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Synapse-specific changes in Arc and BDNF in rat hippocampus following chronic temporal lobe epilepsy

Daniel L. Egbenya [a,b], Suleman Hussain [a,c,*], Yi-Chen Lai [d], Anne E. Anderson [d], Svend Davanger [a]

[a] Laboratory for Synaptic Plasticity, Division of Anatomy, Department of Molecular Medicine, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, Oslo, Norway
[b] Department of Physiology, School of Medical Sciences, College of Health and Allied Sciences, University of Cape Coast, Cape Coast, Ghana
[c] Institute of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway
[d] Jan and Dan Duncan Neurological Research Institute, Baylor College of Medicine, Houston, TX, USA

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ABSTRACT

Expression of immediate early genes (IEGs) in the brain is important for synaptic plasticity, and probably also in neurodegenerative conditions. To understand the cellular mechanisms of the underlying neuropathophysiological processes in epilepsy, we need to pinpoint changes in concentration of synaptic plasticity-related proteins at subsynaptic levels. In this study, we examined changes in synaptic expression of Activity-regulated cytoskeleton-associated (Arc) and Brai Derived Neurotrophic Factor (BDNF) in a rat model of kainate-induced temporal lobe epilepsy (TLE). Western blotting showed reduced concentrations of Arc and increased concentrations of BDNF in hippocampal synaptosomes in chronic TLE rats. Then, using quantitative electron microscopy, we found corresponding changes in subsynaptic regions in the hippocampus. Specifically, we detected significant reductions in the concentrations of Arc in the presynaptic terminal of Schaffer collateral glutamatergic synapses in the stratum radiatum of the CA1 area in TLE, as well as in their adjacent postsynaptic spines. In CA3, there was a significant reduction of Arc only in the presynaptic terminal cytoplasm. Conversely, in CA3, there was a significant increase in the expression of BDNF in the presynaptic terminal, but not in the postsynaptic spine. Significant increase in BDNF concentration in the CA1 postsynaptic density was also obtained. We hypothesize that the observed changes in Arc and BDNF may contribute to both cognitive impairment and increased excitotoxic vulnerability in chronic epilepsy.

1. Introduction

Epilepsy is a chronic brain disease that affects 1–2 % of the world population (Fiest et al., 2017). The disorder is characterised by unpredictable, recurrent generalized or focal seizures (Fisher et al., 2014). Effects of epilepsy are seen in different aspects of the lives of affected persons including their cognitive abilities. The most common form of epilepsy is temporal lobe epilepsy (TLE), which originates in the temporal lobe of the brain. Hippocampal sclerosis is the most frequent histopathological finding in refractory TLE. Consequently, persons with TLE may show severe problems in hippocampus-dependent memory consolidation. A crucial step in the generation and development of epilepsy is altered synaptic transmission, which is influenced by a number of proteins related to synaptic plasticity, including immediate early genes and neurotrophins. However, the state of these affected proteins and synapses during chronic TLE have not been vigorously investigated.

Activity-regulated cytoskeleton-associated (Arc) protein, also known as activity-regulated gene 3.1 (Arg3.1), is a member of the immediate

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AZ, active zone; Arc, activity-regulated cytoskeleton-associated; ACy, astrocytic cytoplasm; BDNF, brain derived neurotrophic factor; IEGs, immediate early genes; EM, electron microscopy; i.p., intraperitoneally; KA, kainic acid; NMDA, N-methyl-D-aspartate; PoCy, postsynaptic cytoplasm; PSD, postsynaptic density; PoL, postsynaptic lateral membrane; PreCy, presynaptic cytoplasm; PreL, presynaptic lateral membrane; SE, status epilepticus; TLE, temporal lobe epilepsy; WB, western blot.

* Correspondence to: Laboratory for Synaptic Plasticity, Division of Anatomy, Department of Molecular Medicine, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo. P.O. Box 1105 Blindern, 0317 Oslo, Norway.
E-mail address: suleman.hussain@medisin.uio.no (S. Hussain).

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early gene (IEG) family, which is activated following robust pathological conditions and physiological stimuli (Szyndler et al., 2013). It mediates the endocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors that are ionotropic transmembrane receptors for glutamate (Chowdhury et al., 2006; DaSilva et al., 2016; Rial Verde et al., 2006; Rogawski, 2011). Thus, alterations of Arc protein levels may play a role in epilepsy (Szyndler et al., 2013), where we have shown that synaptic AMPA receptors are reduced in a TLE model (Egbenya et al., 2018). Additionally, Arc is known to mediate homeostatic plasticity (Shepherd et al., 2006; Turrigiano, 2007); and changes in its expression may affect learning and memory. Arc, therefore, represents a candidate protein underlying epileptogenesis and cognitive deficits experienced by persons with epilepsy.

Brain derived neurotrophic factor (BDNF), a neurotrophin and member of the IEG family, is a low molecular weight protein that is widely distributed in the CNS and has been shown to be present in excitatory presynaptic dense core vesicles (Conner et al., 1997; Dieni et al., 2012; Salio et al., 2007) as well as in postsynaptic spines (Harward et al., 2016; Magby et al., 2006; Song et al., 2017). Moreover, some studies, such as Harward et al. (2016), found BNDF in presynaptic small vesicles. BDNF functions in neuronal survival and maintenance (Kirschenbaum and Goldman, 1995), neuronal morphology, such as modification of dendritic complexity (Danzer et al., 2002), formation and maturation of synapses (Huang et al., 1999; Yamada et al., 2002), and synaptic plasticity (Korte et al., 1998). A disruption of the spatial and temporal expression of BDNF leads to a perturbation in the excitation-inhibition balance of BDNF which causes neurological diseases (Hu and Russek (2008). BDNF mRNA and protein were found to be significantly increased in the hippocampus few days after kainic acid-induced seizures with BDNF protein directly proportional to the extent of seizure severity (Rudge et al., 1998). Similarly, in human TLE patients, BDNF expression was upregulated in the hippocampus (Murray et al., 2000; Takahashi et al., 1999). Within CA3 neurons, BDNF was found to contribute to injury-induced hyperexcitability (Gill et al., 2013). Furthermore, BDNF mediates cognitive processes such as learning and memory; and may function in both early and late phases of LTP (Bekinschtein et al., 2014; Kang et al., 1997; Korte et al., 1998; Lu and Gottschalk, 2000). Together, these observations suggest that BDNF, also, may play a role in epileptogenesis and cognitive changes associated with epilepsy.

In this study, we investigated long-term synapto-pathological changes in the two IEG products, which are synaptic plasticity-related proteins, Arc and BDNF, eight weeks after the induction of status epilepticus in a rat model of TLE. These proteins are activated rapidly and transiently in status epilepticus. However, they were studied in the current project eight weeks after induction of epilepsy. They were studied at this time period for their role in synaptic plasticity and whether or not their actions induced at the time of induction can have continuous implications for epilepsy over a long period of time. While acute, short-term changes may have direct relevance for neuronal or synaptic survival, long-term changes of surviving synapses may be more important for comorbidities, like cognitive and memory impairments. With regard to memory impairments, we would, in fact, expect long-term changes in concentrations of IEGs like Arc and BDNF. To the best of our knowledge, such changes over an eight-week period after induction of seizures have not been examined previously. We found a significant decrease in the concentration of Arc in hippocampal CA1 subsynaptic sites while a significant increase in total BDNF in hippocampal CA3 presynaptic terminals following a rat model of chronic TLE.

2. Materials and methods

2.1. Antibodies

The following antibodies were used in the western blot (WB) experiments: Polyclonal rabbit Arc antibody (Synaptic Systems, Germany, Cat# 156003, RRID:AB_887694), raised against recombinant protein corresponding to amino acids 1 – 396 from mouse Arc, used at 1:2 000; polyclonal rabbit BDNF antibody (N-20) (Santa Cruz, USA, Cat# SC 546, RRID:AB_630940), raised against a peptide mapping within an internal region of BDNF of human origin, used at 1:200; monoclonal actin antibody (Millipore, Germany, Cat# MAB1501, RRID:AB_2223041), raised against amino acids 357–375 at the C-terminus of Actin of human origin, used at 1:20 000; monoclonal anti-β-tubulin (Covance, USA, Cat# MMS-435 P, RRID:AB_2313773), raised against microtubules derived from rat brain, used at 1:10 000.

The following primary antibodies were used for immunogold electron microscopy (EM): Both the anti-Arc and anti-BDNF antibodies, stated above, were used at 1:50 and 1:1 000, respectively. The specificity of both the Arc antibody (Niere et al., 2012) and the BDNF antibody (Talaveron et al., 2013) have been demonstrated previously. The anti-BDNF antibody binds to both forms of BDNF, i.e., proBDNF and mature BDNF (mBDNF).

The following secondary antibodies were used in the WB experiments: Mouse monoclonal anti-rabbit IgG (γ-chain specific) alkaline phosphatase antibody (Sigma, MO, USA, Cat# A2556, RRID: AB_258010) at 1:10 000, and goat polyclonal anti-mouse IgG (whole molecule) alkaline phosphatase (Sigma, MO, USA, Cat# A3562, RRID: AB_258091) at 1:10 000.

The following secondary antibody was used for immunogold electron microscopy: Polyclonal goat anti-rabbit (IgG coupled to 10 nm colloidal gold) (Abcam, Cambridge, UK, Cat# ab27234, RRID:AB_95442) for Arc, and polyclonal goat anti-rabbit (IgG coupled to 15 nm colloidal gold) (Abcam, Cambridge, UK, Cat# ab27236, RRID:AB_954457) for BDNF; both at 1:30.

2.2. Animals

For western blotting experiments, Sprague-Dawley rats weighing 120 – 180 g (Harlan Sprague-Dawley Inc., Indiana, USA) were used. The rats were treated in the animal facility at Baylor College of Medicine, Texas, USA. The animals used in the WB experiments consisted of seven rats in the control group and eight rats in the chronic TLE group. In the case of the postembedding immunogold EM experiments, we used a separate set of six Sprague-Dawley male rats weighing 250 g, supplied by the Møllegaard Breeding centre, Copenhagen, Denmark. Three rats were used in the control group and three rats in the chronic TLE group. The chronic TLE rats were treated with the potent neurotoxin kainic acid (KA) intraperitoneally (i.p.), while the control group received injections of normal saline.

Intraperitoneal administration of kainate 18 mg/kg and 10 mg/kg was carried out to induce status epilepticus in the rats used, respectively, for WB and EM experiments. The Racine Scale (Racine, 1972) was used for monitoring the behavioural dispositions (seizures) of the rats after the kainate administration. A continual limbic seizure with a score of 4 or 5 was termed status epilepticus. This is a model of TLE (Ben-Ari, 1985; Levesque and Avoli, 2013; Nadler, 1981). After an hour of being in the state of status epilepticus, the seizure was terminated using 20 mg/kg pentobarbital intraperitoneal. The control animals were also given pentobarbital 1 h after the saline injection. Though the specific animals included in this study were not monitored for seizures in the chronic phase, a separate set of 14 representative animals in our lab that were treated with the same protocol were monitored and showed a behavioural seizure frequency at eight weeks following status epilepticus (SE): The median seizure frequency was 0.06 seizures per hour, with the interquartile range of 0 – 0.063 seizures per hour. Eight or eleven weeks after induction of the first major status epilepticus, respectively, which corresponds to the chronic phase of TLE, a period during which the long-lasting changes of epilepsy would be manifested, the rats were humanely killed by rapid decapitation. Prior to killing, the rats for WB experiments were anaesthetised with 2 mg/kg of ketamine intraperitoneally. The brains were quickly removed and kept on ice-cold PBS for 5

min. Thereafter, the hippocampi were quickly dissected and immediately frozen. For EM experiments, the rats were deeply anaesthetised with Equithesin (0.4 ml/100 g, i.p.) and transcardially perfused with a flush of about 30 ml of 2 % dextran in 0.1 M sodium-phosphate buffer and thereafter with 1.0 l of a fixative made up of glutaraldehyde (0.5 %) and formaldehyde (4.0 %) in the same buffer. Having left the preparation overnight at 4 ^{0}C, the brains were removed and stored in the above-mentioned fixative after diluting it 1:10 in 0.1 M sodium-phosphate buffer at 4 ^{0}C.

We did not obtain EEG data from these rats. However, using the well-established Racine Scale, their behavioural dispositions were well measured. Also, we have in the past demonstrated behavioural monitoring of seizures in this model of which we reported that majority of the rats started to show spontaneous behavioural seizures at time frames similar to the cohort of animals used in the present study (Brewster et al., 2016).

Animals included in the WB group were treated in conformity with the Guidelines of the National Institute of Health for the Care and Use of Laboratory Animals and were accordingly approved by the Institutional Animal Care and Use Committee of the Baylor College of Medicine, Texas, USA. Treatment of the animals in the EM group was in accordance with the European Convention (ETS 123 of 1986), and accordingly approved by the Norwegian National Animal Research Authority.

Due to difficulties in obtaining Arc and BDNF knockouts, we used their relatively low expression in astrocytes as a test of specificity of these antibodies.

2.3. Preparation of crude synaptosomes

Rat hippocampi were stored in a − 80 °C freezer before they were put into an ice-cold homogenisation buffer (0.32 M sucrose, 4 mM HEPES, pH 7.4) that included a protease inhibitor cocktail (Roche Diagnostics, USA, Cat# 13405700). They were subjected to 8–10 strokes of a glass/Teflon homogenizer in order to homogenise the hippocampi. To remove nuclei and large fragments, the homogenate was centrifuged at 800 x g for 10 min at 4 °C. The supernatant was then centrifuged at 1000 x g for 15 min. The resulting pellet constituted the crude synaptosomes. This was re-suspended in 1 ml of homogenising buffer. Thereafter, the protein concentration of the crude synaptosomes was calculated using the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) with the aid of a Spectrophotometer (Labsystems Multiskan, Canada) at an absorbance value of 562 nm.

2.4. Western blotting

In the WB experiments, protein extracts were run on a 4– 0 % SDS-acrylamide gel (Bio-Rad Laboratories Inc., USA, Cat# 345-0007) at 200 V and 400 mA for 50–55 min followed by electro-blotting for 30 min at 25 V, 1 A. The different protein extract amounts were used to optimize band intensities for quantification. Prior to this, the crude synaptosomes were mixed with sample loading buffer (62.5 mM Tris-HCl, 10 % glycerol, 2% SDS, 5 % 2-mercaptoethanol, 0.025 % bromophenol blue). The membrane was blocked with 5 % skim-milk solution for 60 min followed by an overnight incubation with primary antibody (in 2.5 % skim-milk solution). Secondary antibody incubation (in 1.25 % skim-milk solution), washing (4 ×15 min using TBS-T buffer) and signal detection using ECF substrate (GE Healthcare, UK, Cat# 1067873) were performed. Bio-Rad Scanner (Bio-Rad Laboratories, Inc., USA) was used to visualize the fluorescence signals. Trial blots with the Arc antibody gave only a single band, corresponding to 45 kDa (Supplemental material, Fig. S1A). Trial blots with the BDNF antibody showed more than one band, i.e., primarily the two main bands corresponding to proBDNF at 28 kDa and mBDNF at 16 kDa (Supplemental material, Fig. S1B). We did not see any weaker bands above proBDNF, as some others have found, but we did see one or two weaker bands above the mBDNF band, as has also been seen by others (Lynch et al., 2007). These bands are regarded as related to the main mBDNF protein. Band intensities of the blots were normalized to loading controls (either actin or β-tubulin). The membranes were cut according to the molecular weight markers so that one half contained the experimental protein (Arc or BDNF), while the other half contained the control protein (actin or tubulin, respectively). The two halves of each membrane were then immunostained for their corresponding protein, in 50 ml Falcon tubes.

2.5. Postembedding immunogold EM

2.5.1. Freeze substitution

Tissue pieces (0.5 × 1.0 mm) of the hippocampal CA1 and CA3 regions were freeze substituted, sectioned, and immunolabeled as described previously (Mathiisen et al., 2006). Briefly, the tissue blocks were cryoprotected in glycerol and were immediately frozen in liquid propane at − 170 ^{0}C. The specimens were transferred to 0.5% uranyl acetate that has been dissolved in anhydrous methanol (−90 ^{0}C) in an automatic freeze substitution unit infiltrated with Lowicryl HM20 Resin at − 30 ^{0}C followed with polymerisation by UV light.

2.5.2. Immunolabeling

Small blocks of specimens (0.5–1.0 mm) dissected from the CA1 and CA3 areas of the rat hippocampus were sectioned and immunolabeled using 50 mM glycine in Tris-buffered saline with 0.1 % Triton X-100 (TBST), 2 % Human Serum Antigen (HSA) in TBST and 0.05 % polyethyleneglycol (PEG) as described previously (Mathiisen et al., 2006). Incubation with the primary antibody was performed overnight before subsequent incubation with the secondary antibody in addition to washing, all at room temperature.

2.6. Computation and statistical analysis

Electron micrographs of the sections were acquired with a Tecnai 12 BioTWIN Transmission Electron Microscope (FEI Company, USA). For the Arc experiment, 224 and 438 electron micrographs of asymmetric synapses were taken from the hippocampal CA1 and CA3 areas, respectively. In the case of the BDNF experiment, 180 and 198 electron micrographs of asymmetric synapses were taken from the hippocampal CA1 and CA3 areas, respectively. Electron micrographs of asymmetric synapses were taken from the middle region of the stratum radiatum, i.e., Schaffer collaterals in the CA1, and recurrent collateral synapses in the CA3. Description of the quantification procedure was as stated in Hussain et al. (2016). Briefly, specific plasma membrane and cytoplasmic compartments were defined and used for the quantification (Fig. 1A). They corresponded to: the postsynaptic density (PSD), the active zone (AZ), the postsynaptic lateral membrane (PoL), the presynaptic lateral membrane (PreL), the postsynaptic cytoplasm (PoCy) and the presynaptic cytoplasm (PreCy). Each synaptic profile used for quantification had structurally well-defined PSD and plasma membranes. Immunogold labeling was quantified as number of gold particles/μm of membrane length in asymmetric synapses and as number of gold particles/μm^2 for regions of interest in intracellular compartments. Quantification was done with the aid of our laboratory extension to the analySIS software connected to SPSS (SPSS Inc., Chicago, IL, USA). Two parameters, namely; linear particle density (number per unit length of curve) over membrane regions and area gold particle density (number per unit area) over cytoplasmic compartments were computed using the analySIS software. In the case of the linear particle density, the distance from the centre of each particle to the middle of the membrane was measured. Only gold particles, which were situated within the operator-defined distance of 21 nm from the curve segment, were included in the analysis. All gold particles located in the cytoplasmic compartments were counted to constitute the area gold particle density. Mann-Whitney U test was used to analyse the quantified results because the data were not normally distributed. All values are expressed as mean ± SEM.

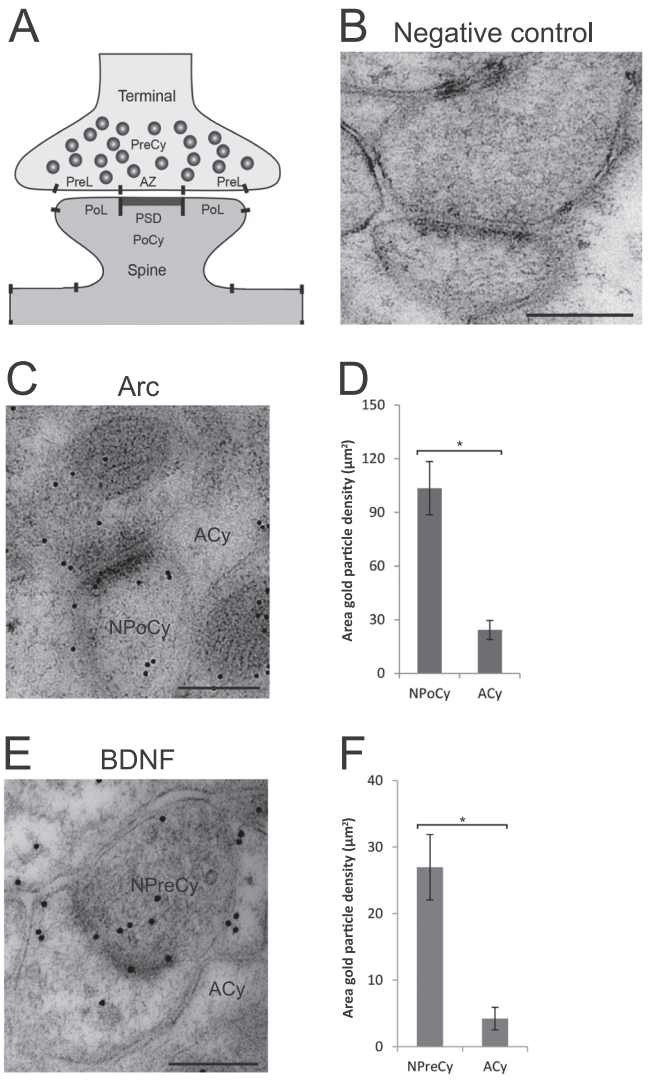

Fig. 1. Regions of interest and antibody controls of hippocampal synapses. (A) Schematic diagram of subsynaptic regions of interest analyzed in the present investigation. PreCy: presynaptic cytoplasm; PoCy: postsynaptic cytoplasm; AZ: active zone; PSD: postsynaptic density; PreL: presynaptic lateral membrane; PoL: postsynaptic lateral membrane. (B) Immunogold labeling of hippocampal section with only secondary antibody. (C) Electron micrograph showing Arc labeling over neuronal postsynaptic cytoplasm (NPoCy) and astrocytic cytoplasm (ACy). (D) Quantitative immunogold analysis of Arc labeling in NPoCy and ACy. (**E**) Electron micrograph demonstrating BDNF labeling over neuronal presynaptic cytoplasm (NPreCy) and ACy. (F) Quantitation of BDNF labeling in NPreCy and ACy.

3. Results

3.1. Regions of interest and antibody controls of hippocampal synapses

Initially, we selected six different synaptic regions of interest for further analyses in the EM preparations: PSD, AZ, PoL, PreL, PoCy and PreCy (Fig. 1A). We then tested the immunolabeling procedure by performing negative control labeling of normal rat brains without primary antibody (Fig. 1B). As expected, this showed no labeling, indicating that gold-conjugated secondary antibodies did not contribute to unspecific labeling. The specificity of the Arc antibody has been characterised previously (Niere et al., 2012). To confirm Arc antibody specificity in our study, we showed that the level of immunogold labeling in neuronal postsynaptic cytoplasm (PoCy) was 326 % higher than in astrocytic cytoplasm (ACy) (103.6 ± 14.9/µm^2 vs 24.3 ± 5.3/µm^2; n = 40 in both groups, $p < 0.05$) (Fig. 1C, D). In comparison with the postsynaptic cytoplasm of controls (presented below), the level of Arc immunogold labeling in astrocyte cytoplasm could not be distinguished from background. This, in addition to the single band obtained for Arc protein in western blotting experiment indicates that the Arc antibody was also specific in our experiments. The specificity of the BDNF antibody has also been characterised previously (Talaveron et al., 2013). We further confirmed this by comparing the labeling intensities of BDNF in neuronal PreCy and ACy (Fig. 1E, F). The value for BDNF labeling was 542 % higher in PreCy than in ACy (27.0 ± 4.9 µm^2 vs 4.2 ± 1.7 µm^2; n = 40 in both groups, $p < 0.05$). This supports the specificity of the BDNF antibody used in this study.

3.2. Arc is reduced in rat hippocampus in chronic TLE

Changes in the concentration of Arc protein in crude hippocampal synaptosomes was examined with following chronic TLE, compared to controls (Fig. 2A). We found a significant decrease (−24 %) in the concentration of Arc in the chronic phase (132.8 ± 9.3 a.u. in the control group vs 101.41 ± 5.5 a.u. in the chronic TLE group; $t(13) = 3.0074$, $p = 0.01$) (Fig. 2B).

3.3. Arc is reduced in Schaffer collateral synapses in CA1 in chronic TLE

We performed postembedding immunogold EM to examine changes in expression of Arc protein in specific synapses and in subsynaptic regions during the chronic phase of epilepsy. Asymmetric synapses in the stratum radiatum of CA1 (Schaffer collateral synapses) (Fig. 3A, B), as well as in CA3 (recurrent collaterals) (Fig. 4A, B) of the hippocampus were examined.

In the CA1, we first compared immunogold levels between controls and chronic TLE in synaptic plasma membrane regions, i.e., in Schaffer collateral synapses in the CA1. See Table 1 for the descriptive statistics. Both pre- and postsynaptic plasma membrane regions of interest showed significant reductions in Arc immunogold labeling in chronic TLE, as

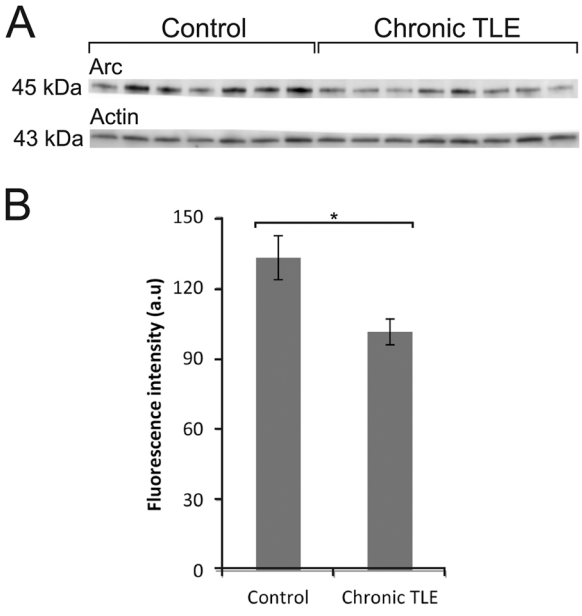

Fig. 2. Western blot of Arc in rat hippocampal synaptosomes. (A) Representative blot of Arc (45 kDa) in chronic TLE and control rats. Each well was loaded with 20 µg of protein. Actin (43 kDa) was used as loading control. (B) Mean band intensities of Arc in hippocampal synaptosomes from control and chronic TLE rats. Error bars represent SEM. Asterisk (*) indicates significant difference between control and chronic TLE groups (independent t test, $p < 0.05$).

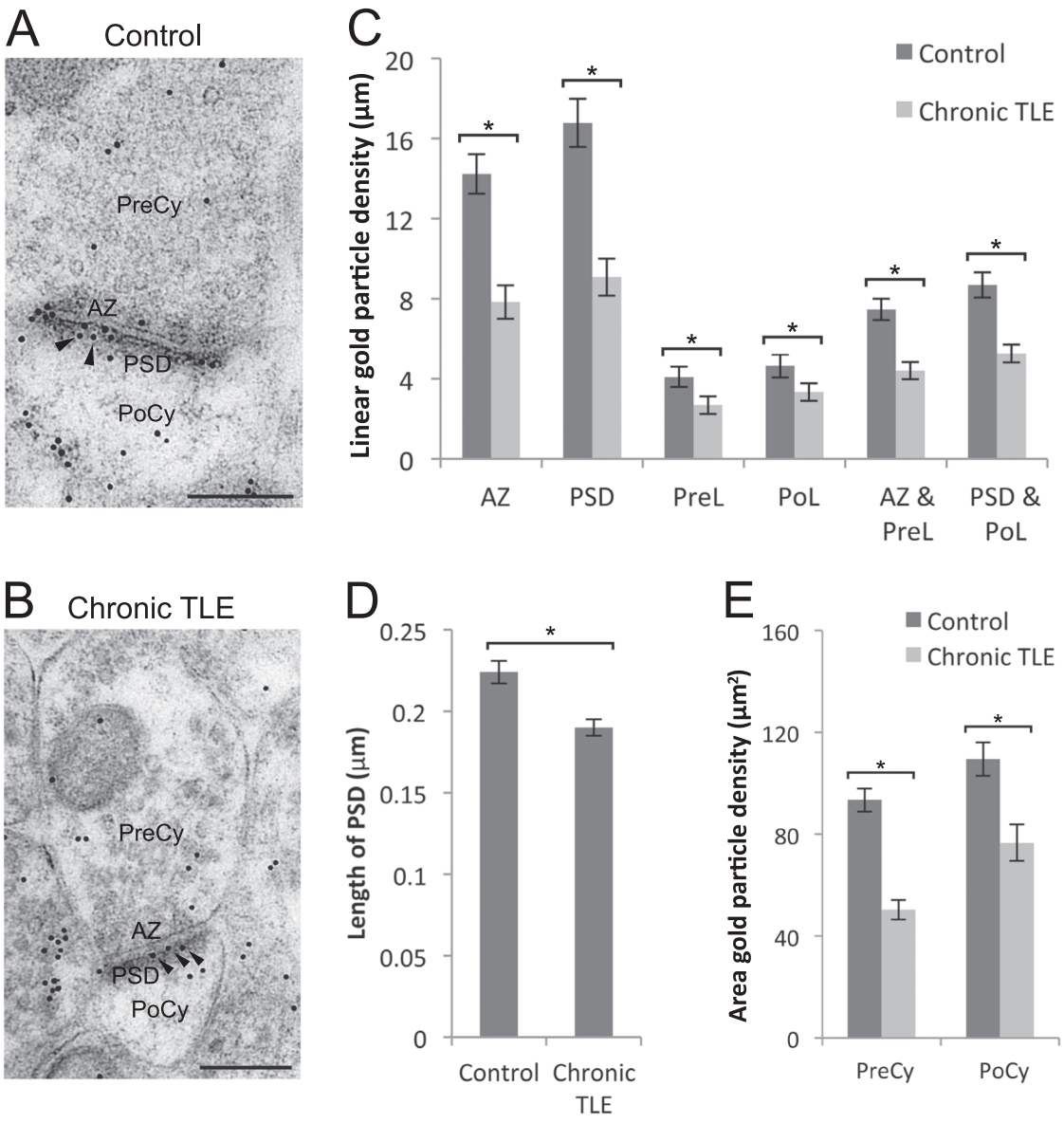

Fig. 3. Arc protein EM immunogold labeling of Schaffer collateral synapses in rat hippocampal CA1 region. (A) Immunogold labeling of control rat. (B) Immunogold labeling of chronic TLE rat. (C) Quantification of labeling along plasma membrane regions of interest. (D) Mean length of the PSD in control and chronic TLE synapses. (E) Quantification of labeling in cytoplasmic regions of interest. Error bars represent SEM. Asterisk (*) indicates significant differences (Mann-Whitney U test, $p < 0.05$) in all graphs. Scale bars: B = 200 nm. Arrowheads point to gold particles over PSD.

specified below (Fig. 3C). The highest concentration of Arc immunogold labeling was seen in the PSD, with a reduction of − 46 % in chronic TLE compared to controls (9.1 ± 0.9/µm vs 16.8 ± 1.2/µm, n = 224 synaptic profiles in both groups, $p < 0.001$). In the active zone (AZ), the corresponding reduction was − 45 % (7.8 ± 0.8/µm vs 14.2 ± 1.0/µm; n = 224 synaptic profiles in both groups, $p < 0.001$). Along the presynaptic lateral plasma membrane (PreL) the reduction was − 34 % (4.1 ± 0.5/µm to 2.7 ± 0.4/µm; n = 448 synaptic profiles in both groups, $p < 0.001$), and in the postsynaptic lateral plasma membrane (PoL) the reduction was − 28 % (4.6 ± 0.6/µm vs 3.3 ± 0.4/µm; n = 448 synaptic profiles in both groups, $p = 0.03$). For the whole presynaptic plasma membrane (AZ + PreL), the reduction was − 41 % (7.5 ± 0.5/µm vs 4.4 ± 0.4/µm; n = 672 synaptic profiles in both groups, $p < 0.001$). The corresponding reduction along the postsynaptic plasma membrane (PSD + PoL) was − 39 % (8.7 ± 0.6/µm vs 5.3 ± 0.5/µm; n = 672 synaptic profiles in both groups, $p < 0.001$).

Due to the detected decrease in Arc concentration in the PSD, we wanted to know whether there was any corresponding effect in the *size* of the PSD. We thus analyzed the mean length of the PSD profile in synaptic sections (Fig. 3D). The length of the PSD was found to be reduced by − 15 % in chronic TLE compared to controls (0.224 ± 0.007 µm vs 0.190 ± 0.005 µm; n = 224 synaptic profiles in both groups, $p < 0.001$).

Next, we examined the expression of Arc in pre- and postsynaptic cytoplasmic regions. Arc immunoreactivity was significantly reduced (−46 %) in presynaptic terminal cytoplasm of the chronic TLE rats compared to controls (50.4 ± 3.8/µm^2 vs 93.4 ± 4.5/µm^2; n = 224 in both groups, $p < 0.001$) (Fig. 3E). In the postsynaptic cytoplasm, the concentration of Arc was also significantly reduced (−30 %) in the chronic TLE rats compared to controls (76.7 ± 7.2/µm^2 vs 109.4 ± 6.6/µm^2; n = 224 in both groups, $p < 0.001$).

In CA3, there was a minor (−13 %), but significant reduction in Arc concentration only in the presynaptic terminal cytoplasm of the recurrent collaterals in the stratum radiatum (Fig. 4A, B) (87.9 ± 3.5/µm vs 76.5 ± 3.2/µm; n = 438 synaptic profiles in both groups, $p = 0.02$). None of the other regions of interest in CA3 showed any significant

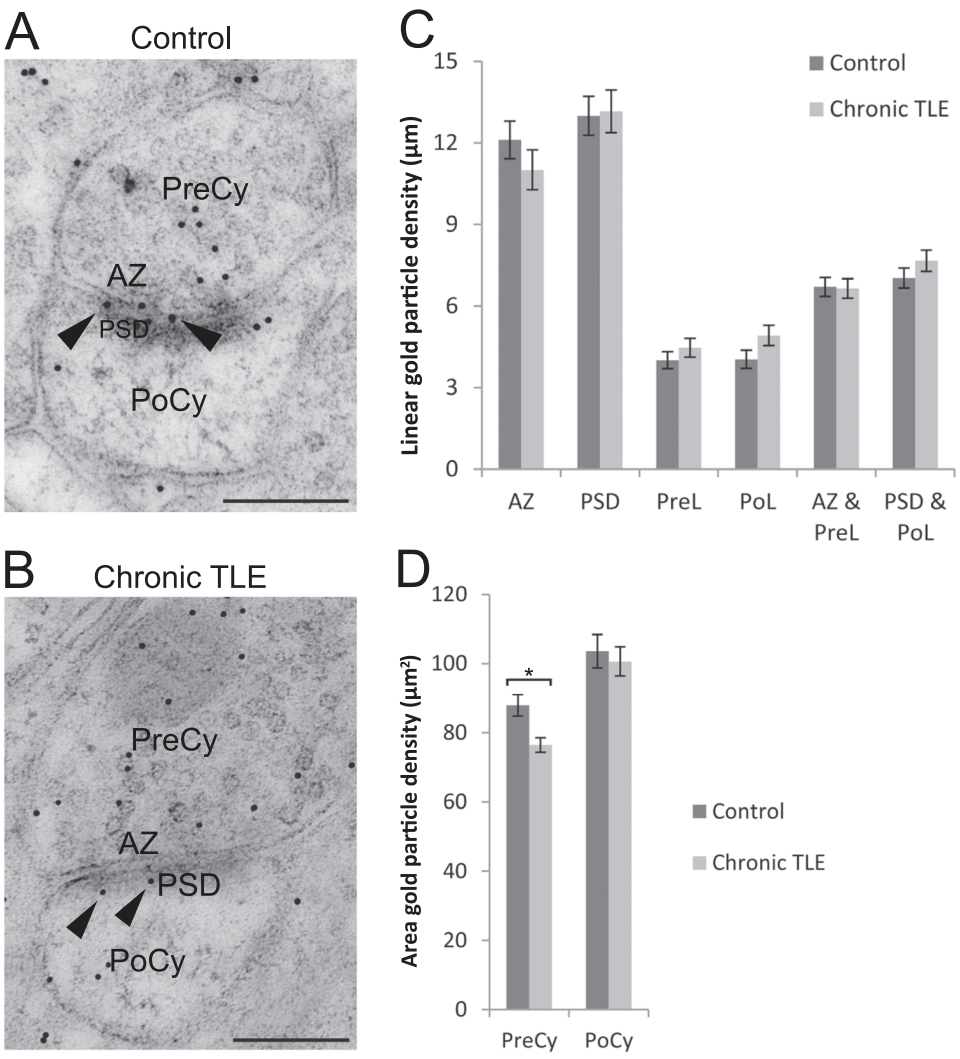

Fig. 4. Arc protein EM immunogold labeling of recurrent collateral synapses in rat hippocampal CA3 region. (A) Immunogold labeling of a control rat. (B) Immunogold labeling of a chronic TLE rat. (C) Quantification of labeling along plasma membrane regions of interest. (D) Quantification of labeling in cytoplasmic regions of interest. Error bars represent SEM. Asterisk (*) indicates significant difference between control and chronic TLE groups (Mann-Whitney U test, $p < 0.05$). Scale bars = 200 nm. Arrowheads point to gold particles over PSD.

Table 1
The table shows descriptive statistics of Arc immunogold labeling in the hippocampal CA1 region. Mean and median values are presented as gold particles/µm and as number of gold particles/µm^2, respectively, in the membrane and cytoplasmic regions of interest.

Group - Region	n	Mean	Median	SD	SEM
CA1 control - AZ	112	14,2	12,0	10,4	1,0
CA1 control - PSD	112	16,8	14,7	12,8	1,2
CA1 control - PreL	224	4,1	0	7,6	0,5
CA1 control - PoL	224	4,6	0	8,5	0,6
CA1 control - AZ & PreL	336	7,5	4,6	9,9	0,5
CA1 control - PSD & PoL	336	8,7	4,4	11,7	0,6
CA1 control - PreCy	112	93,4	85,4	48,1	4,5
CA1 control - PoCy	112	109,4	100,1	69,6	6,6
CA1 epilepsy - AZ	112	7,8	5,2	8,9	0,8
CA1 epilepsy - PSD	112	9,1	6,3	9,8	0,9
CA1 epilepsy - PreL	224	2,7	0	6,6	0,4
CA1 epilepsy - PoL	224	3,3	0	6,6	0,4
CA1 epilepsy - AZ & PreL	336	4,4	0,4	7,8	0,4
CA1 epilepsy - PSD & PoL	336	5,3	0	8,3	0,5
CA1 epilepsy - PreCy	112	50,4	43,7	40,5	3,8
CA1 epilepsy - PoCy	112	76,7	57,4	75,8	7,2

Table 2
The table shows descriptive statistics of Arc immunogold labeling in the hippocampal CA3 region. Mean and median values are presented as gold particles/µm and as number of gold particles/µm^2, respectively, in the membrane and cytoplasmic regions of interest.

Group - Region	n	Mean	Median	SD	SEM
CA3 control - AZ	222	12,1	10,3	10,3	0,7
CA3 control - PSD	222	13,0	11,0	10,7	0,7
CA3 control - PreL	444	4,0	0	6,6	0,3
CA3 control - PoL	444	4,0	0	7,1	0,3
CA3 control - AZ & PreL	666	6,7	4,5	8,9	0,3
CA3 control - PSD & PoL	666	7,0	4,6	9,4	0,4
CA3 control - PreCy	222	87,9	75,9	47,0	3,2
CA3 control - PoCy	222	103,6	92,4	71,9	4,8
CA3 epilepsy - AZ	216	11,0	8,5	10,8	0,7
CA3 epilepsy - PSD	216	13,2	10,4	11,5	0,8
CA3 epilepsy - PreL	432	4,5	0	7,2	0,3
CA3 epilepsy - PoL	432	4,9	0	7,8	0,4
CA3 epilepsy - AZ & PreL	648	6,6	3,7	9,1	0,4
CA3 epilepsy - PSD & PoL	648	7,7	5,3	10,0	0,4
CA3 epilepsy - PreCy	216	76,5	72,6	31,2	2,1
CA3 epilepsy - PoCy	216	100,6	90,7	61,7	4,2

changes in Arc concentrations (Fig. 4C, D). See Table 2 for the descriptive statistics.

3.4. BDNF is increased in rat hippocampus following chronic TLE

Changes in the concentration of BDNF protein in crude hippocampal synaptosomes was examined following chronic TLE, compared to

controls (Fig. 5A). Contrary to the pattern observed for Arc, both mBDNF and proBDNF were *increased* in chronic TLE by 89 % and 60 %, respectively (mBDNF: 53.6 ± 12.2 a.u. in chronic TLE vs 28.3 ± 4.3 a.u. in controls; **t**(13) = −1.8457, p = 0.04) (proBDNF: 96.7 ± 9.9 a.u. in chronic TLE vs 60.5 ± 9.1 in controls, **t**(13) = −2.6535, p = 0.01) (Fig. 5B).

3.5. BDNF is increased in the presynaptic terminal of rat hippocampal CA3

In contrast to the significant increase in the concentration of BDNF in whole hippocampal synaptosomes, there was no consistent change at the synaptic level in the CA1 (Fig. 6A–D). In the chronic TLE rats, total BDNF concentration was significantly increased, by 52 %, only in the PSD (3.5 ± 0.4/μm vs 2.3 ± 0.3/μm; n = 180 synaptic profiles in both groups, p = 0.02) (Fig. 6C) while there was a decrease (−36 %) in the presynaptic cytoplasm (20.7 ± 1.4/μm^2 vs 32.5 ± 1.9/μm^2; n = 180 synaptic profiles in both groups, p < 0.001) (Fig. 6D) and along the PreL membrane (−42 %) (1.1 ± 0.2/μm vs 1.9 ± 0.3/μm; n = 360 synaptic profiles in both groups, p = 0.03) (Fig. 6C). See Table 3 for the descriptive statistics.

On the other hand, analysis of the recurrent collateral synapses of area CA3 (Fig. 7A, B) revealed that total BDNF protein (proBDNF and mBDNF together) was significantly increased in the presynaptic terminal regions (PreCy, AZ and PreL) after chronic TLE (Fig. 7C, E). In the presynaptic cytoplasm, the expression of total BDNF was significantly increased by 26 % in the chronic TLE rats, compared to controls (34.0 ± 2.3/μm^2 vs 27.0 ± 1.4/μm^2; n = 198 synaptic profiles in both groups, p = 0.01). However, the increase in BDNF immunoreactivity in the postsynaptic cytoplasm between the chronic TLE compared to controls (10 %) was not significant (39.4 ± 3.3/μm^2 vs 35.9 ± 3.1/μm^2; n = 198 synaptic profiles in both groups, p = 0.13). Though it did not reach significance, there was a corresponding trend towards increase in BDNF concentrations in postsynaptic spines in the same synapses.

Along the AZ, labeling density of total BDNF was significantly increased by 63 % in chronic TLE rats compared to controls (3.0 ± 0.5/μm vs 4.9 ± 0.9/μm; n = 198 synaptic profiles in both groups, p = 0.04) (Fig. 7C). While there was a major increase by 69 % in the expression of total BDNF along the PreL in chronic TLE rats (2.7 ± 0.5/μm vs 1.6 ± 0.3 μm), n = 396 synaptic profiles in both groups, p = 0.04), no significant change was observed in the PoL between chronic TLE and control rats (2.5 ± 0.5/μm vs 1.8 ± 0.3 μm), n = 396 synaptic profiles in both groups, p = 0.98). Along the overall presynaptic plasma membrane (AZ + PreL), there was a significant increase of 62 % in total BDNF expression in the chronic TLE rats (3.4 ± 0.5/μm vs 2.1 ± 0.2/μm, n = 594 synaptic profiles in both groups, p = 0.01). In contrast, total BDNF expression along the entire postsynaptic plasma membrane (PSD + PoL) was not significant (3.5 ± 0.4/μm vs 2.5 ± 0.3 μm; n = 594 synaptic profiles in both groups, p = 0.1). See Table 4 for the descriptive statistics.

Following chronic TLE, the length of the active zone increased moderately (9 %) but significantly (0.199 ± 0.005 μm vs 0.183 ± 0.004 μm; n = 198 synaptic profiles in both groups, p = 0.02) (Fig. 7D).

In addition, there were a number of synaptic structural observations made regarding the BDNF labeling patterns (Figs. 6A, B; 7A, B). Anti-BDNF labeling was associated mostly with small, clear, synaptic vesicles in presynaptic terminals. There were only very few large dense core vesicles, some of which were labeled with BDNF; others were not. Similarly, large dense core vesicles without BDNF labeling were found on large mossy fibre terminals (not shown). BDNF labeling of dendrites was also observed. The gold particles in the postsynaptic spine were, in most cases, localised close to the PSD.

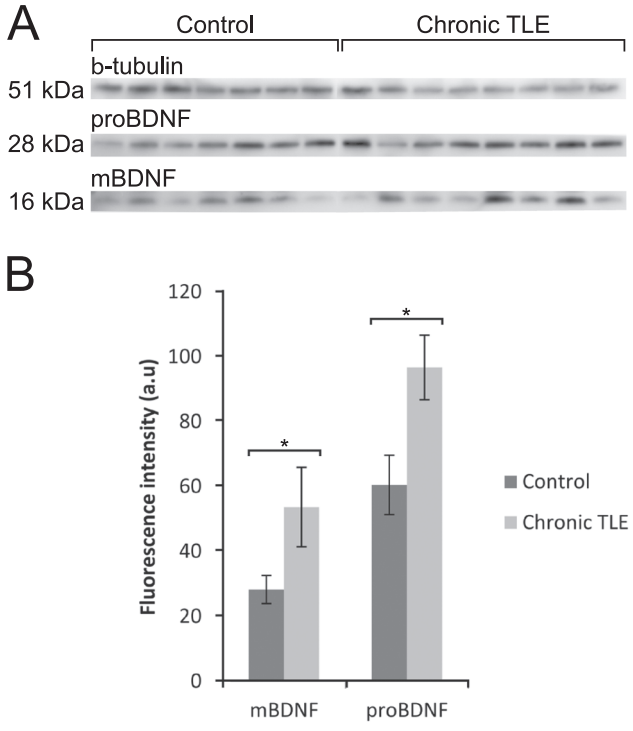

Fig. 5. Western blot of BDNF in rat hippocampal synaptosomes. (A) Representative blots of proBDNF (28 kDa) and mature BDNF (mBDNF) (16 kDa) in chronic TLE and control rats. ß-tubulin (51 kDa) was used as loading control. (B) Mean band intensities of proBDNF and mBDNF in hippocampal synaptosomes from control and chronic TLE rats. Error bars represent SEM. Asterisk (*) indicates significant difference between control and chronic TLE groups (independent t test, p < 0.05).

4. Discussion

We examined changes in Arc and BDNF, because of their established role in regulating synaptic plasticity, by quantifying and localising these proteins in distinct regions within synapses in a rat model of TLE. The hippocampus was chosen for analysis because of its roles in synaptic plasticity-based memory and cognition, and also because it undergoes significant structural changes during TLE. In total hippocampal synaptosomes, examined with western blotting, the concentrations of Arc and BDNF were significantly decreased and increased, respectively (Figs. 2 and 5). At the level of specific synapses, with immunogold EM, we found a significant reduction in Arc protein concentrations in excitatory synapses in the hippocampal CA1 area following chronic TLE (Fig. 3). On the other hand, we found a significant increase in BDNF concentrations in presynaptic terminals in CA3 after chronic TLE, and a corresponding non-significant trend towards increase in postsynaptic spines in the same synapses (Fig. 7). These observations raise important questions about the role of these regulatory proteins in TLE.

Arc has been shown to be essential for the consolidation of synaptic plasticity and memory (Plath et al., 2006). Also, Arc–PSD-95 postsynaptic complexes have been proposed to affect cognitive function (Fernandez et al., 2017). Thus, future studies, specifically testing learning and memory in this rat TLE model, may investigate whether there is a causative link between our observed reduction in Arc and the cognitive impairment in epileptic rats, similar to what has been reported for SNARE proteins (Yu et al., 2018).

Arc protein was most strongly concentrated along the PSD. This fits with its reported function in facilitating the endocytosis of AMPA receptors (Chowdhury et al., 2006; Rial Verde et al., 2006), which are also localised in the same compartment (Feligioni et al., 2006; Takumi et al., 1999). Also, PSD-95 has been shown to be the most abundant Arc-interacting protein (Fernandez et al., 2017). We have shown that AMPA receptors are reduced with comparable percentages as Arc (−46

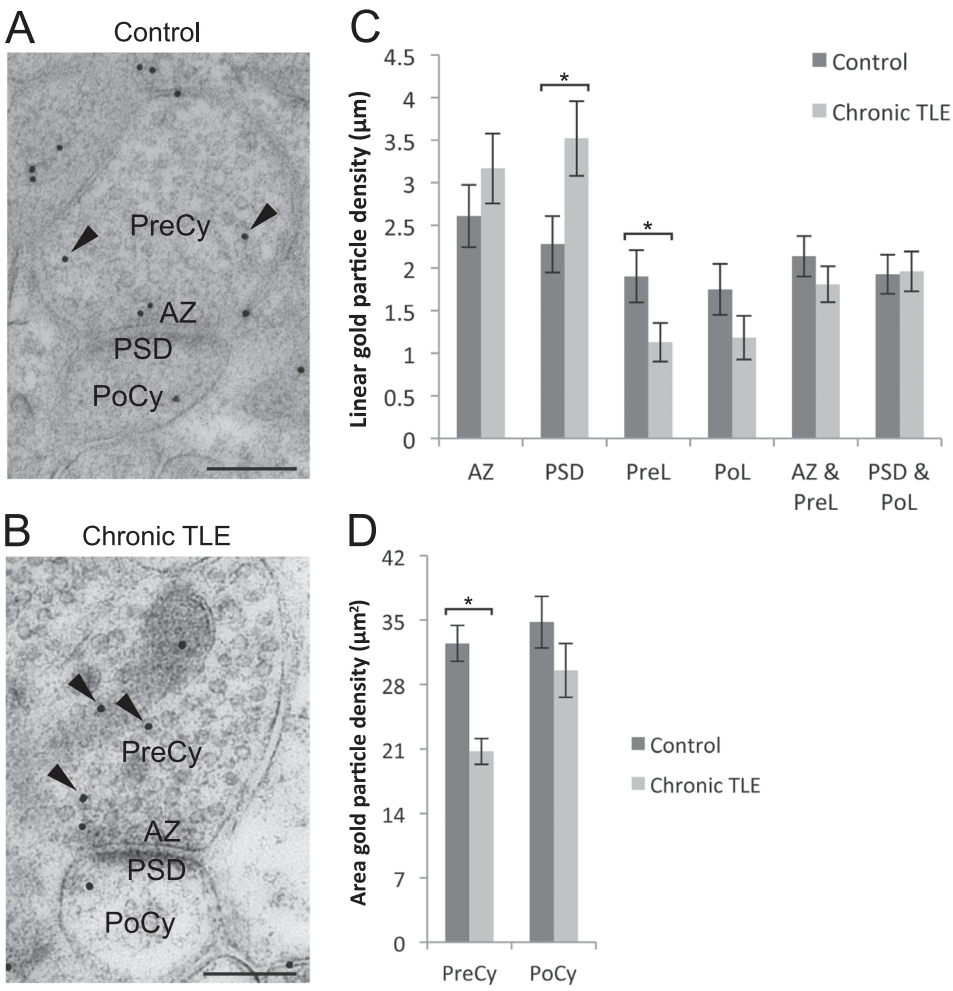

Fig. 6. Total BDNF (proBDNF and mBDNF) immunogold labeling of Schaffer collateral synapses in rat hippocampal CA1 region. (A) Immunogold labeling of control rat. (B) Immunogold labeling of chronic TLE rat. (C) Quantification of labeling along plasma membrane regions of interest. (D) Quantification of labeling in cytoplasmic regions of interest. Error bars represent SEM. Asterisk (*) indicates significant differences (Mann-Whitney U test, $p < 0.05$). Scale bar = 200 nm. Arrowheads point to gold particles over small synaptic vesicles.

Table 3
The table shows descriptive statistics of BDNF immunogold labeling in the hippocampal CA1 region. Mean and median values are presented as gold particles/µm and as number of gold particles/µm^2, respectively, in the membrane and cytoplasmic regions of interest.

Group - Region	n	Mean	Median	SD	SEM
CA1 control - AZ	90	2,6	0	3,5	0,4
CA1 control - PSD	90	2,3	0	3,1	0,3
CA1 control - PreL	180	1,9	0	4,1	0,3
CA1 control - PoL	180	1,7	0	4,0	0,3
CA1 control - AZ & PreL	270	2,1	0	3,9	0,2
CA1 control - PSD & PoL	270	1,9	0	3,8	0,2
CA1 control - PreCy	90	32,5	29,0	18,4	1,9
CA1 control - PoCy	90	34,8	29,5	26,6	2,8
CA1 epilepsy - AZ	90	3,2	0	3,9	0,4
CA1 epilepsy - PSD	90	3,5	4,1	4,1	0,4
CA1 epilepsy - PreL	180	1,1	0	3,0	0,2
CA1 epilepsy - PoL	180	1,2	0	3,4	0,3
CA1 epilepsy - AZ & PreL	270	1,8	0	3,5	0,2
CA1 epilepsy - PSD & PoL	270	2,0	0	3,8	0,2
CA1 epilepsy - PreCy	90	20,7	20,9	13,3	1,4
CA1 epilepsy - PoCy	90	29,5	19,1	27,8	2,9

%) (Fig. 3), in the PSD in the same synapses during TLE (−32 % for GluA1, −52 % for GluA2) (Egbenya et al., 2018). This indicates that the concentration of Arc, at this site, may be regulated to match the concentration of AMPA receptors. We have no information, however, on whether Arc or other regulatory molecules are the ones which initiate the internalization of AMPA receptors during epilepsy in the first place. Others have reported, though, that the loss of Arc leads to network hyperexcitability (Peebles et al., 2010). The marked reduction of Arc at the PSD seen here in the CA1 Schaffer collateral synapse, is in stark contrast to the CA3 recurrent collateral synapse, where no significant change is seen at all (Fig. 3), showing that the regulatory changes in Arc concentrations are highly synapse-specific.

The significant increase in BDNF concentrations found in CA3 presynaptic terminals (presynaptic cytoplasm, active zone and presynaptic lateral areas) in chronic TLE rats (Fig. 7), but not in the CA1 (Fig. 6), is an indication that these changes are synapse-specific. The main factors which trigger changes in BDNF concentrations are the influx of Ca^{2+} and the release of glutamate (Lindvall et al., 1994). Ca^{2+} activity is a major regulator of BDNF, hence changes in neuronal network induced by Ca^{2+} has significant implications for BDNF. Two cardinal lines of pathological molecular signalling in epilepsy are an increase in extracellular glutamate release and in intracellular Ca^{2+} concentrations. Together, these may contribute to the upsurge in the concentration of presynaptic BDNF observed in the CA3 during chronic epilepsy.

BDNF may exert a biphasic effect of excitation and inhibition (Binder and Scharfman, 2004; Hu and Russek, 2008; Koyama and Ikegaya, 2005). It does this by strengthening excitatory synaptic transmission at glutamatergic synapses (Binder and Scharfman, 2004; Kang and Schuman, 1995; Lohof et al., 1993). This may be achieved, for instance, by facilitating the release of neurotransmitters into the synaptic cleft as well as increasing the opening probability of N-methyl-D-aspartate (NMDA) receptors. Similarly, BDNF may enhance inhibition at GABAergic synapses in the epileptic brain (Palma et al., 2005). This is supported by studies reporting that BDNF is involved in the maturation

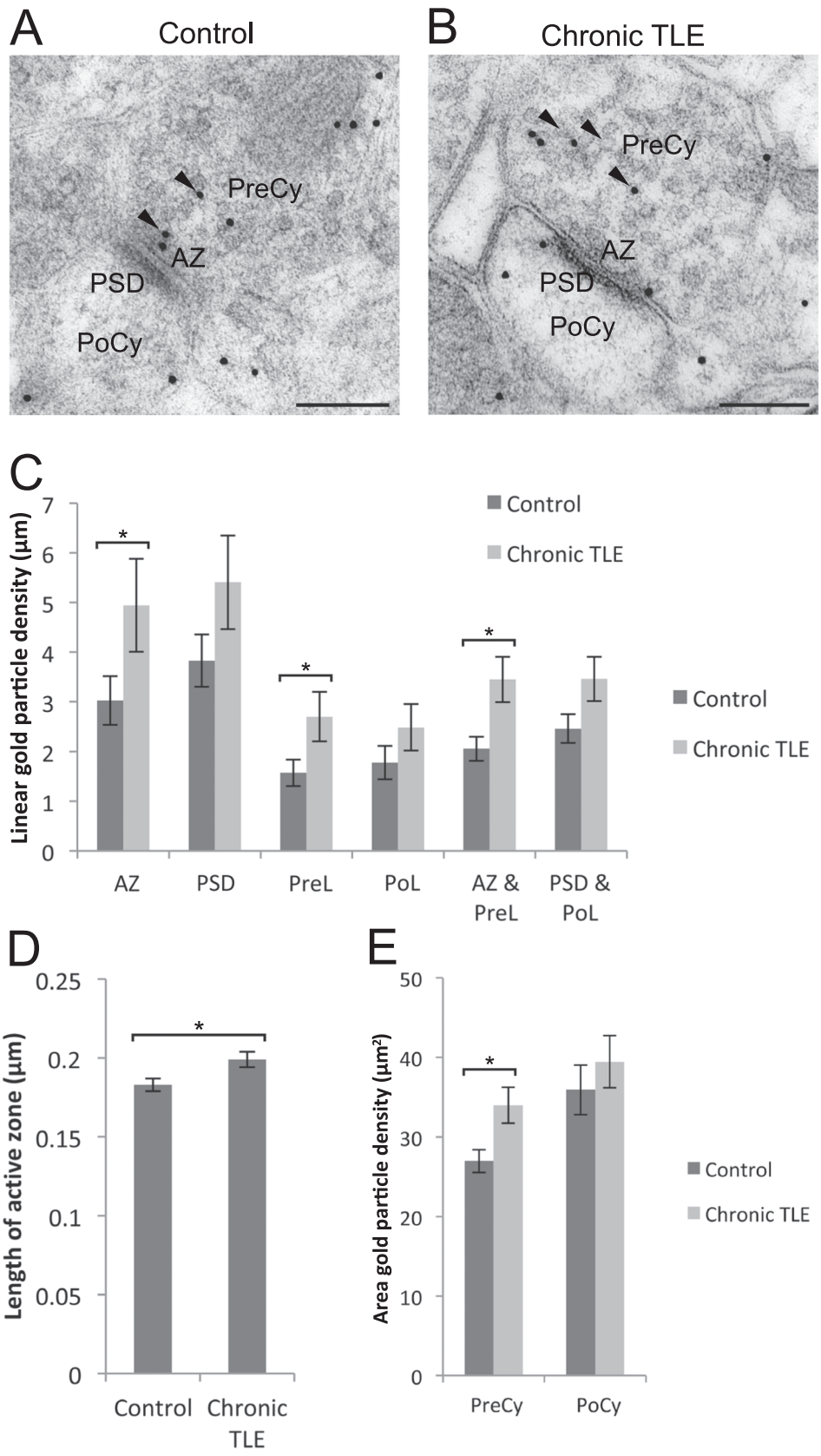

Fig. 7. Total BDNF (proBDNF and mBDNF) immunogold labeling of recurrent collateral synapses in rat hippocampal CA3 region. (A) Immunogold labeling of control rat. (B) Immunogold labeling of chronic TLE rat. (C) Quantification of labeling along plasma membrane regions of interest. (D) Mean length of the AZ in control and chronic TLE synapses. (E) Quantification of labeling in cytoplasmic regions of interest. Error bars represent SEM. Asterisk (*) indicates significant difference between control and chronic TLE groups (Mann-Whitney U test, $p < 0.05$). Scale bar = 200 nm. Arrowheads point to gold particles over small synaptic vesicles.

Table 4
The table shows descriptive statistics of BDNF immunogold labeling in the hippocampal CA3 region. Mean and median values are presented as gold particles/μm and as number of gold particles/μm^2, respectively, in the membrane and cytoplasmic regions of interest.

Group - Region	n	Mean	Median	SD	SEM
CA3 control - AZ	100	3,0	0	4,9	0,5
CA3 control - PSD	100	3,8	0	5,3	0,5
CA3 control - PreL	200	1,6	0	3,8	0,3
CA3 control - PoL	200	1,8	0	4,7	0,3
CA3 control - AZ & PreL	300	2,1	0	4,2	0,2
CA3 control - PSD & PoL	300	2,5	0	5,0	0,3
CA3 control - PreCy	100	27,0	24,0	14,4	1,4
CA3 control - PoCy	100	35,9	29,2	31,3	3,1
CA3 epilepsy - AZ	98	4,9	0	9,3	0,9
CA3 epilepsy - PSD	98	5,4	0	9,4	0,9
CA3 epilepsy - PreL	196	2,7	0	6,9	0,5
CA3 epilepsy - PoL	196	2,5	0	6,5	0,5
CA3 epilepsy - AZ & PreL	294	3,4	0	7,8	0,5
CA3 epilepsy - PSD & PoL	294	3,5	0	7,7	0,4
CA3 epilepsy - PreCy	98	34,0	30,5	22,4	2,3
CA3 epilepsy - PoCy	98	39,4	37,6	32,4	3,3

of inhibitory synapses (Huang et al., 1999; Yamada et al., 2002). However, the hyperexcitatory effect is the predominant role exerted by BDNF (Koyama and Ikegaya, 2005). Whether BDNF exerts hyperexcitation or inhibition at a specific synapse may be dependent on parameters such as neuronal networks, cell specificity and expression timing (Koyama and Ikegaya, 2005). For instance, for single cell activated glutamatergic synapses of postnatal rat hippocampal neurons, BDNF potentiated 30 % and inhibited 10 % of the synapses (Lessmann and Heumann, 1998). This depends, largely, on presynaptic modifications of transmitter release.

In lending further credence to the role of BDNF in epileptogenesis, Binder et al. (2001) suggested that the predominant role of BDNF in epileptogenesis could possibly be by enhancing the excitatory mossy fibers which innervate CA3 pyramidal cells. Additionally, in pathological states such as during epilepsy, the survival-to-death balance of neurotrophins is shifted towards pro-death due to the increased secretion of proneurotrophins such as proBDNF (Friedman, 2010; Volosin et al., 2006). This induces apoptosis via the $p75^{NTR}$, receptor for the proneurotrophins. The controversy surrounding the activities of the ubiquitous BDNF is further complicated by the finding that it attenuates inhibition at GABAergic synapses (Tanaka et al., 1997). Overall, these might result in an increase in excitation.

The effect of BDNF in epilepsy may follow a positive feedback mechanism (Heinrich et al., 2011). Through this mechanism, seizure induction leads to an increase in BDNF signalling, which in turn leads to increased seizure occurrence. According to Binder et al. (2001), the increase in BDNF as well as its receptor activation that characterises epileptogenesis may contribute to the hyperexcitability seen in epilepsy.

The increased concentration of BDNF could thus potentially have two very different implications for persons with epilepsy: While an increase in the concentration of BDNF may be harmful as it may lead to hyperexcitability, it could also facilitate synaptic plasticity, thus supporting learning and memory.

Due to the established cognitive deficit that characterises neurological conditions including epilepsy, it is expected that there will be a reduction in the concentration of BDNF because of its role in synaptic plasticity. However, the increased expression of BDNF, at least in hippocampal CA3 synapses, suggests that there may be an adaptive mechanism that tends to augment the synthesis of BDNF (Iughetti et al., 2018; Lee et al., 2015) during epilepsy. We hypothesise that, in epilepsy, this increase in BDNF concentration could also play an adaptive role to prevent a further worsening of the cognitive challenges such patients experience.

Evidence suggests that BDNF may be involved in increasing the translation of Arc (Yin et al., 2002). Others have shown that a BDNF-induced increment in synaptic activity may serve as a cue to induce synthesis of Arc (Rao et al., 2006). This is further supported by the finding that synaptic activity elicits an increase in Arc mRNA expression (Link et al., 1995; Lyford et al., 1995). In our study, however, there is an increase in BDNF expression, in hippocampal synaptosomes, coupled to a decrease in Arc. Studies suggesting an increase in Arc have been done under physiological conditions, where neurons have been stimulated directly with BDNF, often inducing long-term potentiation (LTP). Our study, however, is done in brains with recurrent seizures, without adding BDNF. During epileptic seizures, neurons are overstimulated through glutamate receptor activation. This is a different mechanism than physiological addition of BDNF to cells or tissues. Further research is needed to investigate to what degree one of the proteins influences the other during epilepsy, and whether such an interaction may occur within single cells or is dependent on synaptic transmission between cells.

We have previously shown that in this TLE rat model there is a decrease in synaptic AMPA receptors in synapses in CA1 in the hippocampus (Egbenya et al., 2018). Though we did see an overall reduction in synaptic AMPA receptor concentrations, the absolute numbers of GluA2-lacking receptors were increased, which may make these synapses more vulnerable to excitotoxicity, as well as providing a molecular background for cognitive impairments. In the present study, we see a decrease in synaptic Arc expression in the same class of CA1 synapses (pre- and post-synaptic). As Arc has been shown to facilitate AMPA receptor endocytosis (Chowdhury et al., 2006), the reduction in Arc concentrations in these synapses may function as a way to curb further endocytosis of AMPA receptor, keeping it in balance.

Despite the findings obtained in this study, there are some limitations. We used the same strain of rats in both parts of the study, but the weights of these rats and concentrations of KA used for rats in the WB experiments differed from those in EM experiments. The two sets of rats (WB and EM rats) were obtained from two different providers specialised for preparation of TLE rats. Also, while the first set of rats were used for screening for changes in concentrations of Arc and BDNF proteins in whole hippocampal synaptosomes (WB), the second set of rats was used for EM investigation in which the subsynaptic profile of both Arc and BDNF proteins were examined. This potential limitation may not have a significant impact on the study, though, since the results obtained from both types of experiments mimic each other closely.

TLE is often described with three different phases, which in chemically induced rat models typically may have the following durations: Acute - hours, latent - 1–2 weeks, and chronic - 3 weeks and onwards (Reddy and Kuruba, 2013). These are characterized by different pathophysiological processes. The chronic phase, which we have studied, is commonly characterized by spontaneous recurrent seizures, though the frequency of these may vary both between animals and in individual animals (Williams et al., 2009). In our material, we have not investigated whether the effects we see are related to specific, recent seizures, we have just acquired data as a mean for the two groups (control and TLE), which is important information in itself. We have previously shown that there is a robust reduction in synaptic glutamate receptors in the hippocampus in the chronic phase, eight and eleven weeks after status epilepticus (Egbenya et al., 2018). In that study, we did in fact see comparable reductions in synaptic glutamate receptors also in the latent phase, where there are none or very few seizures, which may indicate that such changes may occur independently of specific, recent seizures. This important question of pathogenetic mechanisms, however, must be addressed in future studies.

Temporal lobe seizures, also after induction by systemic kainate administration, lead to significant neuronal cell death in the hippocampus, especially in the CA1 and CA3 regions (Ben-Ari, 2012). In the present work, however, we searched for and examined only intact synapses that had survived following the kainate administration. We expect, of course, that neuronal cell death had occurred due to seizure

activity. Thus, it would have been interesting to quantify the numbers of existing synapses in different areas of the epileptic hippocampi, compared to the control animals. Such an EM analysis should be done in a later study. The surviving synapses, however, were of normal structural appearance and did not seem anatomically changed or damaged.

In this study, we examined the synaptic changes that occur in concentrations of upstream regulators of synaptic plasticity, Arc and BDNF, in distinct subsynaptic regions in the hippocampus following a rat model of chronic TLE. We found significant changes in the concentrations of Arc (reduction) and BDNF (increase) in hippocampal synaptosomes in chronic TLE rats. Additionally, we detected significant reductions in the concentration of Arc in the postsynaptic spine and the presynaptic terminal of Schaffer collateral glutamatergic synapses in the stratum radiatum of the CA1 area in chronic TLE. In CA3, there was a significant increase in the expression of total BDNF in only the presynaptic terminals. These changes in concentration in specific hippocampal synapses may affect the development of the disease. Additionally, we suggest that the changes in these proteins may contribute to the cognitive impairment that is associated with persons with epilepsy. This should be examined in future research.

Competing interest

The authors declare that the study was conducted without any relationship that could be considered as a potential conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.neures.2022.12.006.

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