expressing mutant human amyloid precursor protein (*APPswe*) and presenilin 1 (*PS1ΔE9*) genes (Cohen et al. 2013) and age-matched wild-type (WT) WT F344, were obtained from Dr T.Town (Los Angeles, USA). This AD model manifests age-related cerebral amyloidosis preceding gliosis and apoptotic loss of neurons in the cerebral cortex and hippocampus, and presents age-dependent cognitive disturbances that mimic biological and clinical features of AD in humans (Cohen et al. 2013). We chose 18-month-old rats for five lines of observations: (1) plaques are visible at 16 weeks of age; (2) reactive microglia and astrocytes are elevated as early as 6 months of age in Tg; (3) microglia in aged Tg rats is hyperplasic and hypertrophied in close vicinity of amyloid plaques; (4) neuronal loss occurs at 16 months of age; and (5) memory deficits are observed at 15 months of age. Rats were housed with ad libitum access to food and water in a room maintained at a constant temperature (20–22 °C) on a 12 h light–dark cycle, both before and after surgery. This study was approved by the Institute Animal Care Committee. All experiments are in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Surgical procedure and stimulation

All the rats were operated and implanted with bilateral electrodes, but only half of the animals were stimulated (+DBS). Briefly, animals were anesthetized with isoflurane 5 (Isoflo Abott laboratory), and platinium concentric unipolar electrodes were bilaterally implanted in the forniceal region using a rodent stereotactic apparatus. Coordinates relative to bregma were: antero-posterior: −0.6 mm; medio-lateral: ± 0.75 mm; dorso-ventral: −5.8 mm versus dura mera (Paxinos and Watson 2007). Electrodes were connected to the stimulator support fixed on the head of the rats. The microstimulator, with a power supply consisting in two 3 V flat lithium watch batteries, was clipped on the support, as reported by Forni et al. (2012). This device has size and weight compatible for use in freely moving rats (Fig. 1a). Stimulation was applied 10 days after surgery and

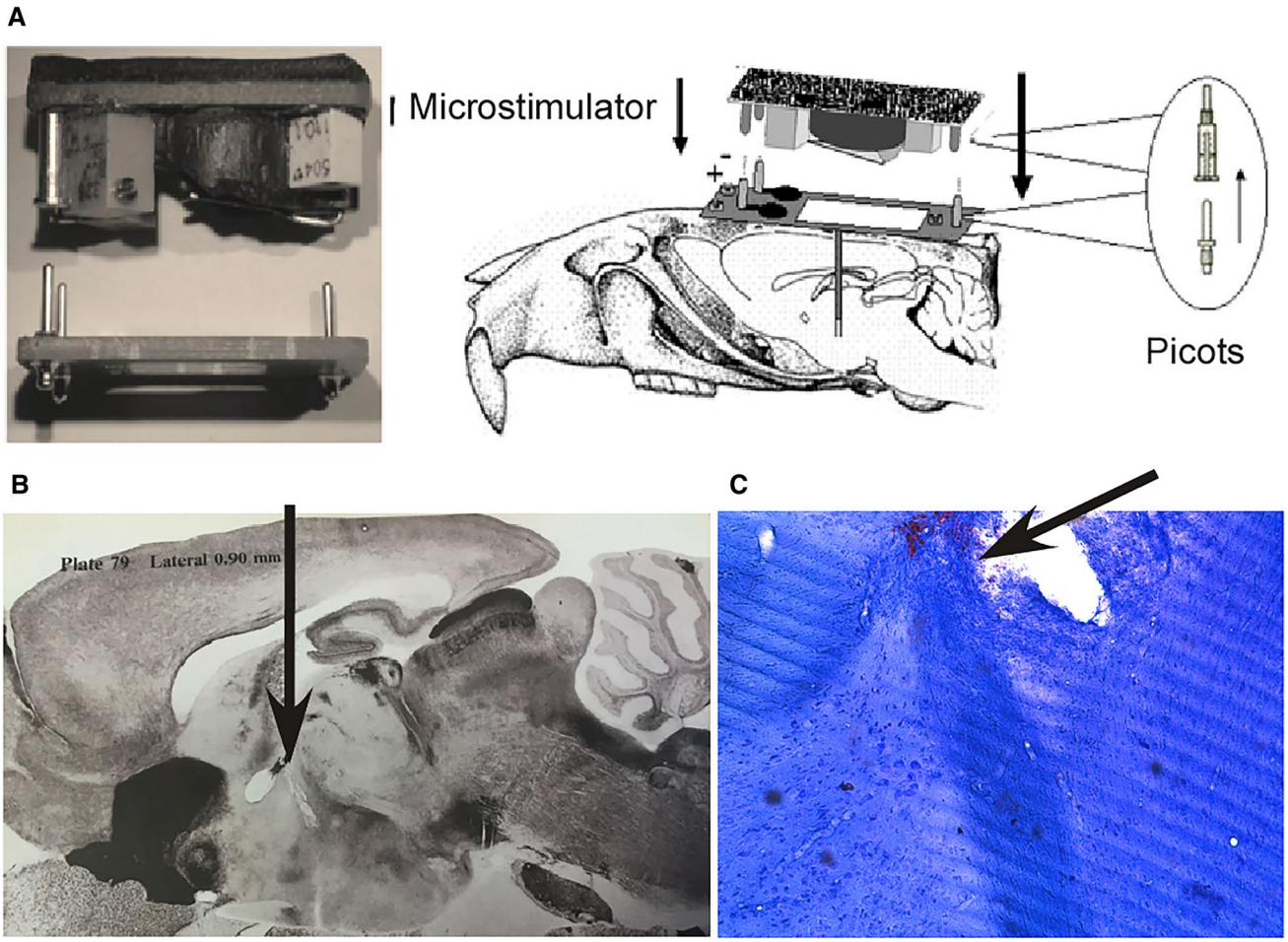
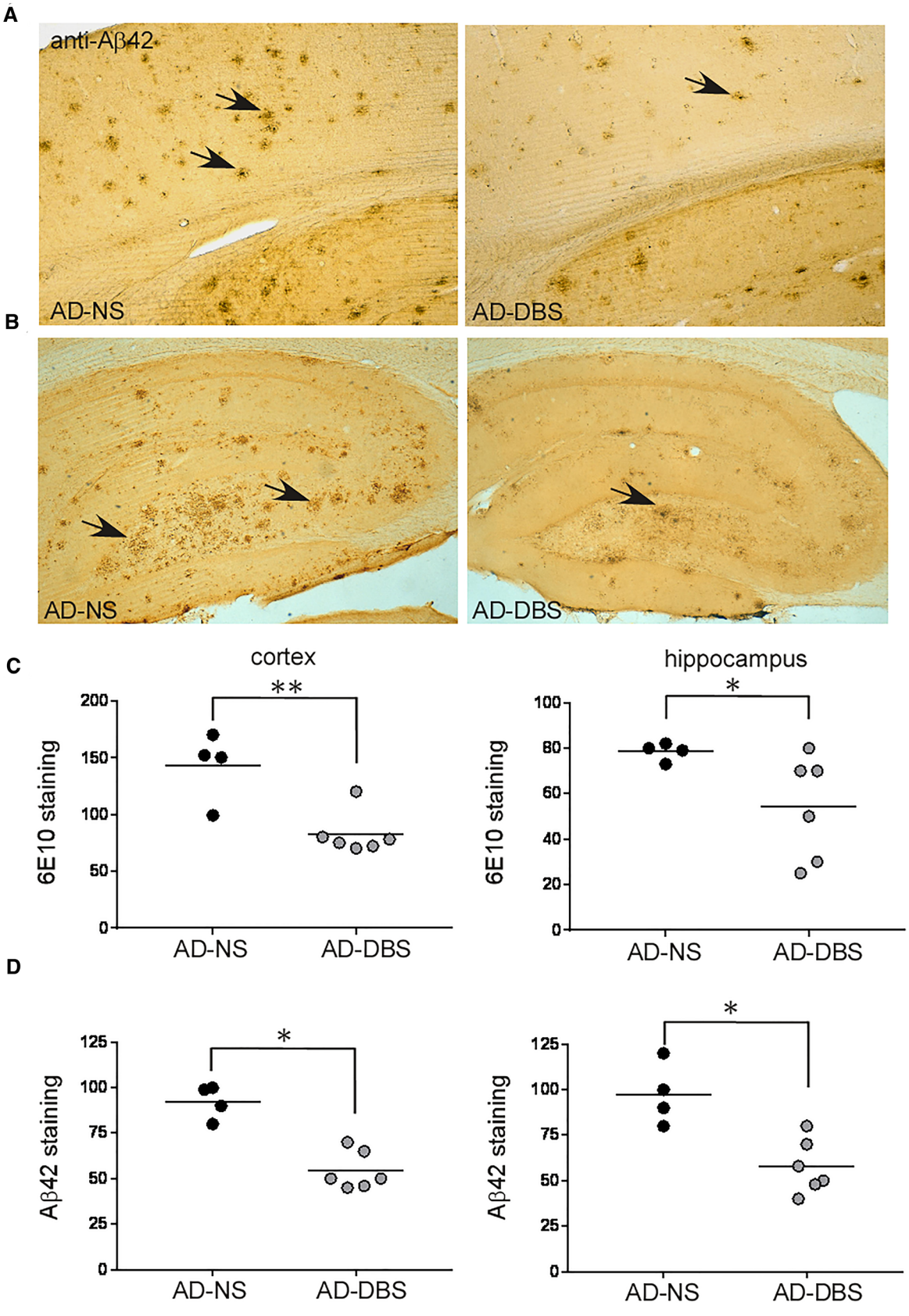


Fig. 1 Deep brain stimulation device and technique. **a** Photograph and schematic drawing illustrating the design of the the portative microstimulator, allowing chronic 5-week stimulation in free moving rats. According to Forni et al. (2012), **b** sagittal section from the rat brain atlas of Paxinos and Watson (2007) showing the location of the fornix. **c** Sagittal slice (cresyl violet coloration) showing the location of the tip of the electrode in close contact with the fornix (arrow) and its trajectory

corresponded to 130 Hz, 80µs, 100 µA stimulation mimicking high-frequency DBS used in clinical studies (Fontaine et al. 2013; Lozano et al. 2016; Ponce et al. 2016) and used in mouse (Mann et al. 2017). Stimulation was permanent, bilateral, unipolar, and maintained during 42 days. Daily inspection of rats was performed throughout the whole procedure to ensure their well-being.

Immunohistochemical staining

Rats were euthanized with a lethal dose of intra-peritoneal injection of pentobarbital (100 mg/kg) and subsequently perfused with 100 ml 0.1 M phosphate buffered saline (PBS) followed by 300 ml cold 4% paraformaldehyde in 0.1 M PBS. Brains were removed from the skull, post-fixed with the same fixative for 12 h and then transferred to PBS solution at 4 °C until slicing. Sagittal sections (40 µm) were obtained using a vibratome (Leica, Wetzlar, Germany) and slices were immediately transferred into a cryopreservation solution and kept at − 20 °C until use. Immunohistochemistry was performed on free-floating sections using the following antibodies: Aβ/APP was detected with the 6E10 antibody (mouse monoclonal, Covance, 1:1000) and Aβ42 with a C-terminal directed antibody (rabbit monoclonal, anti-Aβ42, Invitrogen, 1:1000). Astrocytes were detected with anti-GFAP (rabbit monoclonal, Abcam, 1:2000) and microglia using anti-Iba1 (rabbit polyclonal, Wako, 2000). Neuronal counts were performed by staining with anti-NeuN (rabbit monoclonal, Abcam, 1:1000). Before adding the primary antibodies, sections were permeabilized for 20 min in 0.5% Triton-X100 (for GFAP, Iba1, and NeuN) or treated with 50% formic acid for 5 min (for APP/Aβ staining). Immunostaining was developed using appropriate secondary antibodies peroxidase-conjugated antibodies (Jackson, 1:1000) using


A
anti-Aβ42
AD-NS
AD-DBS
B
AD-NS
AD-DBS
C
cortex
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6E10 staining
200
150
100
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0
AD-NS
AD-DBS
hippocampus
*
6E10 staining
100
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AD-NS
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◂**Fig. 2** Chronic stimulation reduces Ab specific pathological markers in AD rats. Representative images of the cortex (**a**) and hippocampus (**b**) from each AD treatment group, with arrows pointing to Aβ plaques. **c** Quantification of cellular Aβ total (6E10 staining) levels showing that chronic stimulation reduces cellular expression in AD rats compared to AD-NS rat both in cortex and hippocampus. **d** Quantification of cellular Aβ 42 levels in cortex and hippocampus showed reduction of plaques deposition. $*p<0.05$; $**p<0.01$; AD-NS $n=4$, AD-DBS $n=6$

DAB as substrate (DAB impact, Vector Labs.). Slices were viewed using a bright field microscopy (Leica).

Verification of electrode placement

To check for correct electrode implantation, slides were stained with Cresyl violet and inspected using a bright field microscopy. All the animals had electrodes located within the boundaries of the fornix (Fig. 1b, c).

Data analysis and statistics

Using Image J software (NIH), the red channel (for the amyloid stain) was selected and converted into a monochrome signal. Then, a threshold optical density was obtained that discriminated staining from background. The thresholded signal was quantified as percent of the visual field for each image. Data were reported as the percentage of labeled area captured (positive pixels) divided by the full area captured (total pixels). Staining of Iba1, GFAP, and NeuN was measured using the approach described above. A total of 8–12 slices were analyzed for each staining and represented as the average for each animal ($n=6$ for WT-no DBS, WT-DBS, and AD-DBS and $n=4$ for AD-no DBS). For each staining, we analyzed 8 slices out of a total of 20–25 slices for hippocampus and 12 slides out of 40–45 slides for the cortex. Thus, in each case, about 20% of brain per area was quantified.

Statistical analyses were performed with PRISM software (Graph-Pad Software, San Diego. CA) using the Mann–Whitney test for pairwise comparisons or the Tukey one-way ANOVA test for multiple comparisons. Alpha levels were set to 0.05 and all analyses were conducted using prism 5.0.

Results

The aim of our study was to evaluate the effects of continuous forniceal DBS on Aβ deposition, neuroinflammation, and neuronal loss in the transgenic rat AD model TgF344-AD, expressing mutant human amyloid precursor protein (APP-Swe) and presenilin 1 (PS1ΔE9) genes. We implanted electrodes in 18-month-old rats, and sacrificed them after 5 weeks of continuous DBS stimulation. As previously observed, we confirmed significant Aβ load (Fig. 2), neuroinflammatory stigmata (Figs. 3, 4) and neuronal loss (Fig. 5) in both the cortex and hippocampus of the AD rat model.

Chronic forniceal stimulation reduces Aβ burden

To evaluate the effects on DBS on amyloid deposition, we performed immunohistochemistry with different amyloid detecting antibodies. Thus, 6E10 detects full-length APP as well as Aβ and its direct precursor the βCFT (C99) fragment, while an anti-Aβ42 antibody specifically labels Aβ42, the most aggregation-prone Aβ peptide. Using both 6E10 and anti-Aβ42, we observed a significant decreased Aβ load (intracellular and extra cellular) in both the cortex and hippocampus of DBS stimulated animals, compared to non-stimulated Tg-AD rats (Fig. 2). With 6E10, we observed a reduction of − 42% ± 10% in Aβ load in the cortex and of − 32% ± 20% in the hippocampus. Similar reductions of Aβ42 containing Aβ load were monitored in the cortex (− 40.8% ± 7.5%; Fig. 2a, d) and in the hippocampus (− 41.3% ± 11%; Fig. 2b, d).

Reactive gliosis

We next evaluated neuroinflammation by immunohistochemical analyses of activated microglia (using anti-Iba1) (Fig. 3) and astrocytes (using anti-GFAP) (Fig. 4). In Tg-AD rats, we found a significant increase of both activated microglia and astrocytes within both the cortex (Fig. 3a, b) and the hippocampus, as compared to WT rats (Fig. 3c). Iba1 staining increased by 292% ± 80% and 62.6% and ± 10% in the cortex and hippocampus, respectively. In addition to an enhanced Iba1-staining positive cells in the transgenic rat, we also noted a difference in cell morphology and localization. In control rats, Iba1 positive cells were sparse, regularly distributed all over the cortex and had round bodies and thin processes with simple ramification, showing that they were likely in a resting state. On the contrary, these cells were mainly hyperplasic and hypertrophied in TgF344-AD cortex, and many of them were concentrated within specific areas surrounding or in close vicinity to Aβ plaques. Of note, GFAP-positive cells were also modified in the Tg-AD rat. Thus, GFAP-positive staining was higher in both the cortex (76% ± 20%) and hippocampus (209% ± 12%) of Tg-AD rats, as compared to WT rats (Fig. 4). Again, strikingly, many cells were concentrated within specific plaque-bearing areas (Fig. 4a).

We analyzed the influence of DBS on the levels of Iba1 and GFAP-like immunoreactivities. In AD rats, we found that DBS stimulation triggered a significant reduction of the number of both Iba1 and GFAP-positive cells. Thus, the number of Iba1-positive cells was reduced by 67% ± 10%

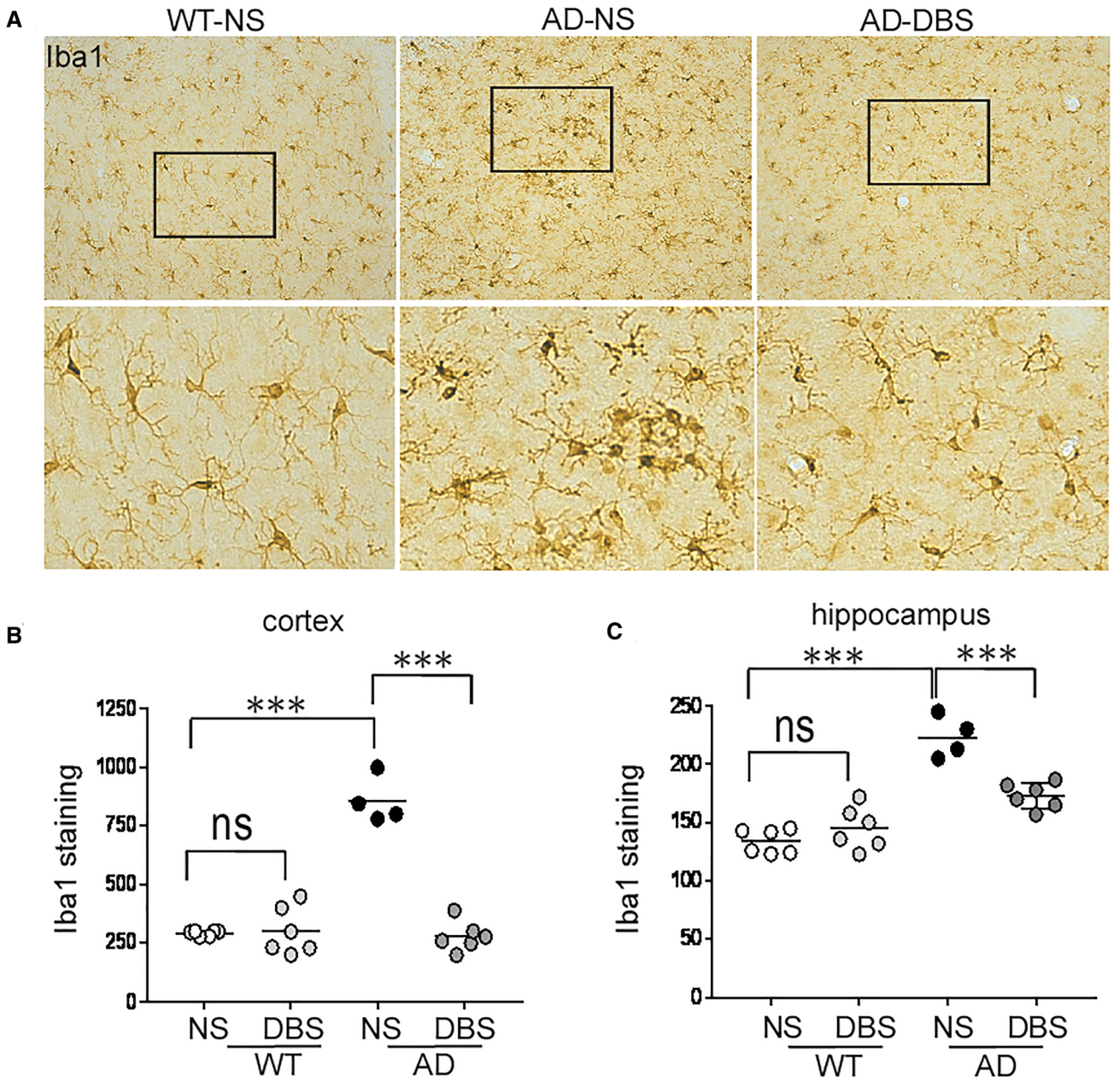

Fig. 3 Chronic forniceal stimulation decreases the number of Iba1-positive cells in AD rats. **a** Low magnification (upper panels) and high magnification (lower panels) photomicrographs of Iba1 staining are shown from brain cortical sections of non-stimulated wild-type (WT-NS), stimulated AD (AD-DBS) or non-stimulated AD (AD-NS) rats. **b, c** Quantitative analysis of IbA1 staining (cortex and hippocampus) show lowered levels in AD-DBS rats compared to AD-NS rats. ***$p < 0.001$ AD-NS $n = 4$, AD-DBS $n = 6$, WT-NS = 6, WT-DBS = 6

and 22.4% ± 6% in the cortex and hippocampus, respectively (Fig. 3b, c). However, the subset of DBS-resistant Iba1-positive cells remained hyperplasic and hypertrophied indicating that chronic forniceal stimulation indeed decreased microglial proliferation, but did not restore morphological alterations of cells altered in the pathology (Fig. 3a). Probably, these findings could be related to the age of animals (18 months) in which neuroinflammation was present, since many months (Cohen et al. 2013). For GFAP, DBS induced 36% and ± 7.5% and 18.4% and ± 8% losses of positive cells in the cortex and hippocampus, respectively (Fig. 4b, c). Finally, we found that there were no statistically significant differences in Iba1 and GFAP stainings in non-stimulated and stimulated non transgenic rats, indicating that DBS per se did not influence neuroinflammatory markers (Figs. 3, 4, respectively).

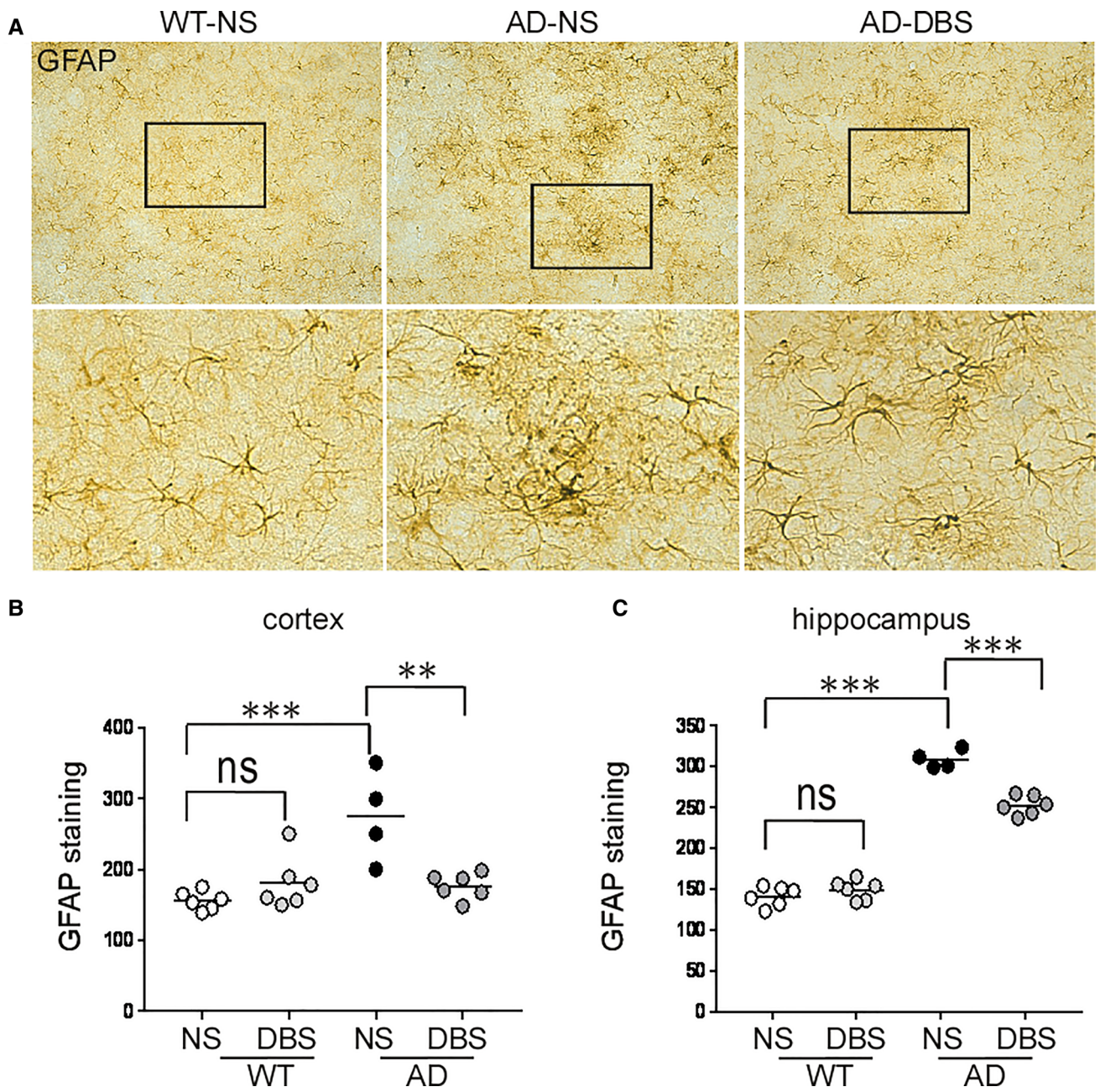

Fig. 4 Chronic forniceal stimulation decreases the number of GFAP-positive cells in AD rats. **a** Low magnification (upper panels) and high magnification (lower panels) photomicrographs of GFAP staining are shown from brain cortical sections of non-stimulated wild-type (WT-NS), stimulated AD (AD-DBS) or non-stimulated AD (AD-NS) rats. **b, c** Quantitative analysis of GFAP staining in the cortex and hippocampus. As predicted GFAP expressing cells were increased in AD-NS rats as compared to WT-NS rats and strikingly, most cells were concentrated within specific to plaque-bearing areas meaning neuropathological neuroinflammation. Moreover, GFAP expressing cells were significantly lower in AD-DBS rats as compared to AD-NS rats in both the cortex and hippocampus. $^{**}p < 0.01$, $^{***}p < 0.001$ AD-NS $n = 4$, AD-DBS $n = 6$, WT-NS = 6, WT-DBS = 6

Neuronal staining

The paper of Cohen et al. (2013) showed that the TgF344-AD rat manifests neurodegeneration occurring particularly in the hippocampus. To monitor neuronal loss, we analyzed the neuronal marker NeuN by immunohistochemistry. When comparing non-stimulated wild-type and Tg-AD rats, we observed a neuronal loss in the dentate gyrus (DG) of the hippocampus (Fig. 5b, reduction of 27.6% ± 7%) as well as in the cortex (Fig. 5c, reduction of 16% ± 5.5%) that agreed well with the range of reduction (30–60% neuronal in 16–26-month-old rats)

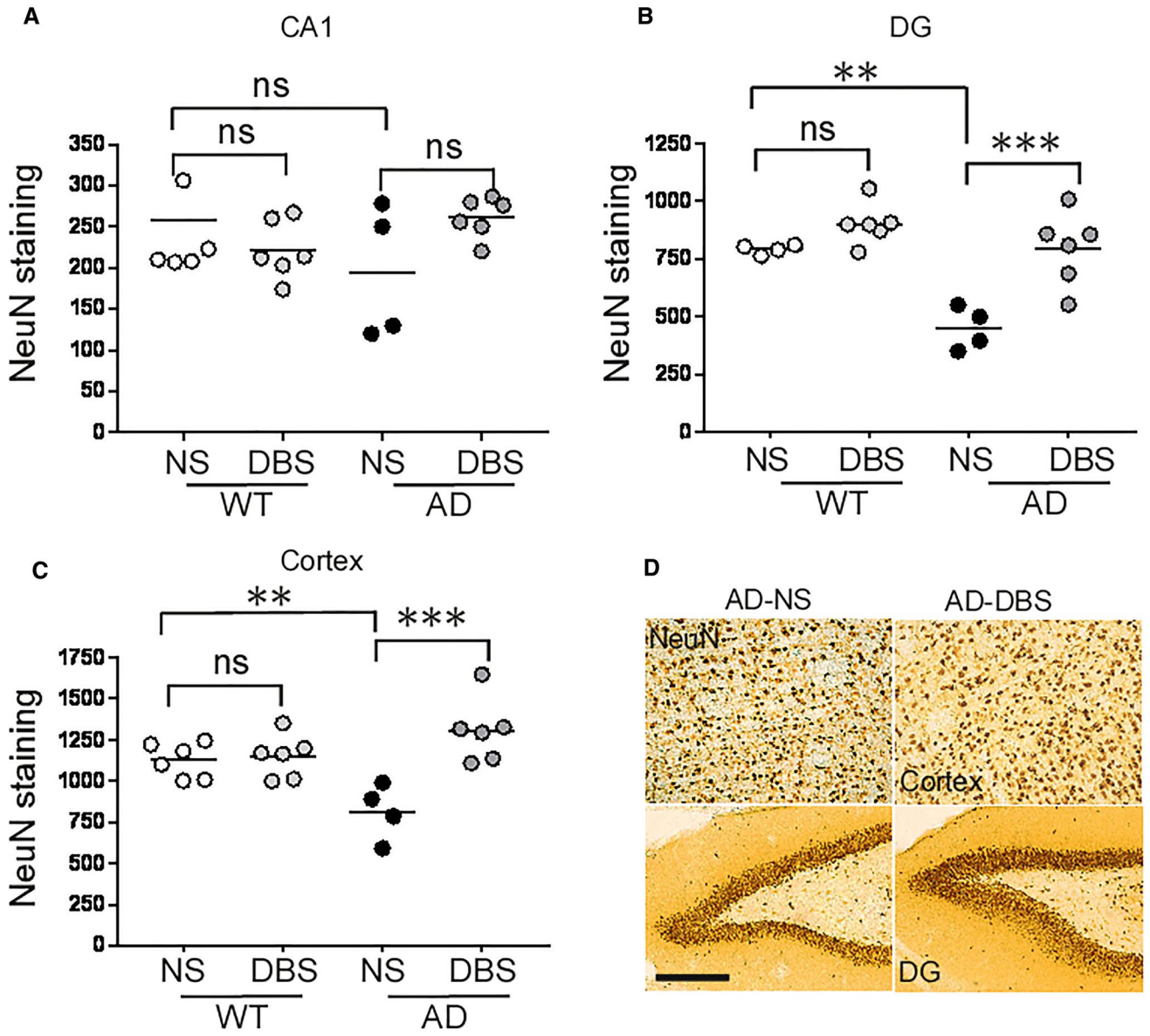

Fig. 5 Forniceal DBS increases neuronal count in AD rats. Quantitative analysis of NeuN staining in the CA1 (**a**), dentate gyrus (**b**) and cortex (**c**). Forniceal DBS increased the numbers of NeuN positive cells in AD rats in both the cortex and DG, but not in WT rats. **d** Representative images of NeuN staining in the cortex and hippocampus from AD-NS and AD-DBS rats. $^{**}p<0.01$, $^{***}p<0.001$; AD-NS $n=4$, AD-DBS $n=6$, WT-NS = 6, WT-DBS = 6

observed in the study of Cohen et al. (2013). No significant reduction in neuronal load was found in the CA1 region (Fig. 5a). In addition, we found that forniceal DBS in the AD rat increased neuronal load by 28% ± 11% in the DG and 23% ± 8% in the cortex versus AD rats without DBS, whereas there were no significant differences between non-stimulated and stimulated WT rats in both brain structures.

Discussion

There is a consensus about the fact that Alzheimer's pathology hallmarks include increased Aβ load and plaques, neuroinflammation, and exacerbated neuronal loss. However, the therapeutic approaches aimed at circumscribing these alterations still await significant

advances. Although not yet perfectly mimicking human disease, transgenic animals are still seen as alternative models to delineate mechanistic alterations taking place in AD and to examine putative pharmacological and non-pharmacological means to thwart disease setting and/or progression.

Among various murine models recently designed to mimic human pathology, a transgenic rat model TgF344-AD displaying *APPswe* and *PS1ΔE9* mutated genes has been developed by the group of Dr. T. Town (Cohen et al. 2013). This rat model appeared particularly adapted to the aims of our study that proposed to examine the influence of forniceal DBS on AD-related stigmata. First, TgF344-AD rats exhibit increased Aβ load, neuroinflammatory signs, and neuronal loss in both cortex and hippocampus. Second, this model allows targeting the fornix, a restricted cerebral area too narrow to envision such approach in transgenic mice models.

Our study is the first demonstration that 5 weeks of chronic forniceal stimulation of freely moving transgenic rats allows significant reduction of Aβ42-related plaques and neuroinflammation and partly rescue neuronal loss. This agrees well a previous study by Lozano et al. that investigated the influence of entorhinal cortex (EC)-DBS in a TgCRND8 mice that harbor pathogenic *APP-swe* and *APP-Ind* mutations (Xia et al. 2017). They found that a 1-h single EC stimulation was sufficient to rescue hippocampal-dependent memory deficits in young (6 weeks, as well as in older Tg-AD mice (6 months). In a second study using triple-transgenic mice (3×Tg-AD) mice (bearing *APPswe, Tau-P301L*, and *PS1M146V mutant proteins*), they found that entorhinal cortex DBS, applied 7 h per day for 25 days, led to memory improvement and reduced hippocampal amyloid burden and total and phosphorylated tau. It should be emphasized that Lozano et al. did not assess the influence of EC-DBS on inflammatory and neuronal markers. However, they were able to link biochemical ameliorations to improved memory function. The low number of Tg-F344-AD rats in our study precluded the possibility to analyze the effects of DBS on behavioral alterations in these animals, but this clearly is a goal that has to be achieved either by means of Tg-F344-AD rats or other available rat models. We found that chronic forniceal DBS decreased the number of plaques in both the cortex and hippocampus. Our study also established that chronic fornix DBS in Tg-AD rat decreased astrogliosis. This glial inflammation is a complex and dynamic response to brain damage characterized by astrocytic hypertrophy, proliferation, and augmented expression of intermediate filaments (Burda and Sofroniew 2014). This physiological response could be considered as a protective reaction, but in AD, it has been shown to contribute directly to a defective Aβ clearance and to interfere with γ-lymphatic flow. Interestingly, DBS-treated rats show decreased number and size of astrocytes, suggesting a contribution to restored homeostasis. As demonstrated by Vedam-mai and al, DBS can act directly on astrocytes resulting in changes in the cerebral blood flow (Vedam-Mai et al. 2012; Etiévant et al. 2015). In our study, forniceal DBS in AD Tg rats was found to reduce migroglia levels in both the cortex and hippocampus. Within the brain, microglia is the phagocytic cell contributing to neuronal apoptosis and inflammatory processes that is a recognized part of the cellular phase in AD. Microglia is in intimate association with senile plaques (McGeer et al. 1988) and neuronal death. Thus, one could envision that DBS-induced reduction of microglia activation could lower inflammation and neuronal death in AD. We estimated this neuronal status using an NeuN directed antibody labeling the core of mature neurons. DBS treatment of AD rats restored the level of both cortical and hippocampal neurons to wild-type control values. In addition to an effect on neuronal death, earlier studies reported a DBS-induced improvement of neurogenesis. Mann et al. (2017) showed that the chronic stimulation of EC increases neurogenesis in the hippocampus of AD mice. Hao et al. (2015) performed a 15-day-long forniceal DBS in a mouse model of Rett syndrome and noted an improvement of neurogenesis in the hippocampus. In a model of acute stimulation, Stone et al. (2011) showed that the stimulation of the entorhinal cortex of the mouse also increased neurogenesis in the dentate gyrus of the hippocampus and that newly formed neurons become mature and functional in hippocampal circuits.

In conclusion, our study is the first reporting on chronic forniceal DBS in a relevant rat model of Alzheimer's disease. This study suggests that DBS is beneficial when major anatomical lesions occurring in AD are used as readout of the pathology. Obviously, work remains to establish the mechanistical aspects underlying these promising observations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards All applicable European guidelines for the care and use of animals were followed. This study was approved by the Institute Animal Care Committee. All experiments are in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Studies involving human and animal participants All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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