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Rapid increases in proBDNF after pilocarpine-induced status epilepticus in mice are associated with reduced proBDNF cleavage machinery

ProBDNF is acutely elevated post-status epilepticus

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Abstract

Brain-derived neurotrophic factor (BDNF) levels are elevated after status epilepticus (SE), leading to activation of multiple signaling pathways, including the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway that mediates a decrease in $GABA_A$ receptor ($GABA_AR$) α1- subunits in the hippocampus (Lund et al., 2008). While BDNF can signal via its pro- or mature form, the relative contribution of these forms to signaling after SE is not fully known. In the current study, we investigate changes in proBDNF levels acutely after SE in C57BL/6J mice. In contrast to previous reports (Unsain et al., 2008; Volosin et al., 2008; VonDran et al., 2014), our studies found that levels of proBDNF in the hippocampus are markedly elevated as early as three hours after SE onset and remain elevated for seven days. Immunohistochemistry studies indicate that seizure-induced BDNF localizes to all hippocampal subfields, predominantly in principal neurons and also astrocytes. Analysis of the proteolytic machinery that cleaves proBDNF to produce mature BDNF demonstrates that acutely after SE there is a decrease in tissue plasminogen activator (tPA) and an increase in plasminogen activator inhibitor-1 (PAI-1), an inhibitor of extracellular and intracellular cleavage, which normalizes over the first week after SE. *In vitro* treatment of hippocampal slices from animals 24 hours after SE with a PAI-1 inhibitor reduces proBDNF levels. These findings suggest that rapid proBDNF increases following SE are due in part to reduced cleavage, and that proBDNF may be part of the initial neurotrophin response driving intracellular signaling during the acute phase of epileptogenesis.

Significance Statement

The studies reported here are the first to demonstrate acute changes in the expression of proBDNF within three hours of onset of status epilpticus (SE) onset that occur within principle cells and glia in all hippocampal subfields. We further found evidence that reduced expression

of tPA, part of the extracellular proteolytic cascade, and increased expression of plasminogen activator inhibitor-1 (PAI-1), an inhibitor of extracellular and intracellular cleavage, may contribute to reduced proBDNF cleavage and elevations in proBDNF levels. These findings suggest that proBDNF may be part of the initial neurotrophin response driving intracellular signaling acutely after SE and during the earliest phase of epileptogenesis.

Introduction

Brain-derived neurotrophic factor (BDNF) promotes growth and differentiation of neurons during development and plays an important role in many physiological processes, such as learning and memory, as well as various pathological processes, such as epileptogenesis (Lu et al., 2014). Synthesis and expression of BDNF are highly regulated throughout the nervous system (Lessmann and Brigadski, 2009). BDNF is initially synthesized as a precursor protein (preproBDNF) in the endoplasmic reticulum and is transported to the Golgi as proBDNF once the signal peptide is cleaved. Mature BDNF can be produced intracellularly by furin-mediated cleavage or by proprotein convertase in immature secretory granules (Mowla et al., 1999). ProBDNF can also be cleaved extracellularly by matrix metalloproteinases (MMP -3, -7, or 9), or by components of the tissue plasminogen activator/plasmin (tPA/plasmin) proteolytic cascade (Lee et al., 2001; Pang et al., 2004). The activity of these proteases is tightly regulated. Plasminogen activator inhibitor-1 (PAI-1) inhibits both tPA and furin, inhibiting both extracellular and intracellular cleavage (Binder et al., 2002; Dupont et al., 2009; Bernot et al., 2011; figure 1). In addition, tissue inhibitor of metalloproteinases (TIMPs) inhibit MMPs while neuroserpin and α2 antiplasmin (A2AP) inhibit the tPA/plasmin proteolytic cascade (Hastings et al., 1997; Krueger et al., 1997; Brew et al., 2000; Yepes and Lawrence, 2004; Coughlin, 2005).

Several studies have demonstrated that a significant portion of BDNF protein is secreted as proBDNF and cleaved extracellularly via the tPA/plasmin proteolytic cascade (Pang et al., 2004; Nagappan et al., 2009). *In vitro*, high frequency neuronal activity triggers simultaneous release of proBDNF and tPA to generate mBDNF extracellularly (Nagappan et al., 2009), suggesting that this could occur *in vivo* after repeated neuronal firing that is observed during seizures. However, the *in vivo* effects of acute seizures on proBDNF levels have not yet been fully elucidated.

Numerous reports suggest that BDNF levels are increased in the hippocampus after seizures induced by kindling (Ernfors et al., 1991), electroconvulsive shock (Altar et al., 2004), kainate (Rudge et al., 1998), and pilocarpine (Roberts et al., 2006). In addition, several studies suggest a pro-epileptogenic effect of BDNF that appears to be mediated at least in part by activation of the tropomyosin-receptor kinase B (TrkB) receptors (McNamara et al., 2006). However, other studies suggest that intrahippocampal infusion of BDNF results in enhanced resistance to kindling and may protect against epileptogenesis (Larmet et al., 1995; Reibel et al., 2000). These contrasting findings may be due, in part, to differential actions of proBDNF and mBDNF during epileptogenesis.

A potential role for the proneurotrophins in epileptogenesis is starting to emerge. Enhancing cleavage of pro-nerve growth factor (proNGF) to generate mature NGF provides neuroprotection after administration of kainate to organotypic slice cultures (Le and Friedman, 2012). In rodents, increases in BDNF mRNA occur as early as three hours after pilocarpine-induced status epilepticus (SE) (Mudò et al., 1996) and increased proBDNF has been detected 24 hours after SE induction (Volosin et al., 2008; VonDran et al., 2014). More recently, it has been reported that high-dose proBDNF applied to cultured hippocampal neurons may cause alterations in GABAergic neurotransmission by promoting $GABA_AR$ endocytosis and

degradation through activation of the RhoA-Rock-PTEN pathway, and may contribute to repression of $GABA_AR$ synthesis through activation of the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Riffault et al., 2014). Addition of exogenous BDNF to neuronal cultures rapidly increases STAT3 phosphorylation (Ng et al., 2006; Lund et al., 2008). BDNF-dependent activation of the JAK/STAT pathway in rat dentate gyrus occurs within six hours of SE onset and drives a decrease in mRNA for the α1 subunit of $GABA_AR$ (Lund et al., 2008; Grabenstatter et al., 2014), suggesting that BDNF-induced activation of the JAK/STAT pathway occurs rapidly after SE onset.

To better understand the potential contribution of proBDNF during the earliest phases of epileptogenesis, we utilized proBDNF specific antibodies in wild-type C57BL/6J mice and knock-in mice on a C57BL/6J background that express a hemagglutinin-tagged BDNF transgene under the control of endogenous *bdnf* promoters (Yang et al., 2009) to assess the levels and localization of BDNF acutely following the induction of pilocarpine-induced SE. The study finds that within the first three hours after SE onset there is an acute increase in proBDNF levels in principal neurons and glia in all hippocampal subfields, as well as altered expression of both tPA and PAI-1 that would be predicted to reduce proBDNF cleavage. Taken together these data suggest that reduced BDNF cleavage acutely after SE leads to proBDNF accumulation, which may be the initial neurotrophin driving cell signaling during early epileptogenesis.

Materials & Methods

Induction of SE:

All animal procedures were performed in accordance with the authors' institutional animal care and use committee's regulations and NIH Guidelines, "Guide for the care and use of laboratory

101 animals". Adult male animals were utilized for all studies and were group housed with up to five
102 age-matched littermates in temperature and humidity controlled rooms with access to food and
103 water *ad libitum* on a 12 hour light/dark cycle.
104
105 Knock-in mice that express a *bdnf* allele with a hemagglutinin tag added to the C-terminus of the
106 murine coding exon of BDNF (BDNF-HA) were generously provided by the Hempstead group
107 (Weill Cornell Medical College) (Yang et al., 2009). BDNF-HA mice were backcrossed on a
108 C57BL/6J background for more than ten generations. Wild-type (WT) C57BL/6J mice (Jackson
109 Laboratory) were utilized for studies with proBDNF antibody detection and the protease
110 machinery studies. SE was induced using repetitive intraperitoneal (i.p.) injections of pilocarpine
111 as previously described (Müller et al., 2009). The mice acquired from Jackson Laboratory were
112 received at five weeks of age and allowed to rest for one week prior to handling in order to
113 acclimate to the environment and altitude. Six- to eight-week-old WT and BDNF-HA mice (18-
114 24g) were handled for at least one week to reduce the stress produced by handling required for
115 induction of SE. The induction protocol was initiated between 7:00-8:00AM to minimize diurnal
116 variation. The mice were transferred to the induction room, marked, weighed, and allowed to
117 rest undisturbed for at least one hour. To block the peripheral muscarinic effects of pilocarpine,
118 each mouse was given an i.p. injection of 1mg/kg scopolamine methyl bromide (Sigma) fifteen
119 minutes before the first pilocarpine injection on the day of seizure induction. An initial dose of
120 pilocarpine HCl (200mg/kg, Sigma) was given, then one hour after the first injection subsequent
121 doses (100mg/kg) were given at 30 min intervals. The animals were group housed with up to
122 five age-matched littermates and then separated into individual cages after the third injection for
123 individual monitoring of behavioral seizures. Injections were discontinued at the onset of SE,
124 which was defined by the appearance of repeated behavioral seizures (stage four or higher with
125 at least one seizure being five or higher) according to a modified Racine scale (Borges et al.,
126 2003).

SE typically initiated approximately three hours after the first injection, requiring at least three injections (400mg/kg total dose) of pilocarpine with an average of five injections (600mg/kg total dose) of pilocarpine. SE persisted for at least 90 minutes with approximately 30% of animals successfully undergoing SE and surviving until their respective time points. The specific cause of death cannot be definitively determined but post-convulsion respiratory failure appeared to be a common cause of acute death after pilocarpine administration (as has been previously described; Boyd and Fulford, 1961). Control mice were given injections of saline at identical time intervals. Mice that were sacrificed more than three hours after SE induction were returned to their housing room and given free access to water, Gatorade, and moistened chow with equal parts sucrose. Mice were sacrificed with rapid isoflurane-induced anesthesia followed by decapitation. Fresh tissue for western blot was collected via rapid hippocampal dissection in ice cold PBS containing phosphatase inhibitors (phosphatase inhibitor cocktail 2, P5726, Sigma) and frozen on dry ice. The two hippocampi from each animal were pooled into a single sample for that animal, and samples were stored at -80°C until lysate preparation. For immunohistochemistry, mice were sacrificed at three hours after SE onset by deep anesthesia with ketamine/xylazine and inhaled isoflurane followed by rapid intracardiac perfusion with ice cold PBS then 4% paraformaldehyde in phosphate buffer pH 7.4. The brains were dissected out, postfixed overnight in 4% paraformaldehyde and underwent cryoprotection in 30% sucrose in PBS, and were then stored at -80°C in Tissue-Tek® O.C.T. Compound (Sakura Finetek, Torrance, CA) until sectioning.

Western blotting: The frozen hippocampi were lysed in RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1mM EDTA) with 10mM phenylmethylsulfonyl fluoride, 10mM sodium orthovanadate, 10mM sodium fluoride, phosphatase inhibitor cocktail 2 (1:250) and protease inhibitor cocktail (1:250, P8340, Sigma)

using an ultrasonic sonifier. Samples were then gently shaken at 4°C for 30 minutes and centrifuged at 14,000 x g for 30 minutes at 4°C. The supernatants were reserved, aliquoted, and stored at -80°C until sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Identical amounts of protein were loaded per lane for each sample on all blots probed with a given antibody, with 20-60ug of protein utilized depending on the specific antibody used. After gel transfer, the nitrocellulose membranes were blocked with 5% milk (furin and MMP-9 were blocked with 5% non-fat dry milk, 2% BSA, 4% FBS, 4% normal horse serum, and 4% normal goat serum). The blots probed with the anti-HA and BDNF antibody were first washed with Tris-buffered saline with Tween-20 (TBST, 50mM Tris-Base, 150mM NaCl, 0.05% Tween-20, pH 7.6) and then fixed with 2.5% glutaraldehyde in PBS, washed twice with PBS, washed twice with TBST, and then blocked with 5% milk in TBST. All membranes were incubated overnight at 4°C with their respective primary antibody in diluted blocking buffer. The following antibodies and concentrations were used: mouse monoclonal HA.11 clone 16B12 antibody (1:3,000, MMS-101P, Covance), mouse monoclonal proBDNF antibody (1:1000, H10001G-MA, GeneCopoeia), rabbit polyclonal to alpha-2 antiplasmin (1:2,000, ab62771, Abcam), rabbit polyclonal furin antibody (1:1,000, sc-20801, Santa Cruz), rabbit polyclonal MMP-9 antibody (1:2,000, AB13458, Millipore), sheep polyclonal neuroserpin antibody (1:2,000, SASMNSP-GF-HT, Molecular Innovations), rabbit polyclonal PAI-1 antibody (1:1,000, ASMPAI-GF-HT, Molecular Innovations), rabbit polyclonal plasminogen antibody (1:3,000, ASMPLG-GF-HT, Molecular Innovations), sheep polyclonal tPA antibody (1:500, SASTPA-GF-HT, Molecular Innovations), and rabbit polyclonal TIMP-1 antibody (1:1,000, AB770, Millipore). Following incubation with the appropriate secondary antibody, membranes were incubated with SuperSignal West Dura Chemiluminescent Substrate (Pierce) with the anti-HA blots being enhanced with Lumigen TMA-6 (Lumigen). Blots were stripped with 50mM glycine (pH 2.3) and reprobed with other primary antibodies or Actin (1:20,000-80,000, A2066, Sigma). Western blotting results presented include representative images of the blots run in duplicate and adjusted for contrast

and the densitometry quantitation of each band normalized to actin that was used as a loading control to ensure consistent protein amounts were loaded across samples using FIJI (Schindelin et al., 2012). The average of the normalized densitometry measurements for the control group was considered 100% with error bars reported as S.E.M and N referring to the number of samples consisting of lysates from both hippocampi from individual animals in each group.

Immunohistochemistry: Brains were sectioned at 30 µm into cryoprotectant (30% sucrose, 30% ethylene glycol, and 0.1M phosphate buffer) and stored at -20°C for floating section staining. Sections were washed in PBS several times and then blocked with 3% BSA, 3% normal goat serum, and 3% normal donkey serum with 0.1% Triton X-100 in PBS for one hour at room temperature and then incubated overnight at 4°C with rabbit polyclonal HA antibody (1:500, A6908, Sigma) in combination with chicken polyclonal MAP2 antibody (1:1000, ab5392, Abcam) and guinea pig polyclonal GFAP antibody (1:500, 174004, Synaptic Systems). After primary antibody washing, sections were incubated for one hour with a biotinylated goat anti-rabbit IgG secondary antibody (1:400, 111-065-144, Jackson Immunoresearch), a goat anti-chicken IgY Alexa Fluor 568, and donkey anti-guinea pig IgG Alexa Fluor 647 to detect MAP2 and GFAP, respectively. Sections were subsequently incubated for one hour with Alexa Fluor 488-Streptavidin (1:800, 016-580-084, Jackson Immunoresearch) to visualize the HA tag. The sections were mounted on glass slides with VECTASHIELD mounting medium with DAPI (Vector Labs), coverslipped, and sealed.

Confocal microscopy: Slide-mounted sections of immunolabeled hippocampi were viewed on an inverted microscope (Axio Examiner Z1, Carl Zeiss) equipped with Plan-Apochromat 20x (0.8 NA) or 63x (oil differential interference contrast; 1.4 NA) objectives and attached to a spectral confocal laser system (LSM 780, Carl Zeiss) powered by ZEN 2012 software (Carl Zeiss). The tissue was scanned at room temperature with a tunable infrared Coherent

Chameleon Ultra II laser tuned to 800nm to detect DAPI staining and 488-, 561-, and 633-nm laser lines to detect the Alexa fluorophores 488, 568, and 647, respectively. Images were acquired as z-stacks using sequential line (mean of four) scanning. Colocalization of two fluorophores with DAPI was simultaneously qualitatively assessed in the x, y, and z planes of each optical section. Average projection images of 5 optical slices spaced 2um in the z-axis were produced and minimal adjustments to image contrast and intensity were made in FIJI (Schindelin et al., 2012) using the levels or contrast/brightness functions. All brains and sections were processed in parallel with images acquired, adjusted and analyzed in an identical manner between SE and control animals. Images were arranged and annotated using Adobe Illustrator (Adobe Corporation).

Acute Hippocampal Section Studies. Animals were anesthetized with isoflurane, sacrificed, the brain swiftly removed, and placed in cold (4°C) oxygenated (95% O_2, 5% CO_2) sucrose-modified aCSF containing (in mM): Sucrose (45), NaCl (87), glucose (25), $NaHCO_3$ (25), KCl (2.5), NaH_2PO_4 (1.25), $MgCl_2$ (7), and $CaCl_2$ (0.5), pH 7.4 and 300–310 mOsm. Transverse hippocampal slices (300 µm) were obtained using a slicing vibratome (Leica VT1200s). Hippocampi were dissected out from slices in the cold sucrose-modified aCSF solution and rinsed briefly in oxygenated aCSF containing (in mM): NaCl (130), Glucose (10), $NaHCO_3$ (25), KCl (3.5), NaH_2PO_4 (1.25), $MgCl_2$ (2), $CaCl_2$ (2), pH 7.4 and 300–305 mOsm. Rinsed slices were then placed on ice in a 6-well plate with 5 mL of aCSF in individual wells modified to allow delivery of oxygen. Alternating slices from the two hippocampi from a single animal were placed in wells for the vehicle or PAI-1 inhibitor group and care was taken to assure that a similar number of sections from each hippocampus was placed in each well. Once sectioning was completed, 25 µL of vehicle (DMSO, Sigma Aldrich) was added to one of the wells (vehicle group) and 25 µL of the PAI-1 inhibitor Tiplaxtinin (Axon 1383) (74 mM stock in DMSO) was added to the other well (final concentration 370 µM; Axon Medchem). Both the vehicle and

inhibitor wells were carefully mixed with repeated aspiration to obtain equal distribution in the aCSF. Once the vehicle and inhibitor were added, the 6-well plate was placed into a water bath and incubated at 36°C for 4 hours with constant, low pressure oxygenation. After incubation, slices were collected, flash frozen using dry ice, and stored at -80°C until lysate preparation.

Statistical Table

	Data Structure	Type of Test	Power
Figure 2a. Increased proBDNF 3h post-SE (HA-immunoreactivity)	Normal distribution	Student T-test	0.9775
Figure 2b. Increased proBDNF 24h post-SE (HA-immunoreactivity)	Normal distribution	Student T-test	0.9917
Figure 3a. Increased proBDNF 3h post-SE (Commercial BDNF antibodies)	Normal distribution	Student T-test	1.0000
Figure 3b. Increased proBDNF 24h post-SE (Commercial BDNF antibodies)	Normal distribution	Student T-test	0.9104
Figure 5a. No change in furin 3h post-SE	Normal distribution	Student T-test	0.0511
Figure 5b. Significant increase in furin 24h post-SE	Normal distribution	Student T-test	0.9198
Figure 5c. No change in plasminogen 3h post-SE	Normal distribution	Student T-test	0.5067
Figure 5d. No change in plasminogen 24h post-SE	Normal distribution	Student T-test	0.6526
Figure 5e. No change in MMP-9 3h post-SE	Normal distribution	Student T-test	0.1905
Figure 5f. No	Normal distribution	Student T-test	0.2765

change in MMP-9 24h post-SE			
Figure 5g. Significant reduction in tPA 3h post-SE	Normal distribution	Student T-test	0.9394
Figure 5h. Significant reduction in tPA 24h post-SE	Normal distribution	Student T-test	1.0000
Figure 6a. No change in A2AP 3h post-SE	Normal distribution	Student T-test	0.5646
Figure 6b. No change in A2AP 24h post-SE	Normal distribution	Student T-test	0.1068
Figure 6c. Reduction in neuroserpin at 3h post-SE.	Normal distribution	Student T-test	0.9961
Figure 6d. No change in neuroserpin at 24h post-SE	Normal distribution	Student T-test	0.0744
Figure 6e. No change in 23kDa non-glycosylated TIMP-1 at 3h post-SE	Normal distribution	Student T-test	0.1867
Figure 6e. No change in 28kDa glycosylated TIMP-1 at 3h post-SE	Normal distribution	Student T-test	0.5007
Figure 6f. No change in 23kDa non-glycosylated TIMP-1 at 24h post-SE	Normal distribution	Student T-test	0.6078
Figure 6f. Significant reduction in 28kDa glycosylated TIMP-1 at 24h post-SE	Normal distribution	Student T-test	0.9987
Figure 6g. Significant increase in PAI-1 at 3h post-SE	Normal distribution	Student T-test	0.9601
Figure 6h. Significant increase in PAI-1 at 24h post-SE	Normal distribution	Student T-test	1.0000
Figure 7a.	Normal distribution	Student T-test	0.971

Increased proBDNF 3d post-SE (Commercial BDNF antibodies)			
Figure 7a. Increased proBDNF 7d post-SE (Commercial BDNF antibodies)	Normal distribution	Student T-test	1.000
Figure 7b. Increased PAI-1 3d post-SE (Commercial BDNF antibodies)	Non-normal distribution	Mann Whitney test	N/A
Figure 7b. No change in PAI-1 7d post-SE (Commercial BDNF antibodies)	Normal distribution	Student T-test	0.985
Figure 7c. No change in tPA 3d post-SE (Commercial BDNF antibodies)	Normal distribution	Student T-test	0.981
Figure 7c. Increased tPA 7d post-SE (Commercial BDNF antibodies)	Normal distribution	Student T-test	1.000
Figure 8. PAI-1 Inhibition reduces proBDNF levels after pilocarpine SE	N/A	Paired T-test	0.995

Results

Levels of proBDNF protein expression were initially assessed in whole hippocampal lysates from age-matched pilocarpine- and saline-treated WT C57BL/6J mice using a mouse monoclonal antibody specific for proBDNF (GeneCopoeia). A significant increase in the immunoreactivity of proBDNF was observed at three (figure 2a; control-100.0 ± 10.6 N=5 vs SE-326.4 ± 12.2 N=5, t-test $p<0.001$) and 24 hours (figure 2b; 100.0 ± 16.6 N=4 vs 180.4 ± 18.0 N=4, t-test $p<0.05$) after SE onset. These data suggest that a significant increase in proBDNF protein levels occurs as soon as three hours after pilocarpine-induced SE.

263

264 To further assess proBDNF levels and cellular localization, we used knock-in mice that express
265 a *bdnf* allele with a hemagglutinin tag added to the C-terminus of the murine coding exon of
266 BDNF (BDNF-HA). Tissue from control BDNF-HA mice (controls) or BDNF-HA mice subjected
267 to pilocarpine induced SE was collected at three and 24 hours after SE onset. Western blot
268 analysis showed a significant increase in proBDNF corresponding to a 34kD HA-
269 immunoreactive band as early as three hours after SE induction (figure 3a; control- 100.0 ±
270 20.5, N=3 vs SE- 300.4 ± 37.4 N=6; t-test $p<0.01$).. In addition, a significant increase in
271 proBDNF immunoreactivity was also observed 24 hours after SE (figure 3b; control- 100.0 ±
272 29.3 N=3 vs SE- 610.1 ± 89.7 N=6; t-test $p<0.01$). In contrast, no difference in HA-
273 immunoreactivity was observed for the 14 kD band corresponding to mBDNF between SE and
274 control animals at either time point (figure 3a&b; three hours: control- 100.0 ±12.2 (N=3) vs SE-
275 96.4 ±5.8 (N=6); 24 hours: control- 100.0 ±4.9 (N=3) vs SE- 98.5 ±5.0 (N=6); t-test $p>0.05$ at
276 both time points). Together, these data provide further evidence that a significant increase in
277 the levels of proBDNF are triggered acutely after SE onset.

278

279 To determine the cellular distribution of BDNF protein after SE, HA immunoreactivity was
280 analyzed in coronal sections co-labeled with a neuronal (MAP2) and an astrocytic (GFAP)
281 marker (figure 4). It is important to note that the HA immunostaining is unable to distinguish
282 between proBDNF and mBDNF, therefore in these experiments the signal detected was
283 considered as total BDNF expression. Another important limitation of these findings is that one
284 cannot distinguish between HA immunoreactivity indicating the site of BDNF pre-release or
285 internalization. Co-localization of HA and MAP2 immunoreactivity was assessed to identify
286 BDNF expression in neurons and co-localization of HA and GFAP immunoreactivity was
287 assessed to identify BDNF expression in astrocytes. The observed pattern of protein expression
288 detected in control animals is consistent with the pattern of expression previously reported by

289 others using the same HA-tagged BDNF knock-in mice (Yang et al., 2009; Dieni et al., 2012).
290 The most prominent HA immunoreactivity signal is detected in the mossy fiber pathway, CA3
291 pyramidal neurons and CA1 pyramidal neurons (figure 4). When compared to controls, mice at
292 three hours post-SE showed an increase in HA immunoreactivity as well as MAP2
293 immunoreactivity that can be detected in all hippocampal regions analyzed. In SE animals, the
294 pattern of HA immunoreactivity is well co-localized with both MAP2 (figure 4a&b) and GFAP
295 immunostaining (figure 4a&c), suggesting that within the hippocampus of animals acutely
296 following SE, BDNF is localized in principal neurons and astrocytes.
297
298 The very rapid elevation of proBDNF levels detected by western blot suggests that in addition to
299 the previously documented increase in BDNF expression, the typically rapid cleavage of
300 proBDNF might also be impaired after SE. Therefore, expression of enzymes involved in
301 proBDNF cleavage were analyzed via western blot (n=5 for each group at three hours and n=4
302 for each group at 24 hours for all assays). Our analyses showed that there is no statistical
303 difference in furin immunoreactivity three hours after SE (figure 5a; control- 100.0 ± 6.5 vs SE-
304 99.9 ± 5.7; t-test $p>0.05$) while there is a modest but significant increase in furin expression at
305 24 hours after SE (figure 5b; control- 100.0 ± 2.202 vs SE- 115.1 ± 3.970; t-test $p<0.05$). In
306 contrast, there is no statistical difference in the immunoreactivity of plasminogen observed at
307 three (figure 5c; control- 100.0 ± 5. 4 vs SE- 113.4 ± 5.0; t-test $p>0.05$) or 24 hours after SE
308 (figure 5d; control- 100.0 ± 11.76 vs SE- 136.8 ± 10.8; t-test $p>0.05$). In addition, there is no
309 statistical difference in the immunoreactivity of MMP-9 observed at three (figure 5e; control-
310 100.0 ± 3.7 vs SE- 104.4 ± 3.7; t-test $p>0.05$) or 24 hours after SE (figure 5f; control- 100.0 ±
311 6.9 vs SE- 112.5 ± 8.0; t-test $p>0.05$). Interestingly, a significant decrease in tPA expression
312 was observed at both three (figure 5g; control- 100.0 ± 9.3 vs SE- 66.3 ± 2.4; t-test $p<0.01$) and
313 24 hours (figure 5h; control- 100.0 ± 5.9 vs SE- 47.9 ± 2.7; t-test $p<0.001$) after SE.
314

In order to further investigate the mechanism of proBDNF cleavage after SE, the levels of protease inhibitors known to inhibit activity of proBDNF cleavage enzymes were analyzed. We observed no change in the immunoreactivity of A2AP, an inhibitor of plasmin activity, at three (figure 6a; control- 100.0 ± 4.8 vs SE- 110.2 ± 1.9; t-test $p>0.05$) or 24 hours after SE (figure 6b; control- 100.0 ± 6.0 vs SE- 96.4 ± 5.3; t-test $p>0.05$). A slight reduction in the immunoreactivity of neuroserpin, a known inhibitor of tPA activity, was observed at three hours after SE (figure 6c; control- 100.0 ± 2.2 vs SE- 72.3 ± 5.4; t-test $p<0.01$) but not at 24 hours after SE (figure 6d; control- 100.0 ± 2.7 vs SE- 101.0 ± 3.6; t-test $p>0.05$). As seen in figure 6e&f, two forms of TIMP-1, an inhibitor of MMP activity, can be detected via western blot, a 23kDa non-glycosylated and a 28kDa glycosylated form. There is no statistical difference in the immunoreactivity of the non-glycosylated form at three (figure 6e; control- 100.0 ± 6.1 vs SE- 93.3 ± 5.4; t-test $p>0.05$) or 24 hours after SE (figure 6f; control- 100.0 ± 4.9 vs SE- 86.41 ± 3.9; t-test $p>0.05$). In addition, there is no statistical difference in the immunoreactivity of the glycosylated form at three hours after SE (figure 6e; control- 100.0 ± 7.9 vs SE- 85.0 ± 2.6; t-test $p>0.05$); however, there is a significant reduction at 24 hours after SE (figure 6f; control- 100.0 ± 5.0 vs SE- 65.4 ± 4.1; t-test $p<0.01$). Most notably, there is a robust increase in the immunoreactivity of PAI-1, an inhibitor of both furin and tPA, at three (figure 6g; control- 100.0 ± 13.1 vs SE- 183.8± 18.2; t-test $p<0.01$) and 24 hours after SE (figure 6h; control- 100.0 ± 12.3 vs SE- 590.8 ± 63.3; t-test $p<0.001$).

To determine if changes in the levels of proBDNF, PAI-1 and tPA persisted beyond 24 hours after SE, levels of these proteins were examined at three and seven days following SE in wild-type C57BL/6J mice. As can be seen in figure 7a, mean proBDNF level appears to peak at three days following SE, although there is more variability at this time point than at earlier time points (figure 7a: control- 100.0 ± 31.8 vs SE- 437.0 ± 122.0; t-test $p<0.05$). At seven days post-SE, proBDNF levels remain elevated, but the relative increase versus control is less than

at three days and variability is lower (figure 7a: control- 100.0 ± 18.8 vs SE- 222.0 ± 28.5; t-test $p<0.05$). PAI-1 levels are variable but overall remain elevated at three days (figure 7b: control- 100.0 ± 9.4 vs SE- 345.0± 117.8; Mann-Whitney test, $p<0.05$), and have returned to control levels at seven days (figure 7b: control- 100.0 ± 5.8 vs SE- 94.0 ± 4.0; t-test $p>0.05$). Of note, the same two samples showed very high levels of proBDNF and PAI-1, and interestingly these two samples both came from animals that had extremely severe SE (multiple stage six seizures). Levels of tPA were not different from controls at three days after SE (figure 7c; control- 100.0 ± 9.2 vs SE- 116.0 ± 8.6; t-test $p>0.05$), and were elevated compared to controls at seven days after SE (figure 7c: control- 100.0 ± 5.0 vs SE- 149.0± 6.0; t-test $p<0.001$).

To better assess if there was a causal relationship between elevated PAI-1 levels and increased proBDNF after SE, we examined the effect of PAI-1 inhibition on proBDNF levels in hippocampal slices from wild-type C57BL/6J mice 24 hours after SE. Hippocampal slices were rapidly removed and incubated for 4 hours in aCSF containing the PAI-1 inhibitor tiplaxtinin (370 μM) or vehicle (DMSO). Tiplaxtinin treatment resulted in a significant reduction in proBDNF levels compared to vehicle treatment in slices from 4 out of the 5 animals (figure 8; $p<0.05$, paired two-tailed t-test), suggesting that a reduction in proBDNF cleavage due to increased PAI-1 may be contributing to elevation of proBDNF levels acutely after SE.

Discussion

These studies provide evidence for an increase in the levels of proBDNF acutely following the induction of SE. In WT C57BL/6J mice as well as in HA-epitope tagged BDNF knock-in C57BL/6J mice there is an increase in proBDNF as early as three hours after SE onset, with levels remaining elevated at 24 hours and peaking at three days post-SE. The epitope tagged knock-in C57BL/6J mice were further utilized to localize early increases in BDNF after SE and HA-immunoreactivity was detected primarily in principal cells but also some astrocytes

367 throughout all hippocampal subfields. Finally, we demonstrated that acute increases in
368 proBDNF after SE are associated with changes in the enzymes involved in the proteolytic
369 processing of proBDNF (reduced tPA and increased PAI-1), and that enhancing proBDNF
370 cleavage by inhibiting PAI-1 reduces proBDNF levels in hippocampal slices from animals 24
371 hours post SE. Taken together these results suggest that inhibition of proBDNF cleavage
372 contributes to acute elevations of proBDNF within hours of SE onset, positioning proBDNF to
373 participate in early cell signaling events after SE such as activation of the JAK/STAT pathway.
374
375 Several groups have previously evaluated the levels of BDNF in epilepsy models (Ernfors et al.,
376 1991; Rudge et al., 1998; Altar et al., 2004; Roberts et al., 2006). The majority of these studies
377 quantitatively evaluated levels of mRNA but did not determine the levels of proBDNF and
378 mBDNF protein. A few studies have evaluated the levels of proBDNF and mBDNF after
379 seizures but none have reported an increase in proBDNF less than 24 hours after SE onset.
380 Unsain et. al analyzed the levels of BDNF following pilocarpine-induced SE in adult rats and
381 demonstrated an increase in proBDNF immunoreactivity three days after SE (Unsain et al.,
382 2008). Using immunohistochemistry, Volosin et. al reported an increase in proBDNF
383 immunoreactivity one day after pilocarpine-induced SE in rats (Volosin et al., 2008). Elevated
384 levels of proNGF protein have also been observed 24 hours after kainate-induced seizures *in*
385 *vivo* (Volosin et al., 2008; Le and Friedman, 2012). This increase in proNGF was not
386 accompanied by increases in mature NGF and resulted from inhibition of MMP-7 by TIMP-1 (Le
387 and Friedman, 2012). Most recently, it was reported that both proBDNF and mBDNF levels
388 were elevated 24 hours after single dose pilocarpine SE in 129SvJ mice (VonDran et al., 2014).
389
390 There may be a number of potential reasons why we were able to identify increases in proBDNF
391 earlier after SE than had been previously reported, including differences in the species (rats vs
392 mice), background strain (129SvJ vs. C57BL/6J), and model of SE (kainate or single high-dose

393 pilocarpine vs repeated low-dose pilocarpine). Repeating the studies utilizing the same
394 techniques on different models would help determine the effect of model selection on the
395 findings. Another distinction between the previous studies and the one presented here is the
396 use of BDNF-epitope tagged knock-in mice that allowed levels of BDNF protein to be probed
397 with high sensitivity and specificity.
398
399 To delineate the spatial expression of BDNF in response to SE, immunohistochemistry for HA
400 was combined with MAP2 and GFAP staining in BDNF-HA tagged mice to evaluate expression
401 in neurons and astrocytes, respectively. The pattern of immunoreactivity for total BDNF in
402 controls is similar to what has been previously reported by others (Dieni et al., 2012; VonDran et
403 al., 2014). BDNF immunoreactivity is increased three hours after SE and colocalizes with MAP2
404 and GFAP, demonstrating that acutely following SE proBDNF is expressed in principal neurons
405 and astrocytes in all hippocampal subfields. BDNF immunoreactivity is most strikingly elevated
406 in the cell bodies of principal neurons of CA3 and CA1 and the mossy fiber pathway.
407 Unfortunately, one is unable to determine if the BDNF localization corresponds with a site of
408 pre-release or internalization. One possibility to explain the presence of BDNF immunoreactivity
409 in non-neuronal cells is that the BDNF localized in the astrocytes may be due to internalization
410 since TrkB.T1 is located primarily in hippocampal astrocytes. The truncated Trk receptors can
411 function as a dominant-negative inhibitor by forming heterodimers with full-length TrkB leading
412 to internalization of BDNF and triggering clearance of BDNF and TrkB (Haapasalo et al., 2002).
413
414 To identify potential mechanisms leading to rapid elevations in proBDNF after SE, we examined
415 the expression of cleavage machinery known to process proBDNF into mBDNF. These studies
416 were based on the hypothesis that the observed rapid elevation in proBDNF levels may be due,
417 at least in part, to a reduction in proneurotrophin cleavage, akin to the inhibition of MMP-7
418 cleavage of proNGF reported to occur after kainate-induced SE in rats (Le and Friedman,

2012). Two potential mechanisms that could contribute to reduced proBDNF cleavage were identified, a significant decrease in tPA levels and a robust increase in PAI-1 levels. The tPA/plasmin proteolytic machinery is a major contributor to extracellular proBDNF cleavage, and PAI-1 inhibits both furin and tPA, thereby inhibiting both intracellular and extracellular cleavage of proBDNF. Elevation in levels of PAI-1 normally corresponds to depression in tPA activity. It is thought that elevation of PAI-1 is an adaptive mechanism to attenuate excessive tPA activity which can contribute to CNS pathology (Melchor and Strickland, 2005). In addition, theta burst stimulation (14,400 pulses, 60 min) triggers simultaneous release of proBDNF and tPA to generate mBDNF extracellularly *in vitro,* and exogenous administration of PAI-1 inhibited tPA activity and attenuated conversion of proBDNF to mBDNF (Nagappan et al., 2009). Taken together with our data demonstrating that PAI-1 inhibition with tiplaxtinin reduces proBDNF levels in hippocampal slices from mice 24 hours after SE, these findings suggest that PAI-1 is a major regulator of proBDNF conversion to mBDNF under both normal physiological conditions and following seizures.

A limitation of the current study is that we were unable to fully evaluate mBDNF levels in parallel with the changes in proBDNF. Unfortunately, in our hands significant intra-lot variability and poor specificity were observed with commercial antibodies that reportedly identify mBDNF, including the antibody used in the recent report by VonDran et al., and therefore studies using mBDNF antibodies were not included in this report. Although we were able to identify clear and specific bands at the reported sizes of both proBDNF and mBDNF on western blots of protein lysates from BDNF-HA tagged mice reacted with an anti-HA antibody, due to difficulty breeding we only had sufficient numbers of these mice to examine the earliest time points (three and 24 hours). Therefore, although we did not find evidence of an increase in mBDNF at these early time points after SE, we were unable to confirm this with a secondary method (using mBDNF antibody detection) and could not evaluate if mBDNF elevations occurred at later time points

445 after SE. An additional potential concern in using BDNF-HA tagged mice to follow endogenous
446 presence of BDNF is the possibility that the HA-tag may alter BDNF expression and/or
447 processing. This seems unlikely, however, as it has been previously reported that the HA-
448 tagged BDNF is expressed, processed and trafficked in the same manner as WT BDNF (Yang
449 et al., 2009, 2014).
450
451 The current study also does not investigate the downstream consequences of elevated
452 proBDNF levels following SE. The rapid increase in proBDNF after onset of SE is temporally
453 positioned to mediate BDNF-induced JAK/STAT activation that begins within an hour of SE
454 onset (Lund et al., 2008). Interestingly, high dose proBDNF has recently been reported to lead
455 to repression of $GABA_AR$ synthesis in cultured hippocampal neurons *in vitro* through activation
456 of the JAK-STAT-ICER pathway (Riffault et al., 2014), suggesting that it might also mediate
457 JAK/STAT activation and subsequent $GABA_AR$ α1 subunit repression *in vivo* after SE. Thus,
458 further studies are required to fully understand the downstream molecular effects of the acute
459 elevation in proBDNF levels demonstrated in these studies and the role of proBDNF signaling in
460 epileptogenesis.
461
462 In summary, we demonstrate an increase in proBDNF levels that occur as early as three hours
463 after SE in principal neurons and their processes, as well as in astrocytes, throughout all
464 hippocampal subfields. We further present evidence that the elevation in proBDNF is due, at
465 least in part, to reductions in proBDNF cleavage that result from acute decreases in tPA
466 expression and increases in PAI-1, an inhibitor of both intracellular and extracellular proBDNF
467 cleavage. These findings suggest that proBDNF is highly abundant immediately following SE
468 onset and may be a key component of neurotrophin signaling during the earliest phases of
469 epileptogenesis.

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Legends

Figure 1. Schematic representation of different proteins involved in the cleavage of BDNF through extracellular and intracellular mechanisms. ProBDNF can be cleaved intracellularly within the endoplasmic reticulum by furin and in regulated secretory vesicles by proconvertase enzymes (PC1/3). ProBDNF can also be cleaved extracellularly by matrix metalloproteinases (MMP -3/-7/-9) or by components of the tissue plasminogen activator/plasmin (tPA/plasmin) proteolytic cascade. The activity of these proteases is tightly regulated by a number of inhibitors including plasminogen activator inhibitor 1 (PAI-1) that inhibits both extracellular and intracellular cleavage, tissue inhibitor of metalloproteinases (TIMPs) that inhibit MMPs and

neuroserpin and α2 antiplasmin (A2AP) that inhibit the tPA/plasmin proteolytic cascade. Red bars indicate inhibition and green bars indicate activation.

Figure 2. ProBDNF protein levels are elevated acutely after pilocarpine induced SE in WT C57BL/6J mice. A) Bottom: Representative western blot of whole hippocampal protein homogenates from WT mice sacrificed 3hr after induction of SE or time-matched saline controls probed with proBDNF (1:1,000) and anti-actin antibodies. Top: densitometry analysis of proBDNF protein abundance. Ratio of proBDNF/Actin at 3hr post-SE (N=5) expressed as percent change relative to mean values (+/- S.E.M.) of control group (N=5; $p<0.001$). B) Bottom: representative western blot of whole of whole hippocampal protein homogenates from WT mice sacrificed 24hr after induction of SE or time-matched saline controls probed with proBDNF (1:1,000) and anti-actin antibodies. Top: ratio of proBDNF/Actin at 24hr post-SE (N=4) expressed as percent change relative to mean values of control group (N=4; $p<0.05$).

Figure 3. ProBDNF levels are elevated in BDNF-HA tagged mice in the first 24 h after pilocarpine induced SE. A) Top: Representative western blot of whole hippocampal protein homogenates from BDNF-HA mice sacrificed 3hr after induction of SE or time-matched saline controls probed with anti-HA (1:3,000) and anti-actin antibodies. Bottom: Densitometry analysis of proBDNF protein abundance. Ratio of proBDNF/Actin at 3hr post-SE (N=6) expressed as percent change relative to mean values (+/- S.E.M.) of control group (N=3; $p<0.001$). B) Top: Representative western blot of whole hippocampal protein homogenates from BDNF-HA mice probed with anti-HA (1:3,000) and anti-actin antibodies sacrificed 24hr after induction of SE or time-matched saline controls. Bottom: Densitometry analysis of proBDNF protein abundance at 24hr post-SE. Ratio of proBDNF/Actin at 24hr post-SE (N=6) expressed as percent change relative to mean values of control group (N=3; $p<0.01$). Densitometry analysis of mBDNF

protein abundance (mBDNF/Actin) showed no significant difference between control and SE group at either time point.

Figure 4. BDNF protein is expressed in neurons and astrocytes of hippocampus after pilocarpine induced SE. A) Representative confocal images of hippocampal subfields from HA-tagged mice 3hr after SE and an age- and handling-matched control (A; 20x magnification, scale bar = 100μm) shows presence of HA immunoreactivity in principal cells, glia and mossy fiber layers. The first column shows anti-HA (green) immunoreactivity with DAPI (blue) in each condition. The second column demonstrates co-localization of immunoreactivity for HA (green) and the neuronal marker MAP2 (red). The third column demonstrates co-localization of immunoreactivity for HA (green) and the glial marker GFAP (red). B) High magnification confocal image of CA3 hippocampal subfield (63x magnification, scale bar = 20 μm). White arrow heads correspond to neuronal localization of HA immunoreactivity in pyramidal cells of CA3; blue arrow heads correspond to localization of HA immunoreactivity in mossy fibers. (SL: stratum lucidum, SP: stratum pyramidale). C) High magnification confocal image of CA3 hippocampal subfield (63x magnification, scale bar = 20 μm) demonstrating glial expression of BDNF.

Figure 5. Enzymes involved in the processing of proBDNF are altered after pilocarpine-induced SE. Representative western blots of whole hippocampal protein homogenates from WT mice sacrificed 3hr (left panels) and 24hr (right panels) after induction of SE or time-matched saline controls. Densitometry analysis of abundance of different cleavage proteins normalized to actin and expressed as percent change relative to mean values of control group (+/- S.E.M.). A and B) anti-furin (1:1,000); C and D) anti-plasminogen (1:3,000); E and F) anti-MMP9 (1:2,000); G and H) anti-tPA (1:1,000). The sample size for 3hr is N=5 in each group and for 24hr N=4 in each group (*p<0.05, **p<0.01, ***p<0.001; t-test)

Figure 6. Inhibitors of proBDNF processing are altered after pilocarpine SE. Representative western blots of whole hippocampal protein homogenates from WT mice sacrificed 3hr (left panels) & 24hr (right panels) after induction of SE or time-matched saline controls. Densitometry analysis of abundance of different inhibitor proteins normalized to actin and expressed as percent change relative to mean values of control group (+/- S.E.M.). A and B) anti-alpha2-antiplasmin (A2AP, 1:2,000); C and D) anti-neuroserpin (1:2,000); E and F) anti-TIMP-1 (1:1,000); G and H) anti-PAI-1 (1:1,000). The sample size for 3hr is N=5 in each group and for 24hr is N=4 in each group (**p<0.01, ***p<0.001; t-test).

Figure 7. ProBDNF, PAI-1 and tPA levels at 3 and 7 days following SE. Right: Representative western blots of whole hippocampal protein homogenates from WT mice sacrificed 3 days (top panels) or 7 days (bottom panels) after induction of SE or time-matched saline controls probed with antibodies against proBDNF (A), PAI-1 (B), or tPA (C). Left: Densitometry analysis of abundance of proBDNF (A), PAI-1 (B), or tPA (C) normalized to actin and expressed as percent change relative to mean values of control group (+/- S.E.M.). A) anti-proBDNF (1:2,000); B) anti-PAI-1 (1:1,000); C) anti-tPA (1:1,1000). N=4 for all control groups, and N=8 for all 7 days SE groups. For 3 days SE groups, N=4 for proBDNF and N=5 for PAI-1 and tPA (*p<0.05, ***p<0.001; t-test used for all analyses except PAI at 3 days for which Mann Whitney (non-parametric) test was used due to non-normal data set).

Figure 8. PAI-1 Inhibition reduces proBDNF levels after pilocarpine SE. Right: representative western blots of protein homogenates from hippocampal slices from individual WT mice removed 24 hours after SE then incubated for four hours in aCSF containing the PAI-1 inhibitor tiplaxtinin (370 μM; TREATED) or vehicle (DMSO; CTRL), probed with anti-proBDNF (1:2,000) or anti-actin antibodies. Left: densitometry analysis of abundance of proBDNF

normalized to actin in homogenates from vehicle-treated (CTRL) and tiplaxtinin-treated (TREATED) slices for each animal (N=5). Tiplaxtinin treatment resulted in a significant reduction in proBDNF levels compared to vehicle treatment ($p<0.05$, t-test).