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REGULAR RESEARCH ARTICLE

Sustained Ultrastructural Changes in Rat Hippocampal Formation After Repeated Electroconvulsive Seizures

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Abstract

Background: Electroconvulsive therapy (ECT) is a highly effective and fast-acting treatment for depression used in the clinic. Its mechanism of therapeutic action remains uncertain. Previous studies have focused on documenting neuroplasticity in the early phase following electroconvulsive seizures (ECS), an animal model of ECT. Here, we investigate whether changes in synaptic plasticity and nonneuronal plasticity (vascular and mitochondria) are sustained 3 months after repeated ECS trials.
Methods: ECS or sham treatment was given daily for 1 day or 10 days to a genetic animal model of depression: the Flinders Sensitive and Resistant Line rats. Stereological principles were employed to quantify numbers of synapses and mitochondria as well as length of microvessels in the hippocampus 24 hours after a single ECS. Three months after 10 ECS treatments (1 per day for 10 days) and sham-treatment, brain-derived neurotrophic factor and vascular endothelial growth factor protein levels were quantified with immunohistochemistry.
Results: A single ECS treatment significantly increased the volume of hippocampal CA1-stratum radiatum, the total length of microvessels, mitochondria number, and synapse number. Observed changes were sustained as shown in the multiple ECS treatment group analyzed 3 months after the last of 10 ECS treatments.
Conclusion: A single ECS caused rapid effects of synaptic plasticity and nonneuronal plasticity, while repeated ECS induced long-lasting changes in the efficacy of synaptic plasticity and nonneuronal plasticity at least up to 3 months after ECS.

Key Words: BDNF, ECS, microvessels, mitochondria, synapse

Introduction

Electroconvulsive therapy (ECT) is a highly effective and fast-acting treatment for depression used in the clinic (Ren et al., 2014). However, its mechanism of therapeutic action remains uncertain. A longitudinal neuroimaging clinical study suggested that neural plasticity may be induced by ECT and partially account for its clinical effectiveness (Cano et al., 2017). Animal studies show

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Significance Statement

Electroconvulsive therapy (ECT) is a highly effective and fast-acting treatment for depression used in the clinic. Its mechanism of therapeutic action remains uncertain. Previous studies have focused on documenting neuroplasticity in the early phase following electroconvulsive seizures (ECS), an animal model of ECT. However, it is less well studied whether changes in synaptic plasticity and nonneuronal plasticity (vascular and mitochondria) are sustained 3 months after repeated ECS trials.

In the present study, our results indicate that a single ECS caused rapid effects of structural plasticity, while repeated ECS induced long-lasting changes in the efficacy of synaptic plasticity and non-neuronal plasticity at least up to 3 months after ECS.

that electroconvulsive seizures (ECS), an animal model of ECT, increased neurogenesis, but newborn neurons seemed not to be associated with relieved depression-like behavior (Olesen et al., 2017; Jonckheere et al., 2018). Thus, increased neurogenesis might not be fully responsible for the rapid efficacy of ECS, implying that other mediators of neural plasticity might, in part, also mediate the antidepressant efficacy of ECS.

Substantial evidence shows that in models of major depression, a disruption of synaptic plasticity results in destabilization and loss of synaptic connections (Popoli et al., 2002; Duman, 2004; Ardalan et al., 2016; Vose and Stanton, 2017). Recovery of synaptic connections and synaptic remodeling is thought to be critical for the clinical efficacy obtained from a rapid antidepressant response (Li et al., 2010, 2011; Kang et al., 2012). Indeed, our previous studies (Chen et al., 2009; Kaae et al., 2012; Chen et al., 2018) have demonstrated that he rapid and efficient therapeutic effect of ECS may be related to changes in neurogenesis, synaptogenesis, angiogenesis, and hippocampal volume, accompanied by brain-derived neurotrophic factor (BDNF) protein level elevation and mitochondrial support.

The vast majority of previous studies (Chen et al., 2009; Kaae et al., 2012; Inta et al., 2013; Nakamura et al., 2013) focused on documenting neuroplasticity in the early phase following ECS, but few studies have investigated the long-term effect of ECS. Some studies (Olesen et al., 2017; Jonckheere et al., 2018) have reported that ECS may produce long-lasting changes in the brain. Few experiments showed ECS induced a long-lasting increase in dentate evoked response in the animals (Stewart and Reid, 1993; Barnes et al., 1994; Stewart et al., 1994). This increase in population-spike amplitude lasted for at least 3 months after the last ECS trial (Burnham et al., 1995; Gombos et al., 1997). A study on the duration of the effects of repeated ECS on long-term potentiation showed that the effect on synaptic function indeed lasted 40 days after ECS (Reid and Stewart, 1997). Previous studies found a significant reduction in the number of bromodeoxyuridine-positive neurons from the initial phase following ECS and up to 3 months after last treatment in rats (Madsen et al., 2000; Malberg et al., 2000) but no further attrition between 3 and 12 months. Almost 50% of newly formed neurons survived at least 12 months following ECS (Olesen et al., 2017).

The aim of the present work was to investigate (1) whether rapid changes of synaptic plasticity and nonneuronal plasticity (vascular and mitochondria) occurred 24 hours after a single ECS, and (2) explore whether the observed changes were sustained at least 3 months after repeated ECS trials in a genetic animal model of depression. The model is the Flinders Sensitive Line (FSL) and their controls, the Flinders Resistant Line (FRL) rats (Overstreet et al., 2005).

MATERIALS AND METHODS

Animals

Adult male FSL (n = 18, 6 in each group: 1 ECS, 10 ECS and sham) and FRL (n = 18, 6 in each group: 1 ECS, 10 ECS and sham) rats (180–200 g, about 2 months old) were breeding colonies maintained in the animal quarters of Translational Neuropsychiatry Unit, Aarhus University Hospital. All rats were pair-housed (2 per cage) and kept on a normal 12-hour-light/-dark cycle and had free access to food and water. The study protocol was approved by the Danish Animal Ethics Council (approval no. 2007/561–1378).

Animals were treated once daily for 1 day or 10 days at 9 AM each day. ECS was bilaterally given via ear clip electrodes using 55–70 mA for 0.5 seconds at a frequency of 100 Hz square wave pulses (UgoBasile, Comerio, Italy). All ECS-treated rats were monitored for seizures, ensuring that clonic movements of the face and forelimbs lasted for at least 10 seconds. The sham-treated group was exposed to the same procedure without current (Ekemohn et al., 2017).

Tissue Preparation and Sampling

Rats were deeply anesthetized with pentobarbital sodium (Unikem A/S, Copenhagen, Denmark) and perfused transcardially by fixatives (4% paraformaldehyde and 2% glutaraldehyde) at 24 hours after a single ECS or 3 months after the 10xECS or sham treatment. Hippocampi were isolated, and left or right hippocampus was selected randomly, embedded in 5% agar, and sectioned at 65-μm thickness on a Vibratome 3000 (Vibratome, St Louis, MO). Four sets of sections were chosen based on a systematic random sampling principle and a section sampling fraction of 1/15. One set was stained with thionin for estimating the volume of subregions of hippocampus and the length of blood vessels with light microscopy. Two sets were stained against BDNF and vascular endothelial growth factor (VEGF) using immunohistochemistry. For electron microscopy, 1 set of sections was embedded in TAAB 812 Epon (TAAB, Berkshire, UK) and 20 consecutive serial ultrathin sections were obtained.

Synapse and Mitochondria Counting

Electron micrographs were taken of the serial Epon embedded sections with a digital camera in a Philips CM 10 electron microscope at an initial magnification of 10 500× and digitally enlarged to a final magnification of 23 850×. The micrographs were saved and later analyzed via iTEM software (Olympus Soft Imaging Solutions) without any postprocessing modifications.

We used the physical disector (Sterio, 1984), which was modified from previous studies (Tang et al., 2001), for estimating synapse and mitochondria numbers. Synapses were identified based on the presence of a postsynaptic density (PSD) with vesicles in proximity to the presynaptic zone (Figure 1). Only spine and shaft synapses of asymmetric synapses were analyzed in this study. The spine synapses are divided into perforated and nonperforated synapses. Perforated synapses display discontinuous or perforated PSD profiles, whereas nonperforated synapses exhibited continuous PSD profiles in all consecutive sections (Figure 1) (Geinisman et al., 2001).

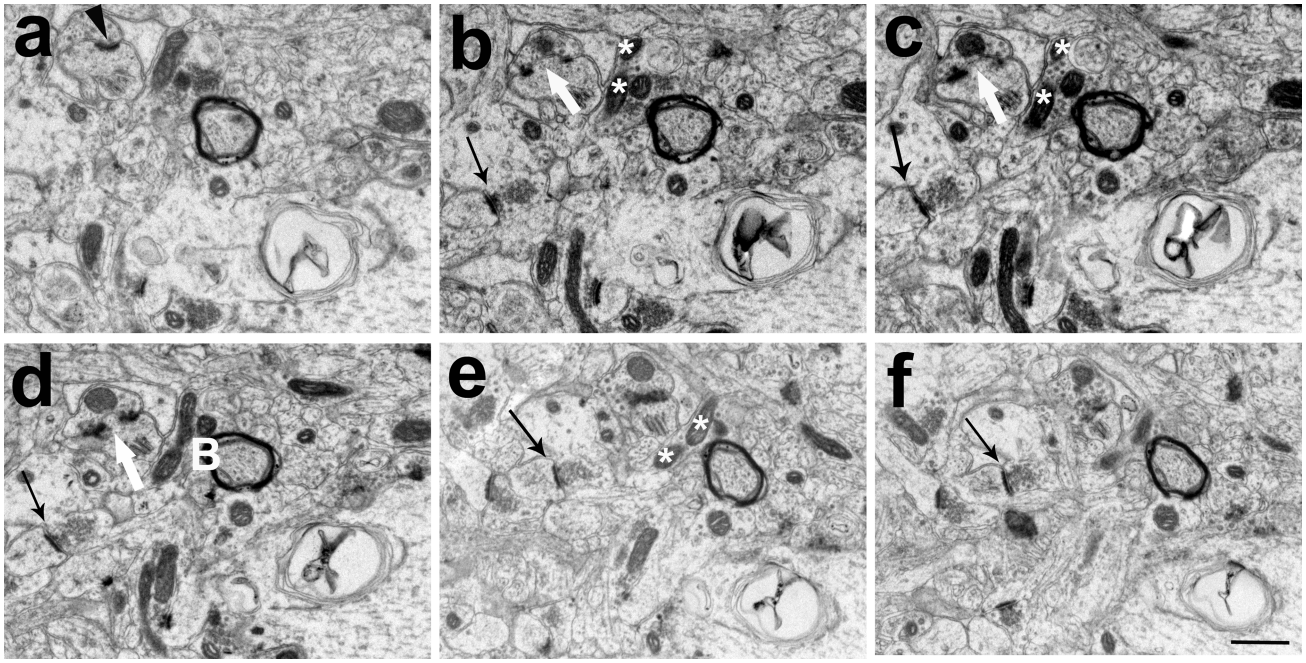

Figure 1. Estimation of synapses and mitochondria in consecutive serial sections. The synapses were identified primarily based on the presence of a postsynaptic density (PSD) with vesicles in proximity to the presynaptic zone. Electron micrographs of consecutive ultrathin sections (a–f) show nonperforated synapses (arrows) and perforated synapse (white arrow). The postsynaptic spine exhibited PSD discontinuities (black arrowhead). The criteria for identifying mitochondria were the presence of distinctive cristae and a double membrane. Axon terminals were identified as presence of 3 or more synaptic vesicles. Dendrites were identified postsynaptic to a synapse or having an attached spine. Mitochondria are identified in each section plane, and a change between planes is deduced as being 1 of 2 significant possibilities: a new isolated part, a so-called "island," *I*, or a new connection between isolated mitochondria, a "bridge," *B*. Branch dividing (white asterisks). Scale bar = 0.5 μm.

The synapse number density was estimated using the PSD as a counting unit. Axo-spinouos perforated synapses and shaft synapses were counted with approximately 120 disectors and axo-spinous nonperforated synapses with approximately 48 disectors in each animal. The total synapse number was estimated as the product of the synapse number density and volume of the CA1 stratum radiatum (CA1-SR). We used the Cavalieri estimator for quantifying the volume of CA1-SR on 1 set of sections stained with thionin using a 4× lens (Dorph-Petersen et al., 2001).

Mitochondria were counted throughout the neuropil and specifically in the axon terminals and dendrites. The criteria for identifying mitochondria were the presence of distinctive cristae and a double membrane (Figure 1). Combining the disector principle with the object's 3D Euler number provides an estimate of the number of mitochondria (Kroustrup and Gundersen, 2001). The total Euler number, Σx, contribution from all disectors is obtained as the sum of islands and bridges (see Figure 1). Detailed information can be found in our previous paper (Chen et al., 2013).

Estimation of Length Density and Total Length of Microvessels

Measurement of length density and total length of microvessels in CA1-SR was done by implementation of the global spatial sampling method (Larsen et al., 1998). Microvessels were defined as a vessel with a 1-celled wall and diameter of ≤10 μm.

Microvessel length was measured on the thionin-stained sections with a 60× oil immersion lens (Olympus, Plan Apochromat, N.A. 1.35). The estimation of the length density of the microvessels was done by counting the total number of intersections between the virtual planes and the microvessels (Figure 2). The total length of microvessels was calculated by the length density of the microvessels multiplied by the volume of CA1-SR. Detailed information can be found in our previous paper (Ardalan et al., 2017).

Immunohistochemistry

Free-floating coronal sections (8–9 per animal) were washed 3 times in Tris-buffered saline (TBS) (pH 7.4), immersed in endogenous peroxidase blocking solution for 30 minutes, and incubated in preheated Target Retrieval solution at 85°C for 40 minutes (Dako, EnVision System HRP). Tissue sections were incubated at 4°C overnight in a solution containing rabbit anti-BDNF polyclonal antibody (1:500) (AB1779, Merck Millipore) or mouse anti-VEGF monoclonal antibody (1:400) (sc-53462, Santa Cruz Biotechnology, Inc.). Then, sections were washed 3 times with buffer (1% bovine serum albumin [BSA] and 0.3% Triton-X in TBS) and incubated in buffer (1% BSA in TBS) added goat anti-rabbit IgG (1:200) or goat anti-mouse IgG (1:200) (for BDNF and VEGF, respectively) for 2 hours at room temperature. Finally, sections were washed 3 times for 10 minutes in TBS and then visualized with 0.1% 3, 3'-diaminobenzidine containing 0.3% H_2O_2 in TBS for 7 minutes and washed with TBS 3 times for 10 minutes. Sections were then mounted on gelatin-coated slides and dehydrated with alcohol gradient and cleared with xylene.

Images of immunostained sections were taken using an Olympus BX61VS Scan microscope (objective: 10×; Hamburg, Germany) equipped with a PIKE digital camera using the software VS ASW OIL 2.7 (Olympus Soft Imaging Solutions). ImageJ software was used for analysis of immunostained sections used for calculation of the mean optical density (MOD) of the BDNF or VEGF positive area in subregions (dentate gyrus [DG], Cornu Ammonis 1 [CA1] and CA2/3) of hippocampus (Figures 3 and 4).

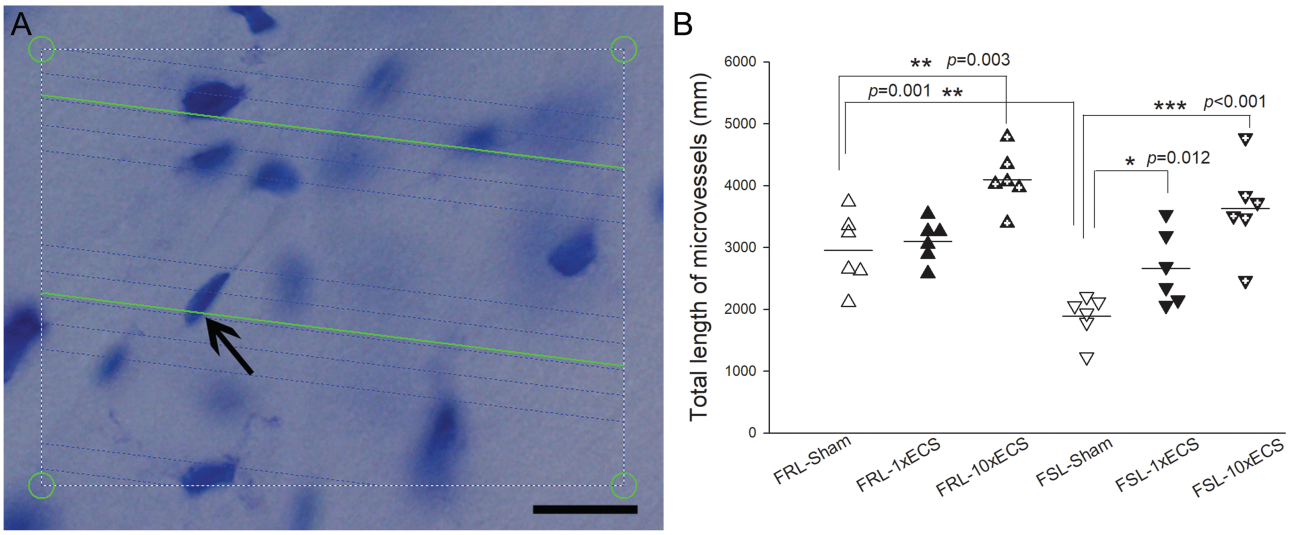

Figure 2. (A) The length of microvessels was measured within a 3-dimensional sampling box. Green test lines were superimposed on the live image by newCAST software and represented the intersection between isotropic virtual planes intersect and the focal plane. The sampling box area was 7200 μm^2 and the box height was 20 μm. When the microvessels are in focus and virtual planes intersect them, they are counted. The 4 box corner points are used to estimate the reference volume. One microvessel is intersecting a green line of the virtual plane (black arrow). (B) Effect of ECS on hippocampal vascular plasticity: length of microvessels (**$P < .01$; *** $P < .001$). The total length of the microvessels in the CA1.SR (CA1 stratum radiatum) was significantly higher in FRL sham rats than FSL sham rats. ECS treatment significantly increased the total length of the microvessels in the CA1.SR in FSL rats.

Statistical Procedures

All values per rat were used for the "comparison of mean" test. Differences across groups were evaluated using 2-way ANOVA between multiple groups with strain and treatment as fixed factors. Turkey's post hoc tests were used to determine specific differences between experimental groups. $P < .05$ was considered statistically significant. Statistical analyses and graphical representations of the findings were carried out using SPSS11 (SPSS Corp, Chicago, IL) and Sigmaplot 10 (SYSTAT Inc, San Jose, CA) software.

RESULTS

Volume of Hippocampal CA1 -SR

After sham treatment, the volumes of hippocampal CA1-SR in FRL and the FSL group did not differ. A single ECS treatment significantly increased the volume of CA1-SR in the FSL-ECS group compared with the FSL sham group ($P = .008$; Table 2). This change was also observed in the FSL group 3 months after repeated ECS ($P = 0.011$; Table 2). In the Flinders "resistant" strain, no differences were observed in hippocampal volume in response to treatment (Figure 5; Table 1). Regarding the volume of hippocampal CA1-SR, a 2-way ANOVA revealed the interaction between 1xECS treatment and strain ($F_{1, 20} = 4.808$; $P = .04$).

Length of Microvessels in CA1-SR

At baseline, the total length of microvessels was significantly longer in FRL sham rats compared with FSL sham rats ($P = .001$) (Figure 2; Table 2). A single ECS treatment significantly affected the total length of microvessels in FSL rats ($P = .012$), but not the FRL rats. However, 10xECS significantly affected the total length of microvessels in both FSL ($P = .003$) and FRL rats ($P < .001$) 3 months after treatment (Figure 2; Table 1 and 2).

Number of Synapses

Single ECS treatment significantly increased total synapse number (FSL: $P < .001$; FRL: $P = .002$) and the number of nonperforated synapses (FSL: $P < .001$; FRL: $P = .021$), perforated synapses (FSL: $P < .001$; FRL: $P = .008$), and shaft synapses (FSL: $P < .001$; FRL: $P = .003$) in both FSL and FRL rat strains (Figure 6; Table 1 and 2). Treatment with 10xECS significantly increased total synapse number ($P < .001$) and the number of nonperforated synapses ($P = .002$), perforated synapses ($P < .001$), and shaft synapses ($P = .002$) in FSL-ECS rats compared with FSL sham rats 3 months after last treatment (Figure 6; Table 2).

Total synapse number ($P = .016$) and the number of both perforated ($P = .01$) and nonperforated ($P = .012$) synapses measured in the sham-treated groups was lower in the FSL group than the FRL group. No difference between the FRL and FSL groups was observed in shaft synapse number (Figure 6; Table 2).

Therefore, 2-way ANOVA revealed a significant interaction between ECS treatment and strain in the number of total synapses ($F_{1, 20} = 8.996$ and 10.261; $P = .007$ and .004), nonperforated synapses ($F_{1, 20} = 5.394$ and 7.4; $P = .031$ and .013), and perforated synapses ($F_{1, 20} = 5.07$ and 9.997; $P = .036$ and .005) after a single ECS and 3 months after 10xECS in both FSL and FRL groups.

Number of Mitochondria

In the sham-treated groups, the number of mitochondria was lower in the FSL group when measured in axon terminals ($P = .023$) and total neuropil ($P = .028$) (Figure 7; Table 2). However, the number of mitochondria in dendrites showed the opposite pattern, that is, a significantly higher number of mitochondria in the FSL group number compared with FRL sham groups ($P = .01$) (Figure 7; Table 2).

In the FSL group, a single ECS treatment significantly increased mitochondria number in axon terminal ($P < .001$) and total neuropil ($P = .032$), but not in dendrites (Figure 7; Table 2). Treatment with 10xECS only increased the mitochondria number in axon

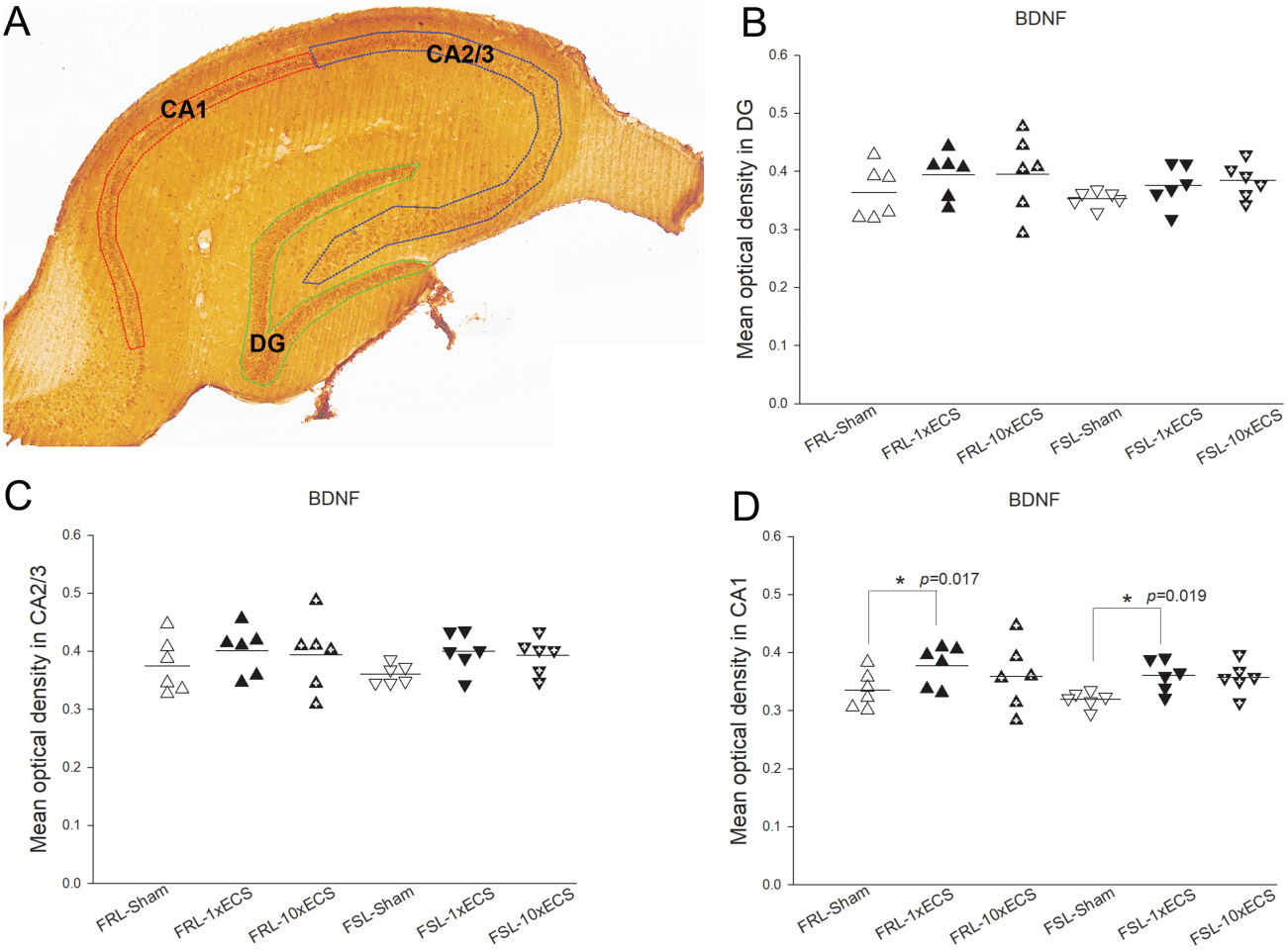

Figure 3. Brain-derived neurotrophic factor (BDNF) expression levels were measured by immunohistochemistry in hippocampus. (A) BDNF expression in the subregions of hippocampus. (B–D) Immunohistochemistry examined BDNF expression levels in each group. Mean optical density (MOD) was calculated with the following formula: OD = log10(max pixel intensity/mean pixel intensity), where max pixel intensity = 255. A single ECS treatment significantly increased the MOD of BDNF expression in both FSL and FRL rat strains in CA1 of hippocampus in contrast to DG and CA2/3 of hippocampus. No difference between the FRL sham and FSL sham rats was observed. Three months after 10xECS, no changes of BDNF expression levels in hippocampal subregions between the FRL and FSL rats were observed.

terminals, but not in total neuropil and dendrites at 3 months after the last treatment ($P = .008$) (Figure 7; Table 2).

In 24 hours after a single ECS groups, a 2-way ANOVA revealed a significant interaction between ECS treatment and strain in the number of total mitochondria ($F_{1, 20} = 12.16$; $P = .002$) and mitochondria in axons ($F_{1, 20} = 7.72$; $P = .012$).

Hippocampus BDNF Expression

No difference in hippocampal BDNF expression between the FRL sham and FSL sham rats was observed. One day after a single ECS, the MOD of BDNF positive immunoreactivity in CA1 (FSL: $P = .019$; FRL: $P = .017$) was significantly higher in both FSL and FRL rats compared with the sham rats (Figure 3; Table 1 and 2).

Three months after 10xECS, no changes of BDNF expression levels in hippocampal subregions were observed between the FRL and FSL rats (Figure 3; Table 1 and 2).

Hippocampus VEGF expression

In 24 hours after a single ECS group, the MOD of VEGF-positive immunoreactivity in DG ($P = .022$), CA2/3 ($P = .02$), and CA1 ($P = .043$) of hippocampus was significantly higher in FSL ECS rats compared with the FSL sham rats (Figure 4; Table 1 and 2). However, there was no difference between the FRL sham and FSL sham rats.

Three months after 10xECS, no changes of VEGF expression levels were found in hippocampal subregions between the FRL and FSL rats (Figure 4; Table 1 and 2).

Discussion

The present study is the first, to our knowledge, to demonstrate that repeated ECS induces long-term changes of structural and ultrastructural plasticity in the hippocampus. A single ECS treatment significantly increased the volume of hippocampal CA1-SR, total length of microvessels, mitochondria number, and synapse number in FSL-ECS rats compared with FSL sham rats accompanied by an increase of BDNF and VEGF expression levels. Multiple ECS treatments significantly increased the volume of hippocampal CA1-SR, total length of microvessels, mitochondria number, and synapse number in FSL ECS rats compared with FSL sham control rats after 3 months of ECS treatment without changing BDNF and VEGF expression levels at the time point measured. Furthermore, the baseline levels of volume, microvessel length, synapses, and mitochondria

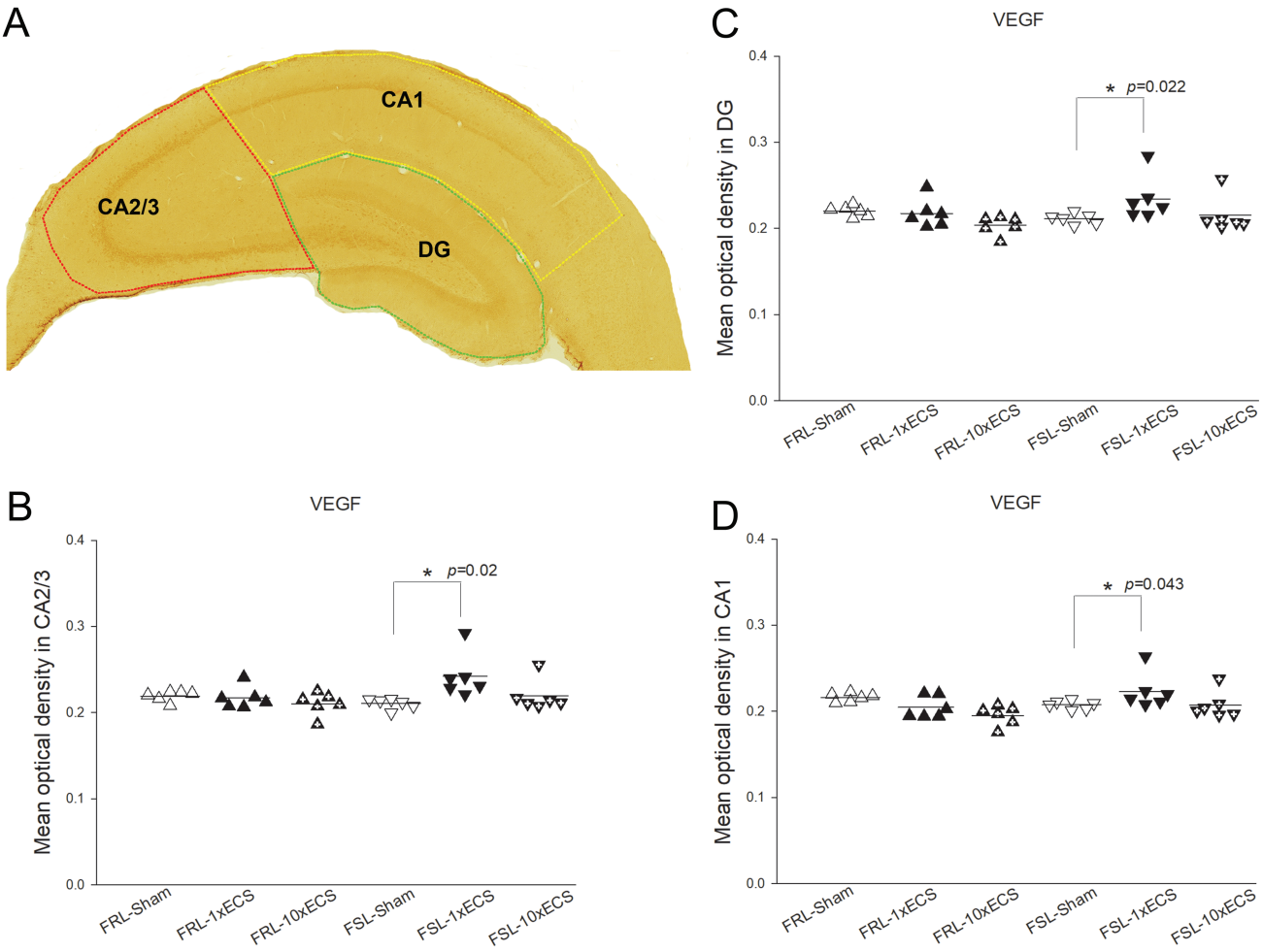

Figure 4. Vascular endothelial growth factor (VEGF) expression levels were measured by immunohistochemistry in hippocampus. (A) VEGF expression in the subregions of hippocampus. (B–D) Immunohistochemistry examined VEGF expression levels in each group. A single ECS treatment significantly increased the MOD (mean optical density) of VEGF expression in both FSL and FRL rat strains in DG, CA2/3, and CA1 of hippocampus. No difference between the FRL sham and FSL sham rats was observed. Three months after 10xECS, no changes of VEGF expression levels in hippocampal subregions between the FRL and FSL rats were observed.

number in the hippocampi of depressive phenotype FSL rats were reduced compared with relevant control FRL rats.

Rapid and Long-Term Effect of ECS on Hippocampal Synaptic Plasticity

Postmortem and animal morphometric studies have demonstrated changes in synapse type and number after antidepressant treatment in hippocampus (Chen et al., 2008, 2009, 2010; Hajszan et al., 2009, 2010; Ardalan et al., 2016). Fluoxetine and S-ketamine induce rapid hippocampal synaptogenesis in the CA1 (Hajszan et al., 2009; Ardalan et al., 2016), whereas onset of DG neurogenesis often happens 3–4 weeks after treatment (Kodama et al., 2004; Marcussen et al., 2008). Furthermore, morphological changes of dendritic spines may be critical for the synaptic plasticity. Therefore, more rapid synaptic plasticity may play an important role in the neurobiology of depression and effects of antidepressant therapy (Levy et al., 2018; Duman et al., 2019). The beneficial effect observed immediately after ECS treatment may be due mainly to improved survival and integration of newborn neurons combined with a rapid increase in synaptic connectivity (Jonckheere et al., 2018).

In the present study, a single ECS treatment after 24 hours significantly increased total synapse number and all subtypes of synapses. In agreement with our study, acute ECS increased the spine density in the apical part of CA1 neurons in nonstressed animals, and there was a trend towards a reduced increase in spine density after a single ECS in restraint stress animals (Kaastrup Muller et al., 2015). The observed increase in CA1 spine density in rats suggested formation of new synapses (Moser et al., 1994). A single-ECS seizure significantly increased cell proliferation in the rat dentate gyrus by 2.3-fold compared with sham treatment (Madsen et al., 2000; Ito et al., 2010). These findings suggest that a single ECS can be beneficial, which is supported by a number of clinical trials with patients receiving a single session of ECT (Thomas and Kellner, 2003; Kellner et al., 2010). Therefore, rapid synaptic plasticity might be partly responsible for the rapid efficacy of ECS. Our findings are supported by the increased expression of a number of genes, which are important for regulating neuronal and synaptic plasticity. Single ECS significantly increased thrombospondin-1 mRNA expression, while thrombospondin-1 is reported to be secreted by astrocytes and to regulate synaptogenesis (Okada-Tsuchioka et al., 2014). Nordgren et al. demonstrated that a single ECS causes transient downregulation of key molecules

Table 1. Results of All Groups in FRL Rats

	FRL sham		FRL 1xECS		FRL 10xECS		FRLsham vs FRL1xECS	FRLsham vs FRL10xECS
	Mean	(SD)	Mean	(SD)	Mean	(SD)	*p*	*p*
Volume (CA1) (mm^3)	4.26	(0.06)	4.4	(0.09)	4.45	(0.14)	.939	.42
Vessel length (mm)	2953	(592)	3098	(335)	4098	(458)	**.012**	**.003**
Optical density BDNF								
DG	0.36	(0.01)	0.39	(0.03)	0.39	(0.06)	.467	.864
CA2/3	0.37	(0.02)	0.40	(0.05)	0.39	(0.06)	.62	.994
CA1	0.34	(0.01)	0.38	(0.03)	0.36	(0.05)	**.017**	.939
Optical density VEGF								
DG	0.22	(0.02)	0.22	(0.01)	0.20	(0.01)	.779	.988
CA2/3	0.22	(0.01)	0.22	(0.02)	0.21	(0.01)	.798	.874
CA1	0.22	(0.01)	0.20	(0.01)	0.20	(0.02)	.13	.669
Synapse number								
Total	9.2	(0.96)	12.2	(0.84)	9.93	(0.74)	**.002**	.374
Non-perforated	6.30	(0.49)	7.57	(0.98)	6.14	(0.62)	**.021**	.736
Perforated	2.34	(0.45)	3.48	(0.81)	2.88	(0.52)	**.008**	.351
Shaft	0.57	(0.07)	1.12	(0.32)	0.92	(0.23)	**.003**	**.005**
Mitochondria number								
Total	4.96	(0.58)	4.21	(0.73)	4.37	(0.64)	.253	.302
In axon	2.54	(0.51)	2.91	(0.25)	2.90	(0.18)	.501	.407
In dendrits	1.38	(0.12)	1.41	(0.38)	1.47	(0.51)	.886	.543

Abbreviations: BDNF, brain-derived neurotrophic factor; CA, Cornu Ammonis; DG, dentate gyrus; SD, standard deviation; VEGF, vascular endothelial growth factor.
P values marked in bold indicate numbers that are statistically significant.

Table 2. Results of All Groups in FSL Rats

	FSL sham		FSL 1xECS		FSL 10xECS		FSLsham vs FSL1xECS	FSLsham vs FSL10xECS	FRLsham vs FSLsham
	Mean	(SD)	Mean	(SD)	Mean	*p*	*p*	*p*	*p*
Volume (CA1) (mm^3)	3.98	(0.15)	4.87	(0.11)	4.62	.653	**.008**	**.011**	.653
Vessel length (mm)	1889	(354)	2662	(593)	3630	**.001**	**.012**	**<.001**	**.001**
Optical density BDNF									
DG	0.35	(0.03)	0.38	(0.03)	0.38	.963	.71	.881	.963
CA2/3	0.36	(0.04)	0.40	(0.03)	0.39	.908	.28	.848	.908
CA1	0.32	(0.03)	0.36	(0.02)	0.36	.775	**.019**	.601	.775
Optical density VEGF									
DG	0.21	(0.01)	0.23	(0.02)	0.22	.36	**.022**	.998	.36
CA2/3	0.21	(0.01)	0.24	(0.01)	0.22	.756	**.02**	.827	.756
CA1	0.21	(0.01)	0.22	(0.01)	0.21	.258	**.043**	.095	.258
Synapse number									
Total	6.88	(0.9)	12.8	(0.94)	10.8	**.016**	**<.001**	**<.001**	**.016**
Non-perforated	4.86	(0.51)	7.83	(0.81)	6.66	**.012**	**<.001**	**.002**	**.012**
Perforated	1.44	(0.34)	3.58	(0.46)	3.15	**.01**	**<.001**	**<.001**	**.01**
Shaft	0.58	(0.22)	1.38	(0.26)	0.98	1	**<.001**	**.002**	1
Mitochondria number									
Total	4.03	(0.43)	5.22	(0.9)	4.49	**.028**	**.032**	.717	**.028**
In axon	1.90	(0.22)	3.28	(0.58)	2.90	**.023**	**<.001**	**.008**	**.023**
In dendrites	1.96	(0.31)	1.94	(0.56)	1.59	**.01**	.907	.858	**.01**

Abbreviations: BDNF, brain-derived neurotrophic factor; CA, Cornu Ammonis; DG, dentate gyrus; SD, standard deviation; VEGF, vascular endothelial growth factor.
P values marked in bold indicate numbers that are statistically significant.

needed to stabilize synaptic structure and to prevent $Ca2^+$ influx, and a simultaneous increase in neurotrophic factors, thus providing a short time window of increased structural synaptic plasticity (Nordgren et al., 2013). Expression of immediate early genes, such as Egr1, Fos, and Arc, is important for regulating neuronal plasticity during memory formation and consolidation (Guzowski et al., 2000; Rodriguez et al., 2005; Bramham et al., 2010). *Egr1* upregulation is thought to initiate a program of gene regulation leading to neuronal plasticity (Kaczmarek and Chaudhuri, 1997). In fact, there was a transient increase in Egr1 and Fos expression immediately after acute ECS (Dyrvig et al., 2012; Calais et al., 2013).

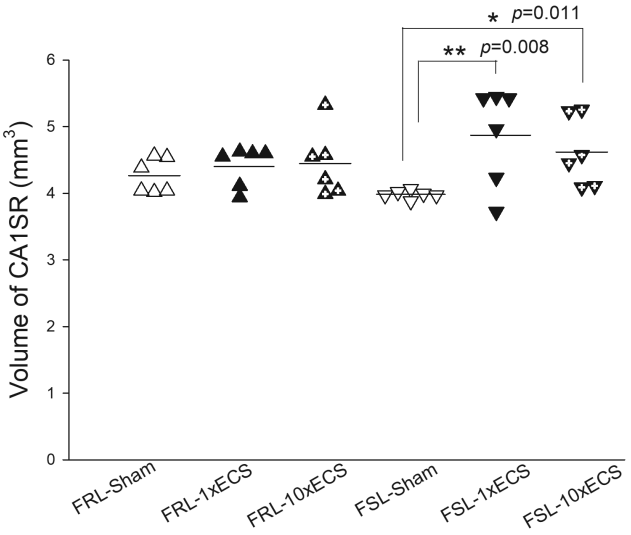

Figure 5. The volume of hippocampal CA1 stratum radium. The volume of hippocampal CA1-SR in the FRL sham rats is significantly larger than that of the FSL sham group. After ECS treatment, the volume of hippocampal CA1-SR in FSL-ECS group significantly increased compared with FSL sham group (*$P < .05$; **$P < .01$).

Our results show an increase in total synapse number and all subtypes of synapses 3 months after multiple ECS treatment. The present study is the first to demonstrate the long-term effect of ECS-induced synaptic plasticity in the adult rat hippocampus. Previous studies have demonstrated that ECS results in long-term survival of newly generated hippocampal neurons in rats (Madsen et al., 2000; Malberg et al., 2000; Olesen et al., 2017). The new neurons induced by ECS have a time-dependent decline from day 1 to 3 months but no further attrition between 3 and 12 months (Olesen et al., 2017). These newborn neurons in DG of the hippocampus need a few weeks to integrate fully and exhibit greater dendritic complexity into the surrounding neural network (Gould and Tanapat, 1999; Zhao et al., 2008).

Rapid and Long-Term Increase of Mitochondria Number After ECS

Mitochondria play important roles in controlling fundamental processes of neuroplasticity (Mattson et al., 1999, 2008; Ruthel and Hollenbeck, 2003). Mitochondria not only provide dynamic energy support for normal synaptic functioning but also directly modulate synaptic structural and functional plasticity (MacAskill et al., 2010; Obashi and Okabe, 2013; Sun et al., 2013; Jonas, 2014). Indeed, altered mitochondrial function has been implicated in alterations in synaptic plasticity (MacAskill and Kittler, 2010). Growing evidence from electron microscopy, imaging, and genetic studies suggest that mitochondrial dysfunction and abnormal mitochondrial structure in neurons affect various aspects of neuronal physiology and contribute to the pathogenesis of neurodegenerative diseases and psychiatric disorders (schizophrenia, bipolar disorder, and major depressive disorder) (Shao et al., 2008; Shao and Vawter, 2008; Chen and Chan, 2009; Scaglia, 2010; Cataldo et al., 2010; Chen et al., 2013).

In the present study, our findings indicate that changes in the mitochondrial number are consistent features of synaptic plasticity. Mitochondrial biogenesis may play an important role in the formation and maintenance of hippocampal synapses and may reflect alterations in neuronal activity associated with variation in abnormal energy demands related to major depression. In addition, the role and functional properties of mitochondria differ in axons and dendrites (Mattson et al., 2008; Palmer et al., 2011). In the axon, mitochondrial ATP production supports the generation of action potentials and trafficking of synaptic vesicles, while in dendrites, it is needed for synaptic transmission and extension/movement of mitochondria into dendritic protrusions in combination with the development and morphological maturation of spines (Zinsmaier et al., 2009). Moreover, twice as many mitochondria are motile in the axons compared with the dendrites of cultured hippocampal neurons, and there is a greater proportion of highly charged, more metabolically active mitochondria in dendrites than in axons (Overly et al., 1996). Single and repeated ECS significantly increased the mitochondria number in axon terminals either 24 hours or 3 months after the last session without changes in dendritic mitochondria number. Moreover, the increased mitochondrial number in axons is twice the number of mitochondria in dendrites in FSL rats after both single and repeated ECS. Since most metabolic activity takes place in axon terminals (Zinsmaier et al., 2009), an increased number of mitochondria in axon terminals after ECS treatment in our study implies that the increased metabolism supports the generation of action potentials and trafficking of synaptic vesicles (neurotransmitter exocytosis and vesicle recruitment).

Short-Term Changes of Hippocampal BDNF and VEGF Expression After ECS

BDNF is suggested to have an important role for the functional and structural synaptic plasticity that occurs after ECS treatment (Vaidya et al., 1999). In support of an antidepressant role of BDNF, decreased serum levels of BDNF have been found in patients with major depression (Karege et al., 2002; Angelucci et al., 2005b). In addition, treatment with ECS increases BDNF protein and mRNA in rat hippocampus (Nibuya et al., 1995; Angelucci et al., 2002, 2005a). A single ECS can induce a complex, transient regulation of levels of Nogo receptors, BDNF, and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors in rat hippocampal formation (Nordgren et al., 2013). Single administration of ECS rapidly upregulates pro-BDNF and t-PA (tissue-type plasminogen activator), leading to mature BDNF production in rat hippocampus (Segawa et al., 2013). In the present study, our results showed that the levels of BDNF-positive immunoreactivity in hippocampal CA1 were significantly increased in both FSL and FRL rats after a single ECS treatment without changes at 3 months after repeated ECS. This is consistent with similar results from Sartorius et al. (2009) showing that BDNF brain tissue levels were mildly increased after a single ECS, especially within the hippocampus. Even though the literature has shown that repeated ECS has long-lasting effects on hippocampal BDNF, the BDNF protein level in the hippocampus remained high for only 7 days after the last treatment (Li et al., 2007). Following multiple ECS, BDNF levels in the hippocampus were strongly elevated for 72 hours after the last ECS session and returned to baseline after 1 week (Sartorius et al., 2009). In addition, BDNF immunoreactivity in our present study showed no differences between FRL sham and FSL sham rats. One possible explanation for the lack of BDNF increase after repeated ECS might be that at 3 months after the treatment, the growth factor response is back at baseline levels. These findings are in line with our previous study (Chen et al., 2018). Similar results from Angelucci et al. (2002, 2003) showed

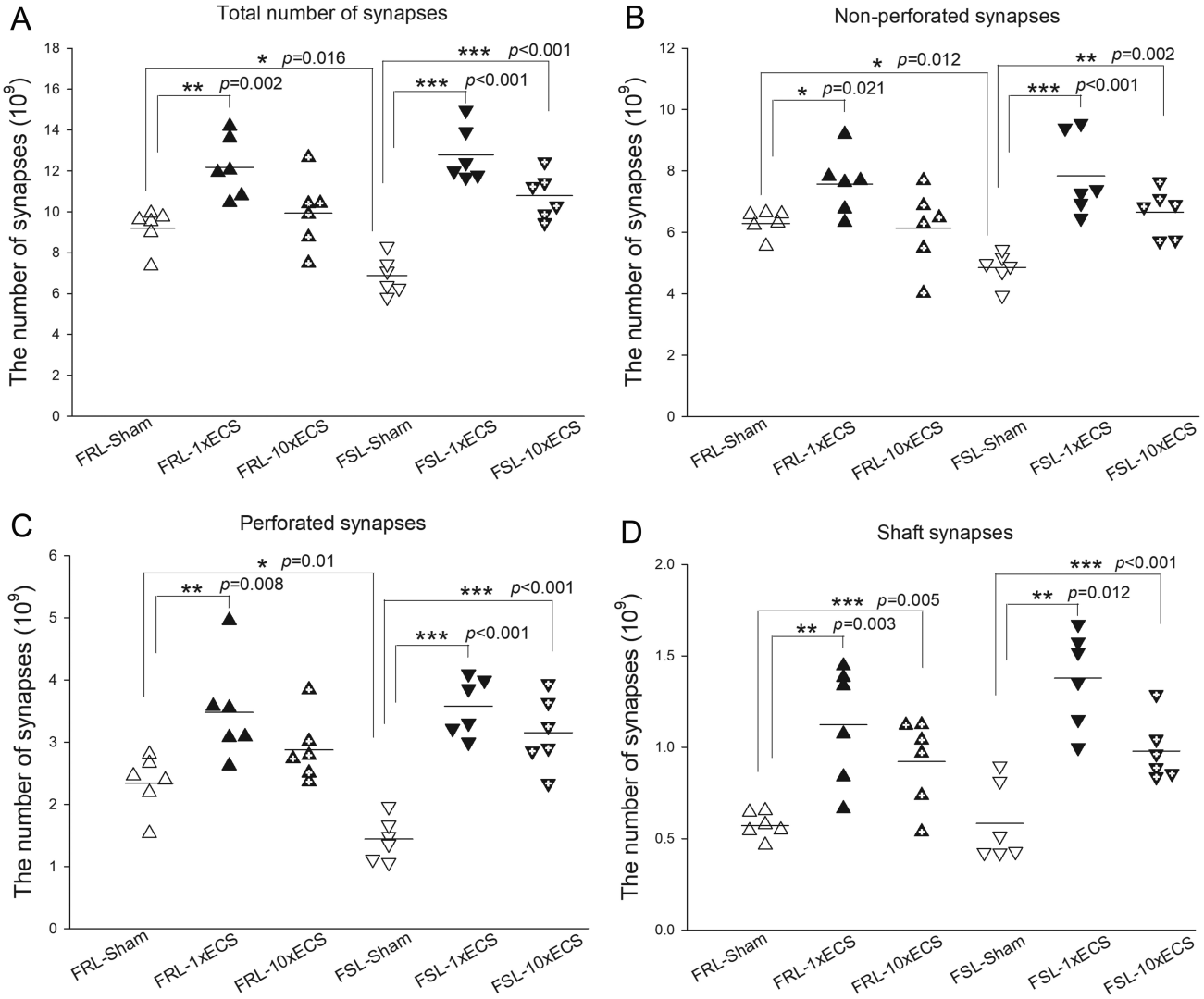

Figure 6. The number of synapses including subtypes of synapses in CA1 (*P < .05; **P < .01, ***P < .001). (A) A single ECS treatment significantly increased total synapse numbers in both FSL and FRL rat strains. 10xECS treatment significantly increased total synapse numbers in FSL ECS rats compared with FSL sham rats in 3 months after last treatment. Total synapse number was significantly higher in FRL sham rats compared with FSL sham rats. (B) The number of nonperforated spine synapses was significantly higher in FRL sham rats compared with FSL sham rats. A single ECS treatment significantly increased the number of nonperforated spine synapses in both strains compared with the respective control. 10xECS treatment significantly increased the number of nonperforated spine synapses in FSL ECS rats compared with FSL sham rats 3 months after last treatment. (C) The number of perforated spine synapses was significantly higher in FRL sham rats compared with FSL sham rats. A single ECS treatment significantly increased the number of perforated spine synapses in FSL ECS rats compared with FSL sham rats. 10xECS treatment significantly increased the number of perforated spine synapses in FSL ECS rats compared with FSL sham rats 3 months after the last treatment. (D) A single ECS treatment significantly increased the shaft synapse number in both FSL and FRL rat strains. 10xECS treatment also increased the shaft synapse number 3 months after the last ECS treatment in both FSL and FRL rat strains.

no difference in the BDNF levels measured by ELISA in the hippocampus of depressed FSL compared with FRL control rats (Li et al., 2007).

VEGF is proven to regulate neuroplasticity as well as promote neurogenesis; thus, it is implicated in the processes of neuronal growth, survival, differentiation, protection, synaptic transmission, and neurobehavioral recovery (Licht et al., 2011). Clinical studies have shown a significant association between baseline serum VEGF levels and a relative reduction in depressive symptomatology after ECT (Minelli et al., 2011; Minelli et al., 2014). Animal studies have further shown that antidepressant induction of hippocampal cell proliferation requires VEGF signaling and VEGF has antidepressant-like properties (Warner-Schmidt and Duman, 2007). These studies suggest that VEGF plays a role in the mechanism of response to ECT, and VEGF-induced antidepressant-like effects may be related to neuronal plasticity. In addition, some evidence has shown that memory-related effects of VEGF may modulate hippocampal synaptic plasticity rather than neurogenesis and improve hippocampal activity related to learning and memory (Cao et al., 2004; Blumberg et al., 2008; Licht et al., 2011). Our results showed that a single ECS treatment significantly increased VEGF expression levels in the hippocampus accompanied by synaptic plasticity.

The short-term effect of ECS on hippocampal BDNF and VEGF expression levels after ECS suggests that temporal changes of BDNF and VEGF may open a time window permissive to synaptic plasticity followed by closure of this window, leading to a lasting alteration of the synaptic circuitry. A clinical study investigated that serum BDNF levels in patients with

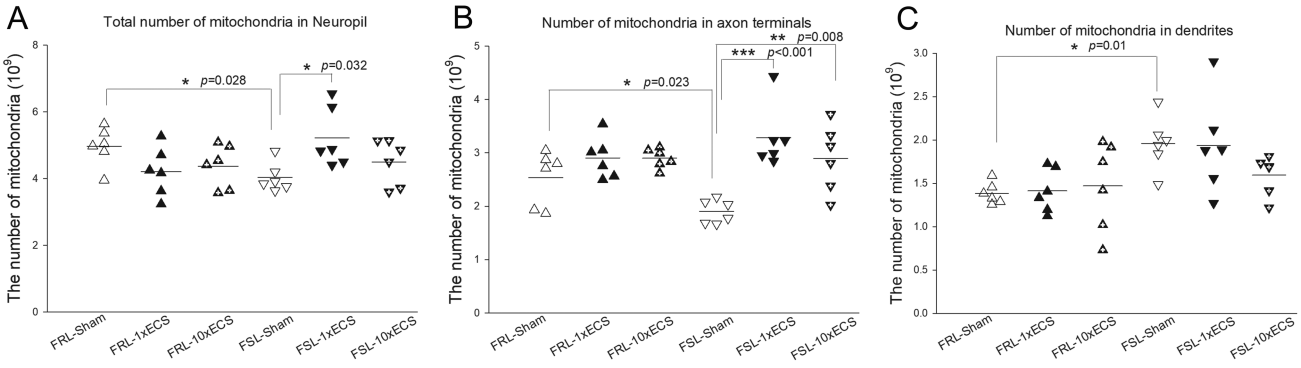

Figure 7. The number of mitochondria in the various structures (neuropil, axons, and dendrites) and the mean volume of mitochondria in CA1 (*P < .05; **P < .01; ***P < .001). (A) The total number of mitochondria in neuropil was significantly smaller in the FSL sham group compared with the FRL sham group. A single ECS treatment significantly increased total mitochondria number in neuropil in FSL ECS rats compared with FSL sham rats. (B) The number of mitochondria in axon terminals was also significantly smaller in the FSL sham group compared with the FRL sham group. A single ECS treatment significantly increased the number of mitochondria in axon terminals in FSL ECS rats compared with FSL sham rats. 10xECS treatment also increased the number of mitochondria in axons 3 months after the last treatment in FSL rats. (C) The number of mitochondria in dendrites was significantly larger in the FSL sham group compared with the FRL sham group. ECS treatment did not make any changes in the number of mitochondria in dendrites between the FSL sham group and the FSL ECS group.

major depression were assessed at 9 time-points before, during, and after acute and subsequent continuation ECT. Their results showed that serum BDNF levels were significantly higher 1 day, 1 week, and 1 month after the last ECT session compared with baseline and returned to baseline level 6 months after the last ECT (Vanicek et al., 2019). Therefore, the changes of hippocampal CA1-SR volume, total length of microvessels, number of synapses, and mitochondria after ECS happened in differential time points after ECS treatment relative to changes of BDNF and VEGF.

Rapid and Long-Term Increase of Length of Microvessels After ECS

Vascular plasticity is another important structural mechanism regulating the replication, survival, and differentiation of cells. Impairment of vascular plasticity of the hippocampus has been shown in hippocampal subregions in animal models of depression (Czeh et al., 2010; Ardalan et al., 2016; Ardalan et al., 2017), and antidepressant treatment increases hippocampal angiogenesis in animals and postmortem studies (Newton et al., 2006; Boldrini et al., 2012). Furthermore, research has revealed that the brain-specific angiogenesis inhibitor plays an important role in synaptogenesis and/or function (Duman et al., 2013; Stephenson et al., 2013). Earlier reports demonstrated that ECS-induced upregulation of angiogenic factors results in increased vascular density in hippocampus (Newton et al., 2006). In agreement with the observations above, our findings showed decreased length of microvessels in the depressed FSL sham rats compared with the FRL control rats. Single and repeated ECS significantly increased length of microvessels either 24 hours or 3 months after the last session.

Limitations of This Study

The main limitation of our study is that we did not investigate the gene and protein expression levels related to the morphological changes. Furthermore, we need to explore a small part of numerous signaling protein molecules implicated in synaptic and mitochondrial plasticity in the present study. To understand the interaction with ECS treatment and synaptic and mitochondrial plasticity, it is very important to reveal the detailed molecular signaling pathways involved in the alterations of the structure and function of synapses and mitochondria. Future human studies should investigate the interplay among BDNF levels, neuroplasticity, and the therapeutic efficacy of ECT in patients with depression.

Taken together, our results indicate that a single ECS causes rapid effects of structural and ultrastructural plasticity, while repeated ECS induces long-lasting changes in the efficacy of synaptic plasticity and nonneuronal plasticity at least up to 3 months after ECS. Overall, this study provides insight into the underlying mechanisms of the rapid and robust therapeutic effect of ECS, which may be related to BDNF and VEGF level elevation, accompanied by mitochondrial and vascular support for the synaptic plasticity.

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Interest Statement

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